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Exploiting the use of 3,4-HPO ligands as nontoxic reagents for the determination of iron in natural waters with a sequential injection approach

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ABSTRACT

In this paper, the use of 3-hydroxy-4-pyridinone (3,4-HPO) chelators as nontoxic chromogenic reagents for iron determination is proposed. The potential application of these compounds was studied in a sequential injection system. The 3,4-HPO ligands used in this work were specially designed to complex iron(III) at physiologic pH for clinical applications. The developed sequential injection method enabled to study the reaction conditions, such as buffering and interferences. Then, to further improve the low consumption levels, a microsequential injection method was developed and effectively applied to iron determination in bathing waters using 3,4-HPO ligands. The formed iron complex has a maximum absorbance at 460 nm. The advantage of using minimal consumption values associated with sequential injection, together with the lack of toxicity of 3,4-HPO ligands, enabled to present a greener chemistry approach for iron determination in environmental samples within the range 0.10–2.00 mg Fe/L with a LOD of 7 μg/L. The overall effluent production was 350 μL corresponding to the consumption of 0.48 mg of 3,4-HPO ligand, 0.11 mg of $NaHCO_3$, 0.16 mg of HNO_3 and 50 μL of sample. Three reference samples were assessed for accuracy studies and a relative deviation $< 5\%$ was obtained. The results obtained for the assessment of iron in inland bathing waters were statistically comparable to those obtained by the reference procedure.

1. Introduction

Iron is an essential element for most forms of life on earth and for that reason it is a micronutrient and not considered a water pollutant. Nevertheless, it is very important to monitor the iron content in waters since high levels of iron may result in aesthetic (odour and taste), cosmetic (colour) and technical (damage to water equipment) effects [1]. The visual impact of the reddish colour in recreational waters, namely bathing waters may result in negative economical impact due to public opinion. Inland bathing waters present a challenging matrix due to the expected diversity of parameters and variability in concentration levels. In fact, these waters are often highly stressed due to recreational activities, so the efficient monitoring of parameters such as iron represents a valuable contribution to the overall environmental assessment. Furthermore, the established relationship between the iron content and algae blooms emphasises on the importance of public acceptance concerning the mentioned recreational waters.

The assessment of iron is often carried out by molecular absorption spectrophotometry using highly toxic species: thiocyanate, 1,10-phenantroline, bathophenantroline, 2,2-bipyridyl, eriochrome cyanine R, and cetyltrimethylammonium [2]. In this context, and aiming the use of nontoxic reagents for spectrophotometric determinations of iron, we are presently exploring the use of 3-hydroxy-4-pyridinone (3,4-HPO) chelators as chromogenic species for iron. These ligands are well known, mainly by reason of their biomedical applications; they are particularly attractive for pharmaceutical purposes since their structure (Table 1) allows tailoring of their hydrophilic/lipophilic balance (HLB) without significantly changing its chelating properties.

Variations in HLB can be achieved by simply introducing appropriate substituents on the endocyclic nitrogen atom of the pyridinone ring thus leading to the optimal lipophilicity for delivery or removal of metal ions in the human body. The 3-hydroxy-4-pyridinones are hard ligands that bear two oxygen

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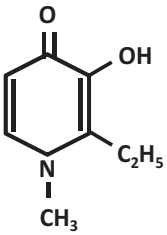
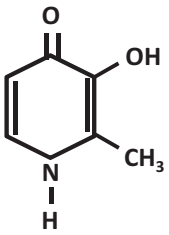
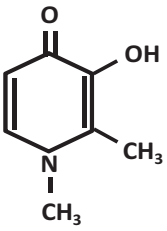
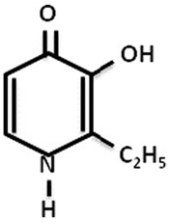
Table 1
The 3,4-HPO bidentate ligands tested as colorimetric reagents for iron determination with the respective chemical formula and molecular weight.

3,4-HPO Ligand	2-methyl-3-hydroxy-4-pyridinone	1,2-dimethyl-3-hydroxy-4-pyridinone	2-ethyl-3-hydroxy-4-pyridinone	1-methyl-2-ethyl-3-hydroxy-4-pyridinone
Abbreviation	Hmpp	Hdmpp	Hetpp	Hempp
Structure				
Chemical formula	$C_6H_7O_2N$	$C_7H_9O_2N$	$C_7H_9O_2N$	$C_8H_{11}O_2N$
Molecular weight	126.14	139.15	139.15	153.16
pKa	$pK_{a_1} = 3.62 \pm 0.05$ $pK_{a_2} = 9.48 \pm 0.05$	$pK_{a_1} = 3.69 \pm 0.01$ $pK_{a_2} = 9.61 \pm 0.03$	$pK_{a_1} = 3.63 \pm 0.04$ $pK_{a_2} = 9.62 \pm 0.05$	$pK_{a_1} = 3.53 \pm 0.02$ $pK_{a_2} = 9.46 \pm 0.05$

coordinating atoms and consequently show a very high capacity to trap iron(III) and comparatively low affinity for iron(II).

The values of iron(III) stability constants are in the range $35.0 < \log \beta_3 < 37.0$ and $12.0 < \log \beta_2 < 15.0$ for iron(II). Affinity for other metal (III) and metal (II) ions can be found in the literature and show that these ligands may be of use in the development of new methods to monitor iron [3,4]. The use of 3,4-HPO ligands as iron colorimetric reagents requires a detailed study of the reaction conditions and a comprehensive interference study. In order to be considered an effective alternative, similar (or better) sensitivity should be obtained when compared to commonly used reagents together with non-significant interferences.

Within this context, in this paper, the potential use of these chelators as chromogenic reagents for iron was studied. Due to the advantages of using flow-based techniques for carrying out spectrophotometric measurements, this study was performed in a sequential injection (SI) system [5]. A sequential injection analysis method for the determination of iron was developed and a thorough study of the 3,4-HPO ligands as iron colorimetric reagents was carried out. The conditions for the colorimetric reaction, the sensitivity and selectivity of the 3,4-HPO/Fe chelates were assessed and critically compared to others involving commonly used chromogenic reagents. Aiming for a greener chemistry approach on the application to natural waters, namely inland bathing waters, downscaling of the SI method led to the development of a microsequential injection lab on a valve (μSI-LOV) method [6]. A detailed assessment of the possible interferences was carried out and the limits of the chelation reaction were tested. The downscaling enabled to decrease both the sample/reagents consumption and effluent. The μSI-LOV method was developed using a lab on valve manifold known for the robustness in handling significantly low volumes, between 5 and 50 μL [7].

2. Materials and methods

2.1. Reagents and solutions

The 3,4-HPO ligands were synthesised according to the methods published in the literature [3]. All solutions were prepared with analytical grade chemicals and boiled deionised water (specific conductance less than 0.1 μS/cm).

Saturated ligand solutions were obtained by dissolution of approximately 2 mg of the synthesised ligand in 100 mL of water corresponding to a concentration of 20 mg/L which is higher than the solubility value thus ensuring the saturation of the solution. The ligand solution used in the μSI-LOV method was a 1.25 dilution of the 20 mg/L solution, resulting in a final concentration of $\approx$ 15 mg/L, also a saturated solution.

The buffer solutions of hydrogen carbonate 0.10 and 0.25 M were prepared by dissolving 420 mg and 1.05 g of sodium hydrogen carbonate in 50 mL of water, respectively. The pH was set to 10.5 with 0.5 M sodium hydroxide.

An iron(III) stock solution, 10 mg/L, was prepared by diluting the atomic absorption standard of 1000 mg/L. Working standards in the dynamic range 0.1–2.0 mg/L were weekly prepared from dilution of the stock solution in 0.03 M of nitric acid.

A stock solution of iron(II) was prepared from the solid iron(II) ammonium sulphate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) to a final concentration of 18.2 mg/L. This stock solution was diluted to obtain an intermediate solution of 4.6 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(III) determination with 3,4-HPO ligand and study of the reaction is depicted in Fig. 1.

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump, equipped with PVC pumping tube connected to the central channel of a ten port selection valve (Valco VICI Cheminert C25-3180EUHB). All tubing connecting the different components was made of PTFE (Omnifit), with 0.8 mm i.d. A personal computer (HP Pavilion zt3000) equipped with a National Instruments DAQcard—DI0 interface card, running a homemade software, was used to control the selection valve position and the peristaltic pump direction and speed.

An Ocean Optics USB 4000 charged coupled device detector (CCD), equipped with a pair of 400 μm fibre optic cable and a Mikropack DH-2000 deuterium halogen light source and a Hellma 178.710-OS flow-cell with 10 mm light path and 80 μL inner volume, was used as the detection system. Data acquisition was performed through the Ocean Optics—Spectrasuite software at 459 nm.

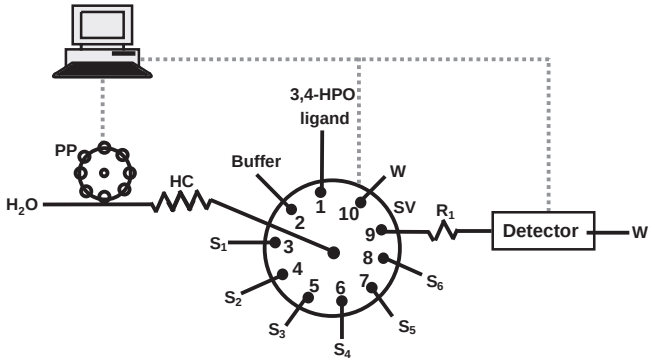


Fig. 1. Sequential injection manifold to study the 3,4-HPO ligand as a colorimetric reagent for iron determination; SV, 10 port selection valve; HC, holding coil with 300 cm of length; PP, peristaltic pump; S_i, iron(III) standards; R_1, reaction coil with 6 cm; and W, waste.

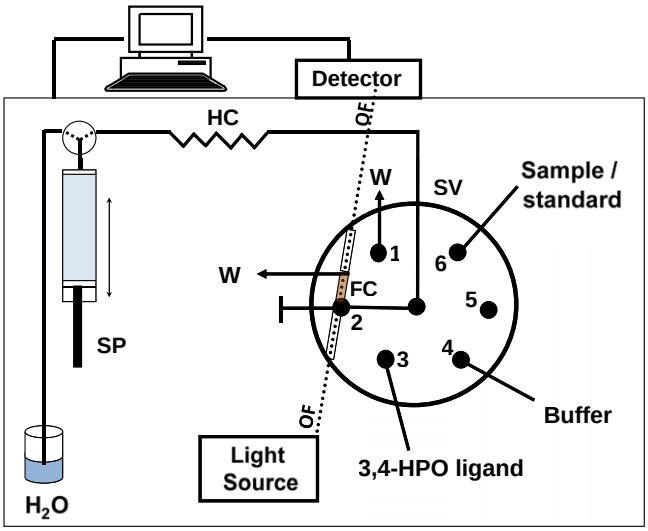


Fig. 2. Microsequential injection-Lab on valve, μSI-LOV, manifold for the determination of iron(III) in bathing waters using the 3,4-HPO ligand as a colorimetric reagent; SV, 6 port selection valve; HC, holding coil with 1.5 m of length; SP, syringe pump; FC, 1 cm flow cell; OF, optical fibbers; W, waste.

2.3. Microsequential injection manifold (lab on valve) and procedure

Aiming for further miniaturisation and decrease of consumption volumes, a microsequential injection lab on valve methodology, μSI-LOV, was also developed and is depicted in Fig. 2.

The μSI-LOV system was a FIAlab-3500 (FIAlab Instruments) consisting of a bi-directional syringe pump (2500 μL of volume), a holding coil and a lab-on-valve manifold mounted on the top of a six-port multi-position valve. As a detection system, a USB 2000 Ocean Optics, a CCD spectrophotometer equipped with fibre optics (FIA-P200-SR, 200 μm), and a Mikropack DH-2000-BAL deuterium halogen light source was used. FIAlab for Windows 5.0 software on a personal computer (HP Compact) was used for flow programming and data acquisition.

The sequence of steps with the respective time and volumes used for both methodologies is shown in Table 2.

The first step was the aspiration to the holding coil of 3,4-HPO ligand (step A), followed by the aspiration of the buffer and the standard (steps B and C). Mixing was promoted by the flow reversal while propelling the aspirated plugs towards the detector (step D). For the μSI-LOV, due to the reduced size of aspiration plugs and absence of reaction coil, mixing mainly occurs in the holding coil.

Table 2
Protocol sequence for both the developed methodologies, SI and μSI-LOV, for iron determination with 3,4-HPO ligand as a colorimetric reagent.

Step	Port		Time (s)	Flow rate (μL/s)		Volume (μL)		Description
	SI	μSI-LOV	SI	SI	μSI-LOV	SI	μSI-LOV	
A	1	3	4	60	25	240	40	Aspiration of ligand
B	2	4	2	15	10	30	5	Aspiration of buffer
C	3–8	6	5	60	25	300	50	Aspiration of standard
D	9	2	20	60	20	1200	350	Propelling to detector

2.4. Water samples—inland bathing waters

Water samples from inland bathing areas were collected in polyethylene plastic bottles of 0.5 L capacity at about 20 cm depth. The samples, acidified at collection according to the collection procedure [8], were introduced directly in the developed system without filtration.

2.5. Accuracy assessment

The collected inland bathing waters were spiked and analysed using the atomic absorption method (APHA 3111B) [8] and the results were compared to those obtained with the developed μSI-LOV method. For further accuracy assessment, results obtained with the proposed μSI-LOV system were compared to the certified values of three certified water samples, ERM-CA010a (hard drinking water), ERM-CA021a (soft drinking water) from LGC standards and NRC-CNR SLRS-4 (River water) from the National Research Council Canada.

3. Results and discussion

3.1. Preliminary studies

The 3,4-HPO bidentate ligands form complexes with iron(III) of the type FeL_3. The stepwise formation of the complex results in a sequence of colour products according to the number of ligands, 1, 2 and 3, bound to the metal ion. The final complex (FeL_3) shows a maximum of absorbance at 460 nm, and was the one used for iron(III) quantification. There were four 3,4-HPO bidentate ligands available with the same affinity for iron(III) and different solubility values. The reaction sensitivity and kinetics were expected to be quite similar but some studies were carried out to ensure the choice of the most appropriate ligand. The tested compounds, used in the initial studies, are shown in Table 1 together with the formulae, molecular weight and acidity constants [3].

Based on the reported solubility for Hdmpp [4] of 14 mg/L, a first approach to the ligand concentration was made. A 6 mg/L ligand solution, about half the maximum concentration, was prepared to ensure that both higher and lower concentrations could be tested. Then, 1 mL of this solution was added to 1 mL of iron(III) standards, 0.5 and 1 mg/L, and no absorbance signal was observed. Because significantly lower concentrations of iron(III) were aimed, another ligand solution was prepared with a concentration of about the reported solubility value, 15 mg/L, a saturated solution. In these conditions, the same procedure was carried out (1 mL of ligand solution added to 1 mL of iron(III) standard) and colour was observed for the 1 mg/L standard ($A=0.035$). Aiming for the highest sensitivity possible, saturated solutions were used for the remaining preliminary

studies and the ligand concentration study was revisited in the flow analysis studies.

Saturated solutions (≈ 20 g/L) were prepared for the four ligands and the first experiments were carried on by mixing 1 mL of ligand solution with 1 mL of the 10 mg/L iron(III) standard solution. Scans were made directly on all the mixtures without any pH adjustment and are shown in Fig. 3. Analysis of the spectra showed that the maximum absorption was observed at 515 nm thus indicating the need for pH adjustment in order to ensure stoichiometric formation of the complex FeL_3, whose maximum of absorption is generally observed at 460 nm. Considering the values of the acidity constants of the ligands (Table 1) we chose to adjust the pH of the ligand solution to 10 prior to the addition of the iron(III) solution. Using the latter procedure, the maximum of absorbance was shifted from 515 nm to 459 nm thus confirming the stoichiometric formation of FeL_3 and the need of including a buffering solution in the system.

Aiming for the application of these ligands for iron quantification in flow analysis, it was important to evaluate the kinetics of the complex formation. The absorbance of a buffered mixture of 1 mL saturated ligand solution and 0.5 mL of 10 mg/L iron(III) standard was measured for 1 min. This procedure was repeated for the four ligand solutions and colour was observed almost immediately after mixing, indicating the complex formation. After the observed initial reaction, there was no significant absorbance increase during the measured time (1 min). In fact, for the ligands Hmpp and Hempp, the final absorbance was the same as the initial value and for Hdmpp and Hetpp ligands there was a slight increase (< 8%).

These preliminary studies enabled to set some basic conditions: the preparation of saturated ligand solutions and the need of buffering the complex formation at pH ≈ 9.5. Although similar results were obtained for all the four tested ligands, given the need of the highest concentration possible the two most hydrophilic ligands (Hmpp and Hdmpp) were used in flow studies.

3.2. Sequential injection method

The advantageous characteristics of sequential injection analysis concept namely versatility, automation and low reagent consumption, made it an appropriate choice for automation of the new analytical application of 3,4-HPO ligands for iron determination. So, a SI manifold was designed for the study of the complex formation.

3.2.1. Physical parameters

Having established the need for three solutions (ligand solution, buffer and sample) the aspiration sequence was assessed. The choice of keeping the buffer in between the ligand and sample ensured buffering the sample prior to the reaction. So, the tested sequences were as follows: ligand–buffer–sample/standard (LBS) and sample/standard–buffer–ligand (SBL). The results obtained with the sequence LBS presented a fourfold increase in sensitivity so that was the chosen sequence. With the established aspiration sequence the volumes of each plug was studied. The buffer volume was the first to be studied with three volumes tested, 30, 60 and 90 μL (Fig. 4). The sensitivity decreased with the increase of the buffer volume, so 30 μL was the volume chosen. Lower volumes

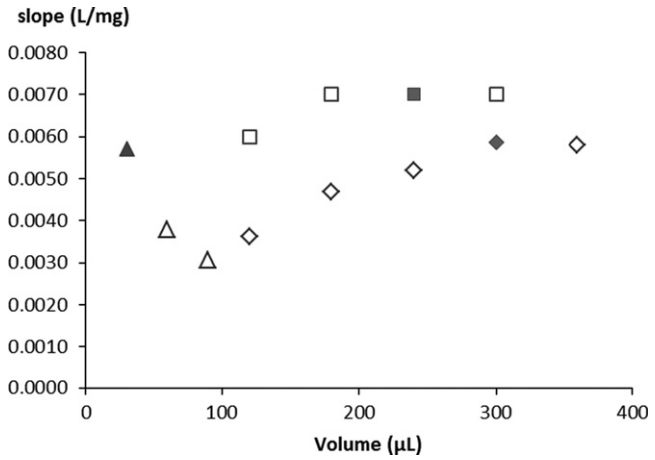


Fig. 4. Study of the influence of the volume of ligand (□), buffer (△) and sample (◇) on the sensitivity of the iron determination; the chosen volumes are represented in full black.

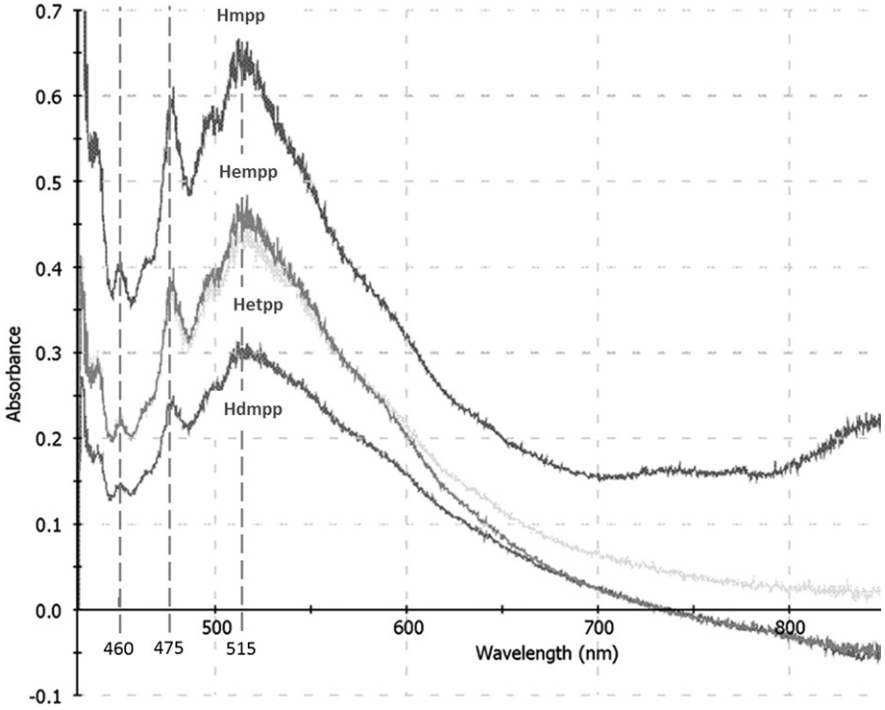


Fig. 3. Visible spectra of the 3,4-HPO ligands.

were not tested as 30 μL normally represents the minimal reproducible value to be used in SI methods [9]. Then, the sample volume was studied within the range 120–360 μL, and the sensitivity increased up to the volume of 300 μL, so that was the chosen value (Fig. 4). Finally, the volume of ligand solution was also studied. From the tested volumes of 120, 180, 240 and 300 μL the volume of 240 μL was chosen to ensure an excess of ligand as the sensitivity increased up to 180 μL (Fig. 4).

In order to reinforce the previous choices, limits of detection and quantification were calculated using two volumes of sample/standard, 300 and 360 μL, and two volumes of ligand, 240 and 300 μL. The previously set values, 300 μL for sample and 240 μL for ligand, proved to be an appropriate choice as they provided the lowest limits.

3.2.2. Chemical parameters

3.2.2.1. Ligand solution. The results observed in the section "Preliminary studies" showed a significant increase in sensitivity when a saturated ligand solution was used. Nevertheless, due to the potential complications from using a saturated solution in a flow system, the study of the ligand concentration was revisited using the SI method.

First, calibration curves with saturated solutions, 20 mg/L, of Hmpp and Hdmpp (two most hydrophilic ligands) were compared to calibration curves with solutions of the same ligands diluted to half and a 42% decrease in sensitivity was observed. Then, for a combined evaluation of the effect in both the sensitivity and the intercept, several dilutions were made from the saturated ligand solutions (Fig. 5).

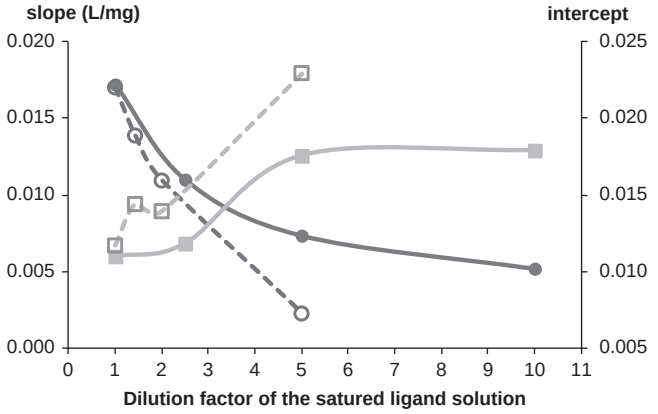


Fig. 5. Study of the influence of the 3,4-HPO concentration; Hmpp, full lines with ●, for the slopes (sensitivity) and ■ for the intercepts; Hdmpp, dashed lines with ○, for the slopes (sensitivity) and □, for the intercepts.

The results, similar for both Hmpp and Hdmpp showed that the increase of the dilution factor resulted not only in a sensitivity (slope) decrease but also an increase of the intercept and consequently of the limit of detection.

The results confirmed the choice of using a saturated solution of ligand in order to obtain the maximum sensitivity. Despite the use of saturated solutions, no clogging problems were observed and the repeatability of the calibration curve slope (RSD = 5.2% for four calibrations in consecutive days) proved that no major problems occurred.

Afterwards, in order to choose one of the two, a comparison was made between the two most soluble 3,4-HPO ligands, Hmpp and Hdmpp, (Table 1). Two calibration curves were made, one with the Hmpp ligand and another with the Hdmpp ligand and the results showed a similar sensitivity. In fact, the obtained slopes were quite comparable (RD = 1.4%) and the intercepts were also quite alike (RD = 1.5%). Without major differences between the two tested ligands, Hmpp was chosen as the most hydrophilic one.

3.2.2.2. Buffer solution. The requirement of buffering the complex formation was established in the section "Preliminary studies". Aiming to buffer the reaction at pH ≈ 9.5, buffer solutions were prepared with higher pH due to the acidity of iron(III) standard solutions (pH ≈ 2). Two buffer compositions, at pH = 10.5, were compared: 0.5 M hydrogen carbonate and 0.5 M hydrogen phosphate. Although the sensitivity doubled with the hydrogen phosphate buffer, the same occurred to the intercept, resulting in a significant increase of the detection and quantification limits. So, a hydrogen carbonate solution was chosen as buffer and a study of its concentration was carried out. The tested concentration range was 0.05–0.5 M and, although the sensitivity increased up to 0.25 M, the intercept increased continuously with the increase in concentrations. In this context, 0.1 M was chosen, as it represented a 72% increase to 0.05 M and was only 12% lower than 0.25 M, maintaining the detection limit below 0.1 mg/L.

3.3. Microsequential injection lab on valve method (μSI-LOV)

Aiming for the application to environmental samples, the concern for a greener analytical procedure led to the down scaling to μSI-LOV. In fact the advantage of μSI-LOV is the substantial reduction in reagent and sample consumption coupled to extremely low effluent production. Consequently, a 6 fold volume reduction was made from the previous set volumes (*physical parameters* section) as shown in Table 2. A further reduction could not be employed, as 5 μL (buffer volume) was previously reported as the minimal volume to produce an acceptable reproducibility [10].

Table 3
Features of the developed methodologies for iron(III) determination in water samples using 3,4-HPO ligand as a colorimetric reagent.

Method	Dynamic range (mg/L)	Typical calibration curve $A = m\,[Fe^{3+}] + b$	LOD (μg/L)	LOQ (μg/L)	Quantification rate (det./h)	Reagent/sample consumption	Effluent production (μL)
SI	0.30–2.00	$y = 0.0251\ (\pm 0.0020)\ [Fe^{3+}] + 0.0155\ (\pm 0.0057)$ $R^2 = 0.999$	83	277	102	3.4 mg Hmpp 0.25 mg $NaHCO_3$ 0.57 mg HNO_3 300 μL	1200
μSI-LOV	0.10–1.00	$y = 0.0488\ (\pm 0.0008)\ [Fe^{3+}] + 0.0030\ (\pm 0.0004)$ $R^2 = 0.999$	7	24	90	0.56 mg Hmpp 0.11 mg $NaHCO_3$ 0.10 mg HNO_3 50 μL	350

The μSI-LOV main characteristic, the detector (flow cell) located in the valve, justifies the study of the flow rate of propelling to the detection as there is no reaction coil. So different flow rates were tested (20, 25 and 30 μL/s), corresponding to different reaction times, and as expected there was a slight increase with the decreasing flow rate so the flow rate of 20 μL/s was chosen. However, as the sensitivity increase was not significant ($<10\%$), lower flow rates were not tested to avoid the decrease of the quantification rate.

The study of carbonate concentration (buffer solution) was revisited, as it had been a compromise solution (*chemical parameters* section). Calibration curves were established for carbonate concentrations of 0.1, 0.25 and 0.5 M and, once again, the sensitivity increased up to 0.25 M (over 30% higher than with 0.1 M). Nevertheless opposite to what was previously observed, the intercept was lower (49% lower than with 0.1 M) so 0.25 M carbonate was chosen as the buffer solution.

The ligand concentration was also revisited because using μSI-LOV implies practically no dispersion raising possibility of lower concentrations. Dilutions from the saturated Hmpp solution were made, dilution factors from 1 to 5, and for a dilution factor of 1.25 the sensitivity was statistically the same (relative deviation 1.4%) decreasing significantly (relative deviation $>5\%$) for the other dilution factors. So the ligand concentration used was a 1.25 dilution of the saturated solution corresponding to ≈ 15 mg/L, still a saturated solution.

3.4. Features of the developed SI and μSI-LOV methodologies

After the detailed studies for the determination of iron based coloured complex formed with 3,4-HPO bidentate ligand, the characteristics of the developed methods were summarised (Table 3).

The limits of detection and quantification, LOD and LOQ, were calculated according to IUPAC recommendations [11,12]. For the SI method, three (LOD) and ten (LOQ) times the standard deviation of ten consecutive injections of deionised water were used for the calculation. As for the μSI-LOV method, four calibration curves were established to calculate the limits based on three (LOD) and ten (LOQ) times the standard deviation of the intercept.

The dynamic range of the SI method was established based on the calculated LOQ and up to the limit of the linear response. For the μSI-LOV method a calibration curve with eight standards, ranging from 0 to 4 mg/L, was made to assess the dynamic linear range defined as 0.1–1.0 mg/L (Table 3).

The determination rate was calculated based on the time spent per cycle. A complete analytical cycle took about 0.59 min for the SI method and 0.67 min for the μSI-LOV method. 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. The presented consumption values, for reagents and sample, and the effluent production were calculated per determination.

Considering the lower limits obtained and the significant reduction of reagents and sample consumption and effluent production, the μSI-LOV was used for the interferences study and sample application.

3.5. Application of the μSI-LOV methodology to iron determination in natural waters

Although in natural waters iron is mostly found as iron(III), evaluating the response of the developed μSI-LOV method to iron(II) was important to assess.

A variety of iron standards, with the same final iron concentration, were prepared: iron(III) standards; iron(II) standards; iron(III) standards with iron(II) concentration constant and iron(II) standards with iron(III) concentration constant. The obtained results proved that both iron forms were effectively complexed with the 3,4-HPO ligands as all the slopes were not significantly different, relative deviations $<5\%$ (ESI, Fig. 1S). This feature clearly indicated that the total dissolved iron was being determined and was probably the result of working with a carbonate buffer at a pH=9.5.

3.5.1. Study of possible interferences

Due to the nature of the colorimetric reaction, the possible interference of several bivalent and trivalent cations was assessed. Besides, the application to water samples justified testing 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 [8]. Exception was made for chloride, as the tested values correspond to the expected values in estuarine and marine waters, 6 g/L and 19.2 g/L, respectively.

The solutions of the tested cations were obtained from proper dilution of atomic absorption standards except for cobalt(II), which were obtained by dilution of a stock solution prepared from solid cobalt sulphate. As for the anions, the tested concentrations were obtained from proper dilution of stock solutions prepared from the respective solids: sodium chloride, sodium nitrate, sodium nitrite and sodium sulphate.

Several standards, with 400 μg/L of iron(III) and the tested concentration of interfering ions, were prepared and analysed with the developed μSI-LOV method. The obtained absorbance values of the standard with and without interfering ion were registered and the interference percentage calculated (Table 4).

Overall, for expected values in natural waters, no significant interferences were observed as most of the interference percentages were below 5%. Exceptions were observed for the highest concentrations tested of calcium, magnesium and cobalt with

Table 4
Assessment of possible interfering ions in the determination of iron according to legislated values; UNFAO, United Nations Food and Agriculture Organisation.

Possible interfering ion	Legislation maximum values		Tested concentration of interfering ion in a standard 0.4 mg Fe^{3+}/L (mg/L)	Interference (%)
	UNFAO (mg/L)	Portugal (mg/L)		
Al^{3+}	5[a]	20[a]	5.10	−0.5
			25.6	−7.6
Ca^{2+}	15[b]	50[b]	10.0	−1.6
			25.0	−12.2
Co^{2+}	0.1[a]	10[a]	0.10	−0.7
			5.0	−14.4
Cu^{2+}	1.3[a]	5[a]	1.00	1.1
			5.00	−9.6
Mg^{2+}	5[b]	50[b]	30.0	−6.9
			50.0	−11.8
Mn^{2+}	0.2[a]	10[a]	0.20	−0.3
			10.0	−1.4
Ni^{2+}	0.2[a]	2[a]	0.20	0.0
			2.0	7.6
Zn^{2+}	2[a]	10[a]	1.00	−0.8
			10.0	1.3
Cl^-	–	–	6000	−4.9
			19,200	5.7
NO_3^-	–	50[a]	25.0	0.9
			50.0	2.5
NO_2^-	–	0.1[b]	0.050	1.6
			0.10	4.8
SO_4^{2-}	–	–	2.00	−0.7
			2000	2.8

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

interference percentages slightly over 10%. However, it is important to emphasise that the concentration ratios of iron/interferent represent over 1:60, 1:120 and 1:10 respectively, values that are not expected to occur in natural waters. In the end, the specificity of the 3,4-HPO ligand to iron was shown even for highly disadvantageous conditions, namely much higher concentrations of possible interfering cations (ESI, Fig. 2S).

It is important to stress the acceptable interference percentage obtained with the expected chloride concentration in sea waters, 19.2 g/L, indicating the possibility for application to those waters.

3.5.2. Method validation and application to bathing water samples

For accuracy assessment of the developed method, three certified water samples were analysed and the results compared to the certified value. Two of the certified samples were drinking waters: a hard drinking water, ERM-CA010a and a soft drinking water, ERM-CA021a with certified values in iron content of 236 ± 6 μg/L and 196 ± 2 μg/L, respectively. The obtained concentrations with the developed μSI-LOV method were 249 ± 9 μg/L for the ERM-CA010a, corresponding to a 5% relative deviation, and 192 ± 9 μg/L for the ERM-CA021a, corresponding to a relative deviation of −2%. Another certified water sample was analysed, a river water sample NRC-CNR SLRS-4, with an iron content of 103 ± 5 μg/L and the relative deviation obtained was 3% as the concentration calculated with the developed method was 107 ± 9 μg/L.

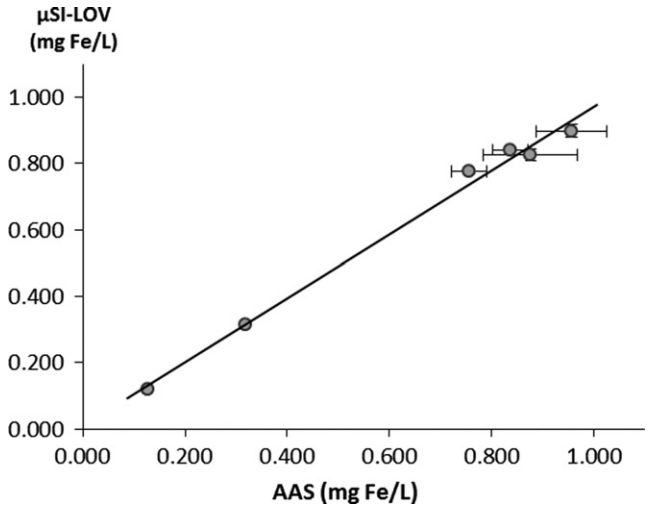


Fig. 6. Scatterplot for the comparison of the results obtained with the developed μSI-LOV method and the atomic absorption method.

For further accuracy assessment, six spiked water samples were analysed by the reference procedure, atomic absorption spectrometry (AAS) [8], and by the developed μSI-LOV methodology and a linear relationship between $C\mu_{\text{SI-LOV}}$ (mg/L) and C_{AAS} (mg/L) was established. The results were plotted (Fig. 6) and the equation found was: $C_{\mu\text{SI-LOV}} = 0.962\ (\pm 0.041) \times C_{\text{AAS}} - 0.009\ (\pm 0.025)$, where the values in parenthesis have 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 [14].

Additionally, several samples of inland bathing waters were collected at pH ≈ 2 [8] and spiked with iron(III) to final concentrations of 250 and 750 μg/L; volumes of 0.25 and 0.75 mL of a 10 mg/L iron(III) standard were used, respectively. Recovery percentages, presented in Table 5, were calculated according to IUPAC [15] and the average was 100% with a standard deviation of 3%. 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 0.094 with a correspondent critical value of 2.593. The statistical results indicate the absence of multiplicative matrix interferences.

The repeatability was assessed by calculation of the relative standard deviation (RSD) obtained by the mean of ten consecutive injections of sample. For a spiked water sample, the calculated RSD was 1.4% (0.814 ± 0.012 TDI mg/L), and for a certified sample it was 4.1% (0.103 ± 0.002 TDI mg/L).

4. Conclusions

The use of 3,4-HPO ligands as iron colorimetric reagents proved to be an environmental friendly effective alternative for the quantification of iron content in natural waters. As far as we know, 3,4-HPO ligands were used for the first time as chromogenic reagents for iron. The sensitivity obtained enabled fairly low detection and quantification limits, namely 7 and 24 μg/L respectively. In fact, although some previously described methods report much lower detection limits [16–18], they include preconcentration steps and rely on pollutant and toxic reagents, namely DPD. Iron being a nontoxic analyte, it is particularly important to have an environmentally friendly reagent as an alternative to those commonly used for its analysis: thiocyanate ($\varepsilon \approx 2.4 \times 10^4$ L/mol cm), 1,10-phenantroline ($\varepsilon \approx 1.1 \times 10^4$ L/mol cm), bathophenantroline ($\varepsilon \approx 2.2 \times 10^4$ L/mol cm),

Table 5
Recovery percentages calculated from the application of the developed μSI-LOV method to the iron determination in inland bathing waters; G, conductance; TDS, total dissolved solids; TDI, total dissolved iron; and SD, standard deviation.

Sample ID	pH	G (μS/cm)	TDS (mg/L)	Initial TDI (mg/L) SD	Added mg Fe^{+3}/L	Found TDI (mg/L) SD	Recovery % SD
Pi1	8.00	45	29	0.043 ± 0.005	0.25	0.283 ± 0.003	96.0 ± 1.2
					0.75	0.815 ± 0.015	103 ± 2
Pi2	7.00	51	33	0.056 ± 0.005	0.25	0.315 ± 0.008	104 ± 3
					0.75	0.830 ± 0.037	103 ± 5
Pi3	7.17	44	28	0.011 ± 0.003	0.25	0.260 ± 0.006	99.6 ± 2.3
					0.75	0.809 ± 0.028	106 ± 4
Pi4	6.82	39	26	0.060 ± 0.022	0.25	0.304 ± 0.014	97.6 ± 5.6
					0.75	0.794 ± 0.047	97.9 ± 6.2
Pi5	6.89	35	23	0.040 ± 0.004	0.25	0.287 ± 0.018	98.8 ± 7.2
					0.75	0.783 ± 0.022	99.1 ± 2.9
Pi6	6.87	33	21	0.032 ± 0.015	0.25	0.272 ± 0.007	96.0 ± 2.8
					0.75	0.782 ± 0.017	100 ± 2

2,2-bipyridyl ($\varepsilon \approx 8.7 \times 10^3$ L/mol cm), eriochrome cyanine R ($\varepsilon \approx 3.3 \times 10^4$ L/mol cm) and eriochrome cyanine R combined with cetyltrimethylammonium ($\varepsilon \approx 1.27 \times 10^5$ L/mol cm) [2]. With the use of the 3,4-HPO ligands ($\varepsilon \approx 4.7 \times 10^3$ L/mol cm), there is a decrease in sensitivity. Even so, some previously described flow methods using those reagents [19–21] present higher detection limits.

The choice of sequential injection as a flow technique enabled to perform the detailed study of the complexation reaction with a fast and automatic method. The complex formation proved to be almost immediate, no absorbance increase was observed after the initial colour formation, which is an excellent characteristic for a flow analysis application. Furthermore, the downsizing to micro-sequential injection Lab on valve method enabled reducing the consumption values to a minimum with only a minor decrease of the determination rate, about 10 determinations per hour. The developed method was successfully applied to natural waters, namely inland bathing waters, after accuracy validation. Although with the microsequential injection Lab on valve method a quantification limit < 30 μg/L was attained (24 μg/L), adequate for inland waters, it was not enough to cover the reported range expected for sea waters (10–100 μg/L [22]). However, the application to these waters was a realistic possibility due to the low interference observed for chloride values expected in those samples.

The developed work enabled to prove the effectiveness of 3,4-HPO ligands as a selective, nontoxic reagents for iron determination as a "more sustainable" alternative. The latter should be further explored, namely with incorporation of preconcentration steps like the mentioned works [16,18,19,21] aiming for lower detection limits.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.02.058.

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