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Rapid Method for Quantification of Iron (Fe^{+3}) from Ferrazone (NaFe-EDTA) in Fortified Wheat Flour

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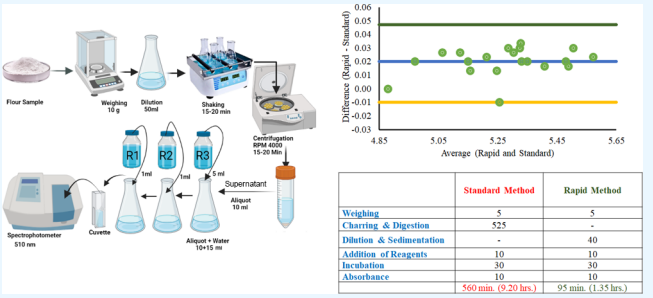
ABSTRACT: Conventional methods for quantifying the added iron in wheat flour are time-consuming and costly. A rapid method (Time/Sample: 95 min) was developed by modifying the conventional standard method (Time/Sample: 560 min) and validated. Linearity and linear regression of the rapid method presented excellent correlation coefficient (R^2) values (0.9976 to 0.9991), which were close to 1, while the limits of agreement (LOA) were in the range of −0.01 to 0.06 mg/kg. The limits of detection (LOD)/specificity and limits of quantitation (LOQ)/sensitivity values were found to be 0.03 and 0.09 mg/kg, respectively. The rapid method was subjected to validation, wherein the precision of intra-assay, inter-assay, and inter-person was determined to be within the range of 1.35−7.25%. These results indicate a high level of accuracy and precision of the method. The percent relative standard deviation (RSD) for recoveries at varying spiking levels, that is, 5, 10, and 15 mg/kg, was determined at 1.33 lying far below the upper limit of acceptability (RSD < 20). Overall, the developed rapid method can be sustainably alternate for conventional methods owing to its ability to produce accurate, precise, robust, and reproducible results.



	Standard Method	Rapid Method
Weighing	5	5
Charring & Digestion	525	-
Dilution & Sedimentation	-	40
Addition of Reagents	10	10
Incubation	30	30
Absorbance	10	10
	560 min. (9.20 hrs.)	95 min. (1.35 hrs.)

1. INTRODUCTION

Malnutrition is prevalent among Pakistani population, especially among preschoolers (age 2−6 years) and women aged 15−49 years mainly due to poor financial status, shortage of food, and deficiency of basic knowledge about nutrition. According to National Nutrition Survey (NNS 2018) of Pakistan, 53.7% of Pakistani children are anemic, 40.2% stunted, 40% are facing Zn and vitamin-D deficiency, and 18.2% women of reproductive age (WRA) have severe iron deficiency.[1] Anemia is associated with iron deficiency,[2] while iron is the building block for hemoglobin, an oxygen carrying protein in red blood cells.[3] According to statistics reported by the Food and Agriculture Organization of the United Nations (FAO), about 50% people in Pakistan is malnourished.[4] An estimated cost of US$ 7.6 billion is lost annually, which accounts for 3% of the national GDP due to a high proportion of undernourishment, and the situation has not improved over the last 40 years.[5]

The fortification of staple foods is one of the most successful strategies for combating malnutrition in underdeveloped nations. Fortification is a cost-effective, large-scale, and simple-to-implement strategy to reduce malnutrition and is incorporated into the food supply chain by adding desirable minerals and vitamins to staple foods in a customized way to fulfill food fortification standards.[6] Wheat flour is a staple food that accounts for 80% of all energy consumption in developing countries.[7] Iron fortification of wheat flour is considered a safe and effective way to prevent, but not cure, iron deficiency anemia (IDA).[8] The Government of Pakistan (GoP), in collaboration with International Non-Governmental Organizations (INGOs), took the initiative of wheat flour fortification with iron fortificant premix, sodium ferric ethylenediaminetetraacetate (NaFe-EDTA), to address anemia.[9] According to the Punjab Pure Food Regulations (PPFR) 2018, iron levels in milled or ground-cleaned wheat flour should not be less than 15 ppm.[10]

The GFDx (2020) guidelines recommended four different forms of iron fortifier premixes for wheat and maize flour fortification: sodium ferric ethylenediaminetetraacetate (NaFe-EDTA), ferrous sulphate ($FeSO_4$), ferrous fumarate (FeFum), and electrolytic iron (EFe).[11] Among these, the NaFe-EDTA premix is being used in Pakistan for wheat flour fortification.[10] This premix has a high iron bioavailability due to chelate formation and also inhibits the absorption of iron with the help of intrinsic phytic acid in wheat flour.[12] Moreover, the NaFe-EDTA premix has 2−3 times higher bioavailability of iron than

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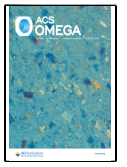

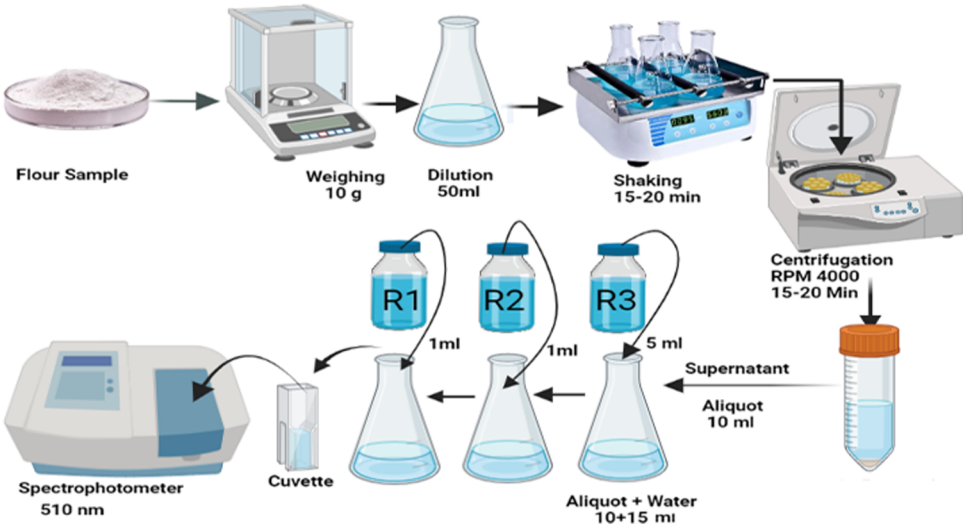

Figure 1. Illustration of the developed rapid method for determination of added Fe^{+3} in wheat flour.

$FeSO_4$,[13] and it can also prevent lipid oxidation in the food matrix, which can instigate the spoilage process.[14]

Different quantitative analytical techniques/methods, for example, gravimetric, titrimetric, induced-coupled plasma (ICP), visible spectrophotometry [atomic absorption spectrophotometry (AAS) and UV−vis], instrumental neutron activation analysis, and so forth, are being utilized to quantify iron [(Total Iron (Fe), Ferrous (Fe^{+2}), and Ferric (Fe^{+3})] in multiple food grains/matrices such as wheat flour, maize flour, rice flour, and mix flour. However, these techniques are tedious, require huge capital investment and human resource costs, and pose serious health and environmental risks during the implementation of different steps, such as sample preparation, ash formation, digestion, solvent handling, and running and maintenance of high-tech equipment.[15] To minimize these problems, there is a dire need to develop a simple, rapid, economical, and safe method for quantification of iron (Fe^{+3}) in wheat flour. The current method has been developed specifically for the quantification of ferric iron (Fe^{+3}) in wheat flour because NaFe-EDTA is used as a fortificant premix for wheat flour, in which iron is present in the ferric form (Fe^{+3}). In addition, there is currently no alternative technique documented for the determination of ferric iron (Fe^{+3}) in wheat flour derived from NaFe-EDTA, other than the AACC 40-41B method. The AACC 40-41B methodology is characterized by a significant lengthiness, with the most arduous stage being the acquisition of a white or grayish ash subsequent to sample digestion. To address these issues, a series of experiments was conducted with the aim (1) to develop a simple, accurate, precise, and inexpensive UV−vis spectrophotometric (due to its elemental specific interaction at a set wavelength and cost-effectiveness) method for quantification of added iron (Fe^{+3}) in wheat flour and (2) to validate the accuracy of this rapid method by comparing it to a standard laboratory method for iron (Fe^{+3}) determination. The method was adapted from a spectrophotometric method used by Hana Ali from the Palestinian university of Birzeit and Omar Dary from the A2Z USAID Project to determine ferrous iron (Fe^{+2}) in different types of flours. The current method measures the iron (Fe^{+3}) concentration in wheat flour by extraction of ferric iron in water: acetone solution, shaking, centrifugation, and readout in the presence of a reducing agent and a chromogen (orange color production).

2. MATERIALS AND METHODS

2.1. Materials. Analytical grade chemicals were purchased for sample analysis: sodium acetate, hydroxylamine hydrochloride (Dae-Jung, Korea), iron standard solution [Fe-$(NH_4)_2(SO4)2.6H_2O$] (Merck), and 1,10 bi-phenanthroline (VWR, USA). Double de-ionized water from the water distillation unit (Thermo Scientific, pressure less Ion Exchanger, D1-425) was used for performing all experiments.

Wheat flour samples were procured randomly from different locations, that is, local markets, grinders, and wheat flour mills of district Peshawar and Nowshera. Samples were tagged and packed in double zip-lock high-density polyethylene (HDPE) bags. For further analysis, zip-packed samples were transferred to the Food Nutrition Lab of the Nuclear Institute for Food and Agriculture (NIFA). The fortificant premix (NaFe-EDTA) was received from the Khyber Pakhtunkhwa (KP) Food Safety and Halal Food Authority, Pakistan.

2.2. Fortification of Wheat Flour. The procured samples were fortified with the fortificant premix (NaFe-EDTA). For uniform mixing, an universal mixer (SECO G.M Climax Co.) was used. At the time of analysis, each sample was tilted for at least 1 min to obtain a homogenous mixture for analysis.

2.3. Reagent Preparation. *2.3.1. Reagent-A: (1,10 Bi-phenanthroline.H_2O) (Chromogen).* 0.1 g of 1−10 bi-phenanthroline.H_2O was dissolved in 80 mL of deionized water at 80 °C, cooled down to 25 °C, diluted to 100 mL with deionized water, and stored in a dark bottle at refrigeration temperature. It was found that the stored solution was stable up to several weeks, and it was used for further experimentations. The solution would be discarded if light pink color appears, as it indicates the presence of iron impurities in the solution.

2.3.2. Reagent-B: (Acetate Buffer Solution) (Buffer to Stabilize pH). 8.3 g of anhydrous sodium acetate (previously dried at 100 °C) was placed in a 250 mL glass beaker and dissolved in 80 mL of deionized water. The pH of the solution was adjusted to 5.7 by adding 2M glacial acetic acid solution dropwise and monitored using a pH meter (JENCO, Model#6231N). The solution was then transferred to a

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volumetric flask with a commensurate capacity, diluted to a volume of 100 mL, and subsequently covered with aluminum foil before being stored at ambient temperature. The solution remained stable for several months.

2.3.3. Reagent-C: (Hydroxylamine Hydrochloride) (Reducing Agent). Accurately weighed 10 g of hydroxylamine hydrochloride was taken in a 250 mL glass beaker and dissolved in 100 mL deionized water using a stirring rod. The solution was then transferred to a 100 mL volumetric flask and stored at room temperature after being covered with aluminum foil. The solution was found to be stable for an indefinite period.

2.4. Working Standards. *2.4.1. Primary Standard Solution of Iron-1000 mg/L: (the Stock Solution).* The primary standard solution was prepared by dissolving 3.512 g of Fe $(NH_4)_2(SO_4)2.6H_2O$ in 500 mL of deionized water, along with the addition of a few drops of conc. HCl in a volumetric flask. The solution could be utilized for analysis until the appearance of a light pink color, indicating contamination.

2.4.2. Secondary Standard Solution of Iron-10 mg/L. A secondary standard solution was prepared by adding 5 mL of primary standard solution (1000 ppm) to another 500 mL volumetric flask, diluted to the desired level, and stored in a cool, dry place. It was noted that the solution was stable for 6 months.

2.4.3. Standard Solutions for the Calibration Curve. Standard solutions for the calibration curve with iron levels of 0.0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 (mg/L, ppm) were prepared by pipetting 2.0, 5.0, 10, 15, 20, 30, 40, and 50 mL of the secondary standard solution (10 mg/L, respectively, into 100 mL volumetric flasks), and 2 mL of concentrated HCl was added to each solution. The desired volume of each solution was prepared using deionized water.

Thorough mixing of the solutions was ensured by turning the flasks upside down several times. The standard solutions were stable for up to 6 months.

2.5. Development of the Rapid Method. A rapid method was developed after slight modifications to the standard method, as shown in Figure 1. The procedure involves measuring the fortified wheat flour sample using an analytical balance (BS-224-S, Sartorius, Germany) with an accuracy of +0.1 mg, followed by transferring the sample into a 250 mL conical volumetric flask. The sample was then diluted with deionized water up to 50 mL and kept in a flask shaker (SEWA, Japan) for 15−20 min. The diluted sample was placed on a benchtop centrifuge machine (Centurion, Scientific Limited, UK) at 4000 RPM for 15−20 min to obtain a clear supernatant. 10 mL of supernatant aliquot was taken in two 25 mL volumetric flasks labeled as flask "A" (sample aliquot with chromogen) and flask "B" (sample aliquot without chromogen). 1 mL of reagent-C was added to both flasks, shaken well, and kept for 5 min (reduction time). After reduction, 5 mL of buffer solution was added to each flask and mixed thoroughly. Finally, a volume of 1 milliliter of reagent-A was introduced solely into flask "A" and agitated with mild shaking. Both flasks were sealed with aluminum foil and kept in dark for 30 min to observe the color change. After 30 min, the volume of both flasks was made up to 25 mL, and readout was observed on a UV−vis spectrophotometer (U-1800, Hitachi, Japan) at 510 nm wavelength (λ) to calculate the iron concentration (ppm) using a regression equation.

2.6. Validation of the Modified Rapid Method. According to the guidelines issued by FDA[16] and ICH,[17] the following parameters are required to validate an analytical method:

2.6.1. Linearity. The linearity of the modified method was established by plotting a graph between the iron concentration (ppm) (x-axis) and absorption (y-axis) and calculating the R^2 value for different concentrations of the fortificant, for example, 5, 10, and 15 ppm. Each sample was analyzed in triplicate.

2.6.2. Accuracy/Recovery. The accuracy was determined using nine determinations for three concentration levels (5, 10, and 15 ppm) in triplicate. The recovery was calculated by using the below formula:

$$\text{Recovery (\%)} = \frac{\text{Obtained Value}}{\text{True Value}} \times 100 \quad (1)$$

2.6.3. Specificity/Limit of Detection (LOD). The LOD was calculated by taking the standard deviation (σ) of ten blanks using the following equation:

$$\text{LOD} = \frac{3.3\sigma}{S} \quad (2)$$

where S denotes the slope of the curve.

2.6.4. Sensitivity/Limit of Quantification (LOQ). LOQ was calculated as mentioned below:

$$\text{LOQ} = \frac{10\sigma}{S} \quad (3)$$

where S denotes the slope of the curve.

To calculate LOD and LOQ, unfortified wheat flour samples were fortified with different concentrations (5, 10, and 15 ppm) of NaFe-EDTA and analyzed, until a coefficient of variance (CV) below 20% was consistently achieved.

2.6.5. Repeatability/Intra-Assay Precision/within-Run Precision. The intra-assay precision is the variability in repeated measurements on the same item realized by one person with the same instrument and under similar conditions. Three concentrations (5, 10, and 15 mg/kg) of fortified wheat flour were analyzed ten times per day, and the CV of the results was calculated.

2.6.6. Reproducibility/Inter-Assay Precision/between-Run Precision. Reproducibility was determined by measuring the same three concentrations (5, 10, and 15 ppm) of fortified wheat flour in triplicate on three different days by one person, and the CV of the results was calculated. The samples were stored in a laboratory under controlled conditions.

2.6.7. Ruggedness/Inter-Person Precision. Inter-person precision was calculated by measuring the same three concentrations (5, 10, and 15 ppm) of fortified wheat flour in triplicate. It was performed by three different technicians on three different days, and the CV of the results was calculated.

2.6.8. Method-Comparison Studies. To validate the modified rapid method against the AACC standard method, 20 samples of the same concentrations (5, 10, and 15 ppm) were analyzed. The sample size was adjusted based on the expected iron concentration in the sample.

2.7. Statistical Analysis. The method comparison studies were statistically analyzed using a balanced factorial design. The relationship between independent and dependent variables was established using regression analysis, and agreement between the two measurement methods was determined by drawing the Bland−Altman Plot. Tukey's

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multiple comparison test was performed (α = 0.05), when the ANOVA was found to be significant.

3. RESULTS AND DISCUSSION

3.1. Development of the Rapid Method. The standard method involves digestion and charring. Charring is employed to burn organic portion of the analyte and requires 10−15 min. The charred sample is transferred to a muffle furnace to get gray ash at 550 °C temperature for 6 h (sufficient time is required for the muffle furnace to attain 550 °C). To avoid any injury/skin burns from the furnace, it is recommended to wait for 30 min before opening the door of the furnace. The crucibles are taken out and kept for 5 min to cool down and then placed inside a desiccator for 60 min to reach room temperature to prevent from environmental contamination. The ashed sample not only contains iron but also other inorganic elements. Acid digestion, which takes around 60 min, is required to burn the other inorganic elements.

For saving time, electricity cost, extra equipment utilization, and tedious efforts, we have modified the standard method by leaving out charring and digestion steps. For extraction of soluble iron (Fe^{+3}), the sample was dissolved in a 20% aqueous acetone solution. After a series of experiments, the laboratory determined 95−99% extraction recovery of soluble iron (Fe^{+3}) from NaFe-EDTA fortified flour as sodium iron-EDTA had good water solubility and high iron extraction efficacy and at the same time prevents lipid oxidation as well. Nonetheless, fortified flour may provide a minimal quantity of intrinsic and elemental iron. However, this contribution is not deemed significant due to the low solubility of other iron sources, and therefore, does not significantly impact the outcome of the experiments. Moreover, as the specificity (LOD) is very low for the rapid method, there are very few chances of detecting residual or impure iron. The extraction rate of different types of flour varies from 72−78% and about 2/3rd of the intrinsic iron is lost during milling in high extraction flours. The solution was then placed on an orbital shaker to get a homogenous mixture. The solution was centrifuged to get the iron (Fe^{+3})-containing supernatant. The rapid method was specifically developed for the detection of the added ferric iron (Fe^{+3}) in wheat flour as illustrated in Figure 1, in which the impure or residual iron is subtracted from the final readings by running a blank sample in parallel.

Similar studies were conducted to develop a rapid method for iron quantification by replacing the dry ash with iron extraction using [1.2 M HCl, 0.6 M TCA, and 0.7 M hydroxylamine hydrochloride] solution followed by heating and addition of reagents to calculate absorbance at 535 nm.[15] In contrast to Kosse et al.[15] study, the current study extracted iron (Fe^{+3}) in an aqueous acetone solution, and the spectrophotometric absorbance was readout at 510 nm. In another study, energy dispersive X-ray fluorescence was used for the determination of Fe, Cu, and Zn in food premixes by microwave acid digestion followed by ICP-optical emission spectroscopy.[18] Szymczycha-Madeja[19] also used the ICP-OES technique for determination of total iron in rye crisp bread, while he used HNO_3/HCl & aqua-regia solution for digestion. A new study was accomplished by Wei et al.[20] to determine the iron content in fortified soy sauce using a rapid technique IP-RP-HPLC as compared to the reference lab method, which saved sufficient time for sample analysis. All of these studies necessitated costly analytical equipment that is lacking in the laboratories of lower-middle income countries due to large capital investment. To address these problems, the current rapid method has been developed that can be performed using readily available and low-cost laboratory equipment, and it requires less time for sample analysis, as shown in Table 1.

Table 1. Comparison of Sample Analysis Time (Approximate) for Standard and Rapid Methods

steps	standard method	rapid method
weighing	5	5
charring & digestion	525	
dilution & sedimentation		40
addition of reagents	10	10
incubation	30	30
absorbance	10	10
	560 min (9.20 h)	95 min (1.35 h)

3.1.1. Validation of the Rapid Method. Method validation is a mandatory requirement for analytical method development according to the guidelines issued by ICH,[17] USP,[21] FDA,[16] International Organization for Standardization ISO/IEC-17025,[22] and EDQM.[23] It is a continual improvement process that ensures the scope of the developed analytical method for evaluating its intended usage efficacy. Moreover, an analytical method can also undergo cross validation over the course of time due to the technological advancements in the field of analytical measurement, which causes restoration of the existing procedure.[24] The following method validation parameters were determined to verify the validity and reliability of the rapid method.

3.1.2. Linearity. The linearity was determined by plotting the iron (Fe^{+3}) concentration against the absorbance to find the value of coefficient of correlation (R^2), which was found to be 0.9998, and the equation of correlation as $y = 0.0795x + 0.1072$ as shown in Figure 2. The findings of the present study

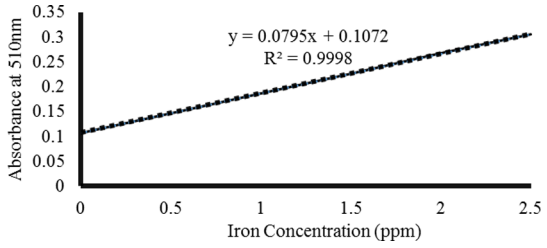

Figure 2. Correlation between Iron (Fe^{+3}) Conc. (ppm) against absorbance for standard solutions.

agreed with those of Kosse et al.,[15] who reported a correlation coefficient (R^2) value of 0.99986 for the formation of a standard curve during the development of a rapid method for determining iron conc. in various fortified meals. Similarly, a linear correlation with an R^2 value of 0.9977 was reported by Tahmouzi et al.[25] during the development and validation of an HPLC-FLD method for rapid estimation of histamine in skipjack tuna fish. The current results are also comparable with those of Rohner et al.,[26] who quantified vitamin-A in palm oil using a hand-held device with a reported R^2 value of 0.996.

3.1.3. Accuracy/Recovery/Trueness. Accuracy refers to the proximity of an analytical method's results to its parent values. According to ICH[17] and USP[21] guidelines for method validation, recovery (%) depends on the spiking concentration of the fortificant. According to the standards formulated by

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Table 2. Linearity, LOD, and LOQ of Rapid Method versus Standard Method[a]

	rapid method				standard method			
fortification Level (ppm)	intercept	R^2	LOD (ppm)	LOQ (ppm)	intercept	R^2	LOD (ppm)	LOQ (ppm)
5	$y = 1.0021x + 0.0022$	0.9976	0.05	0.18	$y = 0.0815x + 0.067$	0.9984	0.04	0.09
10	$y = 0.0788x + 0.0747$	0.9985	0.04	0.12	$y = 0.0812x + 0.0745$	0.9989	0.03	0.08
15	$y = 0.0807x + 0.0867$	0.9991	0.03	0.09	$y = 0.0801x + 0.0868$	0.9994	0.01	0.05

[a]R^2 (co-efficient of regression), limit of detection (LOD), and limit of quantification (LOQ).

Table 3. Precision Levels (Intra & Inter-Day, Inter-Person) for the Analytical Validation of the Rapid Method[a]

	intra-day	inter-day			inter-person		
	analyst[1]	analyst[1]			analyst[1]	analyst[2]	analyst[3]
fortification level (ppm)	RSD %	1st day RSD %	2nd day RSD %	3rd day RSD %	1st day RSD%	2nd day RSD%	3rd day RSD %
5	0.34	0.35	0.36	0.34	0.32	0.39	0.35
10	0.39	0.46	0.48	0.38	0.84	0.95	1.16
15	0.74	0.65	0.69	0.75	1.25	1.05	1.33

[a]RSD: Relative Standard Deviation (%); intra-day: $n = 10$; inter-day: $n = 3$; inter-person: $n = 3$.

USP[21] and findings of Addis and Abebaw,[27] an analytical method should have 70−150% accuracy and <20% RSD during its validation. Results of the current study presented a range of percent recoveries ±S.D. of 93.4−97.2 ± 0.87, 95.5−98.3 ± 1.12, and 96.2−98.1 ± 1.25 for 5, 10, and 15 ppm spiking levels, respectively, with percent RSD values between 0.71 and 1.04. Harke et al.[28] reported RSD values between 2.1 and 6.9% during the development of a nanoparticle-based method for the rapid quantification of Fe^{+3} in food samples. Similarly, Tahmouzi et al.[25] found an RSD % of 1.35, while validating an HPLC method for rapid quantitation of histamines in tuna fish. Moreover, recoveries (%) of NaFe-EDTA were in the range of 94.15 to 101.5 and the RSD value at 0. 89% by Wei et al.,[20] while determining quantity of Fe in fortified soy sauce using IP-RP-HPLC. Szymczycha-Madeja[19] determined total iron in rye crisp bread and found recoveries in the range of 74.7−106% at different spiking levels with high precision (RSD < 5%).

3.1.4. Limits of Detection and Quantification. According to the FDA[16]/ICH[17]/USP[21] guidelines, LOD and LOQ are required parameters to determine the scope of any analytical method. According to the definitions of ISO/IEC-17025[22] and Vert et al.,[29] LOD is the least detectable net concentration/true value of an analyte, whereas LOQ is the minimum amount of an analyte that can be quantitatively determined with suitable precision and accuracy. In the current study, the ranges of R^2 were in between 0.9976 and 0.9991, while LOD values calculated by rapid and conventional methods for all the three concentrations were measured from 0.03 to 0.05 and 0.01 to 0.04 ppm, respectively. Similarly, ranges of LOQ values for both methods were 0.09 to 0.18 and 0.05 to 0.09 ppm, respectively (Table 2). As per ANOVA analysis, the values of LOD and LOQ were found non-significant for both methods. Studies conducted by Harke et al.[28] resulted in significantly high specificity (LOD) and sensitivity (LOQ) values in the range of 2−10 mg/L during detection of Fe^{+3} in food samples by functionalization of biogenic silver nanoparticles. Renaud et al.[30] conducted a study to quantify the amount of vitamin A in fortified rapeseed, groundnut, and soya oils. They found that the LOD and LOQ values for these oils were 3 and 4 mg RE/kg, respectively. When developing and validating a quick method to measure histamine in tuna fish using HPLC-FLD, Tahmouzi et al.[25] reported LOD and LOQ values of 1.5 and 4.5 mg/kg, respectively. The LOD of the sodium ferric-EDTA standard solution was reported 0.03 mg/L, when Wei et al.[20] used IP-RP-HPLC to determine the concentration of iron in fortified soy sauce. Szymczycha-Madeja[19] quantified total Fe content in rye crisp bread with LOD values in the range of 0.12−31.4 ng. mL^{-1} during method validation.

3.1.5. Intra-Assay/Intra-Day Precision/Repeatability. Repeatability was calculated by taking the coefficient of variation (CV) of minimum 10 samples on the same day by the same technician for the same concentrations (5, 10, and 15 mg/kg) of sodium ferric-EDTA. The outcomes of the repeated analysis were found to be 5.14, 3.12, and 4.25%, respectively, with mean values of 5.02, 10.03, and 15.1 mg/kg, which were very much identical to their original values. These results led to the recovery rates (%) for each level being 100.4, 100.3, and 100.7, respectively, along with the RSD (%) values, as shown in Table 3. These results were consistent with those reported by Harke et al.[28] who found 78−88% repeatability in results during Fe^{+3} detection in food samples using biogenic silver nanoparticles. A similarity index was observed between the current findings and those of da Silva Santos et al.,[31] where <5 RSD % in intraday results was obtained during quantification of Fe ion chelating capacity using a newly developed analytical method.

3.1.6. Inter-Assay/Inter-Day Precision/Reproducibility. For reproducibility, the samples were analyzed three times at regular intervals by the same lab personnel under similar conditions. The results were obtained by calculating the CV for each concentration, with values of 7.25, 1.35%, and 3.21%, respectively. The mean concentration values were calculated to be 4.98, 10.05, and 15.2 ppm for each fortification level producing recoveries (%) at 99.6, 100.5, and 101.3%, respectively. The RSD (%) values for these analyses are listed in Table 3. Inter-assay precision was found in the range of 74−94% by Harke et al.[28] during their experiments on different food samples for the detection of Fe^{+3} using novel biogenic techniques. A similar range of inter-day precision with <5 RSD % was reported by da Silva Santos et al.,[31] when they developed and validated a rapid quantitative assay for determination of iron ion chelating capacity.

3.1.7. Inter-Person Precision/Ruggedness. Ruggedness was calculated by subjecting the samples to analysis in triplicate on three different days by different lab personals. The results were expressed as CV, which was found to be 3.52, 4.58, and 5.20%,

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while the mean values were 5.04, 10.3, and 15.6 mg/kg for the corresponding level of fortification. Values for relative standard deviation (%) are displayed in Table 3. The percent recovery of 100.8, 103, and 104 for each level showed a close resemblance with the recoveries (%) stated by da Silva Santos et al.[31] obtained at three concentrations (6.2, 50, and 200 μg/mL) during analytical method validation of iron ion chelating quantification with EDTA. Inter-person precision results were agreed with the findings of da Silva Santos et al.,[31] who reported ruggedness RSD value at <5% during his research work on method validation of iron ion estimation. While investigating the efficacy of two separate analytical methods for simultaneous determination of DS and DC, Mallick et al.[32] calculated the ruggedness value of 0.95% for DS and 0.96% for DC, showing the acceptability between the two methods.

3.1.8. Method-Comparison Studies. The study involved the analysis of 20 samples for each of the three concentrations (5, 10, and 15 ppm) using both rapid and standard methods. The findings indicated that the two methods were compatible across various spiking levels. The rapid method produced non-significant results to its counterpart. At each level of fortification, a good correlation/regression coefficient with R^2 value close to 1 was observed between the two methods (Figures 345). This suggests that the rapid method is an excellent alternative for the standard method for quantification of iron (Fe^{+3}) content in fortified wheat flour.

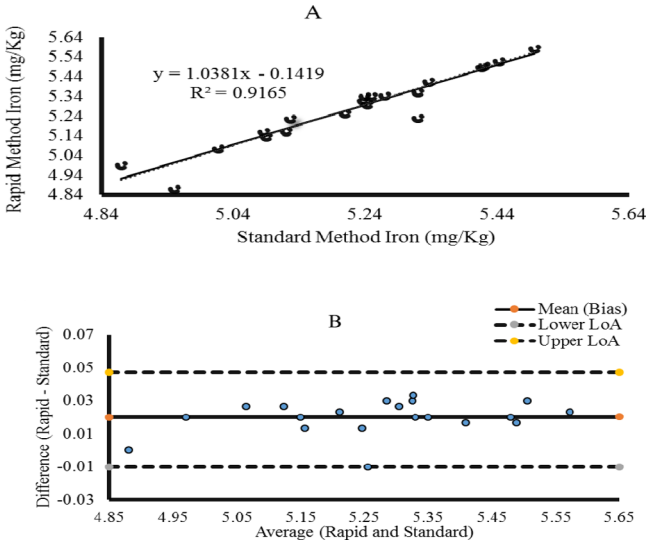

Figure 3. Regression (A) and Bland and Altman plot (B) of the comparison between the rapid and standard method at 5 ppm spiking. The solid line represents Bias, while dashed lines represent Upper and Lower Limits of Agreements at ±1.96 SD.

The regression/correlation between rapid and standard methods at 5 ppm fortification level showed the coefficient of determination (R^2) value of 0.9165, and the equation of correlation was $y = 1.0381x - 0.1419$, where "y" is the AACC standard method as shown in Figure 3A. The distribution/scattering of 5 ppm fortified flour sample analysis results using the Bland and Altman plot presenting the limits of agreements (LOA) for lower and upper values is given in Figure 3B. These results confirmed the lower and upper LOA at −0.01 and 0.0472 mg/kg, respectively, with 1.96 SD line, while the bias/mean (avg. difference) value is at 0.02 mg/kg. This plot explains that the major proportion of data points lies between

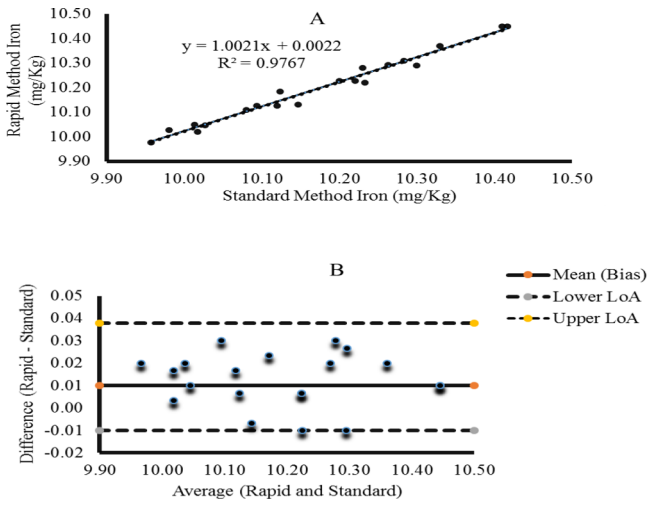

Figure 4. Regression (A) and Bland and Altman plot (B) of the comparison between the rapid and standard method at 10 ppm spiking. The solid line represents Bias (mean), while dashed lines represent Upper and Lower Limits of Agreements at ±1.96 SD.

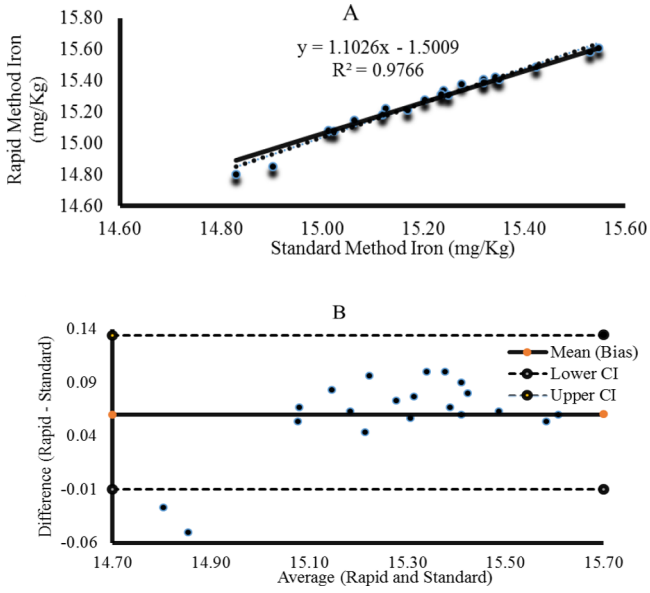

Figure 5. Regression (A) and Bland and Altman plot (B) of the comparison between the rapid and standard method at 15 ppm spiking. The solid line represents Bias, while dashed lines represent Upper and Lower Limits of Agreements at ±1.96 SD.

the corresponding limits with a few exceptions which were outliers, but these points were also not excluded in order to check their significance on the conclusive analytical results. After the final assessment of the overall experimental findings, it was noted that there was a non-significant effect of these outliers on the results, and both the methods produced results within the acceptable range.

Similarly, the regression/correlation and Bland−Altman plot illustration for 10 and 15 ppm spiked flour samples shows that the rapid and standard methods produced good correlation factor (R^2) values of 0.9767 and 0.9766, respectively, at both fortification levels as displayed in Figures 4A and 5A. For 10 ppm spiking concentration, the mean/bias value was calculated at 0.01, while low and high LOA at −0.01 and 0.036 mg/kg, respectively, with 1.96 SD line as shown in the Bland−Altman

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plot (B). Likewise, the bias value for the 15 ppm fortification level was found to be 0.06 mg/kg with low LOA at −0.01 mg/kg and high LOA at 0.134 mg/kg with 1.96 SD line as seen in Figure 5B. Cumulative results indicate that there is a good correlation between the rapid and standard method and a high frequency of sample analysis results was lying within the acceptable range of the experiments. On the basis of these findings, it can be proposed that the rapid method can produce compatible, accurate, and precise (reproducible and repeatable) results with good percent recoveries and robustness.

These findings were in accordance with the study outcomes of Rohner et al.[26] and Renaud et al.[30] who used similar validation parameters for method validation of a simple portable device to standard HPLC method for quantification of vitamin-A in different oils and obtained favorable results. Moreover, Kosse et al.[15] also reported likewise results during development and validation of a rapid method against a reference method for quantitation of Fe in different food commodities. According to Bauman,[33] it is particularly challenging to handle the consistent mixing of a solid matrix with another solid. It could be a significant obstacle to accurately calculate the amount of iron in a sample of wheat flour.

4. CONCLUSIONS/SUMMARY

The conventional method can assess the element of interest accurately and precisely, but the main hindrance to its usage is the cost and time required to complete the sample analysis. The current study has successfully addressed the pre-stated issues by developing a rapid method that has reduced the sample analysis time from 560 to 90 min with excellent percent recoveries (<20 RSD) and good precisions. It showed a good correlation coefficient ($R^2 \approx 1$) with the conventional method, and its LOD and LOQ ranges were also quite lower, which can effectively quantify the spiking level (≥15 ppm) of the added iron (Fe^{+3}) as mandated by the regularity authorities for wheat flour fortification. On the basis of these results, it can be suggested that the rapid method can be an alternative to conventional methods with less time and high accuracy and precision.

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Notes

The authors declare no competing financial interest.

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