File No: 11014/02/2021-QA-Part(1)
Food Safety and Standards Authority of India
(A Statutory Authority established under the Food Safety and Standards Act, 2006)
(Quality Assurance Division)
FDA Bhawan, Kotla Road, New Delhi – 110002

दिनांक: 09 नवंबर,2023

आदेश

Subject: Methods for testing of Fortificants (Iron, Folic Acid and Vitamin B12) in Vitamin Mineral Premix for Fortified Rice Kernel (FRK) and Iron in FRK by AAS - reg.

The Scientific Panel on methods of Sampling and Analysis has approved the following methods:

i. Method for Determination of Iron in Vitamin Mineral Premix for FRK - **FSSAI.VMP-FRK.16.008.2023 (Annexure-I)**
ii. Method for Determination of Folic Acid in Vitamin Mineral Premix for FRK - **FSSAI.VMP-FRK.16.009.2023 (Annexure-II)**
iii. Method for Determination of Vitamin B12 in Vitamin Mineral Premix for FRK - **FSSAI.VMP-FRK.16.010.2023 (Annexure-III)**
iv. Method for Determination of Iron in Fortified Rice Kernel (FRK) by AAS - **FSSAI.FRK.16.007.2023 (Annexure-IV)**

2. The food testing laboratories are directed to use the aforesaid methods with immediate effect.

3. This issues with the approval of competent authority.

Enclosure: As above**.**

Digitally Signed by Sweety Behera
Date: 09-11-2023 17:09:27
Reason: Approved
(स्वीटी बेहरा)
निदेशक (गुणवत्ता आश्वासन)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories

3. ED (QA/QC), FCI
4. CEO, NABL
5. Director DFPD/Quality control cell, Ministry of Consumer affairs, Food & Public Distribution

Copy for information to:

1. SPS to CEO, FSSAI
2. ED(CS), FSSAI
3. Advisor (QA), FSSAI
4. Advisor (S&S), FSSAI

Annexure I

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Determination of Iron in Vitamin Mineral Premix for Preparation of Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.VMP-FRK.16.008.2023	**Revision No. & Date**	0.0
Scope	The Scope of this Method is Applicable for Quantification of Iron in Premix at 5000 mg/kg LOQ Level (with respect to the Sample) by Using Atomic Absorption Spectroscopy (AAS).		
Safety & Precautions	**1. Concentrated Nitric Acid** It is a Chemical which is corrosive to Metals. It causes severe skin burns and eye damage. It is toxic if inhaled and corrosive to the respiratory tract. Following safety measures need to be taken during Handling of Concentrated Nitric Acid: a) Do not breathe dust/fume/gas/mist/vapors/spray b) Wash face, hands and any exposed skin thoroughly after handling c) Wear protective gloves/protective clothing/eye protection/face protection d) Use only outdoors or in a well-ventilated area Keep away from heat/sparks/open flames/hot surfaces. e) Keep/Store away from clothing/ other combustible materials f) Take any precaution to avoid mixing with combustibles g) Keep only in original container h) Wear respiratory protection **2. Hydrogen Peroxide** It is Oxidizing, Corrosive and Irritant chemical. Following safety measures need to be taken during Handling of Hydrogen Peroxide: a) When handling moderate-to-high concentrations of Hydrogen Peroxide in the workplace, ensure eyewash stations and safety showers are accessible, and use splash goggles, gloves, and an approved Vapor Respirator. ***Note:*** *As and when required the corrosive chemicals such as concentrated nitric acid, H_2O_2 etc. should be opened in a chemical fume hood to avoid exposures.*		
Principle	Nitric acid and hydrogen peroxide are added to homogenized Vitamin Premix sample in microwave vessels, and digested using a preprogramed temperature control. Analysis is performed by AAS.		
Apparatus/Instruments	1. Atomic Absorption Spectroscopy (AAS) 2. Microwave Digester 3. Analytical Balance 4. Micro Pipettes (20 -200 µL) & (100 -1000 µL)		
Materials and Reagents	1. Concentrated Nitric Acid (Purity- 69%) - Suprapure 2. Hydrogen Peroxide (Purity -30%) – LR Grade 3. CRM / Standard Stock Solution - Iron (Purity - 1000 mg/kg) 4. Purity of Argon and other gas, if used must fulfill the standard of		

<table>
<tr><td></td><td>instrument requirement</td></tr>
<tr><td>Sample Preparation</td><td><u>PREPARATION OF SAMPLE SOLUTION</u>

1. Weigh 0.50 g (± 0.05 g) of Homogenized Sample.
2. Transfer to Microwave Digestion Closed (MDC) Vessel.
3. Heated Milli Q Water at 60 °C.
4. Add 2.0 mL of Hot Milli-Q water.
5. Add 1.0 mL Hydrogen Peroxide.
6. Add 5.0 mL of Nitric Acid.
7. Close the Microwave Vessel tightly.
8. Keep at Room Temperature for 5 minutes.
9. Keep the Vessel rotor in Microwave Digester.
10. Cool the Vessel at Room Temperature after Digestion.
11. Add 10 mL of Milli Q water.
12. Mixed well.
13. Transfer to 100 mL Volumetric Flask.
14. Volume make-up to 100 mL with Milli-Q water.
15. Filter and use this for injecting on AAS.

Note: If required, dilute the sample for the desired concentration.</td></tr>
<tr><td>Method of Analysis
(a) Preparation of Standard solutions</td><td>A) <u>PREPARATION OF BLANK (5% NITRIC ACID)</u>

Transfer 7.25 mL of Nitric Acid (69%) in 100 mL Milli Q Water in Glass Bottle Mix well. Shake Vigorously.

B) <u>PREPARATION OF CALIBRATION STANDARD SOLUTIONS</u>
Use Intermediate Standard Solution-1 for Preparing Calibration Standard Solutions as mentioned in below Table.

<table>
<tr><th>CAL. STANDARD SOLUTIONS</th><th>SSS (mg/kg)</th><th>VOL. OF SSS (mL)</th><th>VOL. OF NITRIC ACID (mL)</th><th>FINAL VOL. (mL)</th><th>FINAL CONC. (mg/kg)</th></tr>
<tr><td>LS 6</td><td>1000</td><td>1.50</td><td>0.5</td><td>10</td><td>150</td></tr>
<tr><td>LS 5</td><td>1000</td><td>1.25</td><td>0.5</td><td>10</td><td>125</td></tr>
<tr><td>LS 4</td><td>1000</td><td>1.00</td><td>0.5</td><td>10</td><td>100</td></tr>
<tr><td>LS 3</td><td>1000</td><td>0.75</td><td>0.5</td><td>10</td><td>75</td></tr>
<tr><td>LS 2</td><td>1000</td><td>0.50</td><td>0.5</td><td>10</td><td>50</td></tr>
<tr><td>LS 1</td><td>1000</td><td>0.25</td><td>0.5</td><td>10</td><td>25</td></tr>
</table>
CAL : Calibration
SSS : Standard Stock Solution
VOL : Volume
LS : Linearity Solution

NOTE: Use Freshly Prepared Standard solutions for the Analysis.

C) <u>PREPARATION OF BRACKETING STANDARD SOLUTION (50 mg/kg)</u>

1. Transfer 0.5 ml from Standard Stock Solution of Iron (1000 mg/L) in 10 ml volumetric flask.
2. Add 0.5 ml nitric acid and made up the Volume till 10 ml volumetric flask by Milli-Q water and mix by Vortex Shaker Mixer.</td></tr>
</table>

(b) Instrument Details

a) **Instrument**: Atomic Absorption Spectrometer (AAS)

b) **Equipment Conditions**: As detailed in below Table

Hollow cathode Lamp	Iron (Fe)
Lamp Current (mA)	5.0
Absorption Wavelength (nm)	372.0
Slit Width (nm)	0.2
Signal-Type	Atomic Absorption
Signal -Measurement	Integration
Oxidant	Air
Oxidant Flow (L/min)	13.5
Acetylene Flow (L/min)	2
Equation	Linear
Read Parameter	
Time (sec)	10
Delay time (sec)	10

c) Microwave Digestion Program

S.NO	**RAMPING STAGE**	**HOLD TIME (Minutes)**	**TEMP (˚C)**	**POWER (Watt)**
1	1	20	180	800
2	2	10	160	800
3	3	10	140	800
4	COOL DOWN	10	-	-

Note: *The make & model of Instrument can be changed. However, the Instrument should be able to achieve the desired LOD &LOQ value.*

<table>
<tr><td>Batch Organization</td><td><u>Injection Sequence</u>
<table>
<tr><th>S.NO.</th><th>NAME OF INJECTIONS</th><th>NUMBER OF INJECTIONS</th></tr>
<tr><td>1</td><td>Blank</td><td>2</td></tr>
<tr><td>2</td><td>Linearity Solution (LS) - 1</td><td>1</td></tr>
<tr><td>3</td><td>Linearity Solution (LS) - 2</td><td>1</td></tr>
<tr><td>4</td><td>Linearity Solution (LS) - 3</td><td>1</td></tr>
<tr><td>5</td><td>Linearity Solution (LS) - 4</td><td>1</td></tr>
<tr><td>6</td><td>Linearity Solution (LS) - 5</td><td>1</td></tr>
<tr><td>7</td><td>Linearity Solution (LS) - 6</td><td>1</td></tr>
<tr><td>8</td><td>Blank</td><td>2</td></tr>
<tr><td>9</td><td>Sample Solution</td><td>1</td></tr>
<tr><td>10</td><td>Blank</td><td>2</td></tr>
<tr><td>11</td><td>Bracketing Standard Solution</td><td>1</td></tr>
<tr><td colspan="2">TOTAL INJECTIONS</td><td>14</td></tr>
</table></td></tr>
<tr><td>Calculation with Units of Expression</td><td>Carry out analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve, including zero.

Calculate the Iron Content in Vitamin Premix using the following equation:

Iron (mg/kg) = <u>Instrument Conc. (mg/kg) x Make-up Volume (mL)</u>
Sample Weight (gm)</td></tr>
<tr><td>LOD & LOQ</td><td>Limit of Detection 12.5 mg/kg with respective to the Standard.
Limit of Quantification 25.0 mg/kg with respective to the Standard. Limit of Quantification 5000 mg/kg with respective to the Sample.</td></tr>
<tr><td>Reference</td><td>AOAC 2011.14: Determination of Minerals and Trace elements in Milk & Milk Products, Infant Formula, and Adult Nutrition.</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

Annexure II

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Determination of Folic Acid (Vitamin B9) in Vitamin Mineral Premix for Preparation of Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.VMP-FRK.16.009.2023	**Revision No. & Date**	0.0
Scope	The Scope of this Method is applicable for Quantification of Folic Acid (Vitamin B9) at 200 mg/kg LOQ Level (With Respect to the Sample) by using HPLC in Premix.		
Safety & Precautions	**1. Folic Acid:** Folic acid is not considered hazardous by the 2012 OSHA Standard. First Aid: Rise immediately with plenty of water if it is in contact with Eye & skin. Avoid to inhale fume, move to fresh air. If not breathing give artificial respiration. **2. Ammonium Hydroxide:** Routes of Exposure: Inhalation, ingestion, skin contact, eye contact a. Corrosive. May cause damage to mucous membranes in nose, throat, lungs and bronchial system. b. Corrosive. Harmful if swallowed. May produce burns to the lips, oral cavity, upper airway, esophagus and digestive tract. c. Corrosive. Causes severe burns. d. Corrosive. Causes severe burns. May cause eye damage, impaired sight or blindness. **3. Potassium Phosphate Mono Basic:** a. Move to fresh air. Get medical attention if symptoms persist. b. Wash skin thoroughly with soap and water. Get medical attention if symptoms occur. Wash contaminated clothing before reuse. c. Immediately flush with plenty of water for at least 15 minutes. If easy to do, remove contact lenses. Get medical attention if irritation persists after washing. **4. Tetra methyl Ammonium Hydroxide:** a. Rinse thoroughly with plenty of water for at least 15 minutes, lifting lower and upper eyelids. Consult a physician. b. Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required. c. Move to fresh air. If not breathing, give artificial respiration. Do not use mouth-to-mouth method if victim ingested or inhaled the substance; give artificial respiration with the aid of a pocket mask equipped with a one-way valve or other proper respiratory medical device. Immediate medical attention is required. **5. Phosphoric Acid:** a. Seek medical attention immediately. Move exposed individual to fresh air. Loosen clothing as necessary and position individual in a comfortable position.		

	b. Remove contaminated clothing and wash before reuse or discard. Rinse skin for 30 minutes with water or under a shower. Seek immediate medical attention. Wash affected area with soap and water. c. Rinse immediately with plenty of water, also under the eyelids, for at least 30 minutes. Remove contact lens(es) if able to do so during rinsing. Seek medical attention immediately. Protect unexposed eye. d. Seek medical attention immediately. Rinse mouth thoroughly. Do not induce vomiting. Have exposed individual drink sips of water. **6. Methanol:** It is a Flammable and Toxic Liquid. It creates Hazards to Human Health. During handling of Methanol, below safety measures to be followed: a. Wash skin thoroughly after handling. b. Avoid breathing dust/fume/gas/mist/vapours/spray. c. Do not breathe dust/fume/gas/mist/vapours/spray. d. IF ON SKIN: Wash with soap and water. e. Specific measures (see supplemental first aid instructions on this label). f. Wash contaminated clothing before reuse. g. Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. h. Use explosion-proof equipment. i. Keep away from sources of ignition - No smoking
Principle	The Premix Sample is Extracted by Using Potassium Phosphate Mono Basic & Tetra butyl ammonium Buffer Solution for Quantification of Vitamin B9 (Folic Acid) using HPLC.
Apparatus/Instruments	1. HPLC, Binary gradient pump, an auto Sampler. 2. Analytical Balance, Suitable for weighing samples with accuracy up to 0.1 mg 3. Centrifuge 5000 RPM, holding 50 mL tubes 4. Micro Pipettes (100 -1000 µl, 20 -200 µl 10 -100 µl). 5. HPLC C18 ODS Column: 4.6mm X 250 mm X 5 µm; 6. Sonicator for mixing of solution. 7. Vortex for preparation of stock solution. 8. Homogenizer for sample grinding
Materials and Reagents	1. Ammonium Hydroxide, LR Grade 2. Phosphoric Acid, LR Grade 3. Monobasic Potassium Phosphate, LR Grade 4. Tetrabutylammonium Hydroxide, LR Grade 5. Methanol, HPLC Grade. 6. CRM: Folic Acid (CAS No: 593003)
Preparation of Mobile Phase	**PREPARATION OF MOBILE PHASE** **MOBILE PHASE PREPARATION** **1.** Accurately weight 2.0 g of monobasic potassium phosphate into a 1000 ml volumetric flask. **2.** Add 650 mL of Milli-Q Water for Volume make up **3.** Add 15 mL of 0.5 M Tetra butyl ammonium hydroxide in methanol. **4.** Add 7.0 mL of 3 N Phosphoric acid. **5.** Add 270 mL of methanol. **6.** Cool to room temperature. **7.** Adjust pH 5.0 with 3 N Phosphoric Acid or 6 N ammonium hydroxide.

<table>
<tr><td></td><td>8. Finally make the volume 1000 ml with Milli-Q Water.</td></tr>
<tr><td>Sample Preparation</td><td><u>PREPARATION OF SAMPLE SOLUTION</u>
1. Accurately weigh 1 g (± 0.1 g) of Homogenized Sample
2. Add 0.1 ml of 10 % ammonium hydroxide
3. Transfer into a 10 mL Amber Colored Volumetric Flask
4. Add 5 mL Buffer
5. Vortex for 5 minutes
6. Cool the Sample Solution at Room Temperature
7. Do Volume make-up to 10 ml with mobile phase
8. Vortex for 2 minutes
9. Filter the solution through 0.45µm Nylon Syringe Filter
10.Pour the Filtrate into the Vial, and use this for injecting into HPLC

Note: If required, dilute the sample for desired concentration.</td></tr>
<tr><td>Method of Analysis
(a) Preparation of Standards</td><td>PREPARATION OF STANDARD STOCK SOLUTION

a) <u>PREPARATION OF STOCK SOLUTION FOR FOLIC ACID (1000 mg/kg)</u>

1. Accurately weigh 10 mg (± 0.1 mg) of Folic Acid Standard.
2. Add 0.1 ml of 10% Ammonium Hydroxide Solution
3. Transfer to 10 mL Amber Colored Volumetric Flask.
4. Add Buffer for Volume make-up to 10 mL.
5. Vortex for 2 min.
6. Store the Solution at -20°C in the light Protected Area.

b) <u>PREPARATION OF BRACKETING STANDARD SOLUTION (85 mg/kg)</u>

1. Pipette out 0.85 mL of Standard Stock Solution
2. Transfer to 10 mL Amber Colored Volumetric Flask containing 2 mL of Milli Q Water.
3. Add Buffer for Volume make-up to 10 mL.
4. Vortex for 2 min.

c) <u>PREPARATION OF CALIBRATION STANDARD SOLUTIONS</u>

Use Standard Stock Solution for preparing Calibration Standard Solutions as mentioned in below Table.

<table>
<tr><th>CALIBRATION STANDARD SOLUTIONS</th><th>SSS (mg/kg)</th><th>VOL. OF SSS (mL)</th><th>FINAL MAKE UP VOL. DILUENT (mL)</th><th>FINAL CONC. (mg/kg)</th></tr>
<tr><td>LS 6</td><td>1000</td><td>1.50</td><td>10</td><td>150</td></tr>
<tr><td>LS 5</td><td>1000</td><td>1.20</td><td>10</td><td>120</td></tr>
<tr><td>LS 4</td><td>1000</td><td>1.00</td><td>10</td><td>100</td></tr>
<tr><td>LS 3</td><td>1000</td><td>0.85</td><td>10</td><td>85</td></tr>
<tr><td>LS 2</td><td>1000</td><td>0.50</td><td>10</td><td>50</td></tr>
</table>
</td></tr>
</table>

LS 1	1000	0.20	10	20

Note: *Always make Fresh Preparation of Calibration Standard Solutions.*

CAL: Calibration
SSS : Standard Stock Solution
VOL: Volume
LS : Linearity Solution

(b)Chromatographic Conditions

- Instrument : HPLC UV Detector
- Chromatographic Conditions : As detailed in below Table

Instrument	HPLC
Detector	UV 280 nm
Column	C18 ODS Column: 4.6 mm X 250 mm X 5 µm;
Run time	10 min
Flow rate	1.8 ml/min
Injection Volume	10 µl
Column Temperature	25°C

Note: *The make, model of Instrument & Column can be changed. However, Instrument should be able to achieve the desired LOD & LOQ Value & the Column is exactly same in terms of the Composition & Dimensions.*

Batch Organization

Injection Sequence

S.NO.	NAME OF INJECTIONS	NUMBER OF INJECTIONS
1.	Blank	2
2.	Linearity Solution (LS) - 1	1
3.	Linearity Solution (LS) - 2	1
4.	Linearity Solution (LS) - 3	1
5.	Linearity Solution (LS) - 4	1
6.	Linearity Solution (LS) - 5	1
7.	Linearity Solution (LS) - 6	1
8.	Blank	2
9.	Sample Solution	1
10.	Blank	2
11.	Bracketing Standard Solution	1
TOTAL INJECTIONS		**14**

Calculation with units of Expression

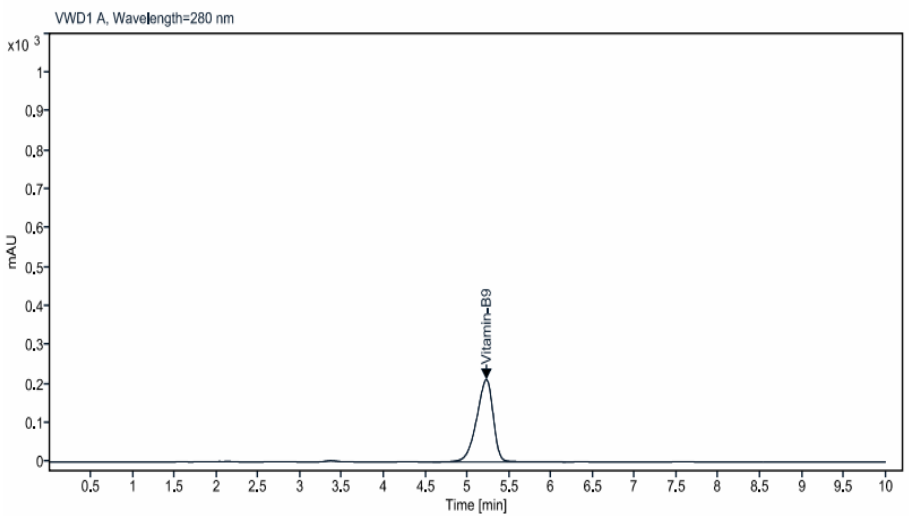
a) Carry out analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve.

Calculate the Folic Acid Content in Premix using the following equation:

Folic Acid (Vitamin B9) (mg/kg) =

$$\frac{\text{Sample Conc.(mg/kg)} \times \text{Make up Volume (mL)}}{\text{Sample Weight (gm)}}$$

b) The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the Folic acid signal in the matrix.

(a) Chromatogram	Chromatogram 
(b) LOD & LOQ	a) Limit of Detection (10mg/kg) With Respective to the Standard b) Limit of Quantification (20mg/kg) With Respective to the Standard c) Limit of Quantification (200mg/kg) With Respective to the Sample
Reference	Method Protocol: PRT/RA/PRM/2023/001, Method Validation Report for Estimation of Folic Acid (Vitamin B9) in Premix using HPLC. United State Pharmacopeia - Folic Acid (Assay)
Approved by	Scientific Panel on Methods of Sampling and Analysis

Annexure III

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Determination of Cyanocobalamin (Vitamin B12) in Vitamin Mineral Premix for Preparation of Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.VMP-FRK.16.010.2023	**Revision No. & Date**	0.0
Scope	The Scope of this Method includes for Quantification of Cyanocobalamin (Vitamin B12) at 2.0 mg/kg LOQ Level (with respect to the Sample) by using HPLC in Premix.		
Safety & Precautions	1) **Methanol:** It is a Flammable and Toxic Liquid. It creates Hazards to Human Health. During handling of Methanol, below safety measures to be followed: a) Wash skin thoroughly after handling. b) Avoid breathing dust/fume/gas/mist/vapours/spray. c) Do not breathe dust/fume/gas/mist/vapours/spray. d) IF ON SKIN: Wash with soap and water. e) Specific measures (see supplemental first aid instructions on this label). f) Wash contaminated clothing before reuse. g) Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. h) Use explosion-proof equipment. i) Keep away from sources of ignition - No smoking **2) Acetonitrile:** It is a Flammable liquid which causes severe skin burns and eye damage. During handling of Acetonitrile, below safety measures to be followed: a) Inhalation: Inhale fresh air. If breathing stops, give mouth-to-mouth breathing or artificial respiration. Provide Oxygen, b) Skin Contact: Take off immediately all contaminated clothing. Rinse skin with water/ shower. c) Eye Contact: Rinse out with plenty of water. Call in ophthalmologist. Remove contact lenses. d) If swallowed: After swallowing, immediately make victim drink water (two glasses at most). 3) **Orthophosphoric Acid:** It is a colorless, crystalline solid, the tribasic acid of pentavalent phosphorus. During handling of Orthophosphoric Acid, below safety measures to be followed: a) Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. b) Wash off immediately with plenty of water for at least 15 minutes. Remove and wash contaminated clothing and gloves, including the inside, before re-use. Call a physician immediately. c) Do NOT induce vomiting. Clean mouth with water. Never give anything by mouth to an unconscious person. d) If not breathing, give artificial respiration. Remove from exposure, lie down. Do not use mouth-to-mouth method if victim ingested or inhaled the substance; give artificial respiration with the aid of a pocket mask equipped with a one-way valve or other proper respiratory medical device.		

	Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination. 4) **Cyanocobalamin:** it is hazardous chemical. During handling of Cyanocobalamin, below Safety Measures to be followed: a) In case of eye Contact, Immediately flush eyes with plenty of water for the least 15 minutes. b) In case of Skin contact, flush skin with plenty of water. Remove contaminated clothing and shoes. c) In case of swallowed, do not induce vomiting unless directed to do so by medical personnel. d) In case of Inhaled, remove to fresh air. If not breathing give artificial Respiration.
Principle	Cyanocobalamin is Extracted from the Sample by Diluent Containing Potassium Dihydrogen Phosphate and Dipotassium Hydrogen Phosphate, Extract & Filtered, and Quantified by HPLC.
Apparatus/Instruments	1. HPLC 2. Analytical Balance, -Suitable for weighing samples with accuracy up to 0.1 mg 3. Centrifuge -5000 rpm, holding 50 mL tubes 4. Micro Pipettes Capable of delivering from 100 -1000 µl, 20 -200 µl 10 -100 µl 5. Column: C8 4.6 mm X 250 mm X 5µm 6. Sonicator for mixing of solution 7. Vortex for preparation of stock solution 8. Homogenizer for sample grinding
Materials and Reagents	1. Methanol, LR Grade 2. CRM Used: Cyanocobalamin (CAS No: 68199) 3. Potassium dihydrogen phosphate, LR Grade 4. Dipotassium hydrogen phosphate, LR Grade 5. Ortho phosphoric Acid, LR Grade 6. Acetonitrile HPLC Grade
Preparation of Reagents	**a) <u>MOBILE PHASE A PREPARATION</u>** 1. Dissolve 2.72 gm Potassium dihydrogen phosphate and 3.48 gm Dipotassium hydrogen phosphate in 1000 ml of water, 2. Adjust pH 6.6 (+/- 0.1) with Ortho phosphoric Acid. **b) <u>MOBILE PHASE B PREPARATION</u>** 1. Prepare a mixture of Mobile Phase A and Acetonitrile (80:20) Ratio and mix well. **c) <u>DILUENT PREPARATION</u>** 1. Mobile Phase A is using as a Diluent.
Sample Preparation	**<u>PREPARATION OF SAMPLE SOLUTION</u>** 1. Weigh 1.0 g (± 0.10 g) of Homogenized Sample. 2. Transfer to a 10 ml amber color volumetric flask. 3. Add 5 mL Mobile phase A. 4. Vortex for 5 minutes.

	5. Do Volume make-up to 10 ml with Mobile phase A. 6. Vortex for 2 minutes 7. Filter the solution through 0.45µm Nylon Syringe Filter. 8. Pour the Filtrate into the Vial, and use this for injecting into HPLC.
Method of Analysis	**A) <u>PREPARATION OF STOCK SOLUTION FOR CYANOCOBALAMIN (1000 mg/kg)</u>** 1. Accurately weigh 10 mg (± 0.1 mg) of Cyanocobalamin Standard. 2. Transfer to 10 mL Amber Colored Volumetric Flask. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. Note: Store the Solution at -20°C in the light Protected Area **B) <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 1 (100 mg/kg)</u>** 1. Pipette out 1.0 mL of Stock Solution 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. **C) <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 2 (10 mg/kg)</u>** 1. Pipette out 1.0 mL of Intermediate Standard Stock Solution – 1. 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. **D) <u>PREPARATION OF BREACKGING STANDARD SOLUTION (0.75 mg/kg)</u>** 1. Pipette out 0.75 mL of Intermediate Standard Stock Solution – 2. 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. **E) <u>PREPARATION OF CALIBRATION STANDARD SOLUTIONS</u>** Use Intermediate Standard Solution – 2 for Preparing Calibration Standard Solution as mentioned in below Table.

CAL. STANDARD SOLUTIONS	ISS - 2 (10 mg/L)	VOL. OF ISS – 2 (mL)	VOL. OF DILUENT (mL)	FINAL VOL. (mL)	FINAL CONC. (mg/L)
LS6	10	2.00	8.00	10	2.00
LS5	10	1.50	8.50	10	1.50
LS4	10	1.00	9.00	10	1.00
LS3	10	0.75	9.25	10	0.75
LS2	10	0.50	9.50	10	0.50
LS1	10	0.20	9.80	10	0.20

Note: *Always make Fresh Preparation of Calibration Standard Solutions*

CAL : Calibration
ISS : Intermediate Stock Solution
VOL : Volume
LS : Linearity Solution

Method of Analysis
(a) Chromatographic Conditions

a) **Instrument** : HPLC
b) **Chromatographic Conditions** : As detailed in below Table

Instrument	HPLC
Detector	DAD
Column	Column: C8 4.6 mm X 250 mm X 5µm
Run time	30 min
Column Temperature	40°C
Flow rate	1.0 mL/min
Injection Volume	100 µl
Mobile Phase A	Dissolve 2.72 gm Potassium Dihydrogen phosphate and 3.48 gm Dipotassium hydrogen phosphate in 1000 ml of water, Adjust pH 6.6 (+/- 0.1) with Ortho phosphoric Acid.
Mobile Phase B	Prepare a mixture of Mobile Phase A and Acetonitrile (80:20) ratio and Mix well.
Diluent	Mobile Phase A
Wavelength	360

c) Gradient Program

TIME	**FLOW RATE**	**MOBILE PHASE A (%)**	**MOBILE PHASE B (%)**
0.01	1.0	90	10
20	1.0	0	100
25	1.0	0	100
28	1.0	90	10
30	1.0	90	10

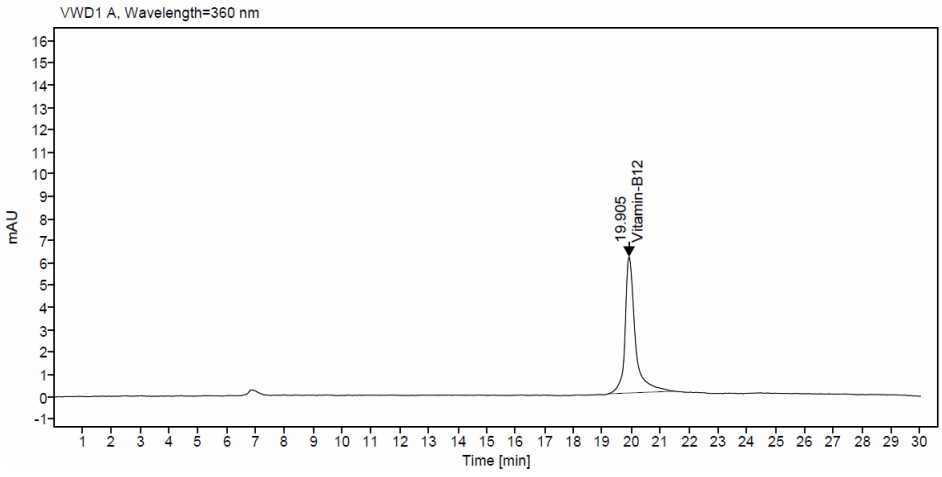
Note: *The make & model of Instrument & Column can be changed. However, the Instrument should be able to achieve the desired LOD value & the Column is exactly same in terms of the Composition & Dimensions.*

Method of Analysis
(b) Batch Organization

INJECTION SEQUENCE

S.NO.	**NAME OF INJECTIONS**	**NUMBER OF INJECTIONS**
1	Blank	2
2	Linearity Solution (LS) - 1	1
3	Linearity Solution (LS) - 2	1

	4	Linearity Solution (LS) - 3	1
	5	Linearity Solution (LS) - 4	1
	6	Linearity Solution (LS) - 5	1
	7	Linearity Solution (LS) - 6	1
	8	Blank	2
	9	Sample Solution	1
	10	Blank	2
	11	Bracketing Standard Solution	1
		TOTAL INJECTIONS	**15**

Calculation with units of Expression	a) Carry out analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve. **Cyanocobalamin (Vitamin B12) (mg/kg)** = Sample Conc.(mg/kg) X Make up Volume(mL) --- Sample Weight (g) b) The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the Cyanocobalamin (Vitamin B12) signal in the matrix.
(a) Chromatograms	
(b) LOD & LOQ	a) Limit of Detection (0.1 mg/kg) With Respective to the Standard. b) Limit of Quantification (0.2 mg/kg) With Respective to the Standard. c) Limit of Quantification (2.0 mg/kg) With Respective to the Sample.
Reference	AOAC 2011.10-AOAC Official method for Vitamin B12 in Indian infant and Pediatric formulas and Adult Nutritionals.
Approved by	Scientific Panel on Methods of Sampling and Analysis

Annexure IV

एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Determination of Iron in Fortified Rice Kernel (FRK) by AAS**		
Method No.	FSSAI.FRK.16.007.2023	**Revision No. & Date**	0.0
Scope	The Scope of this Method is applicable for Quantification of Iron in FRK at 500 mg/kg LOQ Level (with respect to the Sample) by using AAS.		
Safety & Precautions	**1. Concentrated Nitric Acid** It is a Chemical which is corrosive to Metals. It causes severe skin burns and eye damage. It is toxic if inhaled. It is corrosive to the respiratory tract. Following safety measures need to be taken during Handling of Concentrated Nitric Acid: a) Do not breathe dust/fume/gas/mist/vapors/spray b) Wash face, hands and any exposed skin thoroughly after handling c) Wear protective gloves/protective clothing/eye protection/face protection d) Use only outdoors or in a well-ventilated area Keep away from heat/sparks/open flames/hot surfaces. e) No smoking f) Keep/Store away from clothing/ other combustible materials g) Take any precaution to avoid mixing with combustibles h) Keep only in original container i) Wear respiratory protection **2. Hydrogen Peroxide** It is Oxidizing, Corrosive and Irritant chemical. Following safety measures need to be taken during Handling of Hydrogen Peroxide: a) When handling moderate-to-high concentrations of Hydrogen Peroxide in the workplace, ensure eyewash stations and safety showers are accessible, and use splash goggles, gloves, and an approved Vapor Respirator. ***Note:*** *As and when required the corrosive chemicals such as concentrated nitric acid, H_2O_2 etc. should be opened in a chemical fume hood to avoid exposures.*		
Principle	Weigh 0.50 g (± 0.05 g) of Grinded Sample Transfer to Microwave Digestion Cool Vessel. Add 2.0 mL Milli Q Water, 1.0 mL Hydrogen Peroxide, add 5 mL of Nitric Acid digest in microwave digestor, extract the analyte in Nitric acid make up to 50 mL, Filter and Inject in AAS.		
Apparatus/Instruments	1. Atomic Absorption Spectrometry (AAS) 2. Microwave Digester 3. Analytical Balance 4. Micro Pipettes (20 -200 µl) & (100 -1000 µl) ***Note:*** *1. The make & model of Instrument can be changed. However, the Instrument should be able to achieve the desired LOD value.*		

Materials and Reagents

1. Concentrated Nitric Acid (Purity- 69%)
2. Hydrogen Peroxide (Purity -30%)
3. CRM Used: Iron

Preparation of solutions

A) PREPARATION OF INTERMEDIATE STOCK SOLUTION - 1 (100 mg/kg)

1. Transfer 10.0 ml from stock solution of iron (1000 mg/kg) in 100 ml volumetric flask.
2. Add 5.0 ml nitric acid and made up the volume till 100 ml volumetric flask by Milli-Q water and mix by Vortex Shaker Mixer.

B) PREPARATION OF BRACKETING STANDARD SOLUTION (10 mg/kg)

1. Transfer 1.00 ml from Intermediate Standard Solution-1 of Iron (100 mg/Kg) in 10 ml volumetric flask.
2. Add 0.5 ml Nitric Acid and made up the volume till 10ml volumetric flask by Milli-Q water and mix by Vortex Shaker Mixer.

C) PREPARATION OF BLANK (5% NITRIC ACID)

1. Transfer 7.25 mL of Nitric Acid (69%) in 100 mL Milli Q Water in Glass Bottle and Mix well.

D) PREPARATION OF CALIBRATION STANDARD SOLUTIONS

1. Use Intermediate Standard Solution-1 for preparing Calibration Standard Solutions as mentioned in below Table.

CAL. STANDARD SOLUTIONS	ISS - 1 (mg/Kg)	VOL. OF ISS - 1 (mL)	VOL. OF NITRIC ACID (mL)	FINAL VOL. (mL)	FINAL CONC. (mg/Kg)
LS 6	100	8.00	0.5	10	80.0
LS 5	100	6.00	0.5	10	60.0
LS 4	100	4.00	0.5	10	40.0
LS 3	100	2.00	0.5	10	20.0
LS 2	100	1.00	0.5	10	10.0
LS 1	100	0.50	0.5	10	5.0

CAL : Calibration
ISS : Intermediate Stock Solution
VOL : Volume
LS : Linearity Solution

Note: *Use freshly prepared Standard solutions for the analysis.*

Sample Preparation

PREPARATION OF SAMPLE SOLUTION

1. Grind 50g sample as fine as possible.
2. Weigh 0.50 g (± 0.05 g) Grinded Sample.
3. Transfer to Microwave Digestion Closed (MDC) Vessel.
4. Heat Milli Q Water at 60 ^{0}C.
5. Add 2.0 mL of Hot Milli-Q water.
6. Add 1.0 mL Hydrogen Peroxide.
7. Add 5.0 mL of Nitric Acid.
8. Close the Microwave Vessel tightly.
9. Keep at Room Temperature for 5 minutes.
10. Keep the Vessel rotor in Microwave Digester.
11. Cool the Vessel at Room Temperature after Digestion.
12. Add 10 mL of Milli Q water.
13. Mix well.
14. Transfer to 50 mL Volumetric Flask.
15. Volume make-up to 50 mL with Milli-Q water.
16. Filter and use for the injection on AAS.

Method of analysis

a) Instrument : AAS

b) Equipment Conditions : As detailed in below Table

Hallow Cathode Lamp	Iron (as Fe)
Lamp Current	5 (mA)
Absorption Wavelength	372.0
Slit Width(nm)	0.2
Signal – Type	Atomic Absorption
Signal Measurement	Integration
Oxidant	Air
Oxidant Flow(L/Min)	13.5
Acetylene Flow	2
Equation	Linear
Read Parameters	
Time(Sec)	10
Delay time(Sec)	10

c) Microwave Digestion Program

SL. NO	RAMPING STAGE	HOLD TIME (Minutes)	TEMP (^{0}C)	POWER (Watt)
1	01	20	180	800
2	02	10	160	800
3	03	10	140	800
4	COOL DOWN	10	-	-

<table>
<tr><td>Batch Organization</td><td><u>Injection Sequence</u>
<table>
<tr><th>S.NO.</th><th>NAME OF INJECTIONS</th><th>NUMBER OF INJECTIONS</th></tr>
<tr><td>1</td><td>Blank</td><td>2</td></tr>
<tr><td>2</td><td>Linearity Solution (LS) - 1</td><td>1</td></tr>
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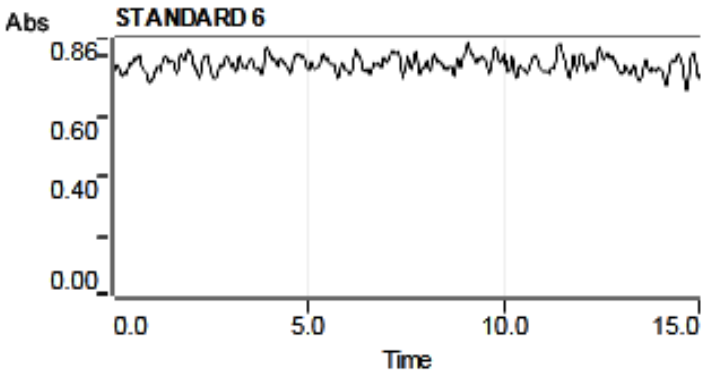
<tr><td>11</td><td>Blank</td><td>2</td></tr>
<tr><td>12</td><td>Bracketing Standard Solution</td><td>1</td></tr>
<tr><td colspan="2">TOTAL INJECTIONS</td><td>14</td></tr>
</table></td></tr>
<tr><td>Calculation with units of expression</td><td>a) Carry out a regression analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve, including zero as the response for the reagent blank.

Iron (mg/kg) = <u>Instrument Conc.(mg/kg) X Make-up Volume (mL)</u>
Sample Weight (g)</td></tr>
<tr><td>Results</td><td> 
</td></tr>
<tr><td>LOD & LOQ</td><td>Limit of Detection 2.5 mg/kg with respective to the Standard.
Limit of Quantification 5.0 mg/kg in with respective to the Standard. Limit of Quantification 500 mg/kg in with respective to the Sample.</td></tr>
<tr><td>Reference</td><td>RPT/MT/FRK/2023/001, Method Validation Report for Estimation of Iron in Fortified Rice Kernel by Using AAS.

AOAC 2011.14: Determination of Minerals and Trace elements in Milk & Milk Products, Infant Formula, and Adult Nutrition.</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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