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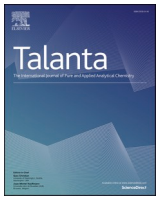

Non-transferrin-bound iron determination in blood serum using microsequential injection solid phase spectrometry– proof of concept

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ABSTRACT

Non-transferrin-bound iron (NTBI) is a group of circulating toxic iron forms, which occur in iron overload or health conditions with dysregulation of iron metabolism. NTBI is responsible for increased oxidative stress and tissue iron loading. Despite its relevance as a biochemical marker in several diseases, a standardized assay is still lacking. Several methods were developed to quantify NTBI, but results show high inter-method and even inter-laboratory variability. Thus, the development of a consistent NTBI assay is a major goal in the management of iron overload and related clinical conditions. In this work, a micro sequential injection lab-on-valve (μSI-LOV) method in a solid phase spectrophotometry (SPS) mode was developed for the quantification of NTBI, using a bidentate 3,4–hydroxypyridinone (3,4-HPO) ligand anchored to sepharose beads as a chromogenic reagent. To attain SPS, the functionalized beads were packed into a column in the flow cell, and the analyte, NTBI retained as iron (III), formed a colored complex at the beads while eliminating the sample matrix. The dynamic concentration range was 1.62–7.16 μmol L^{-1} of iron (III), with a limit of detection of 0.49 μmol L^{-1} and a limit of quantification of 1.62 μmol L^{-1}. The proposed μSI-LOV-SPS method is a contribution to the development of an automatic method for the quantification of the NTBI in serum samples.

1. Introduction

Iron is an essential metal for Human life, due to its role in oxygen transport and its participation in many redox reactions [1]. The main step in body iron homeostasis is recycling haemoglobin and its incorporation in red blood cells during haematopoiesis. However, due to the non-existence of an active excretion mechanism, body iron levels are mainly regulated during intestinal absorption [2]. Iron overload causes organ damage through free radical production and is developed when the mechanisms responsible for regulating iron absorption are altered (genetic mutations, ineffective erythropoiesis) or bypassed (red blood cell transfusion, intravenous iron application) [3].

Iron is transported throughout the body bound to serum transferrin through the bloodstream. Binding to transferrin prevents iron from participating in deleterious redox reactions and ensures a regulated mechanism for the cellular iron acquisition [4]. However, non-transferrin-bound iron (NTBI) occurs when iron influx into the plasma compartment exceeds the iron efflux [5]. The term NTBI denotes the forms of iron in serum that are bound to ligands other than transferrin, ferritin, or heme [6]. This “free” iron can be bound to serum albumin, citrate, and other undefined negatively charged ligands [7]. The presence of NTBI have been reported in several clinical conditions, such as thalassemia, sickle cell anemia, hemochromatosis, diabetes and in patients receiving myeloablative chemotherapy [8]. Hemochromatosis is a high-prevalence disease [9,10], so it is important to have methods that can quantify the NTBI. Being able to determine the NTBI value is important to understand different pathophysiological conditions but can also be potentially useful in the management of iron-overloaded patients [5].

NTBI is normally present at concentrations up to 10 μmol L^{-1} [11]. The methods already developed for the NTBI quantification present several potential problems and low agreement between them [12–14];

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the most common method for the determination employs nitrilotriacetic acid as an iron ligand, chelating NTBI, followed by ultrafiltration, which can lead to false positive errors [8,11,13]. So, an accurate determination of serum NTBI is difficult to attain by employing the available methods.

Chelators such as 3-hydroxy-4-pyridinones (3,4–HPO) have been used as chelator therapy in overload iron patients [15]. Additionally, these ligands have been recently reported as a "more sustainable" alternative as chromogenic and selective reagents to quantify iron in flow analysis techniques [16–19].

Aiming for the development of a method for the determination of the NTBI in serum samples, with sample volume limitation, the micro sequential injection platform could be an effective tool. The micro sequential injection lab-on-valve (μSI-LOV) system [20] was designed to integrate all the necessary sample pre-treatment processes and detection at the selection valve. The configuration and small dimensions of the conduits, and the absence of a reactor, minimize sample and reagents consumption. Moreover, the multipurpose flow cell associated with an additional driving device provides the possibility of handling solid particles, such as beads [21,22]. The combination of miniaturization, programmable flow, and the possibility to handle solid particles, makes this platform a potential privileged tool for NTBI determination with a good repeatability [23].

The determination of the NTBI can be a challenge due to the limited amount of serum sample available and the different forms of "free" iron. To quantify this complexed iron, it is necessary to displace it from these ligands. Nevertheless, it is still necessary to ensure transferrin-bound iron stability. To achieve this objective, in this work a μSI-LOV system comprising sepharose beads functionalized with a 3,4–HPO ligand as a reusable sorbent, is proposed for the NTBI quantification. In these conditions, NTBI is retained in the functionalized beads, with colour formation and detection occurring directly at the solid particles surface; then, the beads are washed and conditioned so that they can be reused in the following cycle. As far as we know, this is the first flow-based method for NTBI determination.

2. Experimental

2.1. Reagents and solutions

All the solutions were prepared with analytical grade chemicals and Milli-Q water (resistivity 18 MΩ cm, Millipore, Bedford, MA, USA).

A commercially available 0.9% NaCl (Labesfal, Fresenius Kabi - Laboratórios Almiro, Portugal) was used.

A physiological pH within the range of 7.35–7.45 was attained with a MOPS buffer solution by dissolving 1.156 g of MOPS sodium salt (3-(N-Morpholino)propanesulfonic acid, ≥99.5%, Sigma-Aldrich, Germany) in 500 mL of 0.9% NaCl solution to a final concentration of 0.01 mol L^{-1} of MOPS. The solution pH value was adjusted to 7.4 using HCl.

A 5 mmol L^{-1} citrate stock solution of was prepared by dissolving 0.029 g of sodium citrate dihydrated (Merck, Germany) in 20 mL of water.

A MOPS buffer solution with citrate was obtained by the dilution of the citrate stock solution (5 mmol L^{-1}) to a concentration of 100 μmol L^{-1} in a MOPS buffer solution.

A 20 g L^{-1} of Bovine Serum Albumin (BSA) (98%, Sigma-Aldrich, Germany) in MOPS buffer solution with citrate was prepared by dissolving 0.40 g of BSA in 20 mL of MOPS buffer solution with citrate.

An iron (III) stock solution of 10.0 mg L^{-1} (0.18 mmol L^{-1}) was obtained by dilution of the atomic absorption standard of 1000 mg L^{-1} (Fluka, Germany). An intermediate solution of 1.0 mg L^{-1} (0.018 mmol L^{-1}) of iron (III) was prepared and left overnight before use, by appropriate dilution of the 10.0 mg L^{-1} (0.18 mmol L^{-1}) stock solution in the different MOPS buffer and synthetic serum solutions. The iron (III) working standards were prepared in the range of: 0.025–0.40 mg L^{-1} (0.45–7.2 μmol L^{-1}) with the different MOPS buffer solutions, namely with citrate and proteins or without.

A 1 mol L^{-1} nitric acid solution was prepared from a suitable dilution of a 5 mol L^{-1} solution, prepared from the concentrated acid (d = 1.4; 65%, Merck, Germany).

2.2. Functionalized beads

The functionalized beads used as chromogenic reagent were prepared as previously reported [24]. The 3,4–HPO ligand, 1-(3′-aminopropyl)-3-hydroxy-2-methyl-4-pyridinone, was anchored to an epoxy-activated sepharose bead with a diameter of 45–165 μm. Beads functionalization was confirmed by elemental analyses. A suspension of 0.125 g of functionalized beads in 3 mL of Milli-Q water was prepared for the daily packing of the beads into a column in the flow cell and kept refrigerated between uses. The beads were used throughout a work week and a batchwise external washing with MOPS buffer was made weekly, enabling its use for a month.

2.3. Sequential injection manifold and procedure

The micro sequential injection lab-on-valve solid phase spectrometry (μSI-LOV-SPS) manifold for NTBI determination using the 3,4-HPO functionalized beads is presented in Fig. 1.

The μSI-LOV consisted of a FIAlab – 3500 (FIAlab Instruments) equipped with a bi-directional syringe pump (2.5 mL of volume), a 3 m holding coil (1.5 mL), and a lab-on-valve head mounted on the top of a six-port selection valve (Fig. 1A). The detection system comprised a USB 2000 Ocean Optics CCD spectrophotometer, a couple of optical fibers (FIA-P400-SR, 400 μm), and a Mikropack DH-2000-BAL deuterium halogen light source. The FIAlab for Windows 5.0 software running on a personal computer (HP Compaq) was used for flow programming and data acquisition. The connection of the different components of the flow system polytetrafluoroethylene (PTFE) tubing with 0.8 mm inner diameter was used, including the 3 m of holding coil.

For packing the functionalized beads into a column in the flow cell, a peristaltic pump (PP in Fig. 1A) was used to circulate the beads suspension through the two-channel port 5 of the selection valve (Fig. 1A). The beads were packed between the two optical fibers positioned and tightened with PEEK (Polyether ether ketone) ferrules, to establish a 3 mm optical path flow cell (Fig. 1B). To prevent beads loss, a PTFE stopper was placed in the port 2 channel aligned with the central channel (Fig. 1B), and a PEEK tube with an inner diameter of 0.06 mm (♯1535 Upchurch scientific) was used in the other channel of port 2, forcing the flow through the 3 mm beads column in the flow cell.

The described μSI-LOV-SPS manifold was used for the NTBI quantification, retained at the beads as iron (III), following the sequence protocol detailed in Table 1.

The initial step consisted of aspirating the carrier (water) into the syringe pump (step A). Then, the MOPS buffer solution and sample/standard were aspirated into the holding coil (steps B and C) and propelled through the column beads in the flow cell and the absorbance measurement was initiated (step D). The propelling of these plugs through the functionalized beads leads to the retention of NTBI as iron (III) by the complex formation with the 3,4-HPO ligand in the beads. To promote the complex formation, a stop period of 20 s was made (step E) prior to the second propelling step (step F) which ensured the complete washing of the sample/standard from the beads column. This washing aimed to minimize the matrix proteins interference and was provided by the initially aspirated MOPS buffer solution, which volume corresponded to threefold the sample/standard volume.

After the determination, the absorbance measurement was stopped, and an acid plug was aspirated (step G) to wash the retained iron by propelling it through the beads column (step H).

Finally, a plug of MOPS buffer solution was aspirated and sent through the beads column for conditioning and preparing the next cycle (steps I and J). Any readjustment of the beads, caused by the washing steps was minimized by performing a reference scan (step K) before

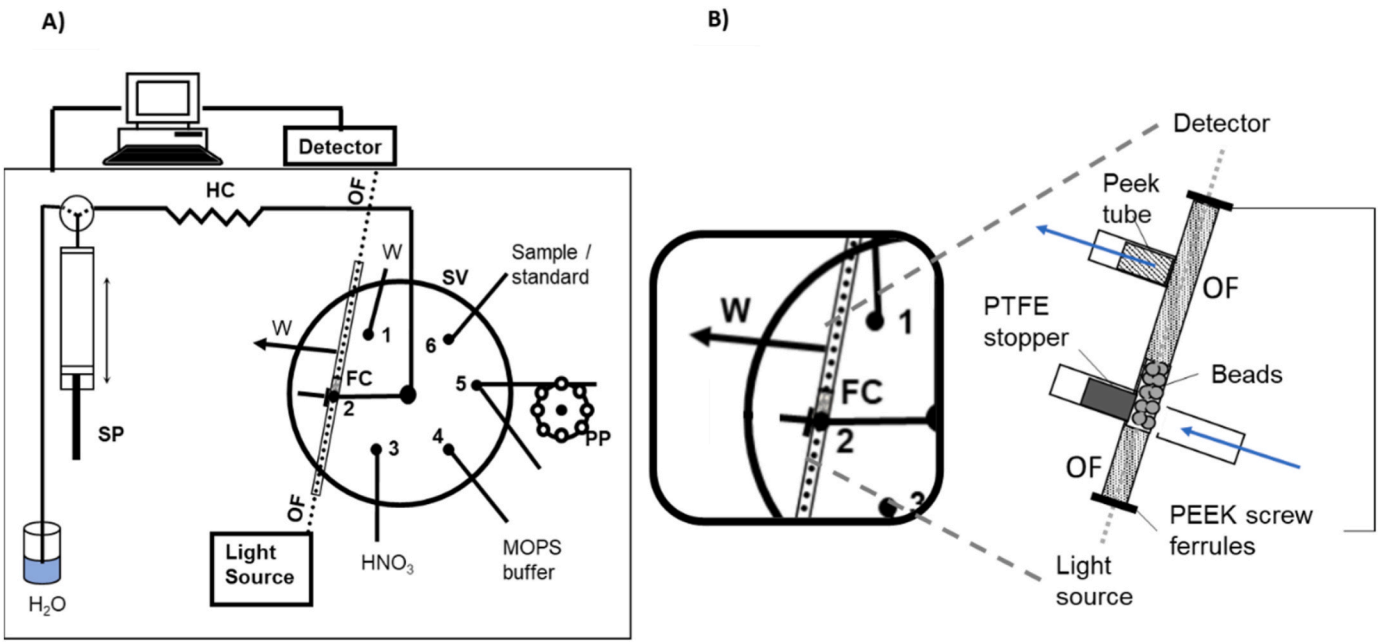

Fig. 1. The micro sequential injection lab-on-valve solid phase spectrometry (μSI-LOV-SPS) manifold: **A)** SP, 2.5 mL syringe pump; HC, holding coil, 1.5 mL (3 m); FC, 3 mm optical path flow cell packed with functionalized beads; OF, optical fibers; SV, 6 port selection valve; W, waste; HNO_3, 1.0 mol L^{-1} nitric acid; MOPS buffer, 0.1 mol L^{-1} MOPS and 0.50 mol L^{-1} NaCl solution, pH 7.4; PP, peristaltic pump and **B)** flow cell (FC) detail, functionalized beads packed between the two optical fibers; the arrows indicate the flow path.

Table 1
Protocol sequence for the developed micro sequential injection lab-on-valve with solid phase spectrophotometry (μSI-LOV-SPS) method for the NTBI quantification, using functionalized beads with a 3,4-HPO ligand as a colorimetric reagent; SV, selection valve.

Step	SV position	Flow rate (μL s^{-1})	Volume (μL)	Description
A	–	200	1000	Aspiration of water (carrier) to the syringe pump
B	4	200	750	Aspiration of MOPS buffer solution
C	6	200	250	Aspiration of sample/standard
D	2	5	170	Propelling trhough the column in the flow cell and initiation of absorbance measurement
E	2	–	–	Stop propelling for 20 s
F	2	5	1080	Continuing the propelling through the column in the flow cell and washing out of sample/standard
G	3	200	500	Stoping of absorbance measurement and aspiration of nitric acid
H	2	10	1000	Propelling through the column for analyte removal, washing the beads column
I	4	200	250	Aspiration of MOPS buffer solution
J	2	10	500	Propelling through the column for conditioning the beads column
K	2	–	–	Reference scan

starting the following cycle.

This protocol does not include setting the beads' column in the flow cell as it was a reusable approach. The beads column was set at the beginning of the workday, and removed at the end and the beads suspension was kept refrigerated overnight.

2.4. Sample collection, preparation and accuracy assessment

Serum samples were collected from homozygous hereditary hemochromatosis patients checking in for regular phlebotomy treatment at the Outpatient Clinic of Centro Hospitalar Universitário do Porto – Hospital de Santo António. Blood donation was voluntary, and all participants provided informed consent.

Blood samples were collected by venesection to vacutainer tubes without any anticoagulant addictive. Serum was separated after clotting for 30 min, by centrifuging the blood at 1000 G, for 10 min. Afterward, the serum was decanted and immediately frozen at −80 °C, for storage until analysis [11,13]. Serum samples were analyzed using the developed μSI-LOV method and the results were compared with those obtained by the microplate reader method described by Evans et al., 2008 [11].

3. Results and discussion

This work aimed to contribute to improving the quantification of NTBI in serum samples as a proof of concept with specially designed functionalized beads for solid phase spectrophotometry (SPS). The idea was to retain NTBI species in the beads, functionalized with an iron ligand, packed into a column in the flow cell of a microfluidic equipment, while not disturbing transferrin-bound iron. One of the main challenges was to ensure the displacing of iron from citrate and serum proteins, and not from transferrin, which was expected to be accomplished by the used 3,4-HPO ligand. Additionally, these 3,4-HPO ligand should act as a chromogenic reagent, allowing the spectrophotometric measurement at the surface of the beads in a SPS approach.

To mimic the conditions from the serum matrix, the studies for the development of a μSI-LOV-SPS method for NTBI determination were performed using iron (III) standards at physiologic pH of 7.4, attained using MOPS buffer and with 0.9% sodium chloride.

To evaluate the effective displacing of the NTBI to the functionalized beads, citrate, albumin, and transferrin were added at different stages of the studies.

The bidentate 3,4-HPO ligand, used to functionalize the sepharose beads, forms a colored complex with iron (III) with a stoichiometry of 3:1 as shown in the spectra analysis from previous works [17], with maximum absorption at 460 nm (for the FeL_3). To minimize the influence of refraction interfaces interference (schlieren effect), the absorbance at 800 nm was also registered and subtracted from the absorbance

at the detection wavelength [17].

The quantification of NTBI in blood serum samples should be carried out using the lowest possible amount of sample, so a maximum volume of 250 μL was set, enabling to have some replicas and still be below 1 mL of sample.

3.1. Length of beads column – optical pathlength

Considering that the functionalized beads were packed in the μSI-LOV flow cell, between the two optical fibers, the optical pathlength corresponds to the distance between the optical fibers and that can be adjusted. The flow cell size (optical pathlength) was studied by varying the set distance between the two optical fibers from 1.5 mm (minimum) to 10 mm (maximum) length (Fig. 2).

The beads column was set by packing the functionalized beads in the flow cell and the absorbance measurement was performed directly on the surface of the beads in a solid phase spectrometry (SPS) approach. The SPS approach requires that the beads have sufficient transparency to enable the light to pass through to set a baseline (reference scan) corresponding to absorbance measurement through the beads column prior complex formation.

As more beads meant a higher reagent amount (3,4-HPO ligand in the functionalized beads) it was expected that higher column length would result in increased absorbance signal and method sensitivity. However, it was only possible to establish calibration curves with 1.5 and 3 mm column lengths because, for higher lengths, the overpacking of the beads blocked the passage of light, making it impossible to set the reference scan. As the sensitivity increased significantly when the column length increased from 1.5 mm to 3 mm (calibration curve slope 20 times higher), the length of 3 mm was chosen.

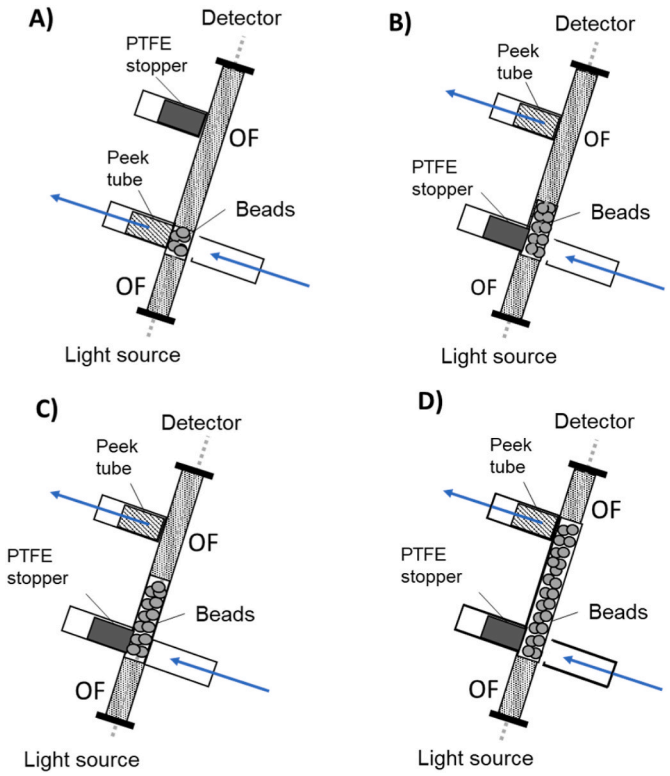

Fig. 2. Scheme of different beads column sizes in the flow cell with consequent different optical pathlength as a result of packing the functionalized beads between the two optical fibres (OF) positioned at different lengths; A) 1.5 mm optical pathlength; B) 3 mm optical pathlength; C) 5 mm optical pathlength and D) 10 mm optical pathlength; the arrows indicate the flow direction.

3.2. Solid phase spectrophotometry studies – color reaction

Having established the sample volume (250 μL) and the length of the functionalized beads column (3 mm), attention was focussed on the removal of NTBI from the matrix and its retention of the beads column. As NTBI can be in serum bound to citrate, and to mimic the average human serum citrate concentration, a final concentration of 100 μmol L^{-1} of citrate was added to the standards with 0.9% sodium chloride in a MOPS buffer at physiologic pH of 7.4. Calibration curves were established using standards with and without citrate, and no significant differences were observed (calibration curve slope deviation <0.1%). This indicated that the NTBI was effectively removed from the citrate to the 3,4-HPO ligand in the functionalized beads.

Aiming to improve the determination sensitivity, and considering that the reagent (3,4-HPO ligand) was anchored to the sepharose beads, there was the possibility of being less accessible for the complex formation. So, a higher residence time of the sample in the beads column was tested. The propelling flow rate was decreased from 10 μL s^{-1} rate (used initially) to 5 μL s^{-1} as the lowest flow rate possible for the μSI-LOV system and a stop period of 20 s was included. The absorbance signal was registered from the initial propelling of the sample/standard until the final propelling of the sample/standard. With this arrangement, a significant sensitivity increase was attained (calibration curve slope over 6 times higher). Longer stop periods were not studied to avoid a significant decrease in the determination rate.

3.3. Solid phase spectrophotometry studies – beads column regeneration

In terms of reagent consumption, the immobilization of the reagent in a solid sorbent, namely the beads, is advantageous over its use in solution. However, the regeneration of the functionalized beads column implies removing the analyte complexed with the ligand in the surface of the beads, before another analytical cycle must be performed. The removal of NTBI, retained as iron (III), from the 3,4-HPO ligand in the beads could be performed using a nitric acid solution. The aspiration and propelling of a 500 μL plug of acid after the signal measurement was set. The influence of the acid concentration was performed using three different concentrations of nitric acid, 0.5, 1.0, and 1.5 mol L^{-1}, and the iron (III) standards were prepared in a MOPS buffer solution with 0.9% sodium chloride and citrate (light grey bars and circles in Fig. 3).

The use of a 1.0 mol L^{-1} nitric acid concentration led to a higher slope (better analyte removal) than 0.5 mol L^{-1}, but no increase was observed when using 1.5 mol L^{-1} (light grey bars and circles in Fig. 3). In fact, for the 0.5 mol L^{-1} of nitric acid solution, it was observed that, for higher iron concentrations, the analyte removal was not effective, as

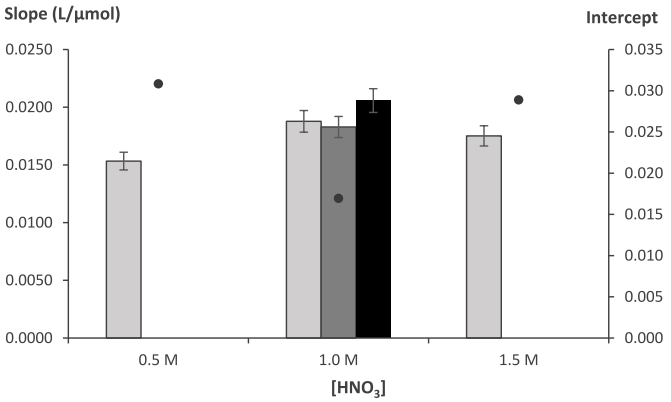

Fig. 3. Study of the influence of washing and conditioning on the analytical curve slope (bars) and intercept (black circles): nitric acid concentration on washing plug (light grey bars), the inclusion of additional MOPS washing plug (dark grey bar), and inclusion of additional MOPS conditioning plug (black bar).

there was still visible color in the column from the iron (III) – 3,4-HPO ligand complex. Therefore, 1.0 mol L^{-1} nitric acid concentration was chosen, as the minimum concentration that allowed an efficient cleaning of beads columns.

Furthermore, the application of the developed method to the analysis of a complex matrix sample, as human serum, using a solid phase approach, may result in the undesirable retention of some serum components, namely proteins, on the packed column. So, efficient column washing must be taken into consideration. A washing step, consisting of an additional 250 μL plug of MOPS buffer solution (with sodium chloride and citrate), was included in the determination step, being propelled through the column after the sample/standard. The volume was set as the same as the sample/standard volume. Additionally, the use of the nitric acid plug, for the analyte removal, leads to the change of pH conditions at the beads column (as the beads would be in an acidic medium) which could affect the complex formation. To guarantee appropriate pH conditions for each cycle, a conditioning step with MOPS buffer solution (with sodium chloride and citrate) following the acid washing, was also included. In the end, two additional MOPS buffer plugs were included, one for washing out the matrix and another one for conditioning the beads column after the iron removal with nitric acid. This conditioning step consisted of a 250 μL plug of MOPS buffer solution (with sodium chloride and citrate). To test the effect of the inclusion of both plugs, calibration curves were established (dark grey and black bars in Fig. 3) and no significant differences (calibration curve relative deviation <10%) were observed.

3.3.1. Testing of a renewable approach

As a potential alternative to the regeneration of the beads column, involving washing, analyte removal, and column conditioning, the use of a new packed column for each determination was tested in an approach named bead injection (BI) [25]. To test the BI approach, the beads column had to be packed and removed at each cycle and in order to be feasible (avoiding excessive sorbent consumption) the optical pathlength was set to 1.5 mm. A calibration curve was attempted but the removal of the beads between determinations was not effective, maybe due to the beads small diameter. Not being able to set a novel beads column after each cycle, the renewable BI approach was disregarded.

3.4. Interferences assessment

Having established the regeneration conditions of the beads column for the SPS approach, the potential interference of serum constituents was tackled. To mimic Human serum samples, the ionic strength should be around 0.15 mol L^{-1} [26,27] which corresponds to 0.9% sodium chloride solution (commercial physiologic serum solution) used throughout the work. The potential interference of serum proteins, namely albumin and transferrin was assessed based on the expected concentrations [28,29]. Albumin, at a concentration of 40 g L^{-1} BSA was added to the MOPS buffer solution for standards preparation, and calibration curves were established with and without BSA. A decrease of about 40% in the sensitivity (calibration curve slope) was observed when the protein was present. The presence of transferrin was also tested because, frequently, patients with “free” iron in serum (NTBI) present a high transferrin saturation rate. This condition can potentially interfere with NTBI determinations, either by sorption at the surface of the beads, a direct increase of the absorbance signal, or by causing part of the iron bound to this protein to be released, complexing at the functionalized beads. To study this possibility, a synthetic serum solution containing 2.5 g L^{-1} of Human holo-transferrin (≥98%, Sigma-Aldrich, Germany) - the common concentration in human serum [29] - was prepared, by adding transferrin to sodium chloride, citrate, and BSA in MOPS buffer. Calibration curves were established using standards with and without saturated transferrin and a decrease in sensitivity (calibration curve slope) of about 60% was observed (Fig. 4).

Aiming to minimize this interference, calibration curves were established increasing the volume of the washing plug, from 250 μL (white bars in Fig. 4) to 500 μL (grey bar in Fig. 4) and to 750 μL (black bar in Fig. 4). With the latter, the protein interference decreased from 60% to about 30% (calibration slope relative deviation) but it was still significantly high.

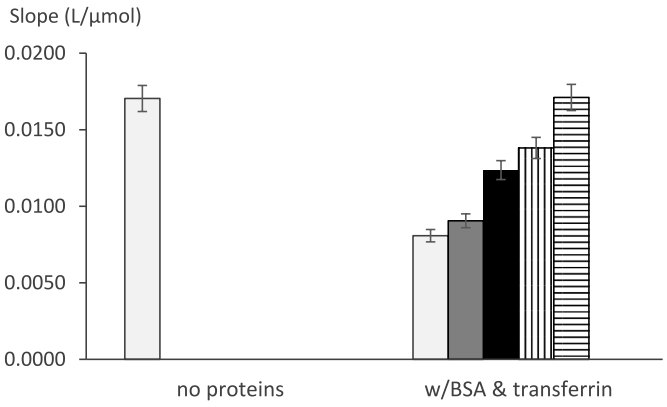

Fig. 4. Study of the minimizing the serum proteins BSA and transferrin interference on the analytical sensitivity; increasing the volume of the washing plug from 250 μL (white bars) to 500 μL (grey bar) and to 750 μL (black bar) and the NaCl concentration from 0.15 mol L^{-1} (white, grey and black bars) to 0.2 mol L^{-1} (vertical stripes bars) and 0.5 mol L^{-1} (horizontal stripes bars).

In this context, to increase the effectiveness of washing, a higher concentration of NaCl in the MOPS buffer solution was tested (stripes bars in Fig. 4). The used 0.9% NaCl corresponds to 0.15 mol L^{-1}, so 0.20 and 0.50 mol L^{-1} NaCl were tested by establishing calibration curves. It was observed that using 0.50 mol L^{-1} NaCl (3% NaCl), there was no difference between the calibration curve slope with and without proteins (Fig. 4).

In the end, the potential interferences from serum samples major protein constituents was minimized using 3% NaCl in the MOPS buffer solution in both the washing and the conditioning plugs.

3.5. Figures of merit

To study the characteristics of the developed method, standards prepared in the abovementioned matrix were used. The characteristics of the developed method were summarized in Table 2.

The limits of detection (LOD) and the limit of quantification (LOQ) were calculated according to IUPAC recommendations [30] as three (LOD) and ten (LOQ) times the standard deviation of the calibration curve intercept divided by the slope (n = 4). The determination rate was calculated as the time spent per analytical cycle, consisting of the sum of the time needed for each step plus the time necessary for the port selection in the selection valve. One calibration curve took about 2 h, including five standards, with 3 replicas each (Fig. 5).

The repeatability was evaluated by calculation of the relative standard deviation (RSD) of different standard/sample within the same day (Table 2). The reproducibility was assessed by calculation of the RSD of the calibration curve slope obtained on different days over five weeks, RSD <6%. This can also assess the intracolumn reproducibility because the beads column is packed daily.

3.6. Accuracy assessment for NTBI determination

The accuracy of the developed μSI-LOV-SPS method to the NTBI quantification was performed by analyzing human serum samples. The restrictions in the amount of sample volume provided by the hospital, approximately 500 μL, and in order to attain at least three replicas for average and standard deviation calculation, a choice was made to dilute to 1 mL of the original sample in MOPS buffer solution (with NaCl and citrate). As the samples were diluted, the composition of the matrix of

Table 2
Features of the developed NTBI determination method; RSD, relative standard deviation; the values are presented with ±standard deviation.

Dynamic range	Typical calibration curve[a] A = Slope x μmol Fe^{3+}/L + b	LOD (μmol L^{-1})	LOQ (μmol L^{-1})	Repeatability %RSD (μmol L^{-1})	Determination rate (h^{-1})
1.6–7.16 μmol L^{-1} (89–400 μg L^{-1})	A = 0.0126 ± 0.0011 x [Fe^{3+}] +0.016 ± 0.002 R2 = 0.997 ± 0.003	0.48	1.6	7.8% (1.12 ± 0.09) 9.3% (3.48 ± 0.33)	7

[a] n = 4.

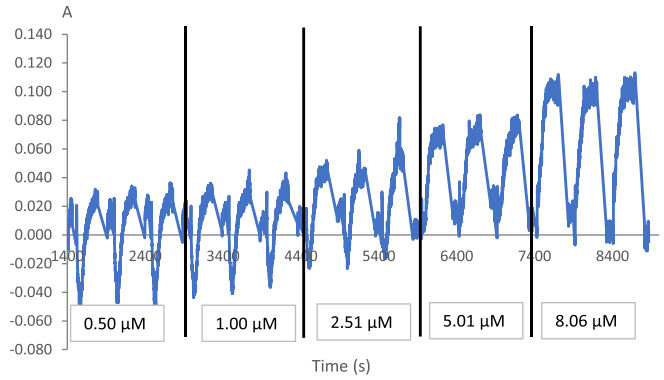

Fig. 5. Absorbance signal register for a calibration curve; the variation in the baseline corresponds to the effect of the stopping and washing periods.

the standards, MOPS buffer with 0.9% NaCl, 100 μmol L^{-1} of citrate, 40 g L^{-1} BSA and 2.5 g L^{-1} transferrin, was also diluted 1:1 in MOPS buffer (with NaCl and citrate) to mimic the diluted samples.

The accuracy assessment was carried out by analyzing seven serum samples with the developed μSI-LOV- SPS method and comparing the results with the NTBI determination method described by Evans et al., 2008 [11] by calculating the relative error (RE) between the two sets of results (Table 3).

Although some NTBI values could not be measured, the results appeared to be comparable (RE < 10%). It is pertinent to point out that, even for the samples with a concentration found lower than the LOD, these values are in line with the results from the comparison method.

4. Conclusions

The use of a microsequential injection lab-on-valve system with a solid phase spectrophotometry approach (μSI-LOV- SPS) proved to be an important contribution to the development of an automatic method for NTBI determination. As far as we know, this is the first flow-based method for NTBI determination and presents advantages over previously described methods [8,11,13], which are based on manual batch procedures, subject to more operator influence, and much more time-consuming.

The choice of the μSI-LOV technique enabled it to easily handle solid particles (sorbent) inside a flow system and carry out the measurement directly at the surface of the beads, displaying relatively good repeatability.

The option to use the bidentate 3,4-HPO ligand, as a reagent immobilized in a solid support, enabled to reduce in the number of analytical steps, and reduce the reagent consumption, being the solid material reused in successive cycles.

The described μSI-LOV-SPS method was a contribution to the development of an automatized method for the quantification of the NTBI in serum samples. To fully validate the method for the NTBI determination in human serum, it is still necessary to analyse a higher number of human serum samples. Anyway, the results presented in this work indicate that the procedure is efficient to discriminate between NTBI and the iron linked to transferrin.

Table 3
Results obtained with the proposed μSI-LOV-SPS method and with a comparison method [11] for NTBI determination, for the analysis of three serum samples; SD, standard deviation; RE, relative error.

Sample ID	μSI-LOV- SPS [NTBI] ± SD (μmol L^{-1})	Comparison method [NTBI] (μmol L^{-1})	RE (%)
#01	0.936[a] ± 0.252	0.90	4%
#02	< LOD	0.20	–
#03	0.685[a] ± 0.172	0.70	−2%
#04	3.71[b]	3.4	9%
#05	3.48[b]	3.4	2%
#06	4.80 ± 0.73	5.2	−3%
#07	< LOD	0.20	–

[a] Value < LOQ.
[b] Not possible to have more than one reading.

Credit author statement

Joana Miranda: Investigation, Formal analysis, Validation, Writing – original draft. André Silva: Conceptualization, Methodology, Writing- Reviewing and Editing. Andreia Leite: Investigation. Raquel Mesquita: Supervision, Conceptualization, Validation, Writing- Reviewing and Editing. Maria Rangel: Conceptualization; Writing - Reviewing and Editing, Funding acquisition. António Rangel: Supervision, Conceptualization, Writing - Reviewing and Editing, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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