



ORIGINAL ARTICLE

Simple solid-phase spectrophotometric method for free iron(III) determination



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Abstract A simple and rapid solid-phase spectrophotometric procedure to determine free Fe(III) in environmental and biological samples is proposed. In particular, a deferoxamine (DFO) self assembled monolayer on mesoporous silica (DFO SAMMS) is developed and here applied as a sensor for iron(III). The solid product became brownish when put in contact with iron(III) solutions; so an immediate application as colorimetric sensor is considered. In order to optimize the DFO SAMMS synthesis and to obtain the best product for iron(III) sensing, a factorial experimental design is performed selecting the maximum absorption at 425 nm as response. The robustness of the spectrophotometric method is also proved.

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1. Introduction

Iron is essential for the proper functioning of all living cells however it is toxic when present in excess: iron overload is the most common metal toxicity condition worldwide (Neilands, 1994).

The presence of Fe(III) in biological systems has to be efficiently moderated as both its deficiency and overloading can induce different biological disorders (Beutler et al., 2001; Cairo and Pietrangelo, 2000). Various human metal

intoxications and overloaded metal-induced pathologies have been treated efficiently by the administration of chelating agents. However, a number of variables and problems have to be examined in the choice of the opportune chelating therapy. The pFe is the semi-empirical quantity, widely used to measure the complexing ability of a chelating drug towards iron(III). It is defined as the negative logarithm of the free iron concentration in solution, as calculated for 10 μM total ligand and 1 μM total metal ion, at pH 7.4 (Abergel and Raymond, 2008). Nowadays, iron chelation therapy is largely based on experimental clinical evidence or on *in silico* studies (performed via computer simulation). This is the reason why we do believe there is room to develop sensors able to directly assess the free iron(III) in biological samples.

Deferoxamine (DFO) is an hydroxamate siderophore which forms 1:1 = Fe:DFO octahedral complex involving the six oxygen atoms of the hydroxamate groups (Domagal-Goldman

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et al., 2009). The terminal amino group is not implicated in iron complexation, therefore it is suitable to anchor DFO onto a solid phase. On the basis of this strategy, we had already prepared two different solid products: the DFO Self-Assembled Monolayers on Mesoporous Silica (DFO SAMMS) and the DFO-immobilized paper (Alberti et al., 2014b; Biesuz et al., 2014). It was noted that these solid phases became brownish after sorption of Fe(III), and the colour has a maximum absorption at 425 nm, similar to the DFO/Fe(III) complex in aqueous solution, with an extinction coefficient of about 2500–2800 M⁻¹ cm⁻¹ (Monzyk and Crumbliss, 1982; Murphy et al., 2003; Duckworth and Sposito, 2005).

In the pursuit of the development of new metal-selective solid phases various research efforts have been exerted to develop alternative materials with high sorption capacity, high preconcentration factor, and low limit of detection (LOD) (Duran et al., 2009; Tuzen et al., 2008). In most of these researches the main focus is the development of an optimal method of solid phase extraction.

The performances of some natural and synthetic sorbents for iron(III) are summarized in Table 1.

Conversely, in the present paper, we have shifted the attention on the possibility of using the solid phase (the DFO SAMMS) as a colorimetric sensor for free Fe(III) determination. The procedure consists in the sorption of Fe(III) on the solid phase and in the direct absorbance measurement of the product, as already done for other analytes by using different materials (Vukovic et al., 2007). Moreover, thanks to the accurate characterization of the sorption equilibria of Fe(III) on DFO SAMMS, from the measured absorbance the free iron concentration and so the pFe can be assessed (see Table 2).

In order to prove the goodness of the proposed method, and to obtain the best product for iron(III) sensing, the synthesis optimization of DFO SAMMS by a factorial experimental design, using the colour of the solid phase as response, is performed.

2. Materials and methods

2.1. Chemicals and apparatus

Mesoporous Silica (MS) MCM-41 type (Sigma–Aldrich, USA), (3-Glycidioxypropyl) trimethoxysilane (GPTMS,

≥98% Sigma–Aldrich, USA), deferoxamine mesylate salt (DFO, Novartis, Italy), acetonitrile (≥99.5% Carlo Erba, Italy), dimethyl sulfoxide (DMSO, ≥99.9%, Sigma–Aldrich, USA), KNO₃ (≥99.0%, Sigma–Aldrich, USA), HNO₃ for trace analysis (≥69.0%, Fluka, USA) and NaOH (Carlo Erba, Italy) were used as received. Iron standard solution for ICP of 1000 mg/L (Fluka, USA) was diluted with ultrapure water (Milli-Q, Millipore, Germany) to obtain the proper Fe(III) concentration in the solution phases.

An Orion 420 pH-metre (Thermo Fisher Scientific Inc., USA), with a combined glass electrode, was used to measure the pH of all solutions. The absorbance spectra were obtained by a Varian Cary 100 UV–vis spectrophotometer (Agilent, USA) using a 1 mm pathlength quartz cuvette. The aqueous solutions were analysed for iron(III) content by an ICP-OES Perkin Elmer Optima 3300 DV (Perkin Elmer, USA). The LOD (3× standard deviation of the blank) and the LOQ (10× standard deviation of the blank) are respectively 0.15 μM and 0.50 μM for Fe(III) at 238.204 nm.

2.2. Synthesis

DFO mesoporous silica (MS) self assembled monolayer (DFO SAMMS) was prepared according to a previously described *one-pot* pathway (Biesuz et al., 2014), as schematically shown in Fig. 1.

Because one of the aims of this paper is the synthesis optimization by a 2³ factorial experimental design, we prepared the solid phase in 8 different conditions as summarized in Table 1.

2.3. UV–vis absorbance measurement

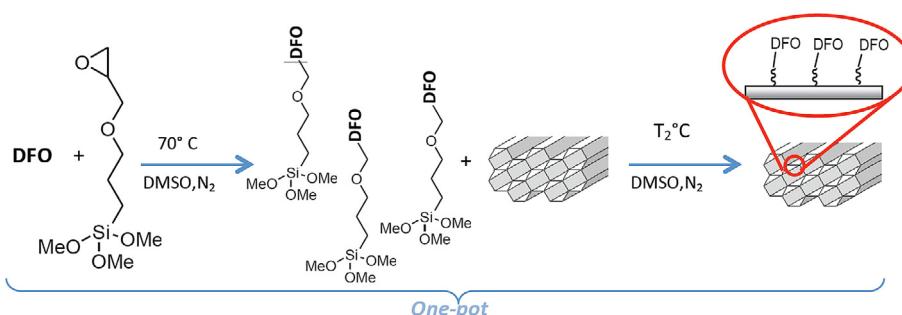
As already described (Vukovic et al., 2007), it is possible to develop a simple solid-phase spectrophotometric method, filling a cuvette with the solid material and registering the absorbance against a proper blank. The experimental procedure is here described. 0.1 M KNO₃ solutions at pH 2.5 and different concentrations of Fe(III), were put in contact with the same amount of DFO SAMMS (50 mg). The mixtures were gently stirred overnight, at room temperature, on an orbital shaker. After equilibration, the Fe(III)/DFO SAMMS was transferred into a 1 mm quartz cuvette until about half of the cuvette was

Table 1 Comparison of maximum sorption capacity (q_{\max}) and limit of detection (LOD) of some sorbents used for the separation and preconcentration of Fe(III).

Sorbent	pH	q_{\max} (mmol g ⁻¹)	LOD (nM)	References
Functionalized halloysite nanotubes with 2-hydroxybenzoic acid		0.81	3.8	Li et al. (2013)
Calcareous soil-alginate compost		0.27		El-Sherbinya et al. (2013)
Functionalized silica with [3-(2,2'-dipyridylamine)propyl] groups	5.0	1.39		Soares et al. (2013)
Ionic liquid modified silica gel	3.0	0.67	8.6	Zhang et al. (2012)
Ion imprinted cyanato-functionalized silica gel	3.0	0.64		Fan and Sun (2012)
Imprinted sorbent with chelating diamines	4.0	0.67		Fazhi et al. (2012)
Natural zeolite	7.0	0.02		Shavandi et al. (2012)
Modified oligosilsesquioxane sorbent	6.0	2.87	4.6	Vieira et al. (2012)
Modified chitosan beads	4.0	0.07		Gandhi et al. (2012)
Natural zeolite	1.9	0.13		Al-Anbera and Al-Anber (2008)
Fe(III)-imprinted amino-functionalized silica gel		0.45	6.1	Chang et al. (2007)
Lignite	2.7	0.21		Mohan and Chander (2006)
Silica – immobilized hydroxypyridinone	3.0	0.18		Esteves et al. (2005)
	7.0	0.25		
2-Mercaptobenzimidazole-silica gel	2.0	0.009	2.7	Bagheri et al. (2000)

Table 2 Experimental plan with the relative responses. Numbers between brackets are the standard error of the slope. Last column: the maximum sorption capacity (q_{\max}) from the sorption isotherms (Biesuz et al., 2014).

2 ³ Experimental design				Experimental plan			Response	q_{\max} (mmol g ⁻¹)
No. Exp.	T_2 (°C)	Type of DFO	Type of silica	T_2 (°C)	Type of DFO	Type of silica	Slope (A M ⁻¹)	
1	-1	-1	1	90	Mesylate	MSU-H	10,438(41)	0.22
2	1	-1	-1	120	Mesylate	MCM-41	7880(33)	0.16
3	-1	1	-1	90	Neutral	MCM-41	23,337(699)	0.33
4	1	1	1	120	Neutral	MSU-H	3069(10)	0.13
5	-1	1	1	90	Neutral	MSU-H	6324(1127)	0.12
6	-1	-1	-1	90	Mesylate	MCM-41	21,502(12)	0.33
7	1	-1	1	120	Mesylate	MSU-H	3308(11)	0.14
8	1	1	-1	120	Neutral	MCM-41	15,676(1)	0.25

**Figure 1** Synthesis of the DFO SAMMS following the novel *one-pot* pathway method (Biesuz et al., 2014).

filled; the solid phase was packed into the cuvette after some minutes. The spectrum was always registered against a blank prepared with the same quantity of DFO SAMMS equilibrated in 0.1 M KNO₃ solution at pH 2.5, without Fe(III). Absorbances at 425 nm and at 700 nm were employed to build up the calibration curves as described below.

3. Results and discussion

The characterization of the Fe(III) sorption on DFO SAMMS and the possibility to apply the functionalized material as sorbent in Solid Phase Extraction (SPE) processes were already discussed (Biesuz et al., 2014; Alberti et al., 2014b).

The aim of the present study is to develop a simple and rapid colorimetric method to dose total and free Fe(III) in environmental and biological samples, using a solid material with high sensitivity. To achieve this goal, a simple factorial experimental design is employed to find out the optimal experimental conditions for the solid phase synthesis (Brereton, 2003; Leardi, 2009).

In a previous study (Biesuz et al., 2014), a similar approach was used and in that case the maximum sorption capacity q_{\max} (mmol g⁻¹) was selected as the response for a factorial experimental design. It was highlighted that, to achieve the q_{\max} value for each experiment, a complete sorption isotherm is needed and the measurements are quite time-consuming. However, it was observed that DFO SAMMS samples, loaded with increasing iron(III) concentrations, became differently coloured. Therefore in the present study, in order to optimize the synthesis, we have decided to use the colour, instead of the q_{\max} , as response for the factorial experimental design;

indeed it seems reasonable to correlate the colour intensity with the maximum sorption capacity of the solid phase.

We decide to perform experiments according to a 2³ factorial design, similar to that presented in the previous work (Biesuz et al., 2014). The model is described by the following equation:

$$R = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

The variables (the same used in the previous optimization) (Biesuz et al., 2014) are: x_1 = temperature of the second stage of the synthesis (T_2 , see Fig. 1), x_2 = form of DFO employed and x_3 = type of MS. For each experiment UV-vis spectra of DFO SAMMS, after equilibration with solutions at different iron(III) content, are registered (vs. blank) from 700 nm to 350 nm.

As an example, spectra of DFO SAMMS (exp. 6, Table 1) after contact with three different iron(III) solutions are reported in Fig. 2. An intense peak is observed at around 425 nm. The measurements of the absorbance at this wavelength and at 700 nm (i.e. in the range where only the silica matrix absorb light), are acquired for data treatment.

The linearity of the response is up to Fe(III) concentration 10⁻⁴ M: at higher metal ion concentration the formation of hydrolysis products interferes significantly, and the absorbance does not follow a Lambert-Beer-like response.

In Table 1, the design and the experimental plan, with the responses (i.e. the slopes of the calibration curves obtained for each experiment plotting the net absorbance ($A = A_{425} - A_{700}$) vs. total iron(III) concentration) are presented. In the last column the response in terms of maximum

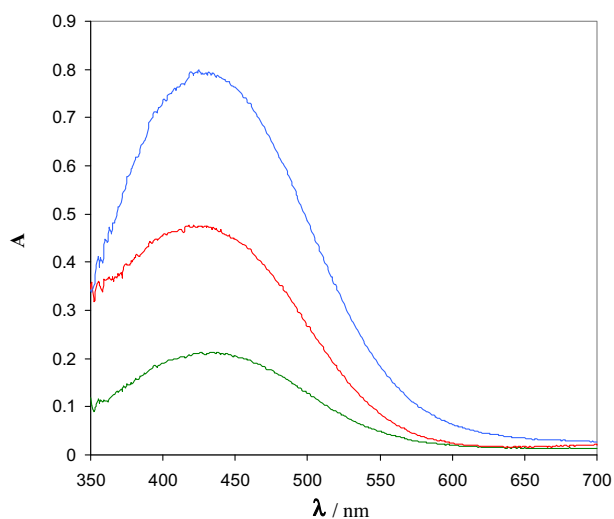


Figure 2 UV-vis spectra of DFO SAMMS (exp. 6) equilibrated with three different Fe(III) solutions: green line 1.8×10^{-5} M, red line 3.5×10^{-5} M and blue line: 8.9×10^{-5} M. Running the m-files, produced by Leardi (2009) in Matlab environment, the coefficients of Eq. (1) were obtained together with the statistical parameters. The values of the coefficients are reported in Fig. 3 where asterisks indicate their significance according to the usual convention: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

sorption capacity, previously obtained (Biesuz et al., 2014), is also reported.

The percentage of explained variance is 99.3% and that in cross validation is 94.3%. The sign of the coefficients indicates in which way the variable has to be set in order to increase the response. In this case, only the variables T_2 and type of silica have an effect on the yield and they should be set at their low level. The variable x_2 , the form under which DFO is employed, is not significant, as well as all the interactions.

The products obtained at low temperature of the second stage T_2 and with the MCM-41 silica show the highest sensitivity i.e. the maximum sorption capacity (according to the previous experimental design (Biesuz et al., 2014): it is expected even if only here proved.

We would like also to highlight that the maximum sorption capacity obtained by the optimized product, 0.33 mmol g^{-1} , is within the wide range of the values achieved with different natural and synthetic sorbents previously studied (see Table 1).

After obtaining the best DFO SAMMS product, the next step is to apply it as sensor for free iron(III) in samples of unknown metal ion speciation. To this purpose, it is necessary to correlate the colour of the solid phase with the pFe. Therefore, we perform calibration curves plotting the intensity of the DFO SAMMS colour, not against the total concentration of iron(III) in solution (as previously done for the syntheses optimization), but against the sorbed quantity of iron(III) on the solid phase, c . In Fig. 4 an example of the calibration curve is reported.

In the following, we describe how, from this information, the pFe can be obtained.

A simple equation, already reported, holds, under the hypothesis that a large excess of active sites on the solid sorbent are present with respect to the total mmol of iron(III).

The sorbed metal ion concentration, c is described by Eq. (2) (Alberti et al., 2003):

$$c = \frac{c_{\text{tot}}}{1 + \frac{\alpha_M \cdot V}{K^* \cdot w}} \quad (2)$$

where c_{tot} is the initial total iron(III) content, $K^* = \frac{[\text{Fe}(\text{HDFO})]}{[\text{Fe}]} = \frac{\beta_{\text{ex}} \cdot c_L \cdot [\text{H}]}{\alpha_L}$ is the partition coefficient of iron(III) between the solution and the solid phase (DFO SAMMS) and α_M is the collateral reaction coefficient of iron(III) in the solution phase, i.e. $\alpha_M = c_{\text{sol}}/[\text{Fe}]$.

c and c_{sol} are respectively the equilibrium concentrations of iron(III) in the solid and in the solution phases. c_L represents the mmol/g of DFO in the solid phase and α_L is the ratio between the total and the free ligand concentration in the solid phase ($\alpha_L = c_L/[\text{DFO}]$).

α_M can be computed for solution of known composition, while K^* depends only on the solid properties (Biesuz et al., 2008). K^* can be calculated by independent experiments, as described in numerous papers concerning the sorption of metal ions on chelating resins, (see for example Alberti et al., 2014a, 2007; Biesuz et al., 2008; Pesavento et al., 2003) and specifically for iron(III) on DFO SAMMS in Biesuz et al. (2014). In this last paper, we verified that the sorption of iron(III) on this solid takes place by the formation of the complex Fe(HDFO), with $\log \beta_{\text{ex}} = 40(1)$. Anyhow, at any value of pH and ionic strength, K^* and consequently c can be computed.

If the partition coefficient K^* cannot be obtained *a priori*, but if it is feasible to measure the amount of iron(III) remained in solution after equilibration with the solid phase (c_{sol}) (for example by ICP-OES), c can be deduced by a simplified method, i.e. purely by the difference from the total iron(III) content.

In Fig. 4, two replicates of calibration curves (obtained by using the optimized DFO SAMMS product) are reported; the empty circles represent the c values estimated from K^* , considering the $\log \beta_{\text{ex}} = 40(1)$ obtained in a previous study (Biesuz et al., 2014), while the gray diamonds are those experimentally determined by the direct measure of iron(III) in solution (c_{sol}).

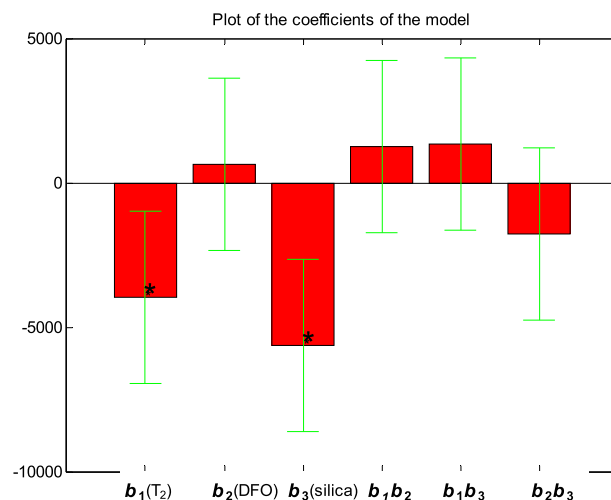


Figure 3 Values of the coefficients obtained from the multiregression of Eq. (1) for the 2^3 full factorial design performed for the DFO SAMMS synthesis.

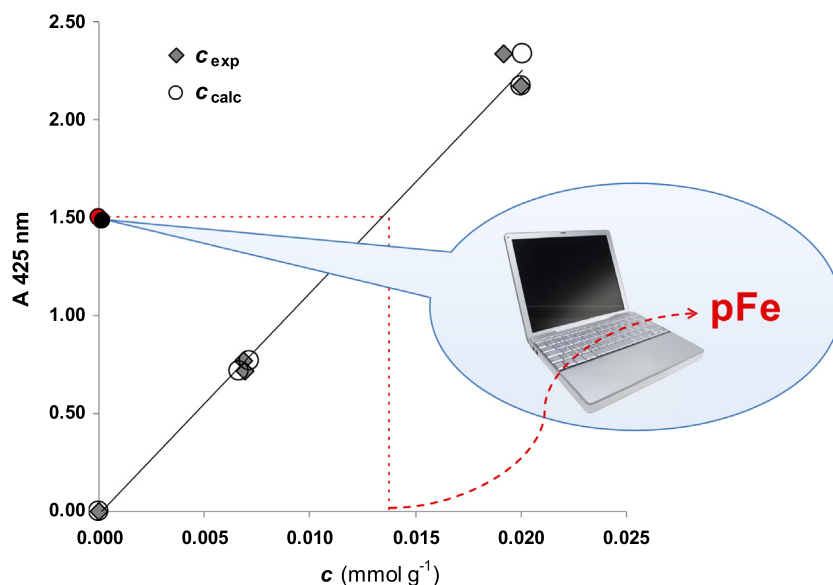


Figure 4 Example of calibration curves (two replicates of calibration curves, obtained by using the optimized DFO SAMMS product). 50 mg of solid phase was allowed to equilibrate with solutions of 10 mL 0.1 M KNO_3 , at $\text{pH} = 2.5$ and increasing amounts of iron(III) (total iron concentrations (c_{tot}) of 0, 3.6×10^{-5} and 1.0×10^{-4} M). The concentration in the resin phase is experimentally determined (grey diamonds) or calculated from the partition coefficient, (empty circles). From the absorbance of an unknown sample (red dot), the iron concentration in the solid phase is evaluated, by which the pFe is computed.

Since a pretty good agreement is obtained, these results confirmed once more that the $\log \beta_{\text{ex}}$ previously found (Biesuz et al., 2014) is reliable. Anyway, the calibration curve, based on the calculated c values from K^* , is more convenient for time and cost saving, and is essential for samples with metal ion concentrations at trace level.

At the end, in order to estimate the pFe in real samples using DFO SAMMS as a sensor, the absorbance of the solid phase, after equilibration with the sample, has to be registered. From an opportune calibration curve (previously prepared checking some parameters on the original sample, for instance: pH, ionic strength, and total iron content), ideally following the red line in Fig. 4, the value of c can be interpolated and, assuming the same K^* , the value of α_M and consequently the pFe, can be easily found.

We are aware that being real samples very complex matrices, the determination could not be very accurate, but surely the free iron(III) concentration will be best approximated with respect to the one computed by simulation programs, that are rigorous and specific, but not exhaustive to correctly describe very complicated real systems like biological samples.

For example, urine is an interesting matrix for the analysis of the free iron because the urine of iron overload patients usually exhibits a high concentration of iron(III), complexed with the ligand employed in the chelation therapy. Information about the speciation of iron and in particular the possibility to assess the free iron in such media is of overwhelming importance.

For these reasons, we decided, as first trial, to apply our method on urine samples; in particular samples were collected from individuals, with no discernible pathology, with iron concentration below the LOD of the ICP-OES; for this reason, Fe(III) and a strong iron chelator (deferiprone) were added to simulate a condition of iron overload patients under

chelation therapy. These samples were called SPU (*Simulating Pathology Urine*).

In particular, the method was tested on four SPU samples (A, B, C and D); their ionic composition is reported in Table 3.

In Table 4 for each sample, the pH, the nominal deferiprone concentration (c_L) and the amount of metal experimentally determined by direct quantification (c_{tot}) are reported (second, third and fourth columns). With these data, it is possible to calculate the theoretic values of α_{Mf} , reported in the fifth column. The partition coefficients K^* between Fe(III) and DFO SAMMS reported in the sixth column, were previously calculated by independent experiments (Alberti et al., 2014b).

As above stated, the gold point of this method is the possibility to determine $\log \alpha_M$. The fairly good accordance between the calculated values ($\log \alpha_{M\text{th}}$) reported in the fifth column and those computed with the procedure ($\log \alpha_M$ seventh column) of Table 4 is extremely important. These last values are found independently, since we only use for the calculation quantities experimentally determined.

The $\log \alpha_M$ became much more informative, using it to obtain the pFe ($-\log[\text{Fe}]$), from the simple relationship $[\text{Fe}^{3+}] = c_{\text{tot}}/\alpha_M$. The values of pFe are reported for the four SPU samples in Table 4, last column. They correspond to

Table 3 Ionic composition of four different SPU samples.

Sample	Na (M)	K (M)	Mg (M)	Ca (M)	I (M)
A	0.09	4.65×10^{-2}	7.1×10^{-4}	8.3×10^{-4}	0.14
B	0.16	8.49×10^{-2}	1.6×10^{-3}	1.5×10^{-3}	0.25
C	0.05	2.86×10^{-2}	5.5×10^{-4}	7.7×10^{-4}	0.08
D	0.07	1.55×10^{-2}	5.7×10^{-4}	4.8×10^{-4}	0.09

Table 4 Results of the experiments on SPU samples.

SPU	pH	c_L (mM)	$c_{tot,dir}$ (μ M)	$\log \alpha_{Mth}$	K^*	c (mmol/g)	$\log \alpha_M$	pFe
A	5.94	6.05	11.0	17.84	3.5×10^{19}	0.0016(2)	17.6(1)	24.33(1)
B	6.10	6.05	13.8	18.33	1.0×10^{20}	0.0019(4)	18.1(1)	24.7(2)
C	7.19	6.20	11.5	21.59	5.0×10^{22}	0.0017(4)	20.59(4)	27.4(6)
D	7.13	6.20	10.2	21.42	1.1×10^{23}	0.0014(3)	21.32(8)	27.9(1)

extremely low concentration, but of same magnitude order to the values that are expected to be found in the urine of patients under chelation therapy.

By the present work an attempt to really measure the pFe is presented; the pFe values are obtained from the competition exerted by designed solid phase: the DFO SAMMS. We are also quite sure that experimental methods devoted to determine free iron(III), at the moment, are not reported in the literature.

It is true that, at the present, we do not have analysed real samples, but with some examples of simulated samples (with real urine) containing known ligand and iron concentrations, we demonstrated that the free iron(III) obtained by the described experimental procedure was in pretty good agreement with the calculated value. These results can be considered as first attempt to a validation of the procedure since it is well known that there are no certified samples to assess speciation.

4. Conclusions

It was verified the possibility to use the colour of DFO SAMMS as a response for an experimental design: the linearity of a Lambert–Beer-kind curve is followed until 1×10^{-4} M of iron(III) concentration. Moreover the possibility to optimize the synthesis of the materials via UV–vis absorbance was demonstrated.

Finally, as already pointed out in the literature, we highlight the opportunity to use this easy determination in order to detect not the total but the free species. We have also reported a first example of application of the optimized DFO SAMMS as sensor for the free iron in urine samples. The pretty good results obtained are promising and the proposed method could definitely open the way to the development of a simple colorimetric sensor for free iron(III) determination.

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