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	**Method for Determination of Cyanocobalamin (Vitamin B12) in Vitamin Mineral Premix for Preparation of Fortified Rice Kernel (FRK)**		
Method No.	FSSAI.VMP-FRK.16.010.2023	**Revision No. & Date**	0.0
Scope	The Scope of this Method includes for Quantification of Cyanocobalamin (Vitamin B12) at 2.0 mg/Kg LOQ Level (with respect to the Sample) by using HPLC in Premix. a) Limit of Detection (0.1 mg/Kg) With Respective to the Standard b) Limit of Quantification (0.2 mg/Kg) With Respective to the Standard. c) Limit of Quantification (2.0 mg/Kg) With Respective to the Sample.		
Caution (Safety & Precautions)	1) **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 **2) 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, b) Skin Contact: Take off immediately all contaminated clothing. Rinse skin with water/ shower. 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). 3) **Orthophosphoric Acid:** It is a colorless, crystalline solid, the tribasic acid of pentavalent phosphorus. During handling of Orthophosphoric Acid, below safety measures to be followed: a) Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. b) Wash off immediately with plenty of water for at least 15 minutes. Remove and wash contaminated clothing and gloves, including the inside, before re-use. Call a physician immediately. c) Do NOT induce vomiting. Clean mouth with water. Never give anything by mouth to an unconscious person.		

	d) If not breathing, give artificial respiration. Remove from exposure, lie down. Do not use mouth-to-mouth method if victim ingested or inhaled the substance; give artificial respiration with the aid of a pocket mask equipped with a one-way valve or other proper respiratory medical device. Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination. 4) **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.
Principle	Cyanocobalamin is Extracted from the Sample by Diluent Containing Potassium Dihydrogen Phosphate and Dipotassium Hydrogen Phosphate, Extract & Filtered, and Quantified by HPLC.
Apparatus/Instruments	1. HPLC. 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. 5. Column: C8 4.6 mm X 250 mm X 5µm; 6. Sonicator for mixing of solution. 7. Vortex for preparation of stock solution. 8. Homogenizer for sample grinding
Materials and Reagents	1. Methanol, LR Grade. 2. CRM Used: Cyanocobalamin (CAS No: 68199) 3. Potassium dihydrogen phosphate, LR Grade. 4. Dipotassium hydrogen phosphate, LR Grade. 5. Ortho phosphoric Acid, LR Grade. 6. Acetonitrile HPLC Grade
Preparation of Reagents	**a) <u>MOBILE PHASE A PREPARATION</u>** 1. Dissolve 2.72 gm Potassium dihydrogen phosphate and 3.48 gm Dipotassium hydrogen phosphate in 1000 ml of water, 2. Adjust pH 6.6 (+/- 0.1) with Ortho phosphoric Acid. **b) <u>MOBILE PHASE B PREPARATION</u>** 1. Prepare a mixture of Mobile Phase A and Acetonitrile (80:20) Ratio and mix well. **c) <u>DILUENT PREPARATION</u>** 1. Mobile Phase A is using as a Diluent.

Sample Preparation	**PREPARATION OF SAMPLE SOLUTION** 1. Weigh 1.0 g (± 0.10 g) of Homogenized Sample. 2. Transfer to a 10 ml amber color volumetric flask. 3. Add 5 mL Mobile phase A. 4. Vortex for 5 minutes. 5. Do Volume make-up to 10 ml with Mobile phase A. 6. Vortex for 2 minutes 7. Filter the solution through 0.45µm Nylon Syringe Filter. 8. Pour the Filtrate into the Vial, and use this for injecting into HPLC.
Method of Analysis	**A) 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 Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. Note: Store the Solution at -20°C in the light Protected Area **B) PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 1 (100 mg/Kg)** 1. Pipette out 1.0 mL of Stock Solution 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. **C) PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 2 (10 mg/Kg)** 1. Pipette out 1.0 mL of Intermediate Standard Stock Solution – 1. 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min. **D) PREPARATION OF BREACKGING STANDARD SOLUTION (0.75 mg/Kg)** 1. Pipette out 0.75 mL of Intermediate Standard Stock Solution – 2. 2. Transfer to 10 mL Amber Colored Volumetric Flask Containing 2 mL of Mobile Phase A. 3. Add Mobile Phase A for Volume make-up to 10 mL. 4. Vortex for 2 min.

E) PREPARATION OF CALIBRATION STANDARD SOLUTIONS

Use Intermediate Standard Solution – 2 for Preparing Calibration Standard Solution as mentioned in below Table.

CAL. STANDARD SOLUTIONS	ISS - 2 (10 mg/L)	VOL. OF ISS – 2 (mL)	VOL. OF DILUENT (mL)	FINAL VOL. (mL)	FINAL CONC. (mg/L)
LS6	10	2.00	8.00	10	2.00
LS5	10	1.50	8.50	10	1.50
LS4	10	1.00	9.00	10	1.00
LS3	10	0.75	9.25	10	0.75
LS2	10	0.50	9.50	10	0.50
LS1	10	0.20	9.80	10	0.20

NOTE: Always make Fresh Preparation of Calibration Standard Solutions

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

Method of Analysis
(a) Chromatographic Conditions

a) **Instrument** : HPLC
b) **Chromatographic Conditions** : As detailed in below Table

Instrument	HPLC
Detector	DAD
Column	Column: C8 4.6 mm X 250 mm X 5µm;
Run time	30 min
Column Temperature	40°C
Flow rate	1.0 mL/min
Injection Volume	100 µl
Mobile Phase A	Dissolve 2.72 gm Potassium Dihydrogen phosphate and 3.48 gm Dipotassium hydrogen phosphate in 1000 ml of water, Adjust pH 6.6 (+/- 0.1) with Ortho phosphoric Acid.
Mobile Phase B	Prepare a mixture of Mobile Phase A and Acetonitrile (80:20) ratio and Mix well.
Diluent	Mobile Phase A
Wavelength	360

c) Gradient Program

TIME	FLOW RATE	MOBILE PHASE A (%)	MOBILE PHASE B (%)
0.01	1.0	90	10
20	1.0	0	100
25	1.0	0	100

	28	1.0	90	10
	30	1.0	90	10

Note: *The make & model of Instrument & Column can be changed. However, the Instrument should be able to achieve the desired LOD value & the Column is exactly same in terms of the Composition & Dimensions.*

Method of Analysis
(b) Batch Organization

INJECTION SEQUENCE

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	Blank	2
9	Sample Solution	1
10	Blank	2
11	Bracketing Standard Solution	1
TOTAL INJECTIONS		**15**

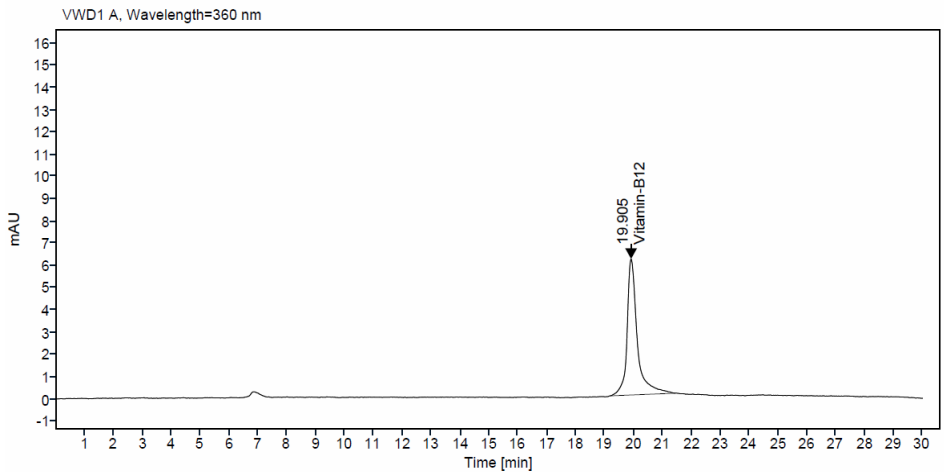
Calculation with units of Expression

a) Carry out analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards by fitting the data into a linear regression curve.

Cyanocobalamin (Vitamin B12) (mg/Kg) =

$$\frac{\text{Sample Conc (mg/Kg) X Make up Volume(mL)}}{\text{Sample Weight (g)}}$$

b) The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the Cyanocobalamin (Vitamin B12) signal in the matrix.

(a) Chromatograms	
(b) LOD & LOQ	a) Limit of Detection (0.1 mg/Kg) With Respective to the Standard. b) Limit of Quantification (0.2 mg/Kg) With Respective to the Standard. c) Limit of Quantification (2.0 mg/Kg) With Respective to the Sample.
Inference (Qualitative Analysis)	This Method is Developed & Validated for Estimation of Vitamin B12 in Premix using HPLC with LOD & LOQ Levels Established at 1.0 mg/Kg & 2.0 mg/Kg (With Respect to the Sample).
Reference	Method Protocol: PRT/RA/FRK/2022/004, Method Validation Report for Estimation of Cyanocobalamin (Vitamin B12) in Premix by HPLC. 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