A new green method for indirect determination of ferric ions in biological samples using Ascorbic acid as reducing agent via the development of CFIA system

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Abstract
A simple, sensitive, automated and environmentally friendly FI/MZ technique was suggested for the indirect determination of iron (III) as ferric chloride in bulk and biological. These method involve the reduction of Fe (III) to Fe (II) using ascorbic acid as reducing agent, the reduced ion was reacted with 1,10- phenanthroline as a selective organic reagent to form red-orange complex, measured at \( \lambda = 510 \) nm. The complexation ratio between metal ion with ligand was 1:3 ratio (M: L). The colored product obeyed Beer’s Law with linear ranges 1-30 and 8-75 \( \mu g / mL \) with detection limits 1.02, 0.28 \( \mu g / mL \) for batch and FIA / MZ methods, respectively. The rate of sampling was 54 sample/h, the %RSD less than 2% and the recovery was about 99%. The developed technique was simply and low cost with high throughput, it provides the use of an aqueous medium as carrier of chemicals in flow system that is nontoxic and causes no pollution, thereby belonging to green chemistry. The proposed procedure can be successfully applied to estimate the content of Iron (III) in biological samples. The results of estimation are satisfactory as compared with those given by a reference method, showed the new method to be accurate and precise at the 95% confidence level.

Keywords: green chemistry, flow injection analysis, Ferric chloride, 1,10- phenanthroline, biological samples, Ascorbic acid.

Introduction
Iron is a chemical element with the symbol Fe and the atomic number 26, atomic mass 55.845 \( g.mol^{-1} \), Density 7.874 \( g/cm^3 \) [1,2]. This metal which is part of the first transition sequence and class (8) of the periodic table. Iron is generally found in water and food such as liver, beef, pork, tofu, soybean, cereals, spinach and watercress. In pharmaceutical it can be found in the iron form such as ferrous fumarate and folic acid. An adult human's body contains about 4 grams of iron, much of which is in hemoglobin and myoglobin both two proteins play important jobs in the metabolism of vertebrates, the transfer of oxygen through blood and the accumulation of oxygen in muscles respectively. The human iron metabolism needs a minimal of iron in the diet to sustain necessary amounts. Iron is also the metal at the active site of many important redox enzymes which deal with cellular respiration and oxidation and plant and animal reduction. Iron is also highly dangerous. Donation and acceptance of electrons ensures it can catalyze conversion of hydrogen peroxide into the free radicals. Free radicals can destroy a wide range of cellular structures and eventually destroy the cell [3]. Its main function is to form hemoglobin, the blood pigment that brings oxygen from the blood to the tissues. Iron deficiency anemia is the most common anemia when iron loss is high in the body and its iron reserves are exhausted as during cycles of rapid development, pregnancy, menstruation or other chronic
blood loss [4-7]. Many of oxidation states of iron was estimation via several spectrophotometric methods using different chelating organic agents [8] were used for the selective determination of Iron (III), (II) ion by using a suitable reducing agents. Miniaturization and automation of analytical techniques based on flow techniques offers many benefits, such as a reduction in human exposure to toxic substances and the production of more environmentally friendly products [9-12]. Process downscaling allows for more cost-effective and environmentally sustainable procedures [13-15]. The reagents are continuously consumed, which is a weakness in continuous flow injection analysis. When this problem is serious, it can be minimized by using the fusing zones method, which can be accomplished by periodic pumping or the use of several injection valves [16, 17]. The injection valves are connected in series, and the sample and reagent are both injected into the same carrier current. The two zones merge and pass downstream until the detector is reached. This technique aims to reduce the use of expensive reagents and create an information-rich composite zone [18, 19]. In this scientific manuscript, a selective, inexpensive and reproducible with high sensitive analytical method via CFIA/ MZ technique with modified spectrophotometric detection unit for indirect estimation of Fe (III) ions in pure forms and biological samples through a manifold is consist of one channel and homemade six-three way valve.

**Experimental**

**Materials and reagent**

A stock solution of Fe (III) was prepared through dissolution 0.025 g of (FeCl$_3$=162.204 g/mol, 250 μg.mL$^{-1}$) (Merck) in a few amount of conc.HCL and completed the solution to 100 ml with distilled water. A stock solution of 1,10- phenanthroline (M.wt=180.2 g/mol, 0.01M (Merck) prepared in 50 ml volumetric flask by dissolving 0.0901 g in 2 ml of ethanol and made up volume with distilled water, tri-sodium citrate 0.1 M (M.wt= 294.1 g/mol, HI-SKY chemicals) was prepared by dissolving 2.941g in 100 ml distilled water, thus ensuring acidic medium. Ascorbic acid (M.wt=176.12g/mol, Sigma-Aldrich) 0.01 M was prepared by dissolving 0.0881 g in 50 ml of distilled water.

**Preparation of biological samples.**

1- **Human serum**

Human serum samples were collected from healthy persons, in plastic tubes separated from blood at 3000 rpm for 15 minutes by centrifugation, dilution with 1 ml distilled water and acidified with 1 ml of HNO$_3$ (1M) to precipitate proteins. A vortex mixer was used to vigorously mix 0.5 mL of the supernatant with 1.0 mL of ethyl acetate in a glass tube for 3 min and the aqueous solution was pipetted into10 ml plastic tube and diluted with distilled water and stored at 20°C until they used [20, 21].

2- **Plasma samples**

Blood samples were collected from healthy persons in glass tubes [contain EDTA] and centrifuged for 20 min at 3000 rpm. Precisely 0.5 ml of plasma was then pipetted into a 10 ml plastic tube and stored at 20 ° C in freeze until it was used [22].

3- **Urine samples**

The samples was collected from different healthy people (two female and one male), directly used after added 2 ml of HClO$_4$ acid (to precipitate the protein) [23] and then centrifuge at 3000 rpm.
Instrumentation

The absorbance measurements in batch procedure were made by using a Shimadzu UV-1800, (Japan) UV-Visible Spectrophotometer double beam and quartz cuvette with an optical length of 1 cm. The suggested FI/MZ system was designed is a simple type with single channel manifold as depicted in Figure (1) was used in developed method of FIA / merging zones system for indirect estimation of ferric ion. A one-channel FIA, Peristaltic pump (Shenchen, LabM1) was used to pump the distilled water as a carrier stream (flow rate= 1.92 ml.min\(^{-1}\)) through the injection valve (six-three-way injection valve, homemade) \[19\], that moves at 90° and three loops of Teflon (I.d=0.5mm). The sample (Fe (III), 30 μg/mL) solution in L\(_1\), reducing agent solution (Ascorbic acid, 6×10\(^{-4}\) M) in L\(_2\), the reagent (1,10- phenanthroline, 2×10\(^{-3}\) M) and trisodium citrate (pH=3) solutions in L\(_3\) were loaded. Mixing in reaction coil made of glass (2 mm, I.D). In FIA procedure absorbance and spectral control, the modifid Optima photometer 301-D', VIS Spectrophotometer single beam (Japan) was used. Kompensograph C1032 (Siemens) was used for measurement of responses expressed as average peak height (n=3) (mV) or optical multimeter absorption (DT9205A, OVA, China) for the absorbance measurements. The detection unit contains a flow cell quartz silica (QS, 1 cm) with an internal volume of 80 μL.

![Figure (1): Single channel manifold of the developed CFIA/MZ technique for indirect determination of Iron (III) in biological samples.](image_url)

Result and discussion

Preliminary studies of batch method

During preliminary studies on a reaction of Ferric chloride with ascorbic acid (1×10\(^{-3}\) M) as a reducing agent to convert Fe(III) to Fe (II), then Fe (II) was react with 1,10-phenanthroline (9×10\(^{-4}\) M). The reaction occurs at 25\(^\circ\)C when a volumetric flask transfers that capacity (20mL) containing 2 mL of FeCl\(_3\) (25 μg.mL\(^{-1}\)), then add 1 mL ascorbic acid (1×10\(^{-3}\)M), then adds 1.2 mL of the reagent 1,10-phenanthroline (9×10\(^{-4}\) M) and 2.8 ml of tri-sodium citrate (0.1M, pH=3.5) , the solutions had been diluted to label with distilled water. The solution was mixed and remain for 5 min, red-orange complex was formed and measured at \(\lambda_{max}\) 510 nm against reagent blank and reagent solution against distilled water, as shown in Figure (2). The proposed mechanism of the reaction between Fe (II) and 1,10-phenanthroline and the stoichiometry of this reaction was investigated by molar ratio methods, that showed (1:3, M:L), as shown in Scheme (1).
Figure (2): A/Absorption spectrum of colored product formed against reagent blank, 25 μg.mL⁻¹ of Fe (III). B/Reagent blank against distilled water.

Scheme (I): The proposed mechanism of the complex formed for indirect determination of Ferric ion using ascorbic acid as reducing agent.

Accuracy and precision
Under the perfect conditions mentioned in the defined method, accuracy and precision were investigated by measuring three different Iron (III) concentrations using the method of work installed in the preceding paragraph, and the results obtained as shown in Table (1) show that the classical method has a good precision and high accuracy.
Table (1): Accuracy and precision of the classical method

| Iron (III) conc. μg.mL⁻¹ | Error | E_rel% | *Rec% | *RSD% |
|--------------------------|-------|--------|-------|-------|
| Present μ                | Found |        |       |       |
| 8                        | 7.92  | -0.08  | -1.00 | 99.00 | 0.85  |
| 16                       | 16.03 | 0.03   | 0.19  | 100.19| 1.62  |
| 25                       | 24.97 | -0.03  | -0.12 | 99.88 | 0.00  |

*Average of three determinations

Calculations of stability constant [24]

The measurement of the stability constant for the proposed interaction (Iron: 1,10-phenanthroline) was performed on the basis of the result obtained from the mole ratio method showing the proportion of 1,10-phenanthroline to Iron (3:1) as defined in the following paragraph. Two groups of solutions were prepared, the first group of solutions was placed to include stoichiometric amounts of Iron (III) to the 1,10-phenanthroline reagent, while the second group was placed to include fivefold excess 1,10-phenanthroline. According to the mechanism and stoichiometry the ratio suggested, between reagent and iron (III) salt (3:1). The reaction of Iron (III) with 1,10-phenanthroline stability constant can be found according to the equation:

\[
K = \frac{1 - \alpha}{27 \alpha ^ 4 C ^ 2}
\]

Where \( C \) is the molar concentration (M) of the product which is equivalent to the concentration of Iron (III) while \( \alpha \) (degree of dissociation) written as follows:

\[
\alpha = \frac{A_m - A_s}{A_m},
\]

Where \( A_m \) and \( A_s \) are the absorbance of the solution containing an excess and stoichiometric amount of reagent 1,10-phenanthroline, as shown in Table (2).

Table (2): Stability constant of the complex between Iron (III) with 1,10-phenanthroline using A. A as reducing agent.

| Iron (III) | *Am | *As | A  | C (M) | \( K \) (L^2.mol⁻²) or (M⁻²) |
|------------|-----|-----|----|-------|-----------------------------|
| 0.736      | 0.548 | 0.225 | 9.305×10⁻⁵ | 6.773×10⁻²⁶ |

Optimization of the experimental conditions

All conditions were explored by adjusting one factor and keeping the other constant by monitoring the effect generated on the colored product's absorbance strength. The concentration 40 μg.ml⁻¹ was taken in the final volume of 10 ml of Fe (III), absorbance of the complex was measured at 510 nm.

In this study was proved the best type of reducing agent in this reaction to convert ferric to ferrous ions by using different types of reducing agents with same concentration prepared (0.02 M), using [SnCl₂.2H₂O, Zn/HCl, Hydroquinone, NH₂OH.HCl and Ascorbic acid] were studied on colored product formation, it was observed that maximum absorbance was got with ascorbic acid that was chosen as the ideal reducing agent for this reaction, as shown in Figure (3a). The effect of concentration ascorbic acid (4×10⁻⁵-1×10⁻³) M was examined on colored product formation, it was observed that absorbance increased with an increase of ascorbic acid concentration but a high concentration of ascorbic acid, thus absorbance decreased, 6×10⁻⁴ M was chosen as the ideal concentration of reducing agent for reduction of Fe (III) as ferric chloride, as shown in Figure (3b).
Figure (3): a-Type of reducing agents (0.02 M), Fe (III) 40 μg.ml⁻¹ with 1,10-phenathroline reagent . b- Effect concentration of ascorbic acid as a reducing agent.

The effect of 1,10-phenathroline concentration (5×10⁻⁵-2×10⁻³) M as selective organic reagent on the resulting absorbance a red-orange complex has been monitored. Throughout the experiment a variable concentrations of 1,10-phenathroline were used. The absorbance increases with increasing concentration of 1,10-phenathroline, shows 3×10⁻⁴ M had was chosen to be the optimum concentration for further experiments; as shown in Figure (4a).

The effect of buffer was investigated carefully due to it was directly effect on the absorbance of the venture formula for Fe (III) with 1,10-phenathroline. A set of volumetric flask (20mL) containing the solutions (pH=2-5) of trisodium citrate solution were used for the experiment which measured by pH meter. The absorbance increase with observed pH up to 2.5 and stays stable to pH (3), which was selected the ideal buffer solution as an appropriate medium for the formation of colored complex, as shown in Figure (4b).

Figure (4): a- Effect concentration of 1,10-phenanthroline reagent. b- Effect of pH.

**Calibration curve of classical method**

Using optimum conditions for construction of calibration curve for Fe (III) determination as ferric chloride with different volumes (0.02, 0.04, 0.12, 0.2, 0.32, 0.48, 0.6, 0.8, 1, 1.2, 2, 3 and 4 mL) while the other parameters were kept constant. A series of volumetric flasks (10 ml) were filled with 1.2 ml of ascorbic acid (6×10⁻⁴) M, A 3.1 ml of sodium citrate buffer (0.1M) and finally 0.6 mL of 1,10-phenanthroline (3×10⁻⁵M), made up the volume with distilled water. The solutions were prepared mixed and remain for 5 min, measured at λmax 510 nm. Standard curve
and linear range for Fe (III) determination is constructed (1-30) μg.mL⁻¹, as shown in Figure (4c).

![Graph](image)

Figure (4): c-Linear calibration curve for indirect determination of Fe (III) as FeCl₃ using ascorbic acid as reducing agent, using batch method.

**Flow injection analysis /Merging Zone technique**

**Optimization of the developed FIA system**

The batch method was used to improve the CFIA procedure, and the CFIA parameters were optimized to obtain a maximum sensitivity with high reproducibility of the results.

Effect of 1,10-phenanthroline concentration was investigated in the range (1×10⁻⁴ – 4×10⁻³) M for determination of Fe (III) (30 μg.ml⁻¹) by using six-three way homemade injection valve, using distilled water as carrier of chemicals in a new FIA system. The results obtained that concentration 2×10⁻³ M had the highest absorbance value which calculated by average peak height in mV (n = 3), as shown in Figure (5a).

Using a six-three way homemade injection valve at 2×10⁻³ M 1,10-phen., the effect of ascorbic acid concentrations in the range (8×10⁻⁵ – 2×10⁻³ M) on the determination of 30 μg.ml⁻¹ Fe (III) was investigated, as shown in Figure (5b), the maximum absorbance value (mV) was found to be 6×10⁻⁴ M and adopted as optimum concentration for the reducing agent in this reaction.

Effect of sodium citrate concentration on the acidity of the medium to choose the best pH in the range (1-5) for determination of Fe (III) (30 μg.ml⁻¹). The best value that equal to 3 had the highest absorbance value, as shown in Figure (5c).

The optimal flow rate was examined by measuring peck height at flow rate (1.6 - 3.16) ml.min⁻¹. Figure (5d) shows that the maximum absorbance value at a flow rate of 1.92 ml.min⁻¹ with low dispersion and regularity of response. At low flow rate, the dispersion will be the highest level while in a greater flow rate, the reaction may be not complete.

The volume of the sample, reagent and reducing agent injected was investigated using a variety of volumes for sample (L₁), reducing agent (L₂) and reagent with buffer (L₃) {58.875, 78.50, 98.125, 127.563 and 147.188} μL, the volumes that provided maximum response; 78.50 μL for L₁, 58.875 μL for L₂ and for L₃ 127.563 μL, were used in subsequent experiments as shown in Figure (5e, f, g).

Different lengths of reaction coil were studied (85, 100, 115, 180, and 250 cm) with (I.d.2 mm) which were directly inserted into the flow system after the injection valve. The length of R.C. with the highest absorbance in (mV) was 115 cm. The absorbance decreased up to 115 cm
while the reaction coil length increased due to that increasing in the dispersion and dilution of sample, as shown in Figure (5h).
Purge time

Purge time of sample segment to injection with distilled water as carrier stream was studied, using the optimum chemical and physical parameters. The purge time (5-10-15-20-25) sec and open valve (injected mode) were used for this study. A 25 sec giving the highest response with less dispersion and selected as an ideal injection time to finish transporting the sample from the sample loop to the flow cell, as shown in Figure (5i).

Calibration curve of Fe (III) via CFIA/MZ

A series of Fe (III) solutions (1, 3, 5, 8, 12, 15, 20, 25, 30, 50 and 75) μg.mL⁻¹ had been prepared with sufficient dilution of stock solution (250 μg.mL⁻¹) injected into L₁, ascorbic acid (6×10⁻⁴ M) in L₂ and a part of 2×10⁻³ M 1,10-phenathroline was mixed with sodium citrate (pH=3) were injected into L₃. Each measurement was repeated three times. The response expressed as average peak height in mV (n=3) was plotted against the concentrations of Fe (III) μg.mL⁻¹, the linear range was (5-75) μg.mL⁻¹, as shown in Figure (6). The high sensitivity of the developed procedures is demonstrated in Table (3). At 95 percent confidence levels, statistical evaluation of the regression line yielded the values of (Sy/x) standard deviation for residuals, (Sb) slope, and (Sa) intercept for n-2 [25, 26], and analytical features like linear range, detection limit, and correlation coefficient [27]. These small points were attributed to the developed
producer of CFIA high reproducibility and repeatability compared to the batch method. Because of a wider linear range of calibration graph with high recovery and speed (sample throughput of 54 sample.h\(^{-1}\)), the FIA/MZ technique is more convenient than the previous process. Others advantages of the proposed method, selectivity, sensitivity, economy of samples and reagents, nontoxic and no pollution, thereby belonging to green chemistry with high sampling/h, cost-effectiveness and can be successfully applied to estimate the content of Iron in biological samples.

![Figure (6): Linear calibration curve for indirect determination of Ferric chloride using A.A as reducing agent via the developed FIA system.](image)

**Table (3): Summary of optical characteristics of the proposed method compared with the classical method for estimation of ferric ion as ferric chloride.**

| Parameters                        | Batch method          | CFIA method         |
|-----------------------------------|-----------------------|---------------------|
| Linear range (μg/mL\(^{-1}\))    | 1-30                  | 5-75                |
| Correlation coefficient,\(r^2\)   | 0.9971                | 0.9984              |
| Regression equation \(y = bx + a\); \(y = \text{absorbance}, x = \text{concentration (μg/mL}\(^{-1}\))\) | \(y = 0.0424x + 0.0796\) | \(y = 16.655x + 31.923\) |
| Linearity (r\(^2\)% )             | 99.71                 | 99.84               |
| Slope (b), L mg\(^{-1}\)          | 0.0424                | 16.655              |
| Intercept (a = y– b x)            | 0.0796                | 31.923              |
| *Relative Standard deviation (RSD %) | 1.96 (at 15 ppm)     | 0.1849 (at 50 ppm)  |
| Standard deviation of the residuals \(S_{yx}\) | 0.01684              | 4.36                |
| Standard deviation of the slope \(S_b\) | 1.3342×10\(^{-3}\)  | 0.136               |
| Standard deviation of the intercept \(S_a\) | 0.0198               | 1.544               |
| Confidence limit of intercept \(a = a \pm S_a\) | 0.0796 ± 0.0364       | 31.923 ± 2.839      |
| Confidence limit of slope \(b = b \pm S_b\) | 0.0424 ± 2.4524×10\(^{-3}\) | 16.655 ± 0.25       |
| Molar absorptivity \(\varepsilon\) \(\text{L.mole}^{-1}.\text{cm}^{-1}\) | \(6835.0496\)        | -                   |
| Sandell’s sensitivity (S) \(\mu g \text{ cm}^{-2}\) | 0.0236               | -                   |
| Sample throughput (h\(^{-1}\))    | 10                    | 54                  |
| Limit of quantification (LOQ) \(\mu g/mL\(^{-1}\)) | 3.39 \(\mu g/mL\(^{-1}\) | 0.946 |
| Limit of detection (LOD), \(\mu g/mL\(^{-1}\)) | 1.02 \(\mu g/mL\(^{-1}\) | 0.284 |

*Average of three determinations*
Analysis of variation (ANOVA) [24]

- Calculate the sum of the squares of the difference of values $y_i$ (response) from $\bar{y}_i$ (appraiser response), (imply error) and called (about regression) to get $\sum (y_i - \bar{y}_i)^2$ for (n-2) of degrees of freedom to get sum of squares $(S0)^2$.

- Calculate the sum of squares of the variance of values $\bar{y}_i$ from average value $\bar{y}$ (due to regression) to get $\sum (\bar{y}_i - \bar{y})^2$ and for (1) of degrees of freedom to obtain sum of squares $(S1)^2$, when dividing the $(S1)^2$ on $(S0)^2$ obtain the value (F), as shown in the Table (4).

Table (4): ANOVA for the equation of a straight line values using the proposed method.

| Source | Sum of squares | Df | Mean Square | Fcal. $=\frac{S1^2}{S0^2}$ |
|--------|----------------|----|-------------|--------------------------|
| Regression | $\sum(\bar{y}_i - \bar{y})^2 = 77332.99$ | V1 = 1 | 93705.94 | 668.531 |
| Error | $\sum(y_i - \bar{y}_i)^2 = 841$ | V2 = 6 | 140.167 |
| Total | 78173.99 | 7 | |

[F critical= 5.987 <$<$ Fstat. = 668.531] so it may be complete which there is an important relation between the concentration of Fe (III) with the response got.

Effect of interferences

The selectivity of proposed method (CFIA/MZ technique) was examined. The interfering of the common ions on an accuracy of a determination of 30 μg.mL$^{-1}$ Fe (III) were assessed by this technique. A sample of pure iron (III) spiked with half, equal and double fold excess of selected interference ions excipients were analyzed. The results mentioned in Table (5). The acceptable recovery values 96-98% demonstrated that, all ions tested have a major impact on iron (III) determination based on recovery values. There were no interferences during the determination of ferric using above technique as summarized in Table (5).

Table (5): Interferences effect of some cations and anions on determination of ferric ions (30 μg.mL$^{-1}$)

| Interference | Conc. μg.mL$^{-1}$ | mV | Erel % | Rec % | Interference | Conc. μg.mL$^{-1}$ | mV | Erel % | Rec % |
|--------------|-------------------|----|--------|-------|--------------|-------------------|----|--------|-------|
| Standard     | 30                | 560 | 0.00   | 100%  | Cr$_2$O$_7$$^-$ | 15              | 560 | 0.00   | 100.00 |
| Na (I)       | 15                | 550 | -1.78  | 98.21 | CrO$_4$$^-$   | 30              | 567 | 1.25   | 101.25 |
|              | 30                | 562 | 0.36   | 100.36|              | 60              | 568 | 1.43   | 101.43 |
|              | 60                | 560 | 0.00   | 100.00|              | 15              | 540 | -3.57  | 96.43  |
| Cu (II)      | 15                | 558 | -0.36  | 99.64 | NO$_3$$^-$    | 30              | 557 | -0.54  | 99.46  |
|              | 30                | 552 | -1.43  | 98.57 |              | 60              | 568 | 1.43   | 101.43 |
|              | 60                | 551 | -1.61  | 98.39 |              | 15              | 565 | 0.89   | 100.89 |
| Zn (II)      | 15                | 563 | 0.54   | 100.54|              | 30              | 561 | 0.17   | 100.17 |
|              | 30                | 550 | -1.78  | 98.21 |              | 60              | 553 | -1.25  | 98.75  |
|              | 60                | 547 | -2.32  | 97.68 |              | 15              | 562 | 0.36   | 100.36 |
| Pb (II)      | 15                | 555 | -0.89  | 99.11 | IO$_4$$^-$    | 30              | 560 | 0.00   | 100.00 |
|              | 30                | 550 | -1.79  | 98.21 |              | 60              | 557 | -0.54  | 99.46  |
|              | 60                | 549 | -1.96  | 98.04 |              | 15              | 559 | -1.78  | 99.82  |
| Bi (III)     | 15                | 560 | 0.00   | 100.00| Br$^-$       | 15              | 553 | -1.25  | 98.75  |
|              | 30                | 562 | 0.36   | 100.36|              | 30              | 560 | 0.00   | 100.00 |
|              | 60                | 559 | -1.78  | 99.82 |              | 60              | 543 | -3.04  | 96.96  |
| Al (III)     | 15                | 556 | -0.71  | 99.28 | Cl$^-$       | 15              | 553 | -1.25  | 98.75  |
|              | 30                | 544 | -2.86  | 97.14 |              | 30              | 551 | -1.61  | 98.39  |
|              | 60                | 540 | -3.57  | 96.43 |              | 15              | 557 | -0.54  | 99.46  |
| Ce (IV)      | 15                | 560 | 0.00   | 100.00| I$^-$        | 30              | 556 | -0.71  | 99.28  |
|              | 30                | 548 | -2.14  | 97.86 |              | 60              | 548 | -2.14  | 97.86  |
Biological samples applications

The FIA/MZ technique was applied for estimation of iron concentration as ferric in spiked human biological samples [21],[23] according to the standard addition method, three types of biological samples (plasma - serum – urine) have been analyzed under proposed method which come from three different patient’s samples. Two different concentration of biological samples (for serum [25 and 50 μg.mL⁻¹], for plasma [30 and 60 μg.mL⁻¹] and for urine [40 and 70 μg.mL⁻¹]) were examined for accuracy and precision. Each concentration was analyzed (n=3). Acceptable accuracy with high repeatability of the results obtained for determination of Fe (III) in biological samples were observed, as shown in Tables (6a, b, c).

Table (6): a- Application of the suggest FIA method for determination of Iron (III) in biological samples (spiked plasma samples).

| Sample | Added conc. μg.mL⁻¹ | Found conc. μg.mL⁻¹ | Error | Ere. % | Rec.% | RSD% |
|--------|---------------------|---------------------|-------|-------|-------|------|
| 1      | 30                  | 29.97               | -3×10⁻² | -0.10 | 99.90 | 1.07 |
|        | 60                  | 60.04               | 4×10⁻²  | 0.07  | 100.07| 0.91 |
| 2      | 30                  | 30.12               | 12×10⁻² | 0.40  | 100.40| 2.04 |
|        | 60                  | 59.90               | -0.1    | -0.17 | 99.83 | 1.06 |
| 3      | 30                  | 29.99               | -1×10⁻² | -0.03 | 99.97 | 0.02 |
|        | 60                  | 59.95               | -5×10⁻² | -0.08 | 99.17 | 0.05 |

Table (6): b- Application of the suggest FIA method for determination of Iron (III) in biological samples (spiked serum samples).

| Sample | Added conc. μg.mL⁻¹ | Found conc. μg.mL⁻¹ | Error | Ere. % | Rec.% | RSD% |
|--------|---------------------|---------------------|-------|-------|-------|------|
| 1      | 25                  | 25.18               | 18×10⁻² | 0.72  | 100.72| 0.24 |
|        | 50                  | 49.89               | -11×10⁻² | -0.22 | 99.78 | 1.20 |
| 2      | 25                  | 24.97               | -3×10⁻² | -0.12 | 99.88 | 0.34 |
|        | 50                  | 49.91               | -9×10⁻² | -0.18 | 99.82 | 0.68 |
| 3      | 25                  | 24.88               | -12×10⁻² | -0.48 | 99.52 | 0.09 |
|        | 50                  | 50.07               | 7×10⁻²  | 0.14  | 100.14| 1.79 |

Table (6): c- Application of the suggest FIA method for determination of Iron (III) in biological samples (spiked urine samples).

| Sample | Added conc. μg.mL⁻¹ | Found conc. μg.mL⁻¹ | Error | Ere. % | Rec.% | RSD% |
|--------|---------------------|---------------------|-------|-------|-------|------|
| 1      | 40                  | 39.92               | -8×10⁻³ | -0.20 | 99.80 | 0.61 |
|        | 70                  | 69.85               | -15×10⁻³ | -0.21 | 99.79 | 2.04 |
| 2      | 40                  | 40.08               | 8×10⁻³  | 0.20  | 100.20| 0.05 |
|        | 70                  | 69.99               | -1×10⁻³ | -0.01 | 99.99 | 1.06 |
| 3      | 40                  | 39.87               | -13×10⁻³ | -0.33 | 99.68 | 0.23 |
|        | 70                  | 70.12               | 12×10⁻³ | 0.17  | 100.17| 1.74 |

Conclusion

The green developed CFIA/merging zones method is automation process for indirect determination of ferric chloride provides accurate and precise, as well as sensitive, and thus can be used for routine analysis of Fe (III) in pure form and biological samples such as serum, plasma and urine with the level μg.ml⁻¹, the proposed homemade CFIA/MZ method as compared
to conventional spectrophotometric technique, similar recorded methods, and has a wider range of linearity, the established method has a number of advantages, including speed and higher sensitivity. Furthermore, because of the lower reagent and chemicals consumption, the FIA spectrophotometric technique is more environmentally friendly compared with official method [22], [28] since it uses less samples and takes less time. The procedure has good linearity, high analytical frequency with throughput 54 sample/h with non-interference to determination of Fe (III) indirectly with minimum error.

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