#### File No.11014/02/2021-QA-Part(1)

### 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 (ग्णवत्ता आश्वासन)

To:

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

### File No.11014/02/2021-QA-Part(1)

- 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

एफएसएसएआइ <u>राइट्राट्र</u> आतीम माप्त सुरवा के समक प्रशिक्तलम माराय और परिवार कल्याण मंत्राखम Ministry Orbath and Panty Webra	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	<ol> <li>Concentrated Nitric Acid         It is a Chemical which is corrosive to damage. It is toxic if inhaled and correst of pollowing safety measures need to Nitric Acid:         <ul> <li>a) Do not breathe dust/fume/gass</li> <li>b) Wash face, hands and any experiments</li> <li>c) Wear protective gloves/protected</li> <li>d) Use only outdoors or in heat/sparks/open flames/hot set (sparks/open flames/hot set)</li> <li>e) Keep/Store away from clothin f) Take any precaution to avoid g) Keep only in original contain h) Wear respiratory protection</li> </ul> </li> <li>Hydrogen Peroxide     <ul> <li>It is Oxidizing, Corrosive and Irrest States and use splash goggles, gloves, and use splash goggles, gl</li></ul></li></ol>	<ul> <li>Metals. It causes severe skin osive to the respiratory tract.</li> <li>be taken during Handling of /mist/vapors/spray posed skin thoroughly after har ctive clothing/eye protection/fa a well-ventilated area Keepurfaces.</li> <li>ng/ other combustible materials mixing with combustibles er</li> <li>ritant chemical.</li> <li>e taken during Handling of Hy high concentrations of Hydrog stations and safety showers and an approved Vapor Respirations such as concechemical fume hood to avoid et al.</li> </ul>	burns and eye Concentrated adling ce protection p away from away from drogen en Peroxide in are accessible, ator. <i>entrated nitric</i> <i>exposures</i> .			
Principle	Nitric acid and hydrogen peroxide sample in microwave vessels, and control. Analysis is performed by AA	are added to homogenized V digested using a preprograme S.	itamin Premix d temperature			
Apparatus/Instruments	<ol> <li>Atomic Absorption Spectrosc</li> <li>Microwave Digester</li> <li>Analytical Balance</li> <li>Micro Pipettes (20 -200 μL) d</li> </ol>	copy (AAS) & (100 -1000 μL)				
Materials and Reagents	<ol> <li>Concentrated Nitric Acid (Pu</li> <li>Hydrogen Peroxide (Purity -3</li> <li>CRM / Standard Stock Soluti</li> <li>Purity of Argon and other</li> </ol>	rity- 69%) - Suprapure 60%) – LR Grade on - Iron (Purity - 1000 mg/kg) gas, if used must fulfill th	) e standard of			

	instrument requirement					
Sample Preparation	PREPARATION	N OF SAMI	PLE SOLUT	ION		
	<ol> <li>Weigh 0.50 g (± 0.05 g) of Homogenized Sample.</li> <li>Transfer to Microwave Digestion Closed (MDC) Vessel.</li> <li>Heated Milli Q Water at 60 °C.</li> <li>Add 2.0 mL of Hot Milli-Q water.</li> <li>Add 1.0 mL Hydrogen Peroxide.</li> <li>Add 5.0 mL of Nitric Acid.</li> <li>Close the Microwave Vessel tightly.</li> <li>Keep at Room Temperature for 5 minutes.</li> <li>Keep the Vessel rotor in Microwave Digester.</li> <li>Cool the Vessel at Room Temperature after Digestion.</li> <li>Add 10 mL of Milli Q water.</li> <li>Mixed well.</li> <li>Transfer to 100 mL Volumetric Flask.</li> <li>Volume make-up to 100 mL with Milli-Q water.</li> <li>Filter and use this for injecting on AAS.</li> </ol>					
Method of Analysis	A) PREPARATI	ION OF BL	ANK (5% N	NITRIC ACID)		
(a) Preparation of Standard solutions	<ul> <li>Transfer 7.25 mL of Nitric Acid (69%) in 100 mL Milli Q Water in Glass Bottle Mix well. Shake Vigorously.</li> <li>B) <u>PREPARATION OF CALIBRATION STANDARD SOLUTIONS</u> Use Intermediate Standard Solution-1 for Preparing Calibration Standard Solutions as mentioned in below Table</li> </ul>					
			VOI	VOL. OF	1	
	CAL.	SSS	OF	NITRIC	FINAL	FINAL
	SIANDARD	(mg/kg)	SSS (mL)	ACID (mL)	(mL)	(mg/kg)
	LS 6	1000	1.50	0.5	10	150
	LS 5	1000	1.25	0.5	10	125
	LS 4	1000	1.00	0.5	10	100
		1000	0.75	0.5	10	75
		1000	0.50	0.5	10	25
	<ul> <li>CAL : Calibration</li> <li>SSS : Standard Stock Solution</li> <li>VOL : Volume</li> <li>LS : Linearity Solution</li> <li><i>NOTE: Use Freshly Prepared Standard solutions for the Analysis.</i></li> <li>C) <u>PREPARATION OF BRACKETING STANDARD SOLUTION</u> (50 mg/kg)</li> <li>1. Transfer 0.5 ml from Standard Stock Solution of Iron (1000 mg/L) in 10 volumetric flask.</li> <li>2. Add 0.5 ml nitric acid and made up the Volume till 10 ml volumetric</li> </ul>					
	flask by M	filli-Q water	and mix by	Vortex Shaker M	lixer.	moure

o) Instrument Details	a) <b>I</b> i b) <b>E</b>	nstrument: Atomic A Equipment Condition	Absorptic <b>ns</b> : As de	on Spectro tailed in l	ometer (AAS pelow Table	)	
	Hollow	cathode Lamp		Iron (Fe	2)		
	Lamp Cu	Lamp Current (mA)4Absorption Wavelength (nm)3					
	Absorpti						
	Slit Wid	th (nm)		0.2			
	Signal-T	ype		Atomic	Absorption		
	Signal -N	Measurement		Integrat	ion		
	Oxidant			Air			
	Oxidant	Flow (L/min)		13.5			
	Acetylen	e Flow (L/min)		2			
	Equation	Equation		Linear			
		Rea	d Parame	ter	э <b>г</b>		
	Time (se	c)		10			
	Delay tir	me (sec)		10			
	c) Micro	owave Digestion Prog	gram	LD	TEMP	POWER	
	5.10	STAGE	(Mi	nutes)	(°C)	(Watt)	
	1	1	20		180	800	
	2	2	10		160	800	
	3	3 3 10			140	800	
	4	COOL DOWN	10		-	-	

<b>Batch Organization</b>	on <u>Injection Sequence</u>						
	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 I	NIECTIONS	14				
	IUIALI	INJECTIONS	14				
	TOTAL	INJECTIONS	14				
Calculation with Units of	Carry out an	alysis and calculate Regression c	Defficient (R <sup>2</sup> ) by analyzing the				
Calculation with Units of Expression	Carry out an calibration st	alysis and calculate Regression calculate data into a l	oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including				
Calculation with Units of Expression	Carry out an calibration st zero.	alysis and calculate Regression c andards by fitting the data into a l	oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including				
Calculation with Units of Expression	Carry out an calibration st zero. Calculate the	alysis and calculate Regression c andards by fitting the data into a l Iron Content in Vitamin Premix usi	ng the following equation:				
Calculation with Units of Expression	Carry out an calibration st zero. Calculate the	alysis and calculate Regression c andards by fitting the data into a l Iron Content in Vitamin Premix usit	oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including ng the following equation:				
Calculation with Units of Expression	Carry out an calibration st zero. Calculate the Iron (mg/	alysis and calculate Regression c andards by fitting the data into a l Iron Content in Vitamin Premix usit kg) = <u>Instrument Conc. (mg/kg) x M</u> Sample Wei	Defficient (R <sup>2</sup> ) by analyzing the inear regression curve, including ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)				
Calculation with Units of Expression	Carry out an calibration st zero. Calculate the Iron (mg/s	alysis and calculate Regression c andards by fitting the data into a l Iron Content in Vitamin Premix usinkg) = <u>Instrument Conc. (mg/kg) x N</u> Sample Wei	oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)				
Calculation with Units of Expression	Carry out an calibration st zero. Calculate the Iron (mg/	alysis and calculate Regression c andards by fitting the data into a l Iron Content in Vitamin Premix usi kg) = <u>Instrument Conc. (mg/kg) x M</u> Sample Wei ection 12.5 mg/kg with respective to	14         oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including         ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)         the Standard.				
Calculation with Units of Expression LOD & LOQ	Carry out an calibration st zero. Calculate the Iron (mg/ Limit of Dete Limit of Qua	alysis and calculate Regression c andards by fitting the data into a 1 Iron Content in Vitamin Premix usit kg) = <u>Instrument Conc. (mg/kg) x N</u> Sample Wei ection 12.5 mg/kg with respective to antification 25.0 mg/kg with respect	14         oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including         ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)         the Standard.         ctive to the Standard. Limit of				
Calculation with Units of Expression LOD & LOQ	Carry out an calibration st zero. Calculate the Iron (mg/ Limit of Dete Limit of Qua Quantification	alysis and calculate Regression c andards by fitting the data into a 1 Iron Content in Vitamin Premix usi kg) = <u>Instrument Conc. (mg/kg) x N</u> Sample Wei ection 12.5 mg/kg with respective to antification 25.0 mg/kg with respect n 5000 mg/kg with respective to the	14         oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including         ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)         the Standard.         ctive to the Standard. Limit of Sample.				
Calculation with Units of Expression LOD & LOQ Reference	Carry out an calibration st zero. Calculate the Iron (mg/ Limit of Dete Limit of Qua Quantification AOAC 2011.	alysis and calculate Regression c andards by fitting the data into a 1 Iron Content in Vitamin Premix usit kg) = Instrument Conc. (mg/kg) x M Sample Wei ection 12.5 mg/kg with respective to antification 25.0 mg/kg with respect n 5000 mg/kg with respective to the 14: Determination of Minerals and	14         oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including         ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)         the Standard.         ctive to the Standard. Limit of Sample.         Trace elements in Milk & Milk				
Calculation with Units of Expression LOD & LOQ Reference	Carry out an calibration st zero. Calculate the Iron (mg/ Limit of Dete Limit of Qua Quantification AOAC 2011. Products, Infa	alysis and calculate Regression c andards by fitting the data into a 1 Iron Content in Vitamin Premix usinkg) = Instrument Conc. (mg/kg) x M Sample Wei ection 12.5 mg/kg with respective to antification 25.0 mg/kg with respect n 5000 mg/kg with respective to the 14: Determination of Minerals and ant Formula, and Adult Nutrition.	14         oefficient (R <sup>2</sup> ) by analyzing the inear regression curve, including         ng the following equation: <u>Make-up Volume (mL)</u> ght (gm)         the Standard.         ctive to the Standard. Limit of Sample.         Trace elements in Milk & Milk				

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Method No.	FSSA	I.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. Foli	ic Acid:						
	Fo Fi & art	blic acid is not considered ha rst Aid: Rise immediately w skin. Avoid to inhale fum tificial respiration.	zardous by the 2012 OSHA Standith plenty of water if it is in contine, move to fresh air. If not br	dard. act with Eye eathing give				
	<b>2. Ammonium Hydroxide:</b> Routes of Exposure: Inhalation, ingestion, skin contact, eye contact							
	<ul> <li>a. Corrosive. May cause damage to mucous membranes in nose, throat, lungs and bronchial system.</li> <li>b. Corrosive. Harmful if swallowed. May produce burns to the lips, oral cavity, upper airway, esophagus and digestive tract.</li> <li>c. Corrosive. Causes severe burns.</li> <li>d. Corrosive. Causes severe burns. May cause eye damage, impaired sight or blindness.</li> </ul>							
	3. Pot	assium Phosphate Mono B	asic:					
	<ul> <li>a. Move to fresh air. Get medical attention if symptoms persist.</li> <li>b. Wash skin thoroughly with soap and water. Get medical attention symptoms occur. Wash contaminated clothing before reuse.</li> <li>c. Immediately flush with plenty of water for at least 15 minutes. If eas do, remove contact lenses. Get medical attention if irritation persists a washing.</li> </ul>							
	4. Tet	ra methyl Ammonium Hyd	lroxide:					
	<ul> <li>a. Rinse thoroughly with plenty of water for at least 15 minutes, lift lower and upper eyelids. Consult a physician.</li> <li>b. Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required.</li> <li>c. Move to fresh air. If not breathing, give artificial respiration. Do not mouth-to-mouth method if victim ingested or inhaled the substance; g artificial respiration with the aid of a pocket mask equipped with a cway valve or other proper respiratory medical device. Immediate mediatemed attention is required.</li> </ul>							
	5. Pho	sphoric Acid:						
	a.	Seek medical attention im air. Loosen clothing as comfortable position.	mediately. Move exposed indivi- necessary and position indiv	dual to fresh vidual in a				

	<ul> <li>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.</li> <li>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.</li> <li>d. Seek medical attention immediately. Rinse mouth thoroughly. Do not induce vomiting. Have exposed individual drink sips of water.</li> </ul>
	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:
	<ul> <li>a. Wash skin thoroughly after handling.</li> <li>b. Avoid breathing dust/fume/gas/mist/vapours/spray.</li> <li>c. Do not breathe dust/fume/gas/mist/vapours/spray.</li> <li>d. IF ON SKIN: Wash with soap and water.</li> <li>e. Specific measures (see supplemental first aid instructions on this label).</li> <li>f. Wash contaminated clothing before reuse.</li> <li>g. Avoid contact with skin and eyes. Avoid inhalation of vapour or mist.</li> <li>h. Use explosion-proof equipment.</li> <li>i. Keep away from sources of ignition - No smoking</li> </ul>
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	<ol> <li>HPLC, Binary gradient pump, an auto Sampler.</li> <li>Analytical Balance, Suitable for weighing samples with accuracy up to 0.1 mg</li> <li>Centrifuge 5000 RPM, holding 50 mL tubes</li> <li>Micro Pipettes (100 -1000 μl, 20 -200 μl 10 -100 μl).</li> <li>HPLC C18 ODS Column: 4.6mm X 250 mm X 5 μm;</li> <li>Sonicator for mixing of solution.</li> <li>Vortex for preparation of stock solution.</li> <li>Homogenizer for sample grinding</li> </ol>
Materials and Reagents	<ol> <li>Ammonium Hydroxide, LR Grade</li> <li>Phosphoric Acid, LR Grade</li> <li>Monobasic Potassium Phosphate, LR Grade</li> <li>Tetrabutylammonium Hydroxide, LR Grade</li> <li>Methanol, HPLC Grade.</li> <li>CRM: Folic Acid (CAS No: 593003)</li> </ol>
Preparation of Mobile Phase	PREPARATION OF MOBILE PHASE
reparation of Widdle r hase	MOBILE PHASE PREPARATION
	<ol> <li>Accurately weight 2.0 g of monobasic potassium phosphate into a 1000 ml volumetric flask.</li> <li>Add 650 mL of Milli-Q Water for Volume make up</li> <li>Add 15 mL of 0.5 M Tetra butyl ammonium hydroxide in methanol.</li> <li>Add 7.0 mL of 3 N Phosphoric acid.</li> <li>Add 270 mL of methanol.</li> <li>Cool to room temperature.</li> <li>Adjust pH 5.0 with 3 N Phosphoric Acid or 6 N ammonium hydroxide.</li> </ol>

	<b>8.</b> Finally make the volume 1000 ml with Milli-Q Water.					
Sample Preparation	PREPARATION C	OF SAMPLI	E SOLUTION	N		
	<ol> <li>Accurately weigh 1 g (± 0.1 g) of Homogenized Sample</li> <li>Add 0.1 ml of 10 % ammonium hydroxide</li> <li>Transfer into a 10 mL Amber Colored Volumetric Flask</li> <li>Add 5 mL Buffer</li> <li>Vortex for 5 minutes</li> <li>Cool the Sample Solution at Room Temperature</li> <li>Do Volume make-up to 10 ml with mobile phase</li> <li>Vortex for 2 minutes</li> <li>Filter the solution through 0.45µm Nylon Syringe Filter</li> <li>Pour the Filtrate into the Vial, and use this for injecting into HPLC</li> </ol>					
	Note: If required, di	lute the sam	ple for desired	l concentration.		
Method of Analysis	PREPARATION C	<b>OF STANDA</b>	ARD STOCK	SOLUTION		
(a) Preparation of Standards	a) <u>PREPARATION OF STOCK SOLUTION FOR FOLIC ACID (1000</u> <u>mg/kg)</u>					
	<ol> <li>Accurately weigh 10 mg (± 0.1 mg) of Folic Acid Standard.</li> <li>Add 0.1 ml of 10% Ammonium Hydroxide Solution</li> <li>Transfer to 10 mL Amber Colored Volumetric Flask.</li> <li>Add Buffer for Volume make-up to 10 mL.</li> <li>Vortex for 2 min.</li> <li>Store the Solution at -20°C in the light Protected Area.</li> </ol>					
	b) <u>PREPARATI(</u> mg/kg)	ON OF BR	ACKETING	STANDARD SO	LUTION (85	
	<ol> <li>Pipette out 0.85 mL of Standard Stock Solution</li> <li>Transfer to 10 mL Amber Colored Volumetric Flask containing 2 mL Milli Q Water.</li> <li>Add Buffer for Volume make-up to 10 mL.</li> <li>Vortex for 2 min.</li> </ol>					
	c) <u>PREPARATI</u>	ON OF CAL	LIBRATION	STANDARD SOI	LUTIONS	
	Use Standard Stoc as mentioned in be	ck Solution f elow Table.	for preparing (	Calibration Standard	d Solutions	
	CALIBRATION	CCC	VOL OF	FINAL MAKE	FINAL	
	SOLUTIONS	888 (mg/kg)	SSS (mL)	DILUENT (mL)	CONC. (mg/kg)	
	LS 6	1000	1.50	10	150	
	LS 5	1000	1.20	10	120	
	LS 4	1000	1.00	10	100	
	LS 3	1000	0.85	10	85	
	LS 2	1000	0.50	10	50	

	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	• Instrument		: HPI	C UV Detector			
Conditions	Chromatographi	ic Conditions	s : As c	letailed in below Ta	ble		
	Instrument	HPLO	2				
	Detector	UV 2	80 nm				
	Column	C18 (	DDS Column:	4.6 mm X 250 mm	X 5 µm;		
	Run time	10 mi	n				
	Flow rate	1.8 m	l/min				
	Injection Volume	10 µl					
	Column Temperatu	ure 25°C					
	<b>Note:</b> The make, model of Instrument & Column can be changed Instrument should be able to achieve the desired LOD & LOQ Value Column is exactly same in terms of the Composition & Dimensions.						
<b>Batch Organization</b>	Injection Sequence						
	S.NO. NAM	IE OF INJE	CTIONS	NUMB INJEC	ER OF		
	1. Blank			2			
	2. Linear	ity Solution	(LS) - 1	1			
	3. Linear	rity Solution	(LS) - 2	1			
	4. Linear	ity Solution	$\frac{(LS) - 3}{(LS) - 4}$	1			
	5. Linear	ity Solution	$\frac{(LS) - 4}{(LS) - 5}$	1			
	0.Emean7.Linear	ity Solution	$\frac{(LS) - 5}{(LS) - 6}$	1			
	8. Blank	10 201401011	(22) 0	2			
	9. Sampl	e Solution		1			
	10. Blank			2			
	11. Bracke	eting Standar	d Solution	1			
	TOTAL INJECT	TOTAL INJECTIONS					
Calculation with units of	a) Carry out analysi	s and calcula	te Regression	coefficient $(R^2)$ by	analyzing the		
Expression	canoration standard	s by mung u	le dala into a l	linear regression cu	.ve.		
	Calculate the Folic	Acid Content	in Premix us	ing the following eq	luation:		
	Folic Acid	(Vitamin B9	) ( <b>mg/kg</b> ) =				
	Sample Con b) The LOD and L respectively, for the	ic.(mg/kg) x Sample OQ are det Folic acid si	Make up Volt Weight (gm) ermined by c gnal in the ma	ume (mL) onsidering the S/N atrix.	of 3 and 10,		



एफएसएसएआई SSSCI माली बच्च इत्या-प्रेर मन्न प्राणिकरण Foot bady and Bandwins Anony of that रखारम और परिवार करनाण मंत्रालय Ministry of Health and Formity 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 So (Vitami HPLC	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) Me Hea	<b>thanol:</b> It is a Flammable alth.	and Toxic Liquid. It creates	Hazards to Human					
	Du a) b) c) d) e) f) g) h) i)	ring handling of Methanol, b Wash skin thoroughly after Avoid breathing dust/fume, Do not breathe dust/fume/g IF ON SKIN: Wash with so Specific measures (see sup) Wash contaminated clothin Avoid contact with skin and Use explosion-proof equip Keep away from sources of	below safety measures to be handling. /gas/mist/vapours/spray. as/mist/vapours/spray. bap and water. plemental first aid instruction g before reuse. d eyes. Avoid inhalation of v nent. 'ignition - No smoking	followed: ns on this label). vapour or mist.					
	2) Acc dar Du a)	etonitrile: It is a Flammable nage. ring handling of Acetonitrile Inhalation: Inhale fresh breathing or artificial respir	e liquid which causes severe e, below safety measures to l air. If breathing stops, gi	e skin burns and eye be followed: ive mouth-to-mouth					
	b) c) d)	Skin Contact: Take off im with water/ shower. Eye Contact: Rinse out v Remove contact lenses. If swallowed: After swallow water (two glasses at most	with plenty of water. Call wing, immediately make vic	clothing. Rinse skin in ophthalmologist. tim drink					
	<ol> <li>Orthophosphoric Acid: It is a colorless, crystalline solid, the tripentavalent phosphorus.</li> </ol>								
	Du foll	ring handling of Orthopholowed:	osphoric Acid, below safe	ety measures to be					
	a) b) c) d)	Rinse immediately with ple 15 minutes. Immediate med Wash off immediately with and wash contaminated clo use. Call a physician imme Do NOT induce vomiting. by mouth to an unconsciou If not breathing, give art down. Do not use mouth-to substance; give artificial re with a one-way valve or oth	enty of water, also under the lical attention is required. a plenty of water for at least thing and gloves, including diately. Clean mouth with water. I s person. ificial respiration. Remove p-mouth method if victim in spiration with the aid of a po- ner proper respiratory medic	eyelids, for at least 15 minutes. Remove the inside, before re- Never give anything from exposure, lie gested or inhaled the ocket mask equipped cal device.					

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

	5. Do Volum	5. Do Volume make-up to 10 ml with Mobile phase A.						
	6. Vortex for	2 minutes	1 0 4 5 1	1 0				
	7. Filter the s	olution throug	h 0.45µm N	ylon Syringe F	ilter.			
	8. Pour the Fi	iltrate into the	Vial, and us	e this for inject	ting into HP	LC.		
Method of Analysis								
	A) <u>PREPARAT</u>	<u>ION OF STO</u>	OCK SOLU	<u>FION FOR C</u>	YANOCOH	<u>BALAMIN</u>		
	<u>(1000 mg/kg</u> )	<u>)</u>						
	1. Accurately	weigh 10 mg	(± 0.1 mg) o	of Cyanocobala	amin Standa	ırd.		
	2. Transfer to	0 10 mL Ambe	r Colored V	olumetric Flash	k.			
	3. Add Mobil	le Phase A for	Volume ma	ke-up to 10 mI				
	4. Vortex for	4. Vortex for 2 min. Note: Store the Solution at $-20^{\circ}$ C in the light Protected Area						
	Note. Store in	Note. Store the Solution at 20 C in the light Flotected Area						
	B) <u>PREPARAT</u> (100 mg/kg)	ION OF IN	<u>TERMEDI</u>	ATE STAND	ARD SOL	LUTION - 1		
	1. Pipette out	1.0 mL of Sto	ock Solution					
	2. Transfer to	o 10 mL Amb	per Colored	Volumetric Fl	lask Contain	ning 2 mL of		
	Mobile Pha	ase A.	<b>X 7 1</b>					
	3. Add Mobil 4. Vortex for	le Phase A for	Volume ma	ke-up to 10 ml	<b></b>			
	1. VOICEATOR	2 11111.						
	C) <u>PREPARAT</u> <u>mg/kg)</u>	ION OF INT	ERMEDIA	TE STANDAI	RD SOLUI	<u>FION - 2 (10</u>		
	1. Pipette o	ut 1.0 mL of Ir	ntermediate	Standard Stock	Solution –	1.		
	2. Transfer	to 10 mL Am	ber Colored	l Volumetric F	lask Contai	ning 2 mL of		
	Mobile P	hase A.			_			
	3. Add Mot	oile Phase A fo	or Volume m	ake-up to 10 n	nL.			
	4. Vonex ic	$\Delta \Gamma \perp \Pi \Pi \Pi$ .						
	D) <u>PREPARAT</u>	ION OF BRE	CACKGING	STANDARD	SOLUTIO	<u>DN</u>		
	<u>(0.75 mg/kg)</u>							
	1 Pinette out 0.75 mL of Intermediate Standard Stock Solution $-2$							
	2. Transfer to	o 10 mL Amb	per Colored	Volumetric Fl	ask Contain	ning 2 mL of		
	Mobile Pha	ase A.				-		
	3. Add Mobil	le Phase A for	Volume ma	ke-up to 10 mI				
	4. Vortex for	2  min.						
	E) DEEDADATION OF CALIDDATION STANDADD SOL LITIONS							
	Lice Intermediate	Standard Salu	tion 2 for	Dremenine Celi	hustion Stor	dand Calution		
	Use Intermediate Standard Solution $-2$ for Preparing Calibration Standard Solut as mentioned in below Table.							
	CAL.	IGG A	VOL.	VOL. OF	FINAL	FINAL		
	STANDARD	188 - 2 (10 mg/T)	OF ISS –	DILUENT	VOL.	CONC.		
	SOLUTIONS		2 (mL)	(mL)	(mL)	(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		
		10	0.75	9.25	10	0.75		
		10	0.50	9.50	10	0.50		
	LOI	10	0.20	2.00	10	0.20		

[	NT-4- Alugua	1 Ela Dream	f Calibration St	1 10 1		
	Note: Always n	ake Fresh Prep	paration of Calibration St	andard Solutions		
	CAL	: Calibration	Staals Solution			
	VOL · Volume					
	LS	: Linearity Sol	ution			
Mothed of Anolysis	a) Instrume	nt . UDI C				
(a) Chromatographic	b) <b>Chromate</b>	nt : HPLC ographic Condi	itions : As detailed in be	low Table		
(a) Chromatographic Conditions						
Conditions	Instrument		HPLC			
	Detector		DAD			
	Column		Column: C8 4.6 mm X	250 mm X 5µm		
	Run time		30 min			
	Column Temp	erature	40°C			
	Flow rate		1.0 mL/min			
	Injection Volu	ime	100 µl			
	Mobile Phase A		Dissolve 2.72 gm phosphate and 3.48 g phosphate in 1000 pH 6.6 (+/- 0.1) with O	Potassium Dihydrogen gm Dipotassium hydrogen ml of water, Adjust rtho 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	t Program	MOBILE PHASE	A MOBILE PHASE B		
		RATE	(%)	(%)		
	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		
	<b>Note:</b> 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>INJECTION S</b>	SEQUENCE				
(b) Batch Organization	S.NO.	NAME (	OF INJECTIONS	NUMBER OF INJECTIONS		
	1	Blank		2		
	2	Linearity	Solution (LS) - 1	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	a) Carry o	ut analysis and calculate Regression c	oefficient (R <sup>2</sup> ) by analyzing	
Expression	the calib	pration standards by fitting the data into	a linear regression curve.	
		Cyanocobalamin (Vitamin B12) (n	ng/kg) =	
	Sample Conc.(mg/kg) X Make up Volume(mL)			
	Sample Weight (g)			
	<ul><li>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.</li></ul>			
(a) Chromotograms				
(a) Chi omatogi anis	VWD1 A, Waveleng	th=360 nm		
	15- 14-			
	13- 12-			
	11- 10-	-812		
	9 9 8 7 6 4 3 2 1 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 20 21 22 23 24 25 26 27 28 29 30 29 20 20 21 22 23 24 25 26 27 28 29 30 20 20 20 20 20 20 20 20 20 2			
		i ime (min)		
(b) LOD & LOQ	a) Limit of Det	ection (0.1 mg/kg) With Respective to	the Standard.	
	b) Limit of Quantification (0.2 mg/kg) With Respective to the Standard.			
Reference	AOAC 2011 10	-AOAC Official method for Vitamir	B12 in Indian infant and	
Mittutt	Pediatric formul	as and Adult Nutritionals.	1 212 in moran main and	
Approved by	Scientific Panel on Methods of Sampling and Analysis			

एफएसएसएआइ जित्र के प्रिया के सामक प्राणिकरण भारतीय बाख सुरक्षा और मामक प्राणिकरण भारतीय बाख सुरक्षा और मामक प्राणिकरण वा सामय और परियार करनाया प्राणंतम् 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. & Date0.0			
Scope	The Scope of this Method is applicable for Q 500 mg/kg LOQ Level (with respect to the S	Quantification of Iron in FRK at Sample) by using AAS.			
Safety & Precautions	1. Concentrated Nitric Acid				
	It is a Chemical which is corrosive to Metals	s. 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/v	vapors/spray			
	b) Wash face, hands and any exposed s	skin thoroughly after handling			
	c) Wear protective gloves/protective cl	othing/eye protection/face protection			
	d) Use only outdoors or in a well-venti	lated area Keep away from			
	heat/sparks/open flames/hot surfaces	S.			
	e) No smoking				
	1) Keep/Store away from clothing/ other combustible materials				
	<ul> <li>b) Keen only in original container</li> </ul>				
	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				
	<i>Note:</i> 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 ( $\pm$ 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				
A provotus/Instruments	up to 50 mL, Filter and Inject in AAS.	45)			
Apparatus/mstruments	Atomic Absorption Spectrometry (A     Microwave Digester	170 <i>)</i>			
	3 Analytical Balance				
	4. Micro Pipettes (20 -200 ul) & (100 -	-1000 µl)			
		· 1. X			
	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. Concentra	ated Nitric A	Acid (Purity- 6	59%)		
	2. Hydrogen Peroxide (Purity -30%)					
	3. CRM Use	ed: Iron	•			
Preparation of solutions	A) PREPARAT	ION OF I	NTERMEDI	ATE STOCK	SOLUTIO	N - 1 (100
-	mg/kg)					
	1. Transfer 10	.0 ml from s	stock solution	of iron (1000 m	g/kg) in 10	0 ml
	volumetric	flask.				
	2. Add 5.0 ml	l nitric acid a	and made up t	the volume till 1	00 ml volui	netric
	flask by Milli-Q water and mix by Vortex Shaker Mixer.					
	B) PREPARAT	ION OF	BRACKETI	NG STANDA	RD SOLU	JTION (10
	mg/kg)					<u> </u>
	1. Transfer	1.00 ml fro	om Intermedi	ate Standard S	olution-1 c	of Iron (100
	mg/Kg) ir	n 10 ml volu	metric flask.			
	2. Add $0.5 \text{ m}$	nl Nitric Aci	id and made u	ip the volume til	l 10ml volu	imetric flask
	by winned		mix by voice	A SHAKET WILLET.		
	C) <u>PREPARATI</u>	ON OF BL	ANK (5% N	ITRIC ACID)		
	1 Transfer 7 25 mL of Nitric Acid (69%) in 100 mL Milli O 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.	ISS - 1	VOL. OF	NITRIC	FINAL	FINAL
	STANDARD	(mg/Kg)	ISS - 1	$\Delta CID (mL)$	VOL.	CONC.
	SOLUTIONS		(mL)	mend (init)	(mL)	(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
	LSI	100	0.50	0.5	10	5.0
	CAI Calibration					
	ISS : Intermediate Stock Solution					
	VOL : Volume					
1	VOL .	LS : Linearity Solution				
	LS :	Linearity Sc	olution			
	LS :	Linearity Sc	olution			

Sample Preparation	PREPARATION OF SAMPLE SOLUTION				
	<ol> <li>Grind 50g sample as fine as possible.</li> <li>Weigh 0.50 g (± 0.05 g) Grinded Sample.</li> <li>Transfer to Microwave Digestion Closed (MDC) Vessel.</li> <li>Heat Milli Q Water at 60 °C.</li> <li>Add 2.0 mL of Hot Milli-Q water.</li> <li>Add 1.0 mL Hydrogen Peroxide.</li> <li>Add 5.0 mL of Nitric Acid.</li> <li>Close the Microwave Vessel tightly.</li> <li>Keep at Room Temperature for 5 minutes.</li> <li>Keep the Vessel rotor in Microwave Digester.</li> <li>Cool the Vessel at Room Temperature after Digestion.</li> <li>Add 10 mL of Milli Q water.</li> <li>Mix well.</li> <li>Transfer to 50 mL Volumetric Flask.</li> <li>Volume make-up to 50 mL with Milli-Q water.</li> <li>Filter and use for the injection on AAS.</li> </ol>				
Method of analysis	a) Instrument : AAS b) Equipment Conditions : As detailed in below Table				
	b) Equipment Conditions : As detailed in below Table				
	Hallow Cathode Lamp Iron (as Fe)				
	Lamp Current     5 (mA)       Absorption Wavalanath     272.0				
	Slit Width(nm) 0.2				
	Sint widui(iiii) 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				
	SI NO	RAMPING	HOLD TIME	TEMP	POWER
	SL. NO STAGE		(Minutes)	( <sup>0</sup> C)	(Watt)
	1	1 01		180	800
	2	02	10	160	800
	3	03	10	140	800
	4	COOL DOW	N 10	-	-

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	
	9	Blank	2	
	10	Sample Solution	1	
	11	Blank	2	
	12	Bracketing Standard Solution	1	
		TOTAL INJECTIONS	14	
expression Results	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) = Instrument Conc.(mg/kg) X Make-up Volume (mL) Sample Weight (g) Abs $5TANDARD 6$ $0.86^{-1}_{0.00}_{0.40^{-1}_{0.00}_{0.00}_{0.00}_{0.00}_{0.00}_{0.00}_{0.00}_{0.00}_{0.00}_{10.0}_{15.0}_{10.0}_{15.0}$			
LOD & LOQ	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.			
Reference	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.			
Approved by	Scientific Panel on Methods of Sampling and Analysis			