QUANTITATIVE ASSESSMENT OF IRON IN FORTIFIED RICE KERNEL: COMPARATIVESTUDY OF VALIDATION PARAMETERS BY ICP-OES AND AAS

[a]*Swapnila Roy*

[a]*School of Basic & Applied Sciences,Lingayas Vidyapeeth*

ABSTRACT

In this investigation, methodology for the estimation of Iron in Fortified Rice Kernel (FRK) was validated and verified. The analysis of Iron(Fe) was performed by ICP-OES (Inductively Coupled Plasma -Optical Emission Spectroscopy) and AAS(Atomic absorption spectrometry). The comparative study has been performed for Iron(Fe) in FRK for the reliability of results. LOD(Limit of Detection), LOQ(Limit of Quantification), recovery(%), accuracy, precision, specificity, suitability, robustness study were carried out for the method validation. The improved techniques demonstrated great recovery, repeatability, relative standard deviation of peak regions, and high coefficients of determination. In the quantification of Iron in Spiked Iron Rice Kernel, the optimised techniques displayed dependability and sensitivity. In this study, validation parameters assessment has been conducted to verify the quantification of fortification as well as comparative study also has been performed for measurement of Iron FRK in ICP-OES and AAS.

KEYWORD

Iron; Quantitative Assessment; Comparative Study; Method Validation; Fortified Rice Kernel; ICP-OES; AAS

INTRODUCTION

A significant global strategy for the prevention of micronutrient deficiencies in many communities is food fortification(Anjos et al.,2002).According to the Food and Agriculture Organisation, Rice is one of the cereals that is produced the most often worldwide and is a staple meal for the majority of people, particularly in developing nations(Bešter et al.,2003; Bozym et al.,2015). Given that Rice is a staple diet for more than 3 billion people globally, Rice has a tremendous amount of promise as a vehicle for vitamin fortification(Angeles-Agdeppa et al.,2011; Mei et al.,2011). Due to the practise of washing and boiling Rice with

excessive water, which causes the leaching of micronutrients used for enrichment, previous attempts to fortify Rice flour have failed(Engle-Stone et al.,2013; Towett et al.,2016). To overcome this obstacle, new technology was developed (Byers et al.,2016; Byers et al.,2019).

Broken and cracked grains can be turned into Rice flour, combined with a binder and other nutrients, and refurbished by extrusion as reconstituted Rice grains with the same size, shape, and texture as conventional Rice(Figueiredo et al.,2016; Turner et al.,2017). Broken and cracked grains typically make up 20% to 30% of the production and are usually intended for animal feed(Losso et al.,2017). Rice grains that have been treated with iron to improve their nutritional value are known as iron-fortified rice kernels(Kaur et al., 2022; Song et al.,2012). The synthesis of haemoglobin, a protein in red blood cells that transports oxygen throughout the body, depends critically on iron, a vital element(Losso et al.,2017). The development of children's cognitive abilities and the correct immune system function both depend on iron(Rodriguez-Iruretagoiena et al.,2015). The WHO recommends ferrous sulphate, ferrous fumarate, encapsulated ferrous sulphate or fumarate, electrolytic iron (at double the iron amount as ferrous sulphate), and ferric pyrophosphate(FePP) (at double the amount of ferrous sulphate) for iron fortification of most foods. Hurrell et al., 2018 evaluated iron compounds used for food fortification most recently. Iron and other nutrients are sprayed onto the surface of the rice grains to create fortified rice kernels. When people who eat rice as a primary diet undergo this process, it is known as fortification, it helps to guarantee that they have access to appropriate quantities of iron(Sanghvi et al.,2010). Demand is higher for iron-fortified foods that are a part of extensive national programmes. They seek to close the gap between the at-risk populations' present iron consumption and their required iron intake by using one or more dietary vehicles(Muthayya et al.,2012) . The WHO has prepared tables for women and children that show the chance of insufficiency at various daily iron intakes in proportion to the predicted dietary iron bioavailability (5%, 10%, or 15%)(Allen et al.,2006). As a result, the WHO recommends the full probability approach. The degree of fortification is then selected to reduce the likelihood of iron deficiency to between 2% and 3%(Radhika et al.,2011).

According to eight effectiveness studies, six used micronized ground FePP (MGFP) (particle size 2.5 m) and two used micronized dispersible FePP (MDFP) (particle size 0.3 m and encapsulated) in women or children who consumed iron-fortified extruded premix rice

(Beinner et al.,2010). The iron status of children in India and Brazil improved significantly in the three studies that provided additional iron intake above the 14 mgFe/d recommended for

FePP, whereas two of the three studies that provided an additional 7–10 mg of additional Fe reported inconsistent effects on iron status(Hackl et al.,2016). More than 50 countries consume more rice than the required 75 g per people per day for a national iron fortification programme. Rice is mostly consumed in Asia, but it has also become a staple in numerous African and Latin American nations(Muthayya et al.,2012). A little bit more than half of the rice used for human consumption is industrially milled and has the potential to be fortified with micronutrients, but the majority is still ground by farmers in tens of thousands of small- and medium-sized mills using antiquated equipment.In areas where iron insufficiency is frequent, fortified rice kernels are a practical and affordable strategy to boost iron consumption following validated guidelines. Thus, the present study aimed to validate methods for the quantitative assessment as well as comparative study of validation of estimation of Iron by ICP-OES and AAS in Fortified Rice Kernel(FRK).

MATERIAL AND METHODS

Determination by ICP-OES

The following chemicals are required for estimation of Iron in ICP-OES(AGILENT 5100). Concentrated Nitric Acid (Purity- 69%, FINAR), Hydrogen Peroxide (Purity -30%, RANKEM), CRM (Certified Reference Materials) / Stock Solution - Iron (Purity - 1000 mg/Kg, NIST, Merck).

The instruments required for the determination of Iron are: Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), Microwave Digester(MARS), Analytical Balance(METTLER-TOLEDO), Micro Pipettes(Eppendorf) (20 -200 µL) & (100 -1000 µL).

Preparation of Sample & Spike Sample Solution

Firstly,50 gm of Rice Sample was grinded and 0.25 g (± 0.05 g) was taken and 2.0 mL of Hot Ultrapure Water and 1.0 mL Hydrogen Peroxide were added. Then it was transferred to Microwave Digestion Closed Vessel and 0.5 mL of Nitric Acid was added, kept at room temp for 5 min to predigest the Sample. Then Microwave Vessel was closed tightly and kept at room temperature for 5 minutes. After digestion the Vessel was cold at room temperature and 10 ml ultrapure water was added. After mixing properly it was transferred

to 100 mL Volumetric Flask(BOROSIL) and volume was made up to 100 ml with ultrapure water. Finally the filtrate(WHATMAN) was used for the injection on ICP-OES.
Similarly, the spike sample solution was prepared by adding 0.5 ml Stock solution of Iron in the 0.25 g (± 0.05 g) grinded rice sample and rest all the steps were same as above.

Preparation of Blank (5% Nitric Acid), Intermediate Stock(100 mg/Kg) andCalibration Standard Solution

7.25 mL of Nitric Acid (69%) was transferred in 100 mL Ultrapure Water in Glass Bottle and mixed vigorously. This solution was treated as Blank. Then 1.0 ml from stock solution of iron (1000 mg/Kg) was transferred in 10 ml volumetric flask and 0.5 ml nitric acid and made up the volume till 10 ml volumetric flask by ultrapure water and mixed very well. This is Intermediate stock solution. From this intermediate stock solution different calibration standard solution are prepared.
For the standard and sample solutions

- Limit of Detection 0.5 mg/Kg with respective to the Standard.
- Limit of Quantification 1.0 mg/Kg in with respective to the Standard.
- Limit of Quantification 400 mg/Kg in with respective to the Sample.

Operating Conditions of ICP-OES

The operating conditions for ICP-OES are mentioned as below. The Plasma flow (Argon 12L/min), Nebulizer flow (0.7 L/min),RF power 1.2 KW, Uptake Delay 25sec,Pump Speed12 rpm, Stabilization 15 sec, Numbers of Replicates 3.0,Resolution Normal

Wavelength 238.204 nm. For Iron Read Time was 5 sec, Aux flow1.0 L/min, Viewing Mode was Radial.

Calculation

The analysis was carried out checking Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve. Iron Content in Fortified Rice Kernel is calculated using the following equation:

$$\text{Iron (mg/Kg)} = \frac{\text{Instrument Conc. (mg/Kg) X Make-up Volume (mL)}}{}$$

Sample Weight**(**gm)

Then the recovery of Iron at Spike level 2000 mg/Kg in Sample is calculated using the following equation:

$$\text{Recovery (\%)} = \frac{(A - B) \times 100}{C}$$

where

A = Concentration of Iron in the spiked sample (mg/Kg)
B = Content of Iron in the control sample (mg/Kg)
C = Spiked concentration of Iron (mg/Kg)

Determination by AAS

The following chemicals are required for estimation of Iron in AAS(AGILENT FS-AA). Concentrated Nitric Acid (Purity- 69%, HONEYWELL), Hydrogen Peroxide (Purity -30%, RANKEM), CRM (Certified Reference Materials)/Stock Solution - Iron (Purity - 1000 mg/Kg, SUPELCO).The instruments required for the measurement of Iron are: Microwave Digester(Mars), Analytical Balance(METTLER-TOLEDO), Micro Pipettes(EPPENDORF) (20-200 µL) & (100 -1000 µL), AAS(AGILENT FS-AA).

Preparation of Sample & Spike Sample Solution

The sample solution preparation method was same as above.

Preparation of Blank(5% Nitric Acid), Intermediate Stock(100 mg/Kg),Bracketing standard and Calibration Standard Solution

7.25 mL of Nitric Acid (69%) was transferred in 100 mL Ultrapure Water in Glass Bottle and mixed vigorously. This solution was treated as Blank. Then 1.0 ml from stock solution of iron (1000 mg/Kg) was transferred in 10 ml volumetric flask and 0.5 ml nitric acid and made up the volume till 10 ml volumetric flask by ultrapure water and mixed very well. This is Intermediate stock solution. The bracketing standard solution and different calibration standard solution are prepared as mentioned below was prepared from this intermediate stock solution.

Operating Conditions for AAS

The operating conditions for AAS is mentioned as below. The Hollow cathode Lamp was Iron (Fe), Absorption Wavelength (nm)was 372.0Slit Width (nm) was 0.2, Signal-Type Atomic Absorption ,Signal -Measurement Integration, Oxidant Air,Oxidant Flow (L/min)13.5,Acetylene Flow (L/min) was 2,Equation was Linear.

Calculation

Iron Content in Fortified Rice Kernel is calculated using the following equation:

$$\textbf{Iron (mg/Kg)} = \frac{\text{Instrument Conc.(mg/Kg) X Make-up Volume(ml)}}{\text{Sample Weight (g)}}$$

Then the recovery of Iron at Spike level in sample is calculated using the following equation:

$$\textbf{\% Recovery} = \frac{\text{Found Sample Conc.(mg/Kg) X 100}}{\text{Actual Sample Conc. (mg/Kg)}}$$

Method Validation

Based on guidelines (AOAC & ICH) for Analytical Method Validation, the below mentioned Method Validation Parameters such as Specificity, System Suitability, Precision at Limit of Detection (LOD),Precision at Limit of Quantification (LOQ),Linearity, Method Precision, Accuracy, Intermediate Precision, Robustness study were conducted.

RESULTS AND DISCUSSION

Quantification by ICP-OES

Specificity

The Specificity of the Method was conducted by Injecting One (1) Blank along with One (1) Linearity Solution-1 to ensure that there is no Significant Interference from other components of similar nature(not mentioned results). From the Specificity Study it is concluded that the % interference is lower than 30% and it meets the acceptance criteria. So the method is specific.

System Suitability

The Suitability of the Instrument (ICP OES) was conducted by Injecting Six (6) Replicate Standard Solutions (of Linearity standard - 2) to ensure that the Intensity variation between all the Six (6) Standards was within 20% of the Relative Standard Deviation (RSD) or in other words the % RSD Value for all Six (6) Standards was not exceed 20%(**Table 1).**From the System Suitability Study, it is concluded that method is suitable.

Table 1: SYSTEM SUITABILITY STUDY

SL.NO.	NAME OF INJECTIONS	INTENSITY
1	Standard Solution - 1	21389.29
2	Standard Solution - 2	21387.73
3	Standard Solution - 3	21211.43
4	Standard Solution - 4	21488.27
5	Standard Solution - 5	21334.29
6	Standard Solution - 6	21415.98

Average	21371.17
Stdev	92.95
% RSD	**0.43**

Precision at Limit of Detection (LOD)

The Precision at Limit of Detection (LOD) of Solution was conducted to ensure that the Intensity variation between all the Six (6) Standards must be within 33% of the Relative Standard Deviation (RSD)(**Table 2**) .

Table 2: PRECISION AT LOD STUDY		
SL.NO.	**NAME OF INJECTIONS**	**INTENSITY**
1	LOD Solution-1	5593.31
2	LOD Solution-2	5572.37
3	LOD Solution-3	5543.65
4	LOD Solution-4	5533.87
5	LOD Solution-5	5520.80
6	LOD Solution-6	5476.98
Average		5540.16
Stdev		40.70
% RSD		**0.73**

So it is concluded that the Precision at Limit of Detection (LOD) Study meets the Requirements of the Acceptance Criteria.

Precision at Limit of Quantitation(LOQ)

The Precision at Limit of Quantitation (LOQ Solution) was conducted to ensure that the Intensity variation between all the Six (6) Standards must be within 20% of the Relative Standard Deviation (RSD)(not mentioned results).

So it is concluded that the Precision at Limit of Quantification(LOQ) Study meets the Requirements of the Acceptance Criteria.

Linearity

The Linearity study was conducted by Injecting Seven (7) Linearity Solutions at Different Level to ensure that the Correlation Coefficient (R^2) value must exceed 0.990(**Table 3**).

So it is concluded that the plot of the graph is linear and the Linearity Study meets the Requirements of the Acceptance Criteria.

Table 3: LINEARITY STUDY			
SL.NO.	**NAME OF INJECTIONS**	**LEVEL, mg/Kg**	**INTENSITY**
1	Linearity Solution (LS) - 1	**0**	23.09
2	Linearity Solution (LS) - 2	**1**	10759.36
3	Linearity Solution (LS) - 3	**2**	21228.14
4	Linearity Solution (LS) - 4	**5**	54457.29
5	Linearity Solution (LS) - 5	**7.5**	80112.55
6	Linearity Solution (LS) - 6	**10**	106582.59
7	Linearity Solution (LS) - 7	**15**	160931.56
8	Linearity Solution (LS) - 8	**20**	214085.9
Squared Correlation Coefficient (R^2)			1.000
Correlation Coefficient (R)			**1.000**

Method Precision

The Method Precision study was conducted by Injecting Six (1) Sample Solutions & Six (6) Spiked Sample Solutions at 2000 mg/Kg to ensure that the % RSD(not mentioned results**).**

So it is concluded that the Method Precision Study meets the Requirements of the Acceptance Criteria(not be more than 20.0%) and it is precise.

Accuracy

The Accuracy study was conducted by Injecting Six (1) Sample Solutions & Six (6) Spiked Sample Solutions at 400 mg/Kg, 2000 mg/Kg and 3000 mg/Kg Level to ensure that the % Recovery of found Concentration against the added concentration should be between 70% to 120 %(**Table 4).** So it is concluded that the Accuracy Study meets the Requirements of the Acceptance Criteria and the method is accurate.

Table 4:ACCURACY STUDY			
SL.NO.	**FOUND CONC.(mg/Kg)**	**ADDED CONC.(mg/Kg)**	**% RECOVERY**
Accuracy at 400 mg/Kg	378.52	400.00	94.63
	391.22	400.00	97.80
	387.32	400.00	96.83
	392.31	400.00	98.08
	384.16	400.00	96.04
	391.37	400.00	97.84
Accuracy at	1996.04	2000.00	99.80
	1997.61	2000.00	99.88
	1992.85	2000.00	99.64

2000 mg/Kg	1995.23	2000.00	99.76
	1999.20	2000.00	99.96
	1979.41	2000.00	98.97
Accuracy at 3000 mg/Kg	2890.17	3000.00	96.34
	2990.84	3000.00	99.69
	2954.73	3000.00	98.49
	2982.46	3000.00	99.42
	2972.54	3000.00	99.08
	2969.00	3000.00	98.97

Intermediate Precision

The Intermediate Precision study was conducted by Injecting Six (1) Sample Solutions & Six (6) Spiked Sample Solutions at 2000 mg/Kg Level by different day and different analyst to ensure that the % RSD of found Concentration in Spiked Sample Solution should not exceed 20.0%(not mentioned results). So it is concluded that the Intermediate Precision Study meets the Requirements of the Acceptance Criteria and the method is rugged.

Robustness

The Robustness study was conducted by Injecting Six (6) Sample Solutions & Six (6) Spiked Sample Solutions at 2000 mg/Kg Level by small changing in method to ensure the robustness of method (2 different conditions) by calculating the % Recovery of found Concentration against the added Concentration should be between 70% to 120 %.

So it is concluded that the Robustness Study meets the Requirements of the Acceptance Criteria and the method is robust.

Quantification by AAS
Specificity

The Specificity of the Method study was conducted by Injecting One (01) Blank along with One (01) Linearity Solution -1 to ensure that there is no significant interference from other components of similar nature(not mentioned results**).**

So the Specificity Study meets the Requirements of the Acceptance Criteria(lower than 30% of the LOQ Area) and the method is specific.

System Suitability

The Suitability of the Instrument (AAS) was conducted by Injecting Six (6) Replicate Standard Solutions to ensure that the Area variation between all the Six (06) Standards must be within

20.0% of the Relative Standard Deviation (RSD) & the % RSD Value for all Six (6) Standards Solution along with Bracketing Standard Solution not exceed 20.0%.

In this case, the Standard Solution was prepared in the following way. Firstly, 0.20 mL from Intermediate Standard Solution – 1 was pipetted out and transferred to a10 mL Volumetric Flask. Then 0.50 mL of Nitric Acid was added and made up the volume upto 10 ml ultrapure water.

The Bracketing Standard Solution was prepared in the following way. Firstly, 1 mL from Intermediate Standard Solution – 1 was pipetted out and transferred to a10 mL Volumetric Flask. Then 0.50 mL of Nitric Acid was added and made up the volume upto 10 ml ultrapure water. This study was performed for Day 1 and Day 2(not mentioned results**)**.

So from this study it was concluded that the System Suitability Study meets the Requirements of the Acceptance Criteria.

Precision at Limit of Detection (LOD)

The Precision at Limit of Detection (of LOD Solution) was conducted to ensure that the Intensity Variation between all the Six (06) Standards must be within 33% of the Relative Standard Deviation (RSD) or in other words the % RSD Value of Intensity for all Six (6) Standards at LOD Level not be more than 33%(not mentioned results).

The Standard solution was prepared in the following way. Firstly, 0.25 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water. So from this study it was concluded that the Precision at Limit of Detection (LOD) Study meet the Requirements of the Acceptance Criteria.

Precision at Limit of Quantitation (LOQ)

The Precision at Limit of Quantitation (of LOQ Solution) shall be established to ensure that the Intensity Variation between all the Six (6) Standards must be within 20% of the Relative Standard Deviation (RSD) or in other words the % RSD Value of Intensity for all Six (6) Standards at LOQ Level must not exceed 20%(not mentioned results).

The Standard solution was prepared in the following way. Firstly,0.5 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Therefore, from this study it was concluded that the Precision at Limit of Quantitation (LOQ) Study meets the Requirements of the Acceptance Criteria.

Linearity

The Linearity study was conducted by Injecting Linearity Solutions at Different Level to ensure that the Correlation Coefficient (R^2) value must exceed 0.99. Here 6 different concentration of solution and bracketing standard solution were prepared as follows (not mentioned results).

Linearity Solution - 1 (5.0 mg/Kg)

0.5 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Linearity Solution - 2 (10.0 mg/Kg)

1.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Linearity Solution – 3 (20.0 mg/Kg)

2.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Linearity Solution – 4 (40.0 mg/Kg)

4.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Linearity Solution – 5 (60.0 mg/Kg)

6.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Linearity Solution – 6 (80.0 mg/Kg)

8.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water.

Bracketing Standard Solution (10.0 mg/Kg)

10.0 ml Intermediate Standard Solution – 1 was pipetted out and transferred it to a 10 mL Volumetric Flask. Then 0.5 mL of Nitric Acid was added and volume was made upto 10 ml ultra-pure water. Therefore, the Linearity Study meets the Requirements of the Acceptance Criteria.

Method Precision

The Method Precision study was conducted by injecting Six (6) Sample Solutions to ensure that the % RSD of found Concentration not exceed 20.0% & recovery be in between 70% to 120%(not mentioned results)

So, the Method Precision Study meets the Requirements of the Acceptance Criteria.

Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as conventional true value or an accepted reference value and the value found. To investigate the effect of the matrix on recovery analysed the different blank sample and spiked at the minimum three different concentration level of analyte and three replicates at each level. It is calculated as the % of recovery by the known amount of analyte in the sample.

The Accuracy study was performed by injecting one (1) Sample Solutions & Six (6) Spiked Sample Solutions at LOQ, 100% and 150% Level (500 mg/Kg, 2000 mg/Kg & 3000 mg/Kg) to

ensure that the % Recovery of found Concentration against the added Concentration should be between 70% to 120 %(not mentioned results).

$$\% \text{ Recovery} = \frac{(\text{Conc. of spiked sample} - \text{Sample conc.}) \times 100}{\text{Spike concentration}}$$

Spike Sample Solution-1: (500 mg/kg w.r.t sample)

Firstly,50 gm of Rice Sample was grinded and 0.25 g (± 0.05 g) was taken, 0.25 ml from Stock Solution of Iron (1000 mg/kg) added and 2.0 mL of Hot Ultrapure Water and 1.0 mL Hydrogen Peroxide were added. Then it was transferred to Microwave Digestion Closed Vessel and 0.5 mL of Nitric Acid was added, kept at room temp for 5 min to predigest the Sample. Then Microwave Vessel was closed tightly and kept at room temperature for 5 minutes. After digestion the Vessel was cold at room temperature and 10 ml ultrapure water was added. After mixing properly it was transferred to 50 mL Volumetric Flask and volume was made upto 50 ml with ultrapure water, then filtrate was used for injection on AAS.

Spike Sample Solution-2: (2000 mg/kg w.r.t sample)

Same as above, only 0.5 ml from Stock Solution of Iron (1000 mg/kg) added.

Spike sample solution-3: (3000 mg/kg w.r.t sample)

Same as above, only 1.5 ml from Stock Solution of Iron (1000 mg/kg) added.
Therefore, the Method Precision Study meets the Requirements of the Acceptance Criteria.

Intermediate Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

The Intermediate Precision study was conducted by injecting Six (6) Sample Solutions to ensure that the % RSD of found Concentration not exceed 20.0% & Recovery be in between 70% to 120%(not mentioned results).

Therefore, the Method Precision Study meets the Requirements of the Acceptance Criteria.

Robustness

The Robustness was performed at two conditions by Changing the Lamp Current at 4.0 Amp & at 6.0 Amp. And by injecting One (1) Sample Solution & Six (6) Spiked Sample Solution at 2000 mg/Kg to ensure that the % Recovery of found Concentration against the added Concentration should be between 70% to 120 %(not mentioned results).

So, the Robustness Study meets the Requirements of the Acceptance Criteria.

COMPARATIVE STUDY

From the comparative study of method validation parameters in ICP-OES and AAS are summarised below in **Table 5.** So it is concluded that this method validation for estimation of Iron in Iron Spiked Rice Kernel by ICP-OES & AAS is justified according to validation parameters. This methodology can be applied in the laboratory scale for the permissible limit of Iron in Fortified Rice Kernel.

Table 5: Comparative Study of Validation Parameters

PARAMETERS	ICP-OES	AAS	ACCEPTANCE CRITERIA
1. SPECIFICITY	0.049%	0.0557(Specificity Std @ LOQ)	such interference should be lower than 30% of the loq area.
2. SUITABILITY	• Intermediate Solution (% RSD 0.43) • Bracketing Solution(%RSD 0.60)	• Intermediate Solution(% RSD 0.3383) • Bracketing Solution(%RSD 3.4286)	(1)% RSD of intensity for six (06) standards (of system suitability solution preparation) should not exceed 20.0 %. (2)% RSD of intensity for six (06) standards (of
			system suitability solution preparation) with bracketing standard solution should not exceed 20.0 %.

3.LOD	% RSD 0.73	% RSD 1.5803	(1) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) The % RSD of Intensity of Six (06) Replicate Injections of Iron at LOD Standard Solutions should not be more than 33.0%
4.LOQ	% RSD 0.20	% RSD 0.5243	(1) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) The % RSD of Intensity of Six (06) Replicate Injections of Iron at LOQ Standard Solutions should not be more than 20.0%
5.LNEARITY	CORRELATION COEFFICIENT 1.0000	Correlation Coefficient (R^2) 0.9998(DAY 1) Correlation Coefficient (R2) 0.9996 (DAY 2)	(1) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) with Bracketing Standard Solution should not exceed 20.0 %. (3) The Correlation Coefficient (R2) value of Linearity Solutions should not be less than 0.99.
6.METHOD PRECISION	% RSD 0.89	% RSD 0.712	(1)% RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation)

			with Bracketing Standard Solution should not exceed 20.0 %. (3) The Correlation Coefficient (R2) value of Linearity Solutions should not be less than 0.990. (4) The % RSD of Iron of Six (06) Sample
			Preparation should not be exceed than 20.0%.
7.ACCURACY	RECOVERY(%) 1.500 PPM(96.87%) 2.2000PPM(98.94%) 3.3000PPM(97.95%)	RECOVERY(%) 1.500 PPM(104.6%) 2.2000PPM(99.44%) 3.4000PPM(99.65%)	(1) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) with Bracketing Standard Solution should not exceed 20.0 %. (3) The Correlation Coefficient (R2) value of Linearity Solutions should not be less than 0.990. (4) % Recovery of three (03) Sample Solutions at each level of accuracy should be in between 70% to 120%.
8.INTERMEDIATE PRECISION	% RSD 1.30	% RSD 2.268	(1) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) should not exceed 20.0 %. (2) % RSD of Intensity for Six (06) Standards (of System Suitability Solution Preparation) with Bracketing Standard Solution

CONCLUSION

The measurement of iron in FRK showed the reliability and sensitivity of the validated methods in ICP-OES and AAS. The improved techniques demonstrated great sensitivity, excellent linearity, outstanding recovery rates, strong repeatability, and low detection and quantification limitations according to the comparative

study. The correlation coefficient forIron in both instruments follow acceptance criteria. Furthermore, the methods were performed in following acceptance criteria(all validation parameters) reflecting positively onthe economy of reagents and analysis times.

REFERENCES

Allen L.H., De Benoist B., Dary O., Hurrell R.F., editors.(2006) Guidelines on Food Fortification with Micronutrients. *World Health Organization and Food and Agricultural Organization of the United Nations*; Geneva, Switzerland.

Angeles-Agdeppa, I., Saises, M., Capanzana, M., Juneja, LR., Sakaguchi, N. (2011) "Pilot- scale commercialization of iron-fortified rice: effects on anemia status", *Food Nutr Bull.* **32(1),**3-12. doi: 10.1177/156482651103200101. PMID: 21560459.

Anjos, M. J., Lopes, R. T., Jesus, E. F. O., Simabuco, S. M., & Cesareo, R. (2002)."Quantitative determination of metals in radish using X-ray fluorescence spectro-Metr,". *X-Ray Spectrometry*, **31(2),** 120–123.

Beinner M.A., Velasquez-Meléndez G., Pessoa M.C., Greiner T.(2010) "Iron-fortified rice is as efficacious as supplemental iron drops in infants and young children". *J. Nutr.* 49–53. doi: 10.3945/jn.109.112623.

Bešter, P. K., Lobnik, F., Eržen, I., Kastelec, D., & Zupan, M. (2013) "Prediction of cadmium concentration in selected home-produced vegetables", *Ecotoxicology and Environmental Safety*, 96, 182–190.

Bozym, M., Florczak, I., Zdanowska, P., Wojdalski, J., & Klimkiewicz, M. (2015) "An analysis of metal concentrations in food wastes for biogas production" *Renewable Energy*, 77,467–472.

Byers, H. L., McHenry, L. J., & Grundl, T. J. (2016) "Forty-nine major and trace element concentrations measured in soil reference

materials NIST SRM 2586, 2587, 2709a,2710a and 2711a using ICP-MS and wavelength dispersive-XRF", *Geostandards and Geoanalytical Research***,** 40(3), 433–445.

Byers, H. L., L. J. McHenry., T. J. Grundl. (2019) "XRF techniques to quantify heavy metals in vegetables at low detection limits," *Food Chemistry,*1,1-10.

Engle-Stone, R., Nankap, M., Ndjebayi, A.O.(2013) "Plasma ferritin and soluble transferrin receptor concentrations and body iron stores identify similar risk factors for iron deficiency but result in different estimates of the national prevalence of iron deficiency and iron-deficiency anemia among women and children in Cameroo",. *J.Nutr.* 143,369–377.

Figueiredo, A., Fernandes, T., Costa, I. M., Gonçalves, L., & Brito, J. (2016) "Feasibility of wavelength dispersive X-ray fluorescence spectrometry for the determination of metal impurities in pharmaceutical products and dietary supplements in view of regulatory guidelines", *Journal of Pharmaceutical and Biomedical Analysis,* 122, 52–58.

Hackl L., Cercamondi C.I., Zeder C., Wild D., Adelmann H., Zimmermann M.B., Moretti D.(2016) "Co-fortification of ferric pyrophosphate and citric acid/trisodium citrate into extruded rice grains doubles iron bioavailability through in situ generation of soluble ferric pyrophosphate citrate complexes." *Am. J. Clin. Nutr.* 1252–1259. doi: 10.3945/ajcn.115.128173.

Hurrell R.F.(2018) "Efficacy and Safety of Iron Fortification. In: Mannar M.G.V., Hurrell R.F.,editors. Food Fortification in a Globalized World." *Academic Press; London, UK*, 196–212.

Kaur, N., Agarwal, A., Sabharwal, M. (2022) "Food fortification strategies to deliver nutrients for the management of iron deficiency anaemia", *Curr Res Food Sci.* 5,2094-2107. doi: 10.1016/j.crfs.2022.10.020. PMID: 36387591; PMCID: PMC9641006.

Losso, JN., Karki, N., Muyonga, J., Wu Y., Fusilier, K., Jacob, G., Yu, Y., Rood, JC., Finley, JW., Greenway, FL. (2017) "Iron retention in iron-fortified rice and use of iron- fortified rice to treat women with iron deficiency: A pilot study", *BBA Clin.* 8,78-83. doi: 10.1016/j.bbacli.2017.09.001. PMID: 28966915; PMCID: PMC5608553.

Mei, Z., Cogswell, ME., Looker, AC., Pfeiffer, CM., Cusick, SE., Lacher, DA., Grummer- Straw, LM.(2011) " Assessment of Iron status in US pregnant women from the National Health and Nutrition Examination Survey (NHANES), 1999–2006," *Am J Clin Nutr.* 93(6),1312-1320.

Muthayya S., Hall J., Bagriansky J., Sugimoto J., Gundry D., Matthias D., Prigge S., Hindle P., Moench-Pfanner R., Maberly G.(2012) Rice fortification: An emerging opportunity to contribute to the elimination of vitamin and mineral deficiency worldwide. *Food Nutr. Bull*. 296–307. doi: 10.1177/156482651203300410.

Radhika M.S., Nair K.M., Kumar R.H., Rao M.V., Ravinder P., Reddy C.G., Brahmam G.N. (2011) Micronized ferric pyrophosphate supplied through extruded rice kernels improves body iron stores in children: A double-blind, randomized, placebo- controlled midday meal feeding trial in Indian schoolchildren. *Am. J. Clin. Nutr.* 1202–1210. doi: 10.3945/ajcn.110.007179.

Rodriguez-Iruretagoiena, A., Trebolazabala, J., Martinez-Arkarazo, I., Diego, A., & Madagascar,M. (2015) "Metals and metalloids in fruits of tomatoes (Solanum perspicuous)and their cultivation soils in the Basque Country: Concentrations and accumulation J. trends", *Food Chemistry*, 173, 1083–1089.

Sanghvi, TG., Harvey, PW., Wainwright, E. (2010) "Maternal Iron–folic acid supplementationprograms: Evidence of impact and

implementation", *Food Nutr Bull,* 31(Supplement 2), 100-107.

Song, X., Hu, X., Ji, P., Li, Y., Chi, G., & Song, Y. (2012) "Phytoremediation of cadmium contaminated farmland soil by the hyperaccumulator Beta vulgaris L. var. cicla",*Bulletin of Environmental Contamination and Toxicology*, 88(4), 623–626.

Towett, E. K., Shepherd, K. D., & Drake, L. B. (2016) "Plant elemental composition andportable X-ray fluorescence (pXRF) spectroscopy: Quantification under different analytical parameters." *X-Ray Spectrometry*, 45(2), 117–124.

Turner, A., Poon, H., Taylor, A., & Brown, M. T. (2017) "In situ determination of trace elements in Fucus spp. by field-portable-XRF", *Science of the Total Environment*,593–594, 227–235.