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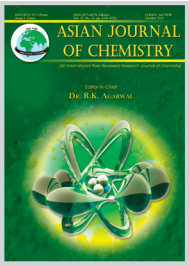

Estimation of Iron in Biological Samples by Atomic Absorption Spectroscopy

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Estimation of iron in many biological samples is difficult as its level is very low. Hence a new methodology is developed for the estimation of total iron present in the sample. This method is based on the formation of iron III-thiocyanate complex using potassium thiocyanate (1.5 M) and hydrochloric acid (2N) as reagents. Estimation procedure is designed in such a way to measure wide range (0.5-5000 µM/mL) without dilution or concentration of sample. Pretreatment may be required as per physical nature of sample but it is same for all solids and that too for fluids. This methodology has the following advantages. It is a photometric method (λ 478) can be applied for solid samples like food material, stool, tissue *etc*. The insoluble organic and inorganic forms of iron can be estimated. It is highly sensitive, even water sample purity can be tested for iron. It has good linearity at low and high ends of concentration. It is a most economical method as it does not require highly sensitive sophisticated instruments and long period for incubation.

Key Words: Iron estimation, Biological samples, AAS.

INTRODUCTION

There is a need for a simple routine method for the measurement of iron in biological materials. Though there are many methods available for the estimation of iron in biological samples, such methods do not cover wider area of research as most of them are specific to particular application. The estimation of iron also has some of the technical difficulties such as, there are two forms of iron (ferric and ferrous), biological fluids have iron associated with other insoluble organic matters (*e.g*., hemoglobin), tissue samples are insoluble in both organic and inorganic solvent systems, as the iron is a trace element their levels are very low in living system but it is important to vital function hence the storage organs have very high levels.

Iron may be determined in biological material by a wet-ash method employing chloric and nitric acids[1]. The conversion of any biological materials in to ash completely eliminates the rich organic content and makes the sample as pure form of inorganic one. Hence the complications of different organic chemicals are avoided at this step and it is ready for the pure inorganic analysis. It is essential to keep it in mind the following factors such as coverage of wide range concentration, cost effective, time requirement, stability of reagents, simple instrumentation *etc*., in the development of a new methodology to solve these problems.

EXPERIMENTAL

1.5 M Potassium thioscyanate (KSCN), standard 1.0 µM/mL $FeCl_3$ (brand MERCK), 2N hydrochloric acid. (10 % hydrogen peroxide or 5 % of sodium hypochlorite may be required). Standards may be prepared from ferrous/ferric sulphate, ferrous/ferric ammonium sulphate, ferrous salts must be converted to ferric form by acidified permanganate solution by titration or standard must be treated like a sample right from the sample preparation procedure for the conversion of Fe^{2+} to Fe^{3+}.

Sample preparation: An approximately measured sample denoted here as sample (A) between 100 and 1000 mg is introduced in to the silica crucible. It is weighed with crucible again to find out the exact net weight of the sample. There is no hard and fast rule is followed to convert the sample into ash as the each sample has the specific physical, chemical nature and different quantities. Using a red hot (450-500 ºC) muffle furnace is better than the open flame technique as the open flame may add shoots at the sides of the crucible. The completion of this conversion can be ensured by keeping the sample crucible repeatedly in a red hot oven for 1 h to produce constant weight at least three times consecutively. The ash obtained may be of any colour like white, black, grey, some of the inorganic matters (minerals and drugs) may produce brown, red *etc*.

TABLE-1
ASSAY PROTOCOL FOR COLORIMETRIC ASSAY

Particulars	Reagent blank	Working standard					Sample blank	Samples
		S_1	S_2	S_3	S_4	S_5		
Vol. of working std. (mL)	-	0.5	1.0	1.5	2.0	2.5	0.0	0.0
Amount of iron (mM)	-	0.5	1.0	1.5	2.0	2.5	-	-
Vol. of prepared digested mix (B) (mL)	-	-	-	-	-	-	0.1–3.0 *Distilled water	0.1–3.0 *Sample
Vol. of 2N HCl (mL)	Make up all the volume to 9.0 mL							
Vol. of KSCN (mL)	1.0 mL added to all the tubes							
OD λ 478	Q	R_1	R_2	R_3	R_4	R_5	S	T

*Take equal volume of sample and water in separate crucibles and process them as per preparation protocol. Take equal volume of digested mix (B) for this assay protocol

To this ash added 5 mL of 10 % hydrogen peroxide or 5 % of sodium hypochlorite (alternatively 0.001 M acidified potassium permanganate or potassium dichromate may be used as oxidants. The decolourization reaction will indicate the presence of residual Fe^{2+} but may need background colour correction in the photometric system) to cover the entire ash in such away adhered at the sides to reach the bottom. It is kept again in a hot oven at 100 ºC to boil this content. This step is important to convert all the Fe^{2+} to Fe^{3+} though the simple boiling with water for 0.5 h is sufficient for oxidation. This treated ash is washed by 2 mL of 10 M hydrochloric acid into a graduated/calibrated 20 mL centrifuge tubes during this wash acid is retained at the crucible for 0.5 h with gentle rotation to dissolve ash particles trapped in the crucible. Rinsing should be at least thrice with about 2 mL of redistilled water. This acid treated ash solution is kept in a boiling water bath for 0.5 h for the conversion of iron to form soluble $FeCl_3$. Finally it was made up to 10 mL with water to become the acid strength to 2N. It is centrifuged at 2000 rpm for 10 min to get clear supernatant. This supernatant is called digested mix (B). A simple flow chart is also given for this sample preparation (Fig . 1).

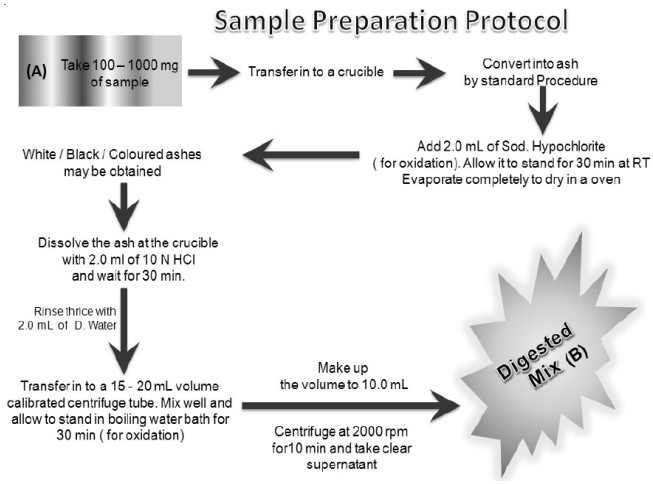


Fig. 1. Simple flow chart-sample preparation protocol/sample pre-treatment process

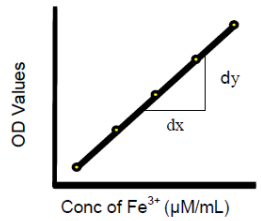
Assay protocol : Table-1 explains the system followed for the estimation of iron by spectrometer. No incubation time and heating are required.

Calculation: According to assay protocol calculate the optical density (OD) as follows:

Standard OD = R–Q

Sample OD = (T–Q)–(S–Q)

Detailed calculation along with standard graph is shown in Fig. 2.



$$\frac{dx}{dy} \times \text{Concentration of Working Stand (mM)} = \mathbf{F}$$

$$\text{The amount of Iron present in the Sample (mg \%)} = \frac{\text{Sample OD X F X 100 X M.Wt}}{\mathbf{A}\text{ (mg) X }\mathbf{B}\text{ (mL)}}$$

Fig. 2. Model standard graph and calculation setps

Colorimeters having measuring range up to the OD 2 should use the 0.5-1 mL of working standard. Single standard in triplicates is sufficient for routine clinical assays. Better result can be made for food and faecal matter analysis. When sample is expected to have high or low level of iron it can be brought to the measuring range of this method of analysis either by taking low or high amount of sample A (mg) and digested mix B (mL) till we get the desired colour intensity. The weight of sample taken A (mg) and quantity of digested mix B (mL) can be directly substituted in the formula for calculation of iron content. A simple flow diagram given in Fig. 3.

Validation: Though there are some methods[2-6] for the estimation of iron colorimetrically. This method is compared with different analytical method and instrument (AAS) as there is a need to measure very low concentration. Sample (10, 20, 30, 40 and 50 ppm) was prepared from the ferric nitrate stock standard (brand MERCK provided for AAS). It was analyzed by this assay protocol colorimetrically (method A) using Lambda 25, UV/VIS spectrometer (Perkin Elmer, USA) and by atomic absorption spectrometer (method B) using A Analyst 400 (Perkin Elmer, USA). The results of 10 repeated analyses showed target values very close to each other with no significant difference ($P < 0.05$). Statistics was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA.

Table-2 exhibits the data analyzed for the variance by unpaired t test and F test. These two tests show no significant difference in measured values. Bland-Altman representation

TABLE-2
VALIDATION DATA ANALYZED FOR THE VARIANCE BY UNPAIRED T TEST AND F TEST

Table analyzed	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm
Column A vs Column B	Method A vs Method B	Method A vs Method B	Method A vs Method B	Method A vs Method B	Method A vs Method B
Unpaired t test					
Are means significantly different? (P < 0.05)	No	No	No	No	No
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
Mean ± SEM of column A	10.09 ± 0.082 N=10	20.02 ± 0.073 N=10	30.06 ± 0.123 N=10	40.04 ± 0.118 N=10	50.31 ± 0.087 N=10
Mean ± SEM of column B	10.02 ± 0.080 N=10	20.11 ± 0.064 N=10	30.17 ± 0.108 N=10	40.01 ± 0.107 N=10	50.22 ± 0.103 N=10
F test to compare variances					
Are variances significantly different?	No	No	No	No	No

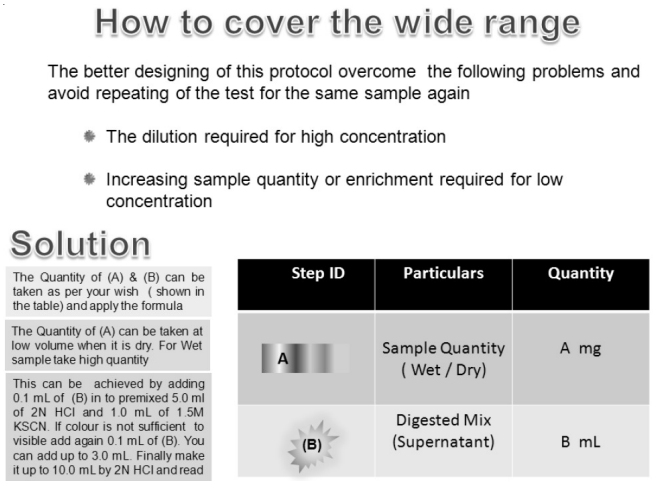


Fig. 3. Simple chart shows, how to bring the sample concentration to the measuring range of this methodology when expected to have extreme levels

(Fig. 4) shows the bias range less than 5 % (-4.17 to 4.17 %) between these methods. There is a good correlation and regression was obtained for methods (R squared: for method A 0.9995 & for Method B 0.9996 in both correlation and regression). It is observed that these two methods are linear to the measured range 10-50 ppm (Figs. 5 and 6).

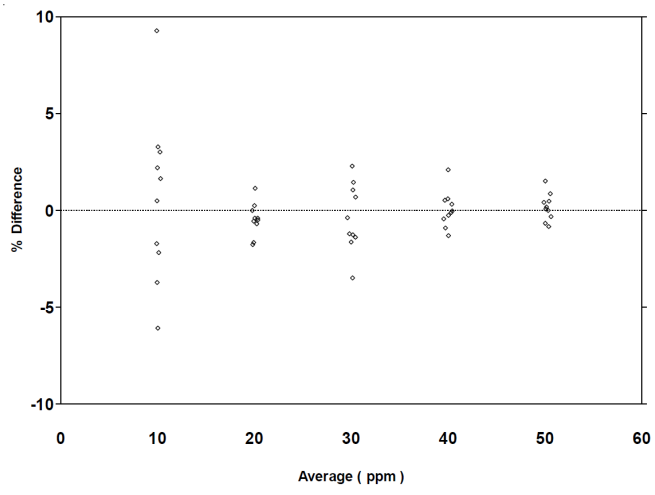
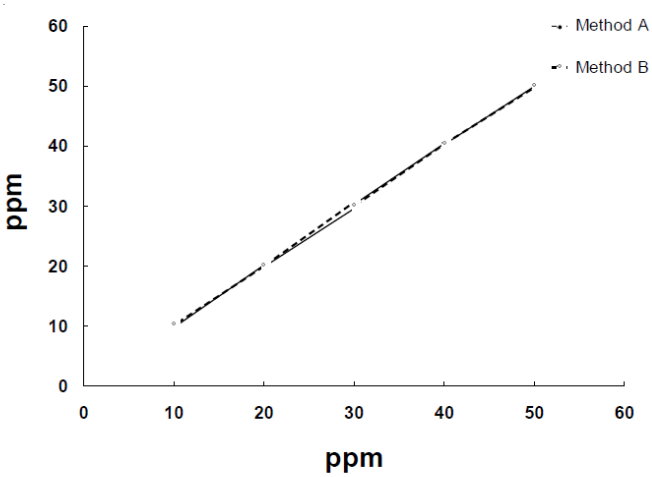
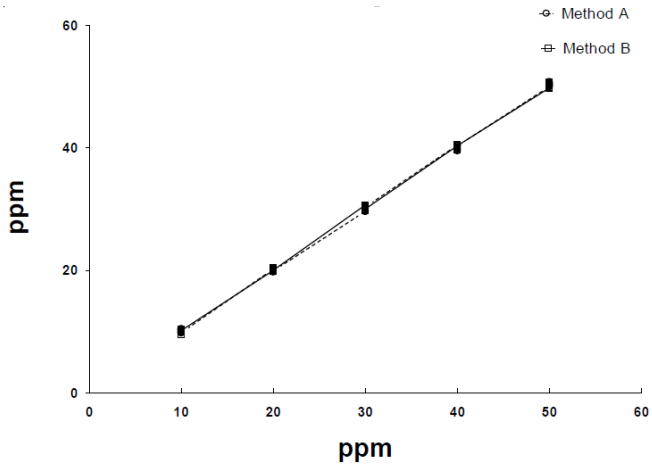


Fig. 4. Bland-Altman representation for method A and B



Correlation	Method A	Method B
Number of XY Pairs	50	50
Pearson r	0.9998	0.9998
95% confidence interval	0.9996 to 0.9999	0.9996 to 0.9999
P value (two-tailed)	< 0.0001	< 0.0001
P value summary	***	***
Is the correlation significant? (alpha=0.05)	Yes	Yes
R squared	0.9995	0.9996

Fig. 5. Correlation between method A and B



Linear Regression Analysis	Method A	Method B
Slope	1.005 ± 0.003	1.003 ± 0.002
1/slope	0.9954	0.9970
r^2	0.9995	0.9996
P value	< 0.0001	< 0.0001
Deviation from zero	Significant	Significant
Total number of values	50	50
Number of missing values	0	0

Fig. 6. Regression analysis of method A and B

RESULTS AND DISCUSSION

Ferric thiocyanate is a brick-red complex ion which is stable in an aqueous environment. In this aqueous environment, the ferric ion (Fe^{3+}) actually exists as a hydrated octahedral complex, more correctly denoted $Fe(H_2O)_6^{3+}$. In presence of thiocyanate ion (SCN^-), one of the water molecule ligands is displaced and the ferric thiocyanate ion $[Fe(H_2O)_5SCN]^{2+}$ is produced. When the reaction is initiated, the concentration of reactants are high and the rate of the forward reaction is great. Once the product concentration start reaches higher concentration than the reactant level the reaction will start to reverse the reactant back. Hence, forward reaction is maintained by taking high concentration of (1.5 M) potassium thiocyanate. This concentration is thousands times higher than that of iron present in the working standard (1.0 µM/mL $FeCl_3$) hence the complete conversion of iron to iron thiocyanate complex is ensured.

Since, the ferric thiocyanate $[Fe(SCN)^{2+}]$ product is brick-red it can be measured colorimetrically. As the experimental (working iron standard concentration) is thousands times lower than the equilibrium concentration it has high linearity. Hence, 1 mole Fe^{3+} consumed = 1 mole $Fe(SCN)^{2+}$ produced, this method is very much suitable to colorimeter.

As this method is designed to solve the following problems it adds further strength to the existing methodology reviewed from the literatures[2-6]. As the organic matters are completely removed from tissue materials by forming ash, iron in complexes and intact is released out, they could take part in estimation. The coverage of wide range iron content samples can be estimated (Fig. 2). This method can also be modified conveniently for the micro volume analysis, instrument free rapid visual screenings.

Points to remember: This method is specific to Fe^{3+} so, for the total iron estimation a strong oxidizing chemical must be added to convert all Fe^{2+} to Fe^{3+}.

Fe^{3+} and KSCN reaction is reversible hence, a very high concentration of KSCN to be maintained in the reaction media to stop the reverse reaction that causes colour fading. A strong acid medium is important for the iron-thiocyanate complex to get the peak absorption at the narrow band.

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