	Method for Determination of Cyanocobalamin (Vitamin B12) in Fortified Rice		
Method No.		**Revision No. & Date**	
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.		

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	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.

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, -5000 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: Kinetex 2.6µm, XB C18 Column, 2.1 x 100 mm
Materials and Reagents	1. Sodium Acetate, LR Grade. 2. Ammonium Formate, MS Grade 3. α-Amylase, (TCI, A0447) 4. Acetic Acid, MS Grade. 5. Methanol, LR Grade. 6. Sodium Hydroxide, LR Grade 7. CRM Used : Cyanocobalamine (CAS No: 68199, P.No: V2876, Sigma Aldrich) 8. Cartridge Details: Name: MAXI-CLEAN SPE 900 mg C-18 Make: S PURE, P/N: 20942, Part: 5122344, Pack Size: 50
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.

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	c) MOBILE PHASE B PREPARATION Transfer 1000 mL Methanol to Mobile Phase Glass Bottle and then Sonicate for 15 minutes. **d) 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	**A) PREPARATION OF STOCK SOLUTION FOR CYANOCOBALAMIN (1000 PPM)** 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 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 °C in the light Protected Area. **B) PREPARATION OF INTERMEDIATE STOCK SOLUTION - 1 (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 for Volume make-up to 10 mL. 4. Vortex for 2 minutes. **C) PREPARATION OF INTERMEDIATE STOCK SOLUTION - 2 (10 PPM)** 1. Pipette out 1.0 mL of Intermediate Stock 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 STOCK SOLUTION - 3 (1 PPM)** 1. Pipette out 1.0 mL of Intermediate Stock 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.

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E) PREPARATION OF INTERMEDIATE STOCK SOLUTION - 4 (100 PPB)

1. Pipette out 1.0 mL of Intermediate Stock 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 STOCK SOLUTION - 4 (5 PPB)

1. Pipette out 0.5 mL of Intermediate Stock 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

1. Pipette out 0.5 mL of Intermediate Stock 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.

H) PREPARATION OF CALIBRATION STANDARD SOLUTIONS

Use Intermediate Stock Solution - 4 for preparing Calibration Standard 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

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	CAL : Calibration ISS : Intermediate Stock 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) Make & Model : Waters & TQ Detector. c) Chromatographic Conditions : As detailed in below Table

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Instrument	WATERS TQD
Detector	Mass Detector
Column	Kinetex 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	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

d) 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

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		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,

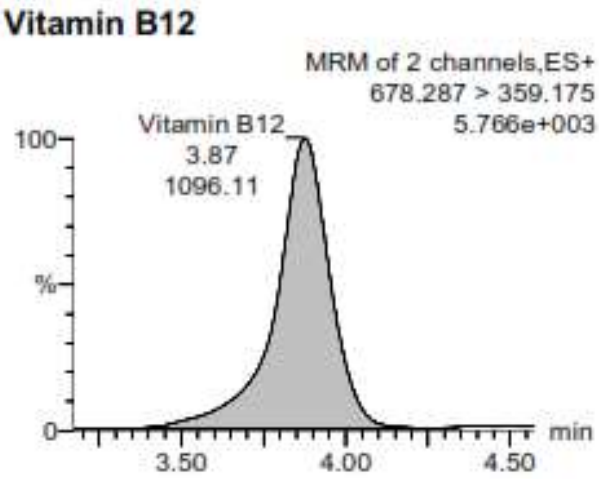
C = Instrument concentration (PPB)
V1 = Volume make-up (mL)
V2 = Volume loaded of Filtrate on Cartridge (mL)
V3 = Volume of diluent added for extract the Vitamin B12 from Cartridge (mL)
W = Sample Weight (g)

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 (ug/Kg)
B = the natural content of Vitamin B12 in the control sample (ug/Kg)
C = the spiked concentration of Vitamin B12 (ug/Kg)

Results (Chromatograms)	
LOD & LOQ	a) Limit of Detection is 0.25 ug/Kg with Respect to the Sample. b) Limit of Quantification is 0.5 ug/Kg with Respect to the Sample.
Reference	Method Protocol: PRT/RA/FTR/2022/004, Method Validation Report for Estimation of Cyanocobalamin (Vitamin B12) in Fortified Rice 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

The following 'note' need to be added in all manuals:

Note: The test methods given in the manual are standardised/ validated/ taken from national or international methods or recognised specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use".

Editorials (For Reference purpose while writing methods)

Abbreviations to be used

Microgram	µg
Milligram	mg
Gram	g

Kilogram	kg
Milliliter	mL
Litre	L
Second	sec
Minute	min
Hour	h
Celsius	°C
Kelvin	°K
Centimeter	cm
Millimeter	mm
Molar	M
Millimolar	mM
Micromolar	µM
Mole	mol
Normal	N
Wavelength	nm

Some Editorials for the manuals

Space between numbers and units

- Mass and volume need spaces 12 g not 12g, 100 mL not 100mL
- Time also needs space 10 h not 10h, 15 min not 15min
- Temperatures need spaces
  - between value and degree sign: **37 ˚C**, not 37˚ C or 37˚C
  - but the degree sign for angles goes with the number: 90º angle
- Centrifugal forces need spaces
  - on both sides of the "×“ (remember not x)
  - 10,000 × g, not 10,000g or 10,000xg
- Other "places for spaces"
  - around equals sign: **n = 3**, not n=3
    - also around >, <, ~, etc
  - around plus/minus: 29 ± 7, not 29±7
- Percentages is the only exception
  - **5%** serum, **0.01%** bromophenol blue
  - This is because % is not really a unit, just an indication that the value is presented as the "ratio to 100"
  - **a <u>space</u> is required**: 10 mM or 6 M, never 10mM or 6M

- Use numerals to express numbers 10 and above.
- Use words to express numbers below 10.
- Use numerals when you have 3 or more numbers in a series, even if each of the numbers is below 10.
- When numbers begin a sentence, you must write them out in words.
- Situations in which Numbers Should be Given as Numerals

General Guideline	Examples
All numbers 10 and above	Trial 14; 35 animals; 16 genera of legumes
All numbers that immediately precede a unit of measurement	A wing 10 cm long; 5 mg of drug; 21days
Numbers with decimals; fractions that include whole numbers	7.38 mm; $4^1/_2$ hours
Numbers that represent statistical or mathematical functions or results, percentages, ratios	Multiply by 5; fewer than 6%; 3.75 times as many; the 2nd quartile
Numbers that represent exact times or dates; ages; size of samples, subsamples or populations; specific numbers of subjects in an experiment; scores and points on a scale; exact sums of money; and numerals as numerals	About 3 weeks ago, at 1:00 a.m. on January 25, 2000, the 25-year-old patients with IQ scores above 125 all awoke simultaneously in the nursing home at 125 Oak Street. They were paid $25 apiece to go back to sleep
Numbers below 10 that are grouped for comparison with numbers 10 and above in the same paragraph	4 of 16 analyses, the 1st and 15th of the 25 responses; lines 2 and 21
Numbers that denote a specific place in a numbered series, parts of books and tables, and each number in a list of four or more numbers	Trial 6; Grade 9 (but the ninth grade); the groups consisted of 5, 9, 1, and 4 animals respectively