Results in Chemistry 7 (2024) 101353

Contents lists available at ScienceDirect

Results in Chemistry

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One-step determination of total iron using deferiprone or kojic acid as colorimetric reagents

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ARTICLE INFO

Keywords:
Iron
Colorimetric reagent
Deferiprone
Kojic acid
Spectrophotometry

ABSTRACT

The role of iron, one of the most common metals in the environment, is fundamental in many biological and geochemical processes, which determine its availability in the two main oxidation states Fe^{2+} and Fe^{3+}. Its relevance in the environment, industrial applications, and human physiology, as well as in many other fields has constantly encouraged the development of analytical techniques for its accurate determination. Spectrophotometric methods are those most frequently applied for iron determination in real samples, with specific reagents for the two existing oxidation state right now. In the present work, two low-cost, non-toxic, colorimetric reagents are proposed: deferiprone and kojic acid. These compounds present peculiar features, in particular the formation of 1:3 complexes with Fe^{3+} of extremely high stability and absorptivity in a wide operative pH range. In this study, we show that both reagents can be used to measure the total iron content. Actually, the extremely low redox potential characterizing the FeL_3 complexes permits to determine the total concentration of iron independently from the starting oxidation state, and assures the complete oxidation in presence of oxygen of any amount of Fe^{2+} to Fe^{3+} complexes. These features constitute a novelty in the analytical determination of total iron not requiring any pretreatment of the sample, contrary to the methods in use, devoted either to Fe^{3+} or to Fe^{2+}, necessitating awkward and error generating oxidative or reductive processes. The analytical performance of the proposed spectrophotometric method has been evaluated for the full compliance with the Lambert-Beer law, the operative range of iron concentration, the influence of pH, and the interfering effects of other metal ions. Finally, it has been validated in terms of LoD, LoQ, linearity, precision, and trueness, and has been tested on total iron determination in natural water certified material and in two biological reference materials, human urine and serum.

1. Introduction

Iron is the most abundant element constituting about 80% of Earth mass. As essential metal, it plays an important role in environmental and biological fields: in living systems it can be traced to the many complexes within which it is found such as oxygen-carrying proteins; it takes part in the photosynthesis process, nitrate reduction and detoxification of reactive oxygen species [1,2]. The presence in the natural waters, plasma or serum and its central role in many diseases promoted the demand for simple, fast and convenient methods for its determination [3]. Although many progresses have been done to better understand its role in humans and environment, the rapid determination remains a big challenge that includes speciation studies and the application of several analytical techniques such as electrochemical methods [4], cyclic voltammetry (CV) [5], inductively coupled plasma (ICP-AES and ICP-MS), atomic absorption spectroscopy (AAS) [6], ion chromatography (IC) [7], ultraviolet–visible spectrophotometry (UV–Vis) [8], and, recently, sequential injection analysis (SIA) [9,10]. Analytical chemists demonstrated particular interest toward spectrophotometric methods due to their high sensitivity, cost-effectiveness, simplicity, and low detection limit. Different reagents, specific for a single iron oxidation state, have been used for the determination of iron: some of them are reported in Tables 1 and 2 while an extensive collection is presented in a recent review [11]. Although the high sensitivity reached with these reagents,

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https://doi.org/10.1016/j.rechem.2024.101353
Received 27 October 2023; Accepted 31 January 2024
Available online 6 February 2024

Table 1
Most used molecules for the determination of Fe^{2+} and spectral properties.

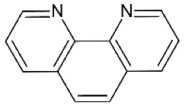
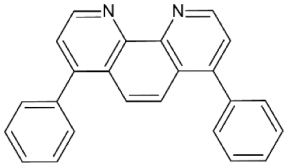
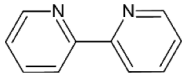
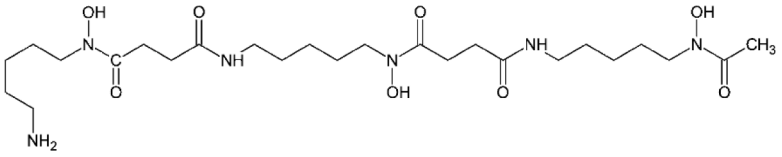
1,10-phenanthroline	(4,7-diphenyl-1,10-phenanthroline, bathophenanthroline)	2,2-bipyridine
		
$\varepsilon = 11000\ M^{-1}cm^{-1}$ $\lambda_{max} = 512$ nm [17]	$\varepsilon = 22400\ M^{-1}cm^{-1}$ $\lambda_{max} = 533$ nm [18]	$\varepsilon = 8700\ M^{-1}cm^{-1}$ $\lambda_{max} = 522$ nm [19]

Table 2
Most used molecules for the determination of Fe^{3+} and spectral properties.

Thiocyanate	Desferal (DFO)
SCN^-	
$\varepsilon = 8530\ M^{-1}cm^{-1}$ $\lambda_{max} = 480$ nm [20]	$\varepsilon = 2764.8\ M^{-1}cm^{-1}$ $\lambda_{max} = 432$ nm [21]

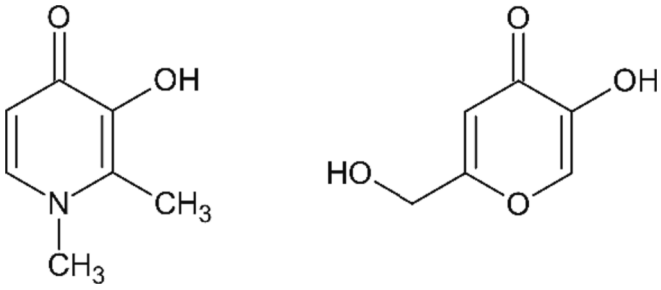


Fig. 1. The proposed colorimetric reagents: deferiprone (DFP) and kojic acid (KA).

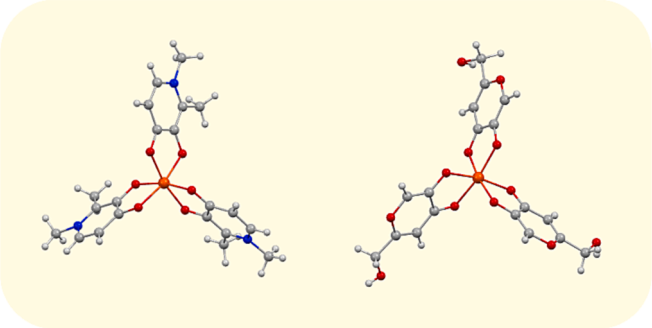


Fig. 2. Structures of Fe^{3+} complexes of DFP and KA: $Fe(DFP)_3$ (left) [22] and $Fe(KA)_3$ (right) [23]. (H white; O red; N blue; C grey; Fe orange). Coordinates obtained from Cambridge Structural Database, images rendered with Mercury 3.5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the methods for total iron determination require awkward and error generating oxidative or reductive processes. The principle of Occam's razor, which states that the simples model as to be preferred in the explanation of scientific results can be extended to the choice of analytical method preferring the simplest procedure. Actually, any unnecessary step introduces both an extension of procedure and use of new reagents that affect the cost of analysis, and above all each step contributes to increasing the final error. Furthermore, some of the employed reagents present toxicity with an undesirable impact on human health. Deferiprone (DFP), the first oral iron chelator in clinical use since 2000, and kojic acid and kojic acid (KA)*, largely used in cosmetics and in food industry, (Fig. 1) are white crystalline non-toxic compounds belonging to the family of hydroxypyridinones (HPs) and hydroxypyrones (HPOs), currently employed in medicine (e. g. treatment of β-thalassemia patients) and in food and cosmetic industry, respectively [12–14]. They are both very strong chelators towards *hard* metal ions [15,13] forming, in a wide range of pH, highly stable and intensely coloured complexes through carbonyl and hydroxy groups, being Fe^{3+} fully coordinated in 1:3 metal:ligand molar ratio complex (Fig. 2). The first attempt of colorimetric determination goes back to the study of fermentation producing kojic acid by means of the reaction with ferric chloride [16]. Indeed, it seemed likely that this process might be reversed by using the organic compound as a reagent for Fe^{3+}. However, in routine determinations, an easy, rapid and low-cost method is very auspicious. Therefore, we propose in this paper the use of DFP and KA as colorimetric complexing agents for the determination of iron, at once in whatever oxidation states, in real samples by an efficient spectrophotometric method, which is selective, sensitive and has a low detection limit.

2. Material and methods

2.1. Chemicals

DFP and KA were purchased from Sigma Aldrich and the purity was

Table 3
General properties of DFP and KA ligands.

	DFP	KA
Molecular formula	$C_7H_9NO_2$	$C_6H_6O_4$
Molecular weight (g/mol)	139.15	142.11
IUPAC name	3-hydroxy-1,2-dimethylpyridin-4-one	5-hydroxy-2-(hydroxymethyl)pyran-4-one
Water solubility (g/L)	16–18 [25]	43.85 [26]
log P_{ow}	−0.77 [25]	−0.64 [27]
Protonation constants at 25 °C	log K_1 9.82; log K_2 3.66 [22]	log K_1 7.70; log K_2 −1.86 [23]

checked by joined potentiometric-spectrophotometric titration. $FeCl_3$, $Fe(ClO_4)_2{\cdot}xH_2O$, $Ga_2(SO_4)_3$, $CuCl_2$, iron standard for ICP (1000 mg/L in Fe^{3+}, from $Fe(NO_3)_3$ in HNO_3 0.3 M, d = 1.015 g/mL) were Sigma Aldrich products used without any purification. The metal ion standard solutions were prepared by dissolving the required amount of salts in pure double distilled water and adding a stoichiometric amount of HCl to prevent hydrolysis. Fe^{3+} solution was standardized by desferal (DFO) [21]; Fe^{2+} solution, prepared under argon, was standardized by Ce^{4+} titration and Cu^{2+} solution by EDTA titration. The used certified materials were: NIST SRM 1643f Natural Water certified reference material, The ClinChek® Control Human Urine - Level II, code 8848, and the ClinChek® Control Blood Serum - Level IA, code 8880 were from Recipe (Munich, Germany).

2.2. Equipment

Potentiometric titrations were performed with a d Electrode plus Metrohm combined glass electrode connected to 888 Titrando Metrohm (Herisau, Switzerland), whereas the spectrophotometric measurements were accomplished using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) with 1 cm path length cell. The electrode was calibrated daily for hydrogen ion concentration via HCl standard titration with NaOH in the used experimental conditions, and data analyzed using Gran's method [24].

3. Results and discussion

3.1. General features of a colorimetric reagent

Before discussing the general features of a colorimetric reagent, let us make some essential considerations. The determination of an analyte by UV–Vis spectrophotometry is based on the Lambert-Beer law, in the form A = ε b C, where A is the measured absorbance, ε the absorptivity, C the concentration of analyte and b the used optical path length. A calibration plot A *vs.* C allows the determination of the absorptivity ε, and furthermore highlights any experimental deviation from the linear

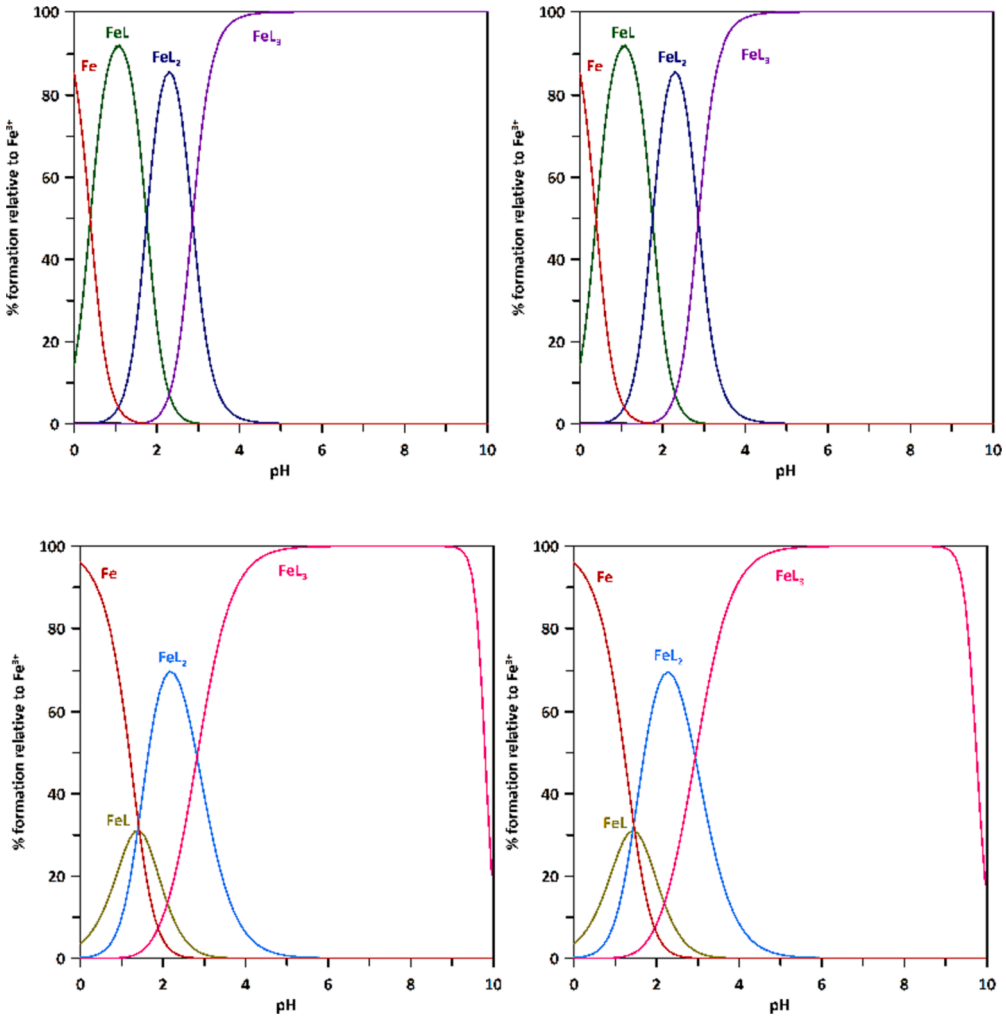


Fig. 3. Speciation plots calculated by HySS program [28] of top) DFP 5 × 10^{-3} M and Fe^{3+} 5 × 10^{-5} M (left) and 5 × 10^{-4} M (right); bottom) KA 6 × 10^{-3} M and Fe^{3+} 6 × 10^{-5} M (left) and 6 × 10^{-4} M (right).

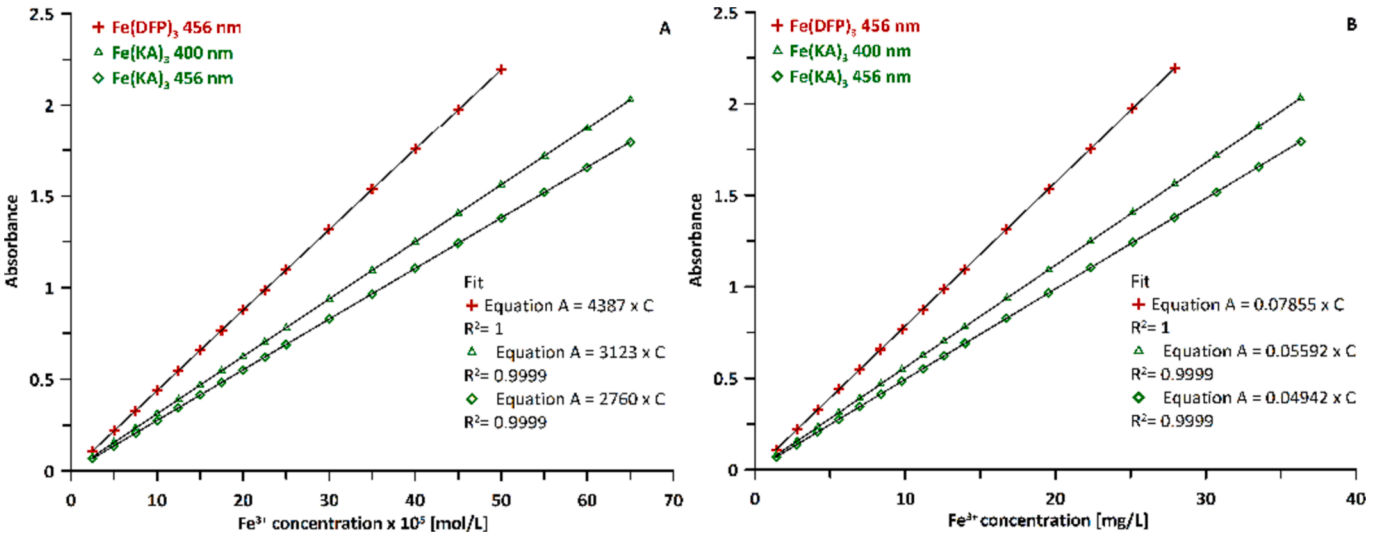


Fig. 4. A) Calibration plots of the absorbance of 15 solutions with [DFP] = 5×10^{-3} M and [Fe^{3+}] ranging from 2.5×10^{-5} M to 5.0×10^{-4} M at 456 nm; of 18 solutions with [KA] = 6.5×10^{-3} M and [Fe^{3+}] ranging from 2.5×10^{-5} M to 6.5×10^{-4} M at 400 nm and at 456 nm; B) The corresponding calibration plots with [Fe^{3+}] expressed in mg/L.

Lambert-Beer law. A good spectrophotometer allows the precise and accurate measurements of absorbance values in the range 0.1 – 2. Therefore, the ε and b values determine the optimal operative range of concentration for measurements of absorbance values in the 0.1 – 2 interval. When a transition metal ion is the target analyte, the visible spectrum of its aquo ion, characterized by ε values ~ 100 M^{-1} cm^{-1}, allows to evaluate concentrations ~ 10^{-2} M using a 1 cm path length. In the case of iron, such molar concentration corresponds to 558 mg/L, surely too high for being of analytical relevance. Therefore, the transformation of the iron aquo ion in highly absorbing iron complexes using a proper ligand is mandatory. A colorimetric reagent to be of analytical interest should demonstrate:

1. formation of a complex of definite stoichiometry;
2. high stability of the formed complex;
3. stability in a wide pH range;
4. high values of the absorptivity (ε);
5. fast reaction of complex formation;
6. selectivity toward the target metal ion.

3.2. Properties of DFP and KA ligands

The general properties of DFP and KA ligands, including the two protonation constants of carbonyl and hydroxyl groups, are shown in Table 3. Concerning the complexation of Fe^{3+}, the bidentate ligands enter one by one until filling the metal ion coordination sphere in a 1:3 metal ligand molar ratio complex, $Fe(DFP)_3$ and $Fe(KA)_3$, characterized by a visible spectrum with λ_{max} at 456 nm and 400 nm and ε 4387 M^{-1} cm^{-1} and 3123 M^{-1} cm^{-1}, respectively.

As can be observed from speciation plots (Fig. 3), calculated using the protonation and complex formation constants from refs [22,23], Fe^{3+} starts to be complexed at strongly acidic pH. Actually, it exists almost completely in $Fe(DFP)_3$ form (99.2%) from pH 4.2 to pH > 10 without any formation of hydroxo species. In the case of KA the pH range is more limited because although total iron is completely in $Fe(KA)_3$ form (99.3%) from pH 5.2, the formation of hydroxo species occurs from pH 9.2.

3.3. Calibration plots

Based on the above absorptivity values, the operative concentration range of the two spectrophotometric reagents has been determined as

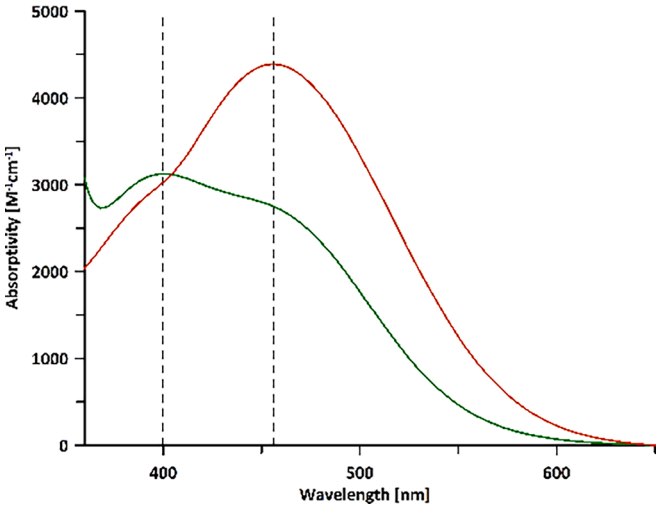


Fig. 5. Absorptivity spectra of the $Fe(DFP)_3$ (red line) and $Fe(KA)_3$ (green line) complexes, calculated as the mean value of the 15 and 18 absorbance spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the concentrations that allow to measure absorbance values in the range 0.2 – 2.0 using a 1 cm cell. This roughly corresponds to iron concentrations in the range 5×10^{-5} M – 5×10^{-4} M for DFP and 6×10^{-5} M – 6×10^{-4} M for KA and the ligand in a tenfold excess with respect to the highest used iron concentrations (DFP 5×10^{-3} M and KA 6×10^{-3} M). Therefore, to evaluate the spectral performance of the systems Fe^{3+}-DFP and Fe^{3+}-KA, we prepared 15 and 18 solutions respectively with iron concentration in the range 2.5×10^{-5} M – 5.0×10^{-4} M for DFP complexes whose absorbance at 456 nm varied from 0.109 to 2.193, and in the range 2.5×10^{-5} M – 6.5×10^{-4} M for KA complexes, whose absorbance at 400 nm varied from 0.078 to 2.030. To comply with the accuracy, we started from a commercial ICP standard $Fe(NO_3)_3$ solution, with declared Fe^{3+} concentration of 1000 mg/L (1.7907 M), with an excess of HNO_3 0.3 M (d = 1.015 g/mL) to prevent the formation of iron hydroxides. Each solution was prepared in a 25 mL volumetric flask by picking up increasing amounts of Fe^{3+} stock solution determined by weighting with a four-digit lab scale. Then, 5 mL of DFP solution 0.025 M were added to the flask, so that in the more concentrated solution the

Table 4
Literature complex formation constants of different metal ions with DFP and KA ligands, in aqueous solution at 25 °C and 0.1 M ionic strength. The charges of the formed complexes are omitted for simplicity. The pM is conventionally defined as − log [$Metal_{free}$], calculated at pH 7.4 for total [Metal] = 10^{-6} and [Ligand] = 10^{-5} M [29]. The pM values are also shown for comparison of the binding strength of the reported metal ions. (*) data at 21 °C.

DFP					
Metal ion	**ML**	**ML_2**	**ML_3**	**pM**	**References**
Fe^{3+}	15.01	27.3	37.43	20.70	[22]
Al^{3+}	12.20	23.25	32.62	15.90	[30]
Ga^{3+}	13.17	25.43	35.76	19.03	[30]
In^{3+}	11.85	22.48	31.71	15.98	[31]
Cu^{2+}	10.42	21.98 CuL_2H 19.09 CuL_2 8.49 CuL_2H_{-1}		10.09	[22]
Zn^{2+} Ni^{2+}	7.19	13.53		6.21	[30]
KA					
Metal ion	**ML**	**ML_2**	**ML_3**	**pM**	**References**
Fe^{3+}	8.5	17.04	24.15	13.28	[23]
Al^{3+}	7.7	14.2	19.5	9.24	[32]*
Ga^{3+}			No data		
In^{3+}			No data		
Cu^{2+}	6.6	11.8		7.26	[32]
Zn^{2+}	4.9	9.1		6.11	
Ni^{2+}	4.9	8.7		6.10	[32]

Table 5
Effects of increasing concentrations of Ga^{3+}, Al^{3+} and Cu^{2+} cations on the formation of the absorbing $Fe(DFP)_3$ complex at pH 6 and 7, being always the Fe^{3+} total concentration 5 × 10^{-4} M and that of DFP 5 × 10^{-3} M. (*) concentration of interfering metal ion used for speciation plot.

	pH 6		pH 7	
10^4 M concentration of interfering Ga^{3+} ion	10^4 M concentration of $Fe(DFP)_3$	$Ga(DFP)_3$	10^4 M concentration of $Fe(DFP)_3$	$Ga(DFP)_3$
0	4.9993	0	4.9999	0
5	4.9988	4.9992	4.9999	4.9999
10	4.9951	9.9938	4.9995	9.9999
15*	2.0954	7.9991	2.0962	8.0014
10^4 M concentration of interfering Al^{3+} ion	$Fe(DFP)_3$	$Al(DFP)_3$	$Fe(DFP)_3$	$Al(DFP)_3$
0	4.9993	0	4.9999	0
5	4.9988	4.9929	4.9999	4.9993
10	4.9951	9.9443	4.9995	9.9944
15*	4.0313	6.1975	4.0343	6.2117
10^4 M concentration of interfering Cu^{2+} ion	$Fe(DFP)_3$	$Cu(DFP)_2$	$Fe(DFP)_3$	$Cu(DFP)_2$
0	4.9993	0	4.9999	0
5	4.9990	4.9678	4.9999	4.9955
10	4.9984	9.8989	4.9998	9.9873
15*	4.9954	14.6036	4.9995	14.9531
20	4.9386	14.6875	4.9438	15.0368

DFP concentration was tenfold that of iron. Moreover, a proper volume of NaOH 0.1 M was necessary to neutralize the H^+ coming from iron standard solution and from DFP, released during complexation. The final volume was checked by weighting. The measured pH of all solutions was between 6.8 and 7.1, and the spectra were recorded in the 360 – 600 nm spectral range. A similar procedure was used for the preparation of the 18 solutions with KA. The calibration curves with both colorimetric reagents in Fig. 4 show a perfect agreement with the Lambert-Beer law in the used concentrations. The absorptivity spectra of the FeL_3 complexes are shown in Fig. 5, calculated as the mean value of the absorptivity spectra of the 15, or 18 solutions respectively. The spectrum of the iron complex with DFP present a maximum at 456 nm with absorptivity 4387(2) M^{-1} cm^{-1} while that of the iron complex with KA present a maximum at 400 nm with absorptivity 3123(1) M^{-1} cm^{-1} and a shoulder at 456 nm with absorptivity 2760(2) M^{-1} cm^{-1}.

Table 6
Effects of increasing concentrations of Al^{3+} and Cu^{2+} cations on the formation of the absorbing $Fe(KA)_3$ complex at pH 6 and 7, being always the Fe^{3+} total concentration 5 × 10^{-4} M and that of KA 5 × 10^{-3} M. (*) concentration of interfering metal ion used for speciation plot.

	pH 6		pH 7	
10^4 M concentration of interfering Al^{3+} ion	10^4 M concentration of $Fe(KA)_3$	$Al(KA)_3$	10^4 M concentration of $Fe(KA)_3$	$Al(KA)_3$
0	4.9934	0	4.9993	0
5	4.9904	4.4424	4.9988	4.9260
10*	4.9741	7.4470	4.9958	9.4811
15*	4.8593	4.8544	4.8875	5.7142
10^4 M concentration of interfering Cu^{2+} ion	$Fe(KA)_3$	$Cu(KA)_2$	$Fe(KA)_3$	$Cu(KA)_2$
0	4.9934	0	4.9993	0
5	4.9922	4.4375	4.9991	4.9254
10	4.9881	8.3673	4.9985	4.7570
15	4.9781	11.0201	4.9966	14.0935
20*	4.9529	11.1354	4.9753	14.1783

3.4. Effects of interfering metal ions

Different metal ions in solution can interfere in iron determination in two ways:

1. by direct competition with Fe^{3+} for DFP (or KA) complexation, depending both on the ratio between the stability constants of the interfering metal ion and Fe^{3+}, and on the ratio of their concentrations;

2. By the formation of an absorbing complex that alters the absorption spectrum of Fe^{3+}-DFP (or KA) complex, being the colorimetric reagent in large excess.

As far as point 1, is concerned, it is useful to examine the data in Table 4 that contains the stability constants of DFP and KA complexes with the trivalent metal ions Al^{3+}, In^{3+} and Ga^{3+}, and the bivalent ones Cu^{2+}, Ni^{2+} and Zn^{2+}. The pM values for the considered metal ions are also reported, for a direct comparison of the stability of their complexes.

The complex formation constants in Table 4 show values for the trivalent competing metal ions lower than those for Fe^{3+}. Among the bivalent metal ions only Cu^{2+} presents remarkable stability constants, however, always lower than those of the corresponding trivalent metal ions. We considered the systems formed by a constant excess of ligand (5 × 10^{-3} M), a constant concentration of Fe^{3+} (5 × 10^{-4} M) and increasing concentrations of competing metal ions, for quantitatively evaluating in which extent a competing metal ion can prevent the Fe^{3+} complexation. The amount of iron complexed as FeL_3 and of complexed competing metal ion were calculated at pH 6 and 7 (Fig. 3). The results for Ga^{3+}, Al^{3+} and Cu^{2+} are reported in Tables 5 and 6 for DFP and KA respectively and the corresponding speciation plots in Figs. 6 and 7.

From the above results, interferences of Ga^{3+} and Al^{3+} in $Fe(DFP)_3$ formation appears when their concentration become three times that of iron, while no interferences of Cu^{2+} occur. In the case of $Fe(KA)_3$ only a negligible interference when Al^{3+} three times in excess is observed.

We chosed Ni^{2+} and Cu^{2+} ions to evaluate the interference depicted in point 2, since they form colored complexes with both DFP and KA. The absorbance of the four systems Ni^{2+}-DFP, Cu^{2+}-DFP, Ni^{2+}-KA and Cu^{2+}-KA at pH 7, measured at 5 × 10^{-4} M metal ion concentration and 5 × 10^{-3} M ligand concentration, were 0.0085 and 0.0226 for Ni^{2+} and Cu^{2+} of DFP complexes at 456 nm, and 0.0113 and 0.0221 for Ni^{2+} and Cu^{2+} of KA complexes at 400 nm, respectively. Therefore, the interferences of both bivalent metal ions on the analyte are 0.4% and 1.0 % for Ni^{2+} and Cu^{2+} in the case of DFP and 0.7% and 1.4% in the case of KA.

3.5. Determination of total iron

The possibility of Fe^{2+} oxidation in the presence of atmospheric oxygen with DFP and KA were checked. The samples were made up of iron complexes, containing only Fe^{2+} or Fe^{3+} (5 × 10^{-4} M) or their

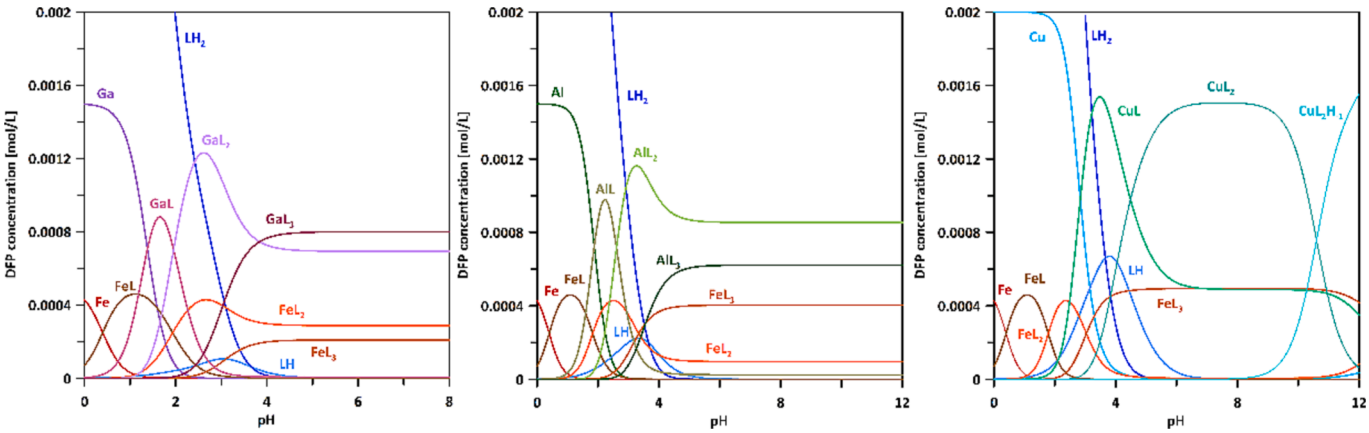


Fig. 6. Speciation plots, calculated by HySS program [28], of iron complexed as $Fe(DFP)_3$ and of complexed competing metal ions Ga^{3+}, Al^{3+} and Cu^{2+}.

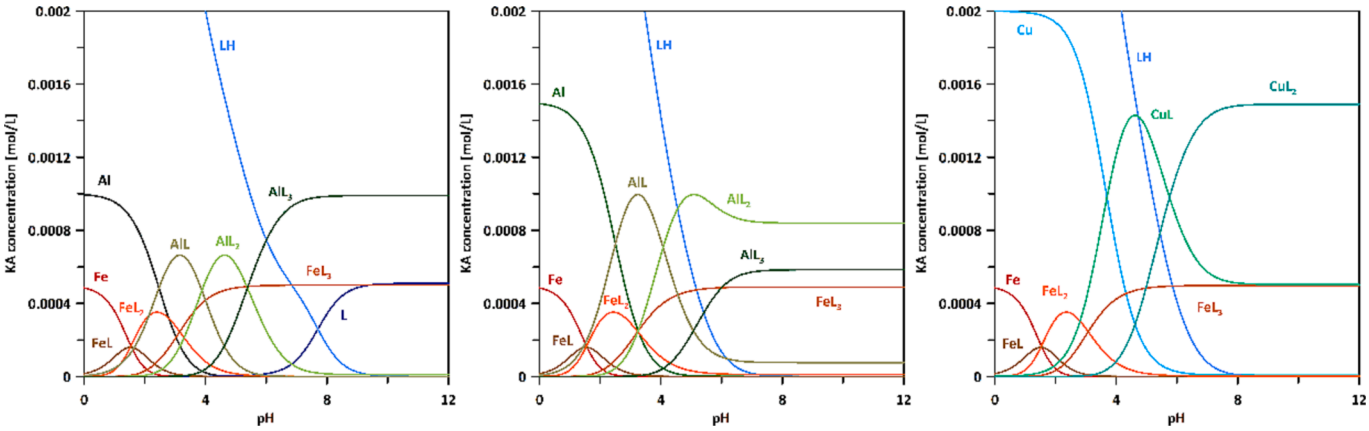


Fig. 7. Speciation plots, calculated by HySS program [28], of iron complexed as $Fe(KA)_3$ and of complexed competing metal ions Al^{3+} and Cu^{2+}.

equimolar mixture (2.5×10^{-4} M) and DFP (5.0×10^{-3} M) or KA (6.5×10^{-3} M). Then the UV–Vis absorption spectra were recorded, of which that at pH 7 equal to what shown in Fig. 5. No significant differences were observed on further spectra collected at pH values ranging between 5 and 8. Therefore, this confirms the suitability of these reagents for the simple and direct analytical determination of total iron, contrary to the methods in use devoted either to Fe^{3+} or to Fe^{2+}, such as thiocynate or phenanthroline ones, which require awkward and error generating oxidative or reductive processes for the determination of total iron.

3.6. Validation: LoD and LoQ

Currie [33] proposed a largely used method for the evaluation of the detection and quantification capabilities LoD and LoQ of analytical procedure in the form.

$$\text{LoD} = (3 \times \sigma_b)/b$$

$$\text{LoQ} = 3.33\ \text{LoD}$$

where b is the slope of calibration curves measured in a range of concentration of the analyte as close as possible to a tentative value of LoD, and σ_b is the standard deviation obtained on a large number of blank measurements. To evaluate the LoD values with DFP at 456 nm, and with KA at 400 nm, first the σ_b values were evaluated as the standard deviations of 25 independent absorbance measurements of the related blank solutions at the proper wavelength. For DFP the blank solution contained DFP 5×10^{-3} M, as in the standard solutions for the calibration plot, and the measurements were performed at 456 nm. The standard deviation of the 25 measurements was 0.0003. For KA the blank solution contained KA 6.5×10^{-3} M and the measurements were performed at 400 nm. The standard deviation of the 25 measurements was 0.0002. As a second step, the b values for the DFP complex at 456 nm and for the KA complex at 456 nm and at 400 nm were evaluated on seven solutions at iron concentrations increasing from 0.1 mg/L to 0.7 mg/L, as conditions described for the calibration plot with the same excess of ligand and neutralized at pH 7. The calculated b values were 0.07874 mg^{-1} L for DFP and 0.05609 mg^{-1} L for KA, somewhat higher than those calculated from the calibration plots presented in Fig. 4b. Based on the overall results the calculated LoD value is 0.01 mg/L for both DFP and KA ligands, being the higher absorptivity of DFP compensated by the lower standard deviation of blank measurements for KA. The obtained LoQ is calculated as 0.033 mg/L for both the colorimetric reagents.

3.7. Precision

An evaluation of the precision of the recommended methods was done both as repeatability and intermediate precision. For repeatability 25 consecutive measurements of absorbance were performed at iron concentrations 2.5×10^{-4} M = 13.96 mg/L (i.e. approximately 1000 times higher than the LoD), a) at 456 nm for $Fe(DFP)_3$ complex, and b) at 400 nm for $Fe(KA)_3$ complex. The intermediate precision was evaluated by repeating ten times the measurement of absorbance on the maximum of $Fe(DFP)_3$ complex (or at 456 nm for the $Fe(KA)_3$ complex) at the above experimental conditions once a week for five consecutive weeks. The repeatability for DFP was always > 0.7% (0.9% for KA), whereas the intermediate precision was 2.1% for DFP and for KA. The acceptability of the precision data was checked according to the Horwitz theory [34].

Table 7
Spectrophotometric determination of iron content in different Certified Reference Materials (CRM) with the two proposed colorimetric reagents (the reported iron concentrations are the mean of five independent measurements).

CRM	Certified iron concentration (mg/L)	DFP colorimetric reagent		KA colorimetric reagent	
		Measured iron concentration (mg/L)	Recovery %	Measured iron concentration (mg/L)	Recovery %
NIST SRM 1643f Natural Water	93.4(8)	92(1)	98	96(3)	103
ClinChek® Control Human Urine Level II	222	229(2)	103	234(3)	105
ClinChek® Control Blood Serum Level I	905	929(2)	103	937(4)	103

3.8. Use of reference materials and trueness

Three certified reference materials (CRM) of abiotic and biotic origin, containing iron at concentrations between 93.44 μg/L and 905 μg/L were used used for evaluating the trueness of the methods. Table 7 reports for each CRM, the certified concentration of iron, the measured concentration with DFP and KA (at 400 nm) and the relevant percent recoveries.

Satisfactory recoveries were found for all the three considered CRM's and for both colorimetric reagents. For the proposed methods, a large applicability in natural water analysis can be predicted, for analyte concentrations high enough to provide direct, fast and reliable measurements. For example, the WHO guidelines [35] for drinking water report "Anaerobic groundwater may contain ferrous iron at concentrations up to several milligrams per litre without discoloration or turbidity in the water when directly pumped from a well. On exposure to the atmosphere, however, the ferrous iron oxidizes to ferric iron, giving an objectionable reddish-brown colour to the water". Its presence may lead, besides aesthetic and taste problems, to accumulation of deposits in the distribution systems. All these problems may be prevented by proper treatments, which require adequate methods, as those proposed, to reliably determine the concentration iron independently from its oxidation state. These methods have also been successfully applied to iron determination in biological fluids.

4. Conclusions

Two colorimetric reagents easily available are proposed, which allow the determination of total iron concentration in different matrices without troublesome procedures. Their complexing ability allows the formation of FeL_3 complexes of high stability in a wide pH range. Further, the main advantage is the use of DFP or KA for the determination of total iron: due to the extremely low redox potential of these complexes, the complete oxidation of Fe^{2+} takes place in presence of air. The absorptivity values for the iron complexes with both the presented ligands are lower than those of the complexes formed with thiocyanate and phenantroline, allowing the determination on samples of higher iron concentration than those of other methods without dilution. The possible interference by the most common trivalent and bivalent metal ions has been investigated, and no significant effects were found in the operative pH range. The LoD of the method is 0.01 mg/L with both colorimetric reagents, being the higher absorptivity of DFP compensated by the lower standard deviation of blank measurements for KA. The calibration plot with both reagents exhibits a full compliance with the Lambert-Beer law. Precision and trueness have been checked on three different certified materials always obtaining satisfactory recoveries.

In conclusion, the anaytical performance of this spectrophotometric method encourages the suitability of DFP and KA for the reliable determination of total iron in environmental and biological samples.

CRediT authorship contribution statement

Rosita Cappai: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Alessandra Fantasia:** Formal analysis, Investigation, Writing – original draft. **Andrea Melchior:** Formal analysis, Validation. **Guido Crisponi:** Data curation, Validation, Writing – review & editing. **Valeria M. Nurchi:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Valeria Marina Nurchi reports financial support was provided by University of Cagliari.

Acknowledgements

This work was supported by Regione Autonoma Sardegna [RASSR79857], Fondazione di Sardegna [CUP F72F20000240007], NECTAR, by COST (European Cooperation in Science and Technology) [CA18202].

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