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

FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Method for Determination of Iron in Fortified Rice**		
Method No.	**FSSAI.FR.16.001.2022**	**Revision No. & Date**	0.0
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: 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.		
Scope	The Scope of this Method is applicable for Quantification of Iron at 10 ppm LOQ Level (with respect to the Sample) by using ICP-MS. a) Limit of Detection 4 mg/kg in with respective to the Sample. Limit of Quantification 10 mg/kg in with respective to the Sample.		
Principle	Weigh 0.25 g (± 0.02 g) Grinded Sample. Transfer to Microwave Digestion Closed (MDC) Vessel. Transfer to Microwave Digestion Cool Vessel. Heat Milli Q Water at 60 ^{0}C. Add 2.0 ml of Hot Milli-Q water, 1.0 ml Hydrogen Peroxide, Add 5 ml of Nitric Acid. Close the Microwave Vessel tightly. Keep at Room Temperature for 5 minutes. Keep the Vessel rotor in Microwave Digester, Cool it, Add 10 ml of Milli Q water &		

<table>
<tr><td></td><td>Mix well. Make upto 50 ml with Milli- Q Water.</td></tr>
<tr><td>Apparatus/Instruments</td><td>1. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)
2. Microwave Digester
3. Analytical Balance</td></tr>
<tr><td>Materials and Reagents</td><td>1. Concentrated Nitric Acid (Purity- 69%)
2. Hydrogen Peroxide (Purity -30%)
3. CRM Used : Iron
4. Purity of Argon and other gas, if used must fulfill the standard of instrument requirement</td></tr>
<tr><td>Preparation of solutions</td><td><u>PREPARATION OF INTERMEDIATE STOCK SOLUTION - 1 (100 PPM)</u>
1. Pipette out 1.027 ml of Stock Solution.
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 for Volume make-up to 10 ml.
5. Mixed by using Vortex Shaker Mixer.

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

<table>
<tr><th>Cal. Standard Solution</th><th>ISS - 1 (100 PPM)</th><th>VOL. OF ISS – 1 (ml)</th><th>VOL. OF NITRIC ACID (ml)</th><th>FINAL VOL. (ml)</th><th>FINAL CONC. (PPM)</th></tr>
<tr><td>LS 7</td><td>100</td><td>2.00</td><td>5</td><td>100</td><td>2.00</td></tr>
<tr><td>LS 6</td><td>100</td><td>1.50</td><td>5</td><td>100</td><td>1.50</td></tr>
<tr><td>LS 5</td><td>100</td><td>1.00</td><td>5</td><td>100</td><td>1.00</td></tr>
<tr><td>LS 4</td><td>100</td><td>0.50</td><td>5</td><td>100</td><td>0.50</td></tr>
<tr><td>LS 3</td><td>100</td><td>0.25</td><td>5</td><td>100</td><td>0.25</td></tr>
<tr><td>LS 2</td><td>100</td><td>0.10</td><td>5</td><td>100</td><td>0.10</td></tr>
<tr><td>LS 1</td><td>100</td><td>0.05</td><td>5</td><td>100</td><td>0.05</td></tr>
</table>
CAL : Calibration
ISS : Intermediate Stock Solution
VOL: Volume
LS : Linearity Solution
NOTE: Use freshly prepared Standard solutions for the analysis.</td></tr>
<tr><td>Sample Preparation</td><td><u>PREPARATION OF SAMPLE SOLUTION</u>

1. Homogenize the Sample by Grinding as finely as possible.
2. Weigh 0.25 g (± 0.02 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 ml of Nitric Acid.
8. Loosely cap the vessel and keep at room temp for 5 min. to predigest the sample.
9. Close the Microwave Vessel tightly.</td></tr>
</table>

10. Keep at Room Temperature for 5 minutes.
11. Keep the Vessel rotor in Microwave Digester
12. Cool the Vessel at Room Temperature after Digestion.
13. Add 10 ml of Milli Q water.
14. Mix well.
15. Transfer to 50 ml Volumetric Flask.
16. Volume make-up to 50 ml with Milli-Q water.

Method of analysis

a) Instrument : ICP-MS Spectrometer.

b) Conditions : As detailed in below Table

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

c) Microwave Digestion Program

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

Calculation with units of expression

$$\text{Iron (ppm)} = \frac{\text{Instrument Conc. X Make-up Volume}}{\text{Sample Weight X1000}}$$

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.

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

c) Determine the recovery of Iron by the external spiking method at three different spike levels (10,20 & 30 mg/kg) in six replicates.
d) Calculate the recovery value using the following equation:
e) Recovery (%) = $\frac{(A - B) \times 100}{C}$

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)

Results

Sample Name	Sample Solution
Sample type	Sample
Comment	—
Prep Dilution	1.0000
Auto Dilution	1.0000
Total Dilution	1.0000
Operator Name	-----
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	02-11-2022 18:41
Bkg File	—
Bkg Mode	Count Substraction except for ISTD
FQ Blank File	—
VIS Fit	Point to point

Full Quant Table

Element	Mass	Tune Mode	Conc.	Units	RSD (%)	CPS	Det.	Rep
Fe	56	He	41.609	ppb	2.3	708422.2	Pulse	3

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	**Method for Determination of Folic Acid (Vitamin B9) in Fortified Rice**		
Method No.	**FSSAI.FR.16.002.2022**	**Revision No. & Date**	0.0
Safety and Precautions	1) **Potassium Hydrogen Phosphate:** It is a Laboratory Chemical. During Handling of Potassium Hydrogen Phosphate, below measures to be followed: a) Eye Contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Get medical attention immediately, if symptoms occur. b) Skin Contact: Wash off immediately with plenty of water for at least 15 minutes. Get medical attention immediately. if symptoms occur. c) Inhalation: Remove to fresh air. Get medical attention immediately if symptoms occur. If not breathing, give artificial respiration. Ingestion Do NOT induce vomiting. Get medical attention. 2) **L-Ascorbic Acid:** It is a Laboratory Chemical. During Handling of L- Ascorbic Acid, the following Safety measures to be followed: a) Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Get medical attention immediately, if symptoms occur. b) Skin Contact: Immediately take off all contaminated clothing. Rinse Skin with Water. c) If Inhalation: Remove to fresh air. Get medical attention immediately if symptoms occur. If not breathing, give artificial respiration. d) If swallowed: Drink water (two glasses at most). Consult doctor if feeling unwell. 3) **α-Amylase:** It is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding shorter chains thereof, Dextrin and Maltose. It is the major form of amylase, found in humans and other mammals. During handling of Methanol, below Safety Measures to be followed: a) Skin Contact: Avoid contact with skin and eyes. b) If Inhalation: Avoid ingestion and inhalation. c) Use adequate ventilation to keep Airborne Concentrations low. 4) **Potassium Hydroxide:** It is a Laboratory Chemical. It may be corrosive to Metals. It is harmful, if swallowed. It causes severe skin burns and eye damage. It may cause Respiratory irritation. During handling of Potassium Hydroxide, below Safety Measures to		

	be followed: a) Wash face, hands and any exposed skin thoroughly after handling b) Do not eat, drink or smoke when using this product c) Do not breathe dust/fume/gas/mist/vapors/spray d) Wear protective gloves/protective clothing/eye protection/face protection. e) Use only outdoors or in a well-ventilated area f) Keep only in original container. 5) **Formic Acid:** It is a Flammable Liquid, which causes severe burns of skin, eye and other exposed surfaces of the human body. During handling of Formic Acid, below safety measures to be followed: a) Wash face, hands and any exposed skin thoroughly after handling b) Do not eat, drink or smoke when using this product c) Use only outdoors or in a well-ventilated area d) Do not breathe dust/fume/gas/mist/vapors/spray e) Wear protective gloves/protective clothing/eye protection/face protection. f) Keep away from heat/sparks/open flames/hot surfaces. g) No smoking. h) Keep container tightly closed Ground/bond container and receiving equipment i) Use explosion-proof electrical/ventilating/lighting equipment. j) Use only non-sparking tools Take precautionary measures against static discharge. k) Keep cool l) Wear respiratory protection. 6) **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, if necessary. Immediately call-in physician. b) Skin Contact: Take off immediately all contaminated clothing. Rinse skin with water/ shower. Consult a physician. 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). Consult a physician. 7) **Folic Acid:** Folic acid is not considered hazardous by the 2012 OSHA Standard. First Aid: Rise immediately with plenty of water if it is contact with Eye & skin. Avoid to inhale fume remove to fresh air. If not breathing give artificial respiration.

Scope	The Scope of this Method is applicable for Quantification of Folic Acid (Vitamin B9) at 10 ppb LOQ Level (with respect to the Sample) by using LC-MS/MS in Fortified Rice. a) Limit of Detection (5 ppb) With Respective to the Sample. Limit of Quantification (10 ppb) With Respective to the Sample.
Principle	Powder samples were reconstituted by dissolving 5 g powder sample and add 0.1 gm of Ascorbic acid and 15 ml of 0.1 M Potassium Hydrogen Phosphate Buffer Maintain the pH of the Sample Solution between 8.0-9.0 using 1M Potassium Hydroxide Solution (KOH). pH of the Sample Solution to 7.0 with 2 N. Add 0.125 g of α-amylase into the Sample Solution. Place 25 ml Amber Colored Volumetric Flask containing Sample Solution on the Water Bath at 55 ^{0}C. Do Volume make-up to 25 ml with 0.1 M Potassium Hydrogen Phosphate Buffer. Shake Vigorously and centrifuge at 6000 rpm. Filter through 0.45 µm membrane into an amber LC Vial for UHPLC MS/MS Analysis.
Apparatus/Instruments	1. LC-MS/MS, system equipped with a quaternary gradient pump, an auto sampler (100 µL maximum loop capacity). 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 Folic Standards, Solvents, Buffers and Extracts. 5. Incubator 6. Column: T3 1.8 µm, 2.1*100mm 7. Sonicator for mixing of solution. 8. Vortex for preparation of stock solution. 9. Homogenizer for sample grinding
Materials and Reagents	1. Potassium Hydrogen Phosphate, 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. CRM Used: Folic Acid
Preparation of Reagents	**PREPARATION OF MOBILE PHASE** **a) <u>BUFFER PREPARATION</u>** 1.Accurately weigh 17.4 g of Potassium Hydrogen Phosphate. 2.Transfer it into 1000 ml of Volumetric Flask. 3.Add Milli Q Water for Volume make up 4.Sonicate for 15 minutes to mix & Dissolve. **b) <u>MOBILE PHASE - A PREPARATION</u>** 1.Transfer 1 ml Formic Acid into 1000 ml Volumetric Flask

	2. Add Milli-Q Water for Volume make up 3. Sonicate to mix & Dissolve well 4. Filter through 0.45 µm Filter Paper **c) <u>MOBILE PHASE - B PREPARATION</u>** 1. Transfer 1000 ml Acetonitrile to Mobile Phase Glass Bottle and then Sonicate.
Preparation of Standards	**A. <u>PREPARATION OF STOCK SOLUTION FOR FOLIC ACID (1000 ppm)</u>** 1. Accurately weigh 10 mg (± 0.1) of Folic Acid 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 min 5. Add Milli Q Water for Volume make-up to 10 ml 6. Store the Solution at 4 ^{0}C in the light Protected Area. **B. <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION – 1 (100 ppm)</u>** 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 Milli Q Water for Volume make-up to 10 ml 4. Vortex for 2 minutes. **C. <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 2 (10 ppm)</u>** **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 for Volume make-up to 10 ml 4. Vortex for 2 minutes. **D. <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION – 3 (1 ppm)</u>** 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 for Volume make-up to 10 ml 4. Vortex for 2 minutes. **E. <u>PREPARATION OF STANDARD SOLUTION - 3 (10 ppb)</u>** 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 for Volume make-up to 10 ml

F. <u>PREPARATION OF BRACKETING STANDARD SOLUTION</u>

Standard Solution - 3 (10 ppb) shall be used for Bracketing Standard Solution.

<u>PREPARATION OF CALIBRATION STANDARD SOLUTION</u>

1. Use Intermediate Standard Solution - 3 for preparing Calibration Standard Solutions as mentioned in below Table.

CAL. STD. SOLUTION	ISS 3 (1000 ppb)	VOL. OF ISS 3 (ml)	VOL. OF MILLI Q WATER (ml)	FINAL VOL. (ml)	FINAL CONC. (ppb)
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 Standard solutions for the analysis.

Preparation of Test Samples

1. Take 1 Kg of Rice Sample and Homogenize the whole Sample using Homogenizer.
2. Accurately weigh 5 g (± 0.5 g) of Homogenized 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 Potassium Hydrogen Phosphate Buffer into the Sample
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. Keep the Sample Solution on an Orbital Shaker & shake at 20 rpm for one hour at 37 ºC.
8. Maintain the pH of the Sample Solution to 7.0 with 2 N Hydrochloric Acid Solution.
9. Add 0.125 g of α-amylase into the Sample Solution and shake for 5 minutes.
10. Place 25 ml Amber Colored Volumetric Flask containing Sample Solution on the Water Bath at 55 ^{0}C for 30 minutes.

11. Cool the Sample Solution at Room Temperature.
12. Do Volume make-up to 25 ml with 0.1 M Potassium Hydrogen Phosphate Buffer.
13. Transfer the Sample Solution into the Centrifuge Tube for shaking vigorously for 2 minutes using Vortex.
14. Centrifuge the Sample Solution at 6000 rpm for 5 minutes.
15. Collect the Supernatant layer and filter it through 0.45µm Nylon Syringe Filter.
16. Pour the Filtrate into the Vial, and use this for injecting into LC-MS/MS.

Chromatographic Conditions

- Instrument : LC-MS/MS Spectrometer
- Chromatographic 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

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

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
12	Blank	2
13	Bracketing Standard Solution	1
TOTAL INJECTIONS		**22**

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.

b) Folic Acid (Vitamin B9) (ppb) = $\frac{\text{Instrument Conc. X Make up Volume}}{\text{Sample Weight (g)}}$

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

d) 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.

e) Calculate the recovery value using the following equation:

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

	C 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	**Method for Determination of Cyanocobalamin (Vitamin B12) in Fortified Rice**		
Method No.	**FSSAI.FR.16.003.2022**	**Revision No. & Date**	0.0
Safety & Precautions	1) **Sodium Acetate:** It is a Laboratory Chemical. During handling of Sodium Acetate, below safety measures to be followed: a) Eye/Face Protection: Wear safety glasses or goggles. b) Skin Protection: Wear appropriate clothing to prevent repeated or prolonged skin contact. c) Keep out reach of Children d) Do not eat, drink or smoke when using this Chemical. 2) **Ammonium Formate:** It is a Laboratory Chemical which causes Skin Corrosion/Irritation, serious Eye Damage/Eye Irritation, Specific target organ toxicity (single exposure) and can harm to Respiratory system. During handling of Sodium Acetate, below safety measures to be followed: a) Wash face, hands and any exposed skin thoroughly after Handling b) Wear protective gloves/protective clothing/eye protection/face protection c) Avoid breathing dust/fume/gas/mist/vapours/spray d) Use only outdoors or in a well-ventilated area. 3) **Acetic Acid:** It is a Chemical which is corrosive that causes severe burns of skin, eye and other exposed surfaces of the human body. Long-term exposure to the Vapors of this substance causes chronic bronchitis and other respiratory effects, erosion of tooth enamel, and cracking and darkening of the exposed skin. During handling of Acetic Acid, below safety measures to be followed: a) Never add water to this chemical, and always keep acetic acid away from sources of heat, sparks or flame. b) Wear suitable respiratory equipment if handling acetic acid in an area that isn't well-ventilated. c) Wash face, hands and any exposed skin thoroughly after Handling d) Wear protective gloves/protective clothing/eye protection/face protection. 4) **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		

	Sodium Hydroxide: It is odorless and white solid. During handling of Sodium Hydroxide, below Safety Measures to be followed: a) Avoid contact with eyes, skin, and clothing. b) Do not inhale gases, fumes, dust, mist, vapor, and aerosols. c) Wear protective safety goggles, gloves, and clothing. d) Do not mix with Acids. e) Do not eat, drink, smoke, or use personal products when handling chemical substances. **6) α-Amylase:** It is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding shorter chains thereof, Dextrin and Maltose. It is the major form of amylase, found in humans and other mammals. During handling of Methanol, below Safety Measures to be followed: a) Avoid contact with skin and eyes. b) Avoid ingestion and inhalation. c) Use adequate ventilation to keep Airborne Concentrations low. 7) **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.
Scope	The Scope of this Method includes for Quantification of Cyanocobalamin (Vitamin B12) at 0.5 ppb LOQ Level (with respect to the Sample) by using LC-MS/MS. a) 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.
Principle	Weigh 10 g (± 0.5 g) of Homogenized Sample. Add 50 mg α-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. Vertex & Sonicate for 20 minutes, add 50 ml of 0.25 M Sodium Acetate Buffer. Sonicate & Centrifuge @ 6000rpm at 4 ºC, Pass through 900 mg of C18 SPE cartridge, Pass 20 ml of filtrate. Elute the solution and Transfer the collected Sample Solution in to the Vial and use this for Injecting into LC-MS/MS.
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: 2.6µm, 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. CRM Used : Cyanocobalamine 8. Cartridge Details: C18 60Å 50µm SPE Cartridge, 900mg
Preparation of Reagents	**a) <u>BUFFER PREPARATION</u>** 1. Weigh accurately 20.5 g of Sodium Acetate. 2. Transfer it into 1000 ml of Volumetric Flask. 3. Add Milli Q Water for Volume make-up to 1000 ml. 4. Sonicate for 15 minutes to Dissolve. **b) <u>MOBILE PHASE A PREPARATION</u>** 1. Weigh accurately1.261 g of Ammonium Formate. 2. Transfer it into 1000 ml of Volumetric Flask. 3. Add Milli-Q Water for Volume make-up to 1000 ml. 4. Sonicate for 15 minutes to mix well. 5. Filter through 0.45 µm Filter Paper. **c) <u>MOBILE PHASE B PREPARATION</u>** Transfer 1000 ml Methanol to Mobile Phase Glass Bottle and then Sonicate for 15 minutes. **d) <u>DILUENT PREPARATION</u>** 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	**A) <u>PREPARATION OF STOCK SOLUTION FOR CYANOCOBALAMIN (1000 ppm)</u>** 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 for Volume make-up to 10 ml. 6. Vortex for 2 minutes. 7. Store the Solution at 4 ^{o}C in the light Protected Area. **B) <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION – 1 (100 ppm)</u>** 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 for Volume make-up to 10 ml.
4. Vortex for 2 minutes.

C) PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 2(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 for Volume make-up to 10 ml.
4. Vortex for 2 minutes.

D) PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 3 (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 for Volume make-up to 10 ml.
4. Vortex for 2 minutes.

E) PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 4 (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 for Volume make-up to 10 ml.
4. Vortex for 2 minutes.

F) PREPARATION OF STANDARD SOLUTION - 4 (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.

G) 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 ppb)	VOL. OF ISS – 4 (ml)	VOL. OF DILUENT (ml)	FINAL VOL. (ml)	FINAL CONC. (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: Use freshly prepared Standard solutions for the analysis.

Preparation of Test Samples

1. Take 1 kg of Rice Sample. Homogenize the Whole Sample using Homogenizer.
2. Accurately weigh 10 g (± 0.5 g) of Homogenized Sample.
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. Volume 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 for shaking vigorously for 2 minutes using Vortex.
10. Centrifuge the Sample Solution at 6000 rpm for 5 minutes at 4 ºC.
11. Collect the supernatant layer of the Sample Solution and filter it through 0.45 µm filter paper.
12. Insert 900 mg C18 Solid Phase Extraction Cartridge onto the Stopcock of the Vacuum manifold.
13. Attach a 10 ml disposable Syringe Barrel to the top of the Cartridge.
14. Condition the Cartridge with 20 ml Methanol by allowing Methanol to gravity filter through the Cartridge.
15. Rinse with 10 ml Water.
16. Transfer 20 ml of Filtered Sample Solution into the Cartridge
17. (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 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.

Chromatographic Conditions

a) Instrument : LC-MS/MS Spectrometer.

b) Chromatographic Conditions : As detailed in below Table

Detector	Mass Detector
Column	2.6µm, 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	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

c) 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

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	**Cyanocobalamin (Vitamin B12) (ppb)** = $\frac{C \times V1 \times V3}{W \times V2}$ Where, C = Instrument concentration (ppb) V1 = Volume make-up (ml) V2 = Volume loaded of Filtrate 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) by analyzing 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 folic acid signal in the matrix. c) Determine the recovery of folic acid by the external spiking method at three different spike levels (0.5, 2.0, 5.0 and 10.0 µg/kg) in six replicates. d) Calculate the recovery value using the following equation: e) Recovery (%) = $\frac{(A - B) \times 100}{C}$ 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