File No: 11014/02/2021-QA (e-file no.1238)
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

Dated: 8th September 2022

ORDER

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

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

i. Method for determination of **Iron** in Fortified Rice: **FSSAI.FR.16.001.2022. (Annexure-I)**
ii. Method for determination of **Folic Acid** in Fortified Rice: **FSSAI.FR.16.002.2022. (Annexure-II)**
iii. Method for determination of **Vitamin B12** in Fortified Rice: **FSSAI.FR.16.003.2022. (Annexure-III)**

2. The food testing laboratories are hereby requested 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: 08-09-2022 09:54:15
Reason: Approved

(Sweety Behera)
Director (Quality Assurance Division)

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

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Annexure-I

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Iron in Fortified Rice**		
Method No.	**FSSAI.FR.16.001.2022**	**Revision No. & Date**	0.0
Scope	This method is applicable for the quantitative analysis of Iron in fortified rice.		
Safety & Precautions	**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:** **Microwave operation** 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 inductively coupled plasma (ICP)-MS. Polyatomic interferences with low mass elements are reduced or eliminated by analysis in He collision mode using kinetic energy discrimination. 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. ICP MS.—With quartz spray chamber, quartz torch, Ni/Pt sample cone, Ni/Pt skimmer cone, autosampler, and printer. The ICP-MS must have collision/reaction cells. 2. Microwave digestor. —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. Repipetter: 50 mL. 6. Bottle-top dispenser. —PTFE; Adjustable volume 0.5–5 mL. 7. Volumetric pipets. —Class A, assorted sizes. 8. 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. Methanol —99.99%, analytical reagent grade. 2. Nitric acid—Concentrated trace metal grade/Suprapure/ultrapure. 3. Hydrogen peroxide, 30%—ACS reagent grade. 4. Laboratory water—metal-free, organic-free, pyrogen-free, filtered 18 MV cm quality. 5. Argon gas—≥99.996% purity.		

	6. Helium gas—≥99.9999% purity 7. Fe Standard (974 mg/L)
Preparation of standard solutions	**Preparation of intermediate stock solution* - 1 (100 mg/L)** 1. Pipette out 1.027 ml# of Fe standard (974 mg/L). 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Add 0.5 ml Nitric Acid. 4. Add Milli Q Water and make-up to 10 ml. 5. Mixed by using Vortex Shaker Mixer. *# Volume of stock standard solution required for preparing the intermediate stock solutions of 100 mg/L will vary depending on the concentration of the concentration of the Fe standard solution* **Preparation of calibration standard solutions** 1. Use Intermediate stock solution – 1 (100 mg/L) for preparing calibration standard solutions as mentioned in below Table.

Cal. Standard Solution	ISS - 1 (100 mg/mL	VOL. OF ISS – 1 (ml)	VOL. OF NITRIC ACID (ml)	FINAL VOL. (ml)	FINAL CONC. (mg/mL (ppM)
LS 7	100	2.00	5	100	2.00
LS 6	100	1.50	5	100	1.50
LS 5	100	1.00	5	100	1.00
LS 4	100	0.50	5	100	0.50
LS 3	100	0.25	5	100	0.25
LS 2	100	0.10	5	100	0.10
LS 1	100	0.05	5	100	0.05

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

NOTE: Use freshly prepared calibration Standard solutions daily for the analysis.

Sample Preparation	1. Homogenize the sample by grinding as finely as possible. 2. Weigh 0.25 g (± 0.02 g) of ground sample by difference. 3. Transfer to Microwave Digestion Closed (MDC) Vessel. 4. Add 2.0 ml of hot (60 °C) Milli-Q water. 5. Add 1.0 ml Hydrogen peroxide. 6. Add 5 ml of Nitric Acid. 7. Loosely cap the vessel and keep at room temp for 5 min. to predigest the sample. 8. Seal the vessels and place into the rotor. 9. 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	-	-

10. After digestion, place the vessels in a fume hood, unscrew the cap/venting nut slowly to gradually release pressure, and then completely remove the cap.
11. Slowly add approximately 10 mL Milli Q water to the contents of the vessel, swirl to mix
12. Transfer contents to a 50 mL volumetric flask and make-up the volume with Milli-Q water.

Method of analysis

1. Using the appropriate tuning solutions, tune the ICP-MS instrument for optimal sensitivity in KED mode and/or reaction mode according to the instrument design.

2. Also, tune the instrument to find the P/A calibration factors needed for those calibration curves that will extend above roughly 100 µg/L (depending on instrument type). Table below summarizes typical instrument parameters for analysis using Agilent 77001 (for reference only*).

Plasma condition	a) Plasma Flow-Argon (15L /min) b) Nebulizer pump uptake speed (0.5 rps) c) RF power 1550 watts	
S/C Temperature	2°C	
Uptake Time	40 Sec	
Delay Time	40 Sec	
Stabilize Time	40 Sec	
Nebulizer Flow	1.0 ml/Min	
Reaction Cell	ORS and KED with helium flow:3.8 ml/Min	
Numbers of Replicates	3.0	
Detector's parameters	5 mV	
Mode	He	
Recommended mass for Iron	56	
TMP Revolution	100 %	
Auto sampler conditions	Working Mode	Continuous
	Wash	Between runs

*The laboratory may use their own ICP-MS instrument parameters after appropriate optimization.

3. Analyze test solutions using an ICP-MS instrument standardized with the

	indicated standard solutions. 4. The order of analysis should be calibration standards, followed by rinse, blank check, check standard, control sample, sample, sample duplicate, and, finally, a repeated check standard
Calculation with units of expression	Sample concentrations are automatically calculated by the software using a non-weighted least-squares linear regression calibration analysis to produce a best-fit line: y = ax + blank Note that, the sample blank is identical to the Cal Blk in this method and is essentially zero because high-purity reagents are used $$Iron\ \frac{(Fe)\ mg}{kg} = \frac{C\ \times Makeup\ volume}{Sample\ weight\ (g) \times 1000}$$ C= concentration from instrument software 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. Should be >0.99 b) 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 = 4 mg/kg in with respective to the Sample. ii. Limit of Quantification = 10 mg/kg in with respective to the Sample c) Determine the recovery of Iron by the external spiking method at three different spike levels (10, 20 & 30 mg/kg) in six replicates. Calculate the recovery value using the following equation: $$Recovery(\%) = \frac{(A - B)}{C} \times 100$$ 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	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

FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Folic Acid (Vitamin B9) in Fortified Rice**		
Method No.	**FSSAI.FR.16.002.2022**	**Revision No. & Date**	0.0
Scope	This method is applicable for quantitative analysis of Folic Acid (Vitamin B9) in fortified rice by using LC-MS/MS.		
Safety and Precautions	**Potassium 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		
Principle	Extraction of folic acid using phosphate buffer and then quantitative analysis using reverse phase liquid chromatography followed by tandem mass spectrometry (LC-MS/MS).		
Apparatus/Instruments	1. Ultra-High Performance Liquid Chromatograph with Tandem Mass Spectrometer (LC-MS/MS), system equipped with a quaternary gradient pump, an auto sampler. 2. Analytical Balance, -Suitable for weighing samples with accuracy up to 0.0001 g. 3. Centrifuge 6000 rpm, holding 50 ml tubes. 4. Volumetric Flasks-Class A. 5. Micro Pipettes Capable of delivering from 100 -1000 µl, 20 -200 µl 100 µl. of liquids. 6. Incubator shaker set at 37 ^{0}C. 7. Water bath at 55 ^{0}C. 8. Column: C-18 1.8 µm, 2.1×100 mm. 9. Sonicator 10. Vortex 11. Homogenizer with steel blades.		
Materials and Reagents	1. Anhydrous Dipotassium Hydrogen Phosphate(K_2HPO_4), LR Grade 2. L-Ascorbic Acid, LR Grade 3. α-Amylase 4. Potassium Hydroxide, LR Grade 5. Formic Acid, MS Grade 6. Acetonitrile, MS Grade 7. Folic Acid (Purity>90%)		
Preparation of Reagents	**a) Phosphate buffer (0.1 M)** 1. Accurately weigh 17.4 g of anhydrous K_2HPO_4. 2. Transfer it into 1000 ml of volumetric flask. 3. Add 200 mL MilliQ water		

	4. Sonicate for 15 minutes to dissolvelve. 5. Makeup with MilliQ water **b) Mobile phase -A (0.1% Formic acid)** 1. Transfer 1 ml Formic acid into 1000 ml Volumetric Flask 2. Add Milli-Q Water to make up volume 3. Filter through 0.45 µm filter **c) Mobile phase - B (100% acetonitrile)** 1. Transfer 1000 mL Acetonitrile to mobile phase glass bottle and then Sonicate.
Preparation of Standards	**A. Preparation of stock Folic acid Standard (1000 mg/L)** 1. Accurately weigh 10 mg (± 0.1) of standard Folica 2. Transfer to 10 ml amber colored volumetric flask 3. Add 2 ml of 0.1 N Potassium hydroxide 4. Vortex for 2 min 5. Add Milli Q Water make-up to 10 ml 6. Store the Solution at 4 ^{0}C in the dark. **B. Preparation of intermediate standard solution – 1 (100 mg/L)** 1. Pipette out 1.0 ml of Stock Solution. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Make-up the volume to 10 ml 4. Vortex for 2 minutes. **C. Preparation of intermediate standard solution - 2 (10 mg/L)** **1.** Pipette out 1.0 ml of intermediate standard solution – 1. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Add Milli Q Water and make-up to 10 ml 4. Vortex for 2 minutes. **D. Preparation of intermediate standard solution – 3 (1 mg/mL)** 1. Pipette out 1.0 ml of Intermediate standard solution – 2. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Add Milli Q Water and make-up to 10 ml 4. Vortex for 2 minutes. 5. Use this solution for making the calibration standards **E. Preparation of standard solution - 4 (10 µg/L)** 1. Pipette out 0.10 ml of Intermediate Standard Solution - 3 2. Transfer to 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water 3. Add Milli Q Water and make-up to 10 ml **F. Preparation of bracketing standard solution** Standard Solution – 4 (10 **µg/L**) shall be used for Bracketing Standard Solution. **Preparation of calibration standard solutions** 1. Use Intermediate Standard Solution – 3 (10 µg/L) for preparing Calibration Standard Solutions as mentioned in below Table.

CAL. STD. SOLUTION	ISS 3 (1000 µg/L)	VOL. OF ISS 3 (ml)	VOL. OF MILLI Q WATER (ml)	FINAL VOL. (ml)	FINAL CONC. (µg/L)
LS 6	1000	0.40	9.60	10	40
LS 5	1000	0.20	9.80	10	20
LS 4	1000	0.15	9.85	10	15
LS 3	1000	0.10	9.90	10	10
LS 2	1000	0.05	9.95	10	5
LS 1	1000	0.02	9.98	10	2

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

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

Preparation of Test Samples

1. Take 1 Kg of rice sample and grind the whole sample to a fine powder using the Homogenizer.
2. Accurately weigh 5 g (± 0.5 g) of powdered sample.
3. Transfer into a 25 ml amber colored volumetric flask.
4. Add 0.1 g L-Ascorbic acid and 15 ml of 0.1 M phosphate buffer
5. Vortex for 5 minutes.
6. Maintain the pH of the sample solution between 8.0-9.0 using 1M Potassium hydroxide solution (KOH).
7. Shake the sample at 37 ºC, 20 rpm using an orbital shaker for 60 mins.
8. Maintain the pH of the solution at 7.0 using 2 N HCl
9. Add 0.125 g of α-amylase and continue shaking for 5 minutes.
10. Incubate the sample in a water bath at 55 ^{0}C for 30 minutes.
11. Cool to Room Temperature.
12. Make-up to 25 ml with 0.1 M phosphate buffer.
13. Transfer to centrifuge tube for shaking vigorously for 2 minutes using Vortex.
14. Centrifuge at 6000 rpm for 5 minutes.
15. Filter supernatant through 0.45µm Nylon Syringe Filter.
16. Transfer filtrate to Vial, and use this for injecting into LC-MS/MS.

Chromatographic Conditions

Instrument: LC-MS/MS Spectrometer

Chromatographic and instrument Conditions : As detailed in below Table*

Detector	Mass Detector
Column	T3 1.8 µm, 2.1*100mm
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
Buffer	Potassium Hydrogen Phosphate
Source Temperature	140 ^{0}C
MRM (Quantifier)	442.2 > 295.1
MRM (Qualifier)	442.2 > 176
CE	12.00
CV	40.00
De-solvation Temperature	450 ^{0}C
Source	ESI +ve

*The laboratory may use their own LC-MS/MS instrument parameters after appropriate optimization

Gradient Program

TIME	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

*The laboratory may use their own LC-MS/MS instrument parameters after appropriate optimization.

Method of Analysis/ Batch Organization

Injection Sequence

SL.NO	NAME OF INJECTIONS	NUMBER OF INJECTIONS
1	Blank	2
2	Standard Solution - 3 (100%)	6
3	Blank	2
4	Linearity Solution (LS) - 1	1
5	Linearity Solution (LS) - 2	1
6	Linearity Solution (LS) - 3	1
7	Linearity Solution (LS) - 4	1
8	Linearity Solution (LS) - 5	1
9	Linearity Solution (LS) - 6	1
10	Blank	2
11	Sample Solution	1

	<table><tr><td>12</td><td>Blank</td><td>2</td></tr><tr><td>13</td><td>Bracketing Standard Solution</td><td>1</td></tr><tr><td colspan="2">TOTAL INJECTIONS</td><td>22</td></tr></table>
Calculation with units of expression	a) Carry out a regression analysis and calculate Regression coefficient (R2) by analyzing the calibration standards by fitting the data into a linear regression curve, including zero as the response for the reagent blank. $$Folic\ acid\ \frac{\mu g}{kg} = \frac{C\ \times Makeup\ volume}{Sample\ weight\ (g)}$$ C= Concentration obtained from instrument software 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. • Limit of Detection (5 µg/kg) with respective to the Sample. • Limit of Quantification (10 µg/kg) with respective to the sample. c) Determine the recovery of folic acid by the external spiking method at three different spike levels (10, 25, 50 and 100 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 natural content of folic acid in the control sample (µg/kg) C = the spiked concentration of folic acid (µg/kg)
Reference	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

FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Cyanocobalamin (Vitamin B12) in Fortified Rice**		
Method No.	**FSSAI.FR.16.003.2022**	**Revision No. & Date**	0.0
Scope	The scope of this method includes the quantitative analysis of Cyanocobalamin (Vitamin B12) at 0.5 ppb LOQ Level (with respect to the Sample) by using LC-MS/MS. Limit of Detection is 0.25 µg/kg with Respect to the Sample. Limit of Quantification is 0.5 µg/kg with Respect to the Sample.		
Safety Precautions	**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.		
Principle	Cyanocobalamin (Vitamin B12) a water-soluble vitamin is extracted from the matrix using acetate buffer. The extract is cleaned up using a C18 cartridge and then separated by reverse phase chromatography and detected in a Tandem mass spectrometer and Multiple Reaction Monitoring (MRM).		
Apparatus/Instruments	1. LC-MS/MS, system equipped with a quaternary gradient pump, an auto sampler (100 µL maximum loop capacity) and Mass spectrometer. 2. Analytical Balance, -Suitable for weighing samples with accuracy up to 0.1 mg. 3. Centrifuge, 6000 rpm, holding 50 ml tubes. 4. Micro Pipettes Capable of delivering from 100 -1000 µl, 20 -200 µl 10 -100 µl. of liquids such as vitamin B12 Standards, Solvents, Buffers and Extracts. 5. Incubator 6. Column: 2.6 µm, C18 Column, 2.1 x 100 mm. 7. Homogenizer for sample grinding.		
Materials and Reagents	1. Sodium acetate, LR Grade. 2. Ammonium formate, MS Grade 3. α-Amylase, 4. Acetic acid, MS Grade. 5. Methanol, LR Grade. 6. Sodium hydroxide, LR Grade 7. Cyanocobalamin (>98% pure)		

	8. Cartridge Details: C18 60Å 50µm SPE Cartridge, 900 mg 9. Milli Q Water
Preparation of Reagents	**Buffer preparation:** Weigh accurately 20.5 g of Sodium acetate. Transfer it into 1000 mL volumetric flask. Sonicate to dissolve in about 100 mL water and make up to 1000 mL mark. **Mobile phase A**: Weigh accurately1.261 g of Ammonium formate. Transfer it into 1000 ml volumetric Flask. Dissolve with Milli-Q Water and make-up to 1000 ml. Sonicate for 15 minutes to mix well. Filter through 0.45 µm filter. **Mobile phase B**: Transfer 1000 ml methanol to mobile phase glass bottle and then sonicate for 15 minutes. **Diluent preparation:** Transfer 500 mL methanol and 500 ml Milli Q Water into 1000 ml glass bottle. Mix well and sonicate for 15 minutes.
Preparation of Standards	**Preparation of stock solution for cyanocobalamin** **(1000 mg/L (1000 ppm))** 1. Accurately weigh 10 mg (± 0.1 mg) of Cyanocobalamin Standard 3 (100%) 2. Transfer to 10 ml amber colored volumetric Flask. 3. Add 2 ml of 0.1 N sodium hydroxide. 4. Vortex for 2 minutes. 5. Add Milli Q Water and make-up to 10 ml. 6. Vortex for 2 minutes. 7. Store the solution at 4 °C in the dark. **Preparation of intermediate standard solution – 1** **(100 mg/L (100 ppm))** 1. Pipette out 1.0 ml of stock solution. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Add diluent to make-up to 10 mL. 4. Vortex for 2 minutes. **Preparation of intermediate standard solution – 2** **(10 mg/L (10 ppm))** 1. Pipette out 1.0 mL of intermediate standard solution – 1. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water. 3. Add Diluent to make-up to 10 mL. 4. Vortex for 2 minutes. **Preparation of intermediate standard solution - 3** **(1mg/L (1 ppm))** 1. Pipette out 1.0 mL of intermediate standard solution – 2. 2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml

of Milli Q Water.

3. Add Diluent to make-up to 10 mL.
4. Vortex for 2 minutes.

Preparation of intermediate standard solution - 4
(100 µg/L (100 ppb))

1. Pipette out 1.0 ml of Intermediate Standard Solution – 3.
2. Transfer to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q Water.
3. Add diluent to make-up to 10 ml.
4. Vortex for 2 minutes.

Preparation of working standard solution
(5 µg/L 5 ppb)

1. Pipette out 0.5 ml of intermediate standard solution – 4.
2. Transfer to 10 ml amber colored volumetric flask containing 2 ml of Milli Q water.
3. Add diluent for volume make-up to 10 ml.
4. Vortex for 2 minutes.

Preparation of bracketing standard solution

Standard Solution - 4 (5 ppb) shall be used for Bracketing Standard Solution

Preparation of calibration standard solutions

Use Intermediate Standard Solution - 4 for preparing Calibration Standard Solution as mentioned in below Table.

CAL. STANDARD SOLUTIONS	ISS - 4 (100 µg/L 100 ppb)	VOL. OF ISS – 4 (ml)	VOL. OF DILUENT (ml)	FINAL VOL. (ml)	FINAL CONC. (µg/L ppb)
LS 6	100	2	8.00	10	20
LS 5	100	1	9.00	10	10
LS 4	100	0.5	9.50	10	5
LS 3	100	0.2	9.80	10	2
LS 2	100	0.1	9.90	10	1
LS 1	100	0.05	9.95	10	0.5

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

NOTE: Prepare calibration standard solutions freshly every day for the

<table>
<tr><td></td><td>analysis.</td></tr>
<tr><td>Preparation of Test Samples</td><td>1. Take 1 kg of rice. Grind the whole sample to a fine powder using homogenizer.
2. Accurately weigh 10 g (± 0.5 g) of finely ground powder.
3. Transfer into a 50 ml amber colored volumetric flask.
4. Add 50 mg α-amylase and 20 ml of 0.25 M Sodium Acetate Buffer.
5. Vortex for 5 minutes.
6. Sonicate the solution for 20 minutes.
7. Make-up to 50 ml using 0.25 M sodium acetate buffer.
8. Sonicate for 20 minutes.
9. Transfer the Sample Solution into the 50 ml Centrifuge tube
10. Shaking vigorously for 2 minutes using Vortex.
11. Centrifuge the sample solution at 6000 rpm for 5 minutes at 4 ºC.
12. Collect the supernatant layer filter through 0.45 µm filter paper.
13. Insert 900 mg C18 Solid Phase Extraction Cartridge onto the stopcock of the vacuum manifold.
14. Attach a 10 mL disposables barrel to the top of the Cartridge.
15. Condition the cartridge with 20 ml Methanol by allowing Methanol to gravity filter through the Cartridge.
16. Rinse with 10 ml Milli Q water.
17. Transfer 20 ml of filtered sample solution into the cartridge
(Note: If necessary, apply enough vacuum, so that the sample will drip steadily through the cartridge).
18. Pass the sample solution through the cartridge.
19. Rinse the cartridge with 5 ml of water
20. Discard eluent.
21. Air-Dry the cartridge by pulling a vacuum until no more effluent is observed.
22. Close each stopcock.
23. Place 5 ml receiving Ria vial under the Cartridge.
24. Add 4 ml diluent to the cartridge.
25. Open Stopcock.
26. Elute the solution into the Ria Vial.
27. Transfer the collected sample solution in to the vial and use this for injecting into LC-MS/MS.</td></tr>
<tr><td>Chromatographic Conditions</td><td>a) Instrument : LC-MS/MS Spectrometer.
b) Chromatographic Conditions: As detailed in below Table*

<table><tr><td>Detector</td><td>Mass Detector</td></tr><tr><td>Column</td><td>2.6 µm, C18 Column, 2.1 x 100</td></tr></table></td></tr>
</table>

	mm
Run time	7 min
Column Temperature	35°C
Flow rate	0.25 ml/min
Injection Volume	20 µl
Mobile Phase A	20 mM Ammonium Formate in Water
Mobile Phase B	Methanol
Buffer	Sodium Acetate
Diluent	Milli Q Water
Source Temperature	140°C
Desolvation Temperature	300°C
MRM (QUANTIFIER)	678.29 > 359.17
MRM (QUALIFIER)	678.29 > 665.00
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

*The laboratory may use their own LC-MS/MS instrument parameters after appropriate optimization.

Method of Analysis

Injection sequence

SL.NO.	NAME OF INJECTIONS	NUMBER OF INJECTIONS
1	Blank	2
2	Standard Solution - 4 (100%)	6
3	Blank	2
4	Linearity Solution (LS) - 1	1

5	Linearity Solution (LS) - 2	1
6	Linearity Solution (LS) - 3	1
7	Linearity Solution (LS) - 4	1
8	Linearity Solution (LS) - 5	1
9	Linearity Solution (LS) - 6	1
10	Blank	2
11	Sample Solution	1
12	Blank	2
13	Bracketing Standard Solution	1
Total Injections		**22**

Calculation with units of expression

$$Cyanocobal\ amine\left(\frac{\mu g}{kg}\right) = \frac{C \times V1 \times V3}{W \times V2}$$

Where:

C = Instrument concentration (µg/kg (ppb))

V1 = Volume make-up (ml)

V2 = Volume of filtrate loaded on Cartridge (ml)

V3 = Volume of diluent added for extract the Vitamin B12 from cartridge (ml)

W = Sample weight (g)

a) Carry out a regression analysis and calculate Regression coefficient (R2) of the calibration standards by fitting the data into a linear regression curve, including zero as the response for the reagent blank.

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

c) Determine the recovery of Cyanocobalamin by the external spiking method at three different spike levels (0.5, 2.0, 5.0 and 10.0 µg/kg) 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 natural content of Vitamin B12 in the control sample (µg/kg)

C = the spiked concentration of Vitamin B12 (µg/kg)

Reference	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