File No: 11014/07/2021-QA
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: ०७ नवंबर, 2023

आदेश

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

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

i. Method for determination of **Iron** in Fortified Rice Kernel: **FSSAI.FRK.16.004.2023 (Annexure-I)**
ii. Method for determination of **Folic Acid** in Fortified Rice Kernel: **FSSAI.FRK.16.005.2023 (Annexure-II)**
iii. Method for determination of **Vitamin B12** in Fortified Rice Kernel: **FSSAI.FRK.16.006.2023 (Annexure-III)**

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: 07-11-2023 13:40:07
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 CP, FSSAI
2. SPS to CEO, FSSAI
3. Advisor (QA), FSSAI
4. ED(CS), FSSAI
5. Head (Regulations/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 as Fe in Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.FRK.16.004.2023	**Revision No. & Date**	0.0
Scope	The method is applicable for estimating the iron content in FRK using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).		
Caution	**Concentrated Nitric Acid** is highly corrosive and can cause irritation to the eyes, skin, and mucous membrane. Always add acid to water to prevent splattering from overheating and boiling. Clean-up spills promptly with appropriate materials. Handle only inside a fume hood **Hydrogen Peroxide:** Hydrogen Peroxide is a strong oxidising agent that also has corrosive properties. Keep hydrogen peroxide away from sources of ignition, heat, and moisture, storing in a tightly closed container. Keep away from incompatible materials such as organic materials, metals, acids, alkalis, combustible materials, and oxidizing agents. **Operation of Microwave Digester** involves a hot pressurized acid solution. Use appropriate personal protective equipment, face protection such as a laboratory coat, safety glasses, rubber gloves, and a fume hood.		
Principle	Nitric acid and hydrogen peroxide are added to the sample in microwave vessels, and the samples are digested using preprogrammed temperature control. The addition of hydrogen peroxide helps reduce carbon and nitrous oxide levels in the digestate. Analysis is performed by an ICP-OES. Quantitation of Fe is achieved essentially simultaneously by comparing the analyte–ISTD response ratios in the unknown samples with a standard curve constructed from the response ratios of calibration standards.		
Apparatus/Instruments	1. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) 2. Microwave digester - A commercial microwave designed for laboratory use at 0–300°C with a closed-vessel system and controlled temperature ramping capability. Use manufacturer recommended vessels 3. Analytical Balance (capable of weighing 0.0001 g) 4. Fume hood 5. Bottle-top dispenser - PTFE; Adjustable volume 0.5–5 mL 6. Volumetric pipets - Class A, assorted sizes 7. Digital pipets - 1 mL adjustable, to deliver 500 µL with accuracy tolerance of better than 0.8% and precision of better than 0.2% RSD		
Materials and Reagents	1. Concentrated Nitric acid (Purity - 69%) 2. Hydrogen peroxide (Purity - 30%) 3. CRM / Stock Solution - Iron (Purity - 1000 mg/kg) 4. Purity of Argon and other gas, if used must fulfill the standard of instrument requirement		

Sample Preparation

1. Grind 50 g of FRK sample.
2. Weigh 0.25 g (± 0.05 g) of ground kernels.
3. Transfer to microwave digestion closed vessel.
4. Add 2.0 mL of Hot (60 °C) Milli-Q Water.
5. Add 1.0 mL H_2O_2.
6. Add 0.5 mL of Nitric acid.
7. Loosely cap the vessel and keep at 25 °C for 5 min to predigest the sample.
8. Close the microwave vessel tightly.
9. Keep at 25 °C for 5 min.
10. Place the vessel rotor in microwave digester.
11. Keep the vessel rotor in microwave digester and execute a heating program equivalent to that shown in the Table below for total digestion of the sample.

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	-	-

12. Cool the vessel to 25 °C after digestion.
13. Add 10 mL of Milli Q water and mix well using a vortex.
14. Transfer to a 100 mL volumetric Flask.
15. Make-up the volume to 100 mL with Milli-Q water.
16. Filter and use for ICP-OES analysis.

Preparation of Standard solutions

A) Preparation of intermediate stock solution - 1 (ISS-1) (100 mg/kg)

1. Transfer 1.0 mL from stock solution of iron (1000 mg/kg) in 10 mL volumetric flask.
2. Add 0.5 mL Nitric acid and make up the volume to 10 mL using Milli-Q water and mix using a vortex.

B) Preparation of blank (5% Nitric acid)

1. Transfer 7.25 mL of Nitric Acid (69%) into 92.75 mL of Milli Q water in a glass bottle. Mix well**.**

C) Preparation of calibration standard solutions

Prepare the calibration standard solutions using the ISS-1 as indicated in the Table below.

Cal. Standard Solution	ISS - 1 (100 mg/L	VOL. OF ISS – 1 (mL)	VOL. OF Nitric acid (mL)	Final vol. (mL)	Final Conc. (mg/L)
LS 7	100	2.00	0.5	10	20.0

LS 6	100	1.50	0.5	10	15.0
LS 5	100	1.00	0.5	10	10.0
LS 4	100	0.75	0.5	10	7.5
LS 3	100	0.50	0.5	10	5.0
LS 2	100	0.20	0.5	10	2.0
LS 1	100	0.10	0.5	10	1.0

NOTE: Use freshly prepared calibration standard solutions for the analysis.

Method of analysis

Instrument: ICP-OES

Equipment conditions:

Plasma condition	Plasma flow (Argon 12 L/min) Nebulizer flow (0.7 L/min) RF power 1.2 kW
Uptake Delay	25 sec
Pump Speed	12 rpm
Stabilization	15 sec
Numbers of Replicates	3.0
Resolution	Normal
Wavelength	238.204 nm For Iron
Read Time	5 sec
Aux flow	1.0 L/min
Viewing Mode	Radial

Note: ***The make & model of instrument may be changed. Instrument tuning varies with make and model. Set parameter as per manufacturer's instructions and optimize for best resolution to obtain the desired LOD.***

Sequence of Injection

The injection sequence for standards and sample is given below:

SL.NO.	Sample	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	Linearity Solution (LS) - 7	1
9	Blank	2
10	Sample Solution	1
11	Blank	2
12	Spike sample	1
	TOTAL INJECTIONS	**15**

Calculation with units of expression	a) Carry out a regression analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards including zero as the response for the reagent blank. Should be >0.99. Calculate the Fe content in FRK using the following equation: $$Iron\ \frac{(Fe)\ mg}{kg} = \frac{C\ \times Makeup\ volume}{Sample\ weight\ (g)}$$ Where C= concentration from instrument software The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the Iron in the matrix. i. Limit of Detection 0.5 mg/kg with respective to the Standard. ii. Limit of Quantification 1.0 mg/kg in with respective to the Standard. iii. Limit of Quantification 400 mg/kg in with respective to the sample b) Determine the recovery of Iron by the external spiking method at a spike level of 2000 mg/Kg in six replicates. Calculate the recovery value using the following equation: $$Recovery(\%) = \frac{(\mathrm{A} - \mathrm{B})}{\mathrm{C}} \times 100$$ where A = the concentration of Iron in the spiked sample (mg/kg) B = the natural content of Iron in the control sample (mg/kg) C = the spiked concentration of Iron (mg/kg)
Reference	PRT/MT/FRK/2022/006, Method Validation Protocol for Estimation of Iron in Fortified Rice Kernel by Using ICP OES. 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

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 Fortified Rice Kernel**		
Method No.	FSSAI.FRK.16.005.2023	**Revision No. & Date**	0.0
Scope	This method is only applicable for quantitative analysis of Folic acid (Vitamin B9) in fortified rice kernels using LC-MS/MS.		
Caution (Safety & Precautions)	**Sodium hydroxide** is caustic. Contact with very high concentrations of sodium hydroxide can cause severe burns to the eyes, skin, digestive system or lungs. Prolonged or repeated skin contact may cause dermatitis. Handle with care. **Formic acid** is a corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Inhaling formic acid can irritate the nose and throat. Use in fume hood. **Acetonitrile:** Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Keep away from sources of ignition as it is flammable. **Hydrochloric acid**: Handle with extreme care. Concentrated HCl is corrosive. Avoid breathing vapors and avoid contact with skin and eyes. Handle only inside a fume hood.		
Principle	Extraction of folic acid using acetate buffer in the presence of α-amylase and then quantitative analysis using reverse phase liquid chromatography followed by tandem mass spectrometry (LC-MS/MS).		
Apparatus/Instruments	1. Liquid Chromatograph with Tandem Mass Spectrometer (LC-MS/MS), system equipped with a binary gradient pump, an auto sampler 2. Analytical Balance -Suitable for weighing samples with accuracy up to 0.0001 g. 3. Centrifuge 6000 rpm, capable of accommodating 50 mL tubes. 4. Volumetric flasks-Class A 1000 mL 5. Amber colored volumetric flask: 100 mL 6. Micro Pipettes capable of delivering from 100 -1000 µl, 20 -200 µl 100 µl. of liquids 7. Incubator shaker set at 37 ^{0}C 8. Water bath set at 55 ^{0}C 9. Column: XB C18 Column, 2.6 µm, 2.1 x 100 mm or equivalent 10. Sonicator 11. Vortex mixer 12. Homogenizer with steel blades		
Materials and Reagents	1. L-Ascorbic Acid, LR Grade 2. α-Amylase (TCI, A0447) 3. Sodium hydroxide, LR Grade 4. Formic Acid, MS Grade 5. Acetonitrile, MS Grade 6. Sodium acetate (anhydrous) LR Grade 7. Hydrochloric Acid, LR Grade 8. CRM: Folic Acid (CAS No: 593003)		

Preparation of Reagents	**Sodium acetate buffer (0.1 M)** 1. Weigh accurately 8.2 g of anhydrous sodium acetate. 2. Transfer it into 1000 mL of volumetric flask. 3. Add Milli Q Water, dissolve and make-up to 1000 mL. 4. Sonicate for 15 min to dissolve. **Sodium hydroxide (1 M)** Weigh 40 g of NaOH pellets and dissolve in 1000 mL of water. Cool and store **Mobile phase A (0.1% Formic acid)** 1. Transfer 1 mL Formic Acid into 1000 mL Volumetric Flask. 2. Add Milli-Q Water and make up to mark. 3. Sonicate to mix 4. Filter through 0.45 µm filter **Mobile phase B (100% acetonitrile)** Transfer 1000 mL MS grade acetonitrile to solvent reservoir sonicate for 1-2 mins.
Sample Preparation	1. Grind 50 g of fortified rice kernels to a fine powder. 2. Accurately weigh 1 g (± 0.1 g) of the powder. 3. Transfer into a 100 mL Amber colored volumetric flask. 4. Add 0.1 g L-Ascorbic acid and 50 mL of 0.1 M sodium acetate buffer. 5. Vortex for 5 min. 6. Adjust the pH of the solution to between 8.0-9.0 using 1 M NaOH. 7. Shake at 20 rpm for 60 min at 37 ºC using an orbital shaker. 8. Adjust the pH of the to 7.0 with 2 N HCl. 9. Add 0.05 g of α-amylase and shake for 5 minutes. 10. Incubate the sample at 55 ^{0}C for 30 mins using a water bath. 11. Cool the sample to 25 ^{0}C. 12. Make-up the volume to100 ml with 0.1 M Sodium Acetate. 13. Transfer the sample to a centrifuge tube after vigorous vortexing for two min. 14. Centrifuge at 6000 rpm for 5 min. 15. Filter the supernatant using a 0.45µm Nylon syringe filter. 16. Use the filtrate for LC-MS/MS. Prepare all samples as described above.
Preparation of Standard	**A) Preparation of stock solution for folic acid (1000 mg/kg)** 1. Accurately weigh 10 mg (± 0.1) of Folic acid standard. 2. Transfer to 10 mL amber colored volumetric flask. 3. Add 2 mL of 0.1 N NaOH. 4. Vortex for 2 min. 5. Add Milli Q Water and make-up to 10 mL. 6. Vortex for 2 min. 7. Store at -20 ^{0}C, protected from light. **B) Preparation of intermediate stock solution-1 for folic acid (100 mg/kg)** 1. Pipette out 1.0 mL of stock solution. 2. Transfer to 10 mL amber colored volumetric flask.

3. Add Milli Q Water and make-up to 10 mL.
4. Vortex for 2 min.

C) Preparation of intermediate stock solution-2 for folic acid (10 mg/kg)

1. Pipette out 1.0 mL of intermediate stock solution-1.
2. Transfer to 10 mL amber colored volumetric flask.
3. Add Milli Q Water and make-up to 10 mL.
4. Vortex for 2 min.

D) Preparation of intermediate stock solution-3 for folic acid (1 mg/kg)

1. Pipette out 1.0 mL of intermediate stock solution-2.
2. Transfer to 10 mL amber colored volumetric flask.
3. Add Milli Q Water and make-up to 10 mL.
4. Vortex for 2 min.

Preparation of calibration standards

Use Intermediate Stock Solution (ISS) – 3 (1 mg/kg) for preparing calibration standards as described in below Table.

Cal. standard solutions	ISS 3 (µg/kg)	Vol. of ISS 3 (mL)	Vol. of Milliq water (mL)	Final volume (mL)	Final conc. (µg/kg)
LS7	1000	2.00	8.00	10	200
LS6	1000	1.50	8.50	10	150
LS5	1000	1.00	9.00	10	100
LS4	1000	0.75	9.25	10	75
LS3	1000	0.50	9.50	10	50
LS2	1000	0.25	9.75	10	25
LS1	1000	0.10	9.90	10	10

NOTE: Prepare Calibration Standards fresh everyday

Chromatographic Conditions

Instrument : LC-MS/MS

Chromatographic Conditions: As detailed in below Table

Instrument	LC-MS/MS
Detector	Mass Detector
Column	2.6µm, XB C18 Column, 2.1 x 100 mm
Run time	7 min
Column temperature	35 °C
Flow rate	0.25 mL/min
Injection Volume	20 µL
Mobile Phase A	0.1 % Formic acid in water
Mobile Phase B	Acetonitrile
Water	Milli Q Water
Source Temperature	140°C
Desolvation Temperature	300ºC
MRM (Quantifier)	442 > 295
MRM (Qualifier)	442 > 176
CE	26 V
CV	35 V
Source	ESI + VE

Gradient Program

Time (min)	FLOW (mL/min)	% A	% B
0.00	0.25	90	10
2.00	0.25	90	10
4.00	0.25	10	90
5.00	0.25	90	10
7.00	0.25	90	10

Note: The laboratory may use any model of LC-MS/MS instrument after appropriate tuning and optimization. Instrument tuning and settings vary with make and model. Set parameter as per manufacturer's instructions and optimize the method to achieve the desired LOD and LOQ.

Sequence of Injection

SL.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	Linearity Solution (LS) – 7	1
	9	Blank	2
	10	Sample Solution	1
	11	Blank	2
	12	Spike Sample Solution	1
		TOTAL INJECTIONS	**15**

Calculation with units of Expression

a) Carry out LC-MS/MS analysis and calculate regression coefficient (R^2) of the calibration curve.

Calculate the Folic acid content in Fortified Rice Kernel using the following equation:

$$Folic\ acid\ \frac{\mu g}{kg} = \frac{C\ \times Makeup\ volume}{Sample\ weight\ (g)}$$

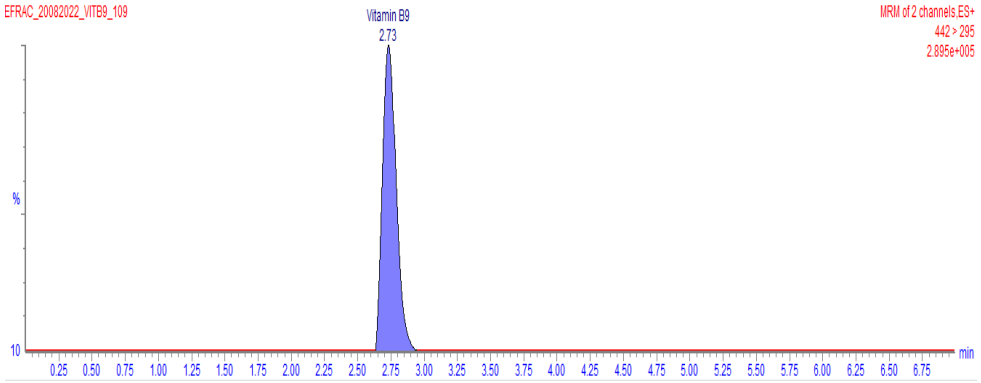
Wherein
C= Concentration obtained from instrument software

The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the folic acid signal in the matrix.
Limit of Detection (10 µg/kg)
Limit of Quantification (25 µg/kg)

Determine the recovery of folic acid by the external spiking method at 5000 µg/kg) in six replicates. Calculate the recovery value using the following equation:

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

where
A = the concentration of folic acid in the spiked sample (µg/kg)
B = the folic acid content in the control sample (µg/kg)
C = the spiked concentration of folic acid (µg/kg)

A representative chromatogram	Chromatograms 
Reference	Method Protocol: PRT/RA/FRK/2022/005, Method Validation Report for Estimation of Folic Acid (Vitamin B9) in Fortified Rice Kernel using LC-MS/MS. Journal of AOAC International, Vol 103, No 1, 2020- HPLC UV Estimation of Folic acid in fortified Rice and Wheat flour.
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 Vitamin B12 (Cyanocobalamin) in Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.FRK.16.006.2023	**Revision No. & Date**	0.0
Scope	This method is applicable for the quantitative analysis of Vitamin B12 as Cyanocobalamin at an LOQ of 25 µg/kg using Liquid chromatography coupled with Tandem Mass Spectrometer (LC-MS/MS).		
Caution	**Methanol** is a flammable Liquid. Handle in a hood away from flames. **Sodium hydroxide** is caustic. Contact with very high concentrations of sodium hydroxide can cause severe burns to the eyes, skin, digestive system or lungs. Prolonged or repeated skin contact may cause dermatitis. Handle with care. **Formic Acid** is a corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Inhaling formic acid can irritate the nose and throat. Use in a fume hood.		
Principle	Cyanocobalamin is extracted with the diluent (50% Methanol containing 0.1% Formic acid) and α-amylase. The extract is then diluted with water, filtered, diluted with diluent and the analysed by LC-MS/MS.		
Apparatus/Instruments	1. LC-MS/MS, System equipped with a Binary gradient pump, an auto sampler and tandem mass spectrometer. 2. Analytical Balance, -Suitable for weighing samples with accuracy up to 0.0.0001 g 3. Centrifuge -5000 rpm, that can accommodate 50 mL tubes 4. Amber colored volumetric flask (25 mL) 5. Volumetric flask: 1000 mL 6. Measuring cylinder 1000 mL 7. Micropipettes capable of delivering from 100 -1000 µl, 20 -200 µl10 -100 µl. 8. Shaker incubator 9. Column: 2.6 µm, XB C18 Column, 2.1 × 100 mm or equivalent 10. Sonicator. 11. Vortex mixer. 12. Homogenizer for sample grinding		
Materials and Reagents	1. Ammonium formate, MS Grade 2. Methanol, LR Grade. 3. Formic acid, MS Grade. 4. Sodium hydroxide, LR Grade 5. α-Amylase, (TCI, A0447) or equivalent 6. CRM Cyanocobalamin (CAS No: 68199) or equivalent		
Preparation of Reagents	**a) Mobile phase A (5 mM Ammonium formate in water)** 1. Weigh accurately 0.315 g of Ammonium formate. 2. Transfer into a 1000 mL of volumetric flask.		

	3. Add Milli-Q Water to dissolve and make-up to 1000 mL. 4. Sonicate for 15 mins. 5. Filter through 0.45 µm filter. **b) Mobile phase B (100% Methanol)** Transfer 1000 mL Methanol to mobile phase glass reservoir and sonicate for 15 min. **c) Diluent (50% Methanol containing 0.1 % Formic acid)** 1. Transfer 500 mL Methanol into 1000 mL measuring cylinder. 2. Add 1 mL Formic acid. 3. Add water up to mark 1000 mL. 4. Mix well and sonicate for 15 min.
Sample Preparation	1. Grind 50 g of FRK into a fine powder. 2. Accurately weigh 5 g (± 0.5 g) of ground sample into a 25 mL amber colored volumetric Flask. 3. Add 50 mg α-amylase and 20 mL of diluent. 4. Vortex for 5 min. 5. Make-up the volume to 25 mL using diluent. 6. Sonicate for 20 min. 7. Allow the sample to come to room temperature (25 °C). 8. Filter the sample using a syringe filter it (0.45 µm). 9. Use the filtrate for LC-MS/MS analysis. 10. Prepare the spike sample solution in a similar manner.
Preparation of Standards	**Preparation of stock solution for cyanocobalamin (1000 mg/kg)** 1. Accurately weigh 10 mg (± 0.1 mg) of Cyanocobalamin standard. 2. Transfer to 10 mL amber colored volumetric flask. 3. Add 2 mL of 0.1 N NaOH. 4. Vortex for 2 minutes. 5. Add Milli Q Water and make-up to 10 mL. 6. Vortex for 2 min. 7. Store the Solution at -20 ^{o}C away from light. **Preparation of intermediate standard solution (ISS) - 1 (100 mg/kg)** 1. Pipette out 1.0 mL of stock standard. 2. Transfer to a 10 mL amber colored volumetric flask 2 mL of Milli Q Water. 3. Add diluent and make-up to 10 mL. 4. Vortex for 2 min. **Preparation of intermediate standard solution – 2 (ISS-2) (10 mg/kg)** 1. Pipette out 1.0 mL of ISS-1. 2. Transfer to a 10 mL amber colored volumetric flask 2 mL of Milli Q Water. 3. Add diluent and make-up to 10 mL. 4. Vortex for 2 min.

Preparation of intermediate standard solution – 3 (ISS-3) (1 mg/kg)

1. Pipette out 1.0 mL of ISS-2.
2. Transfer to a 10 mL amber colored volumetric flask 2 mL of Milli Q Water.
3. Add diluent and make-up to 10 mL.
4. Vortex for 2 min.

Preparation of calibration standard solutions

Use ISS – 3 (1 mg/kg) for preparing Calibration standard solution as indicated Table below.

Cal. Standard	ISS - 3 (1 mg/kg))	Vol of ISS – 3 (mL)	Vol of diluent (mL)	Final vol. (mL)	Final conc. (µg/Kg)
LS7	1000	2.000	8.000	10	200
LS6	1000	1.000	9.000	10	100
LS5	1000	0.500	9.500	10	50
LS4	1000	0.200	9.800	10	20
LS3	1000	0.100	9.900	10	10
LS2	1000	0.050	9.950	10	5.0
LS1	1000	0.025	9.975	10	2.5

NOTE: Always use freshly prepared calibration standards

Chromatographic Conditions

a) Instrument: LC-MS/MS Spectrometer

b) Chromatographic Conditions: As detailed in below Table

Instrument	LC-MS/MS
Detector	Mass Detector
Column	2.6µm, XB C18 Column, 2.1 x 100 mm
Run time	7 min
Column Temperature	35°C
Flow rate	0.25 mL/min
Injection Volume	20 µl
Mobile Phase A	5 mM Ammonium formate
Mobile Phase B	Methanol
Diluent	50% Methanol containing 0.1 % Formic acid
Source Temperature	140 °C
Desolvation Temperature	300 °C
MRM (Quantifier)	678>147
MRM (Qualifier)	678>359
CE	26 V
CV	35 V
Source	ESI + VE

c) LC-Gradient Program

Time (min)	Flow rate (mL/min)	A (%)	(B)%
0.00	0.25	90	10
2.00	0.25	90	10
4.00	0.25	10	90
5.00	0.25	90	10
7.00	0.25	90	10

Note: ***The laboratory may use any model of LC-MS/MS instrument after appropriate tuning and optimization. Instrument tuning and settings vary with make and model. Set parameter as per manufacturer's instructions and optimize the method to achieve the desired LOD and LOQ.***

Sequence of Injection

The sequence of analysis is listed below

SL.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	Linearity Solution (LS) - 7	1
9	Blank	2
10	Sample Solution	1
11	Blank	2
12	Spike Sample Solution	1
	TOTAL INJECTIONS	**15**

Calculation with units of Expression

a) Construct a calibration curve and carry out a regression analysis. by fitting the data into a linear regression curve, including zero as the response for the reagent blank. The Regression coefficient (R^2) of should be >0.99

b) Calculate the concentration of Cyanocobalamin using the formula

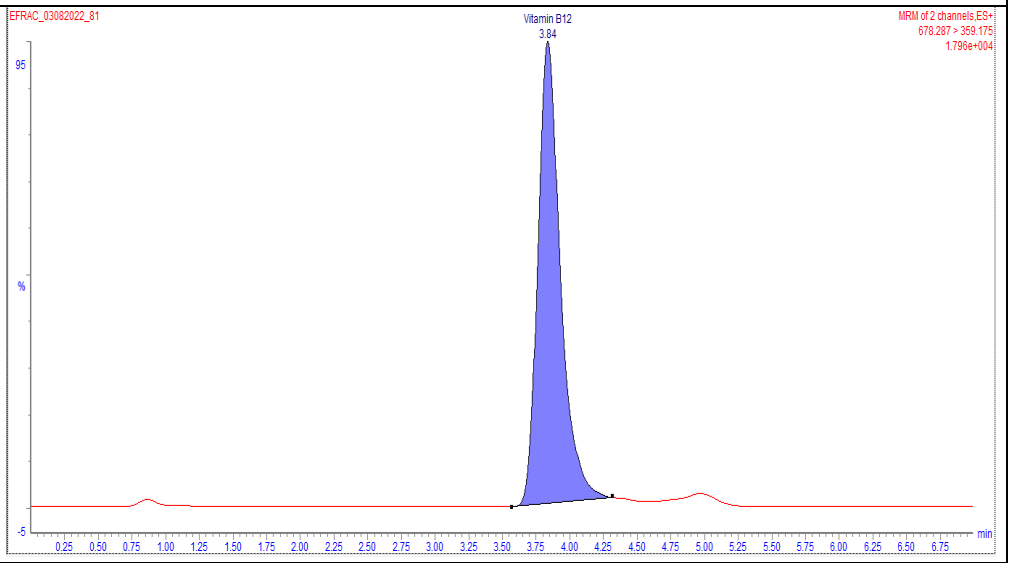
$$Cyanocobalamine\left(\frac{\mu g}{kg}\right) = \frac{C \times V}{W}$$

Where

C= concentration cyanocobalamin in sample

V=Make-up volume

W= Mass of sample taken in g

	c) 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. d) Determine the recovery of Cyanocobalamin (Vitamin B12) at spike level (50 µg/kg) in sample in six replicates. Calculate the recovery value using the following equation: $$Recovery(\%) = \frac{(A - B)}{C} \times 100$$ Where: A = the concentration of Vitamin B12 in the spiked sample (µg/kg) B = the content of Vitamin B12 in the control sample (µg/kg) C = the spiked concentration of Vitamin B12 (µg/kg)
A representative chromatogram	
Reference	Method Protocol: PRT/RA/FRK/2022/004, Method Validation Report for Estimation of Cyanocobalamin (Vitamin B12) in Fortified Rice Kernel by LC-MS/MS. AOAC 2011.10 – Single Laboratory Validation of AOAC Official method 2011.10 for Vitamin B12 in Indian infant and Pediatric formulas and Adult Nutritionals.
Approved by	Scientific Panel on Methods of Sampling and Analysis