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 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: Kinetex 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, P.No: V2876, Sigma Aldrich) 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 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	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	5 mM Ammonium formate
Mobile Phase B	Methanol
Diluent	50% Methanol containing 0.1 % Formic acid
Source Temperature	140 ºC
Desolvation Temperature	300 ºC
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***

Chromatographic

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

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

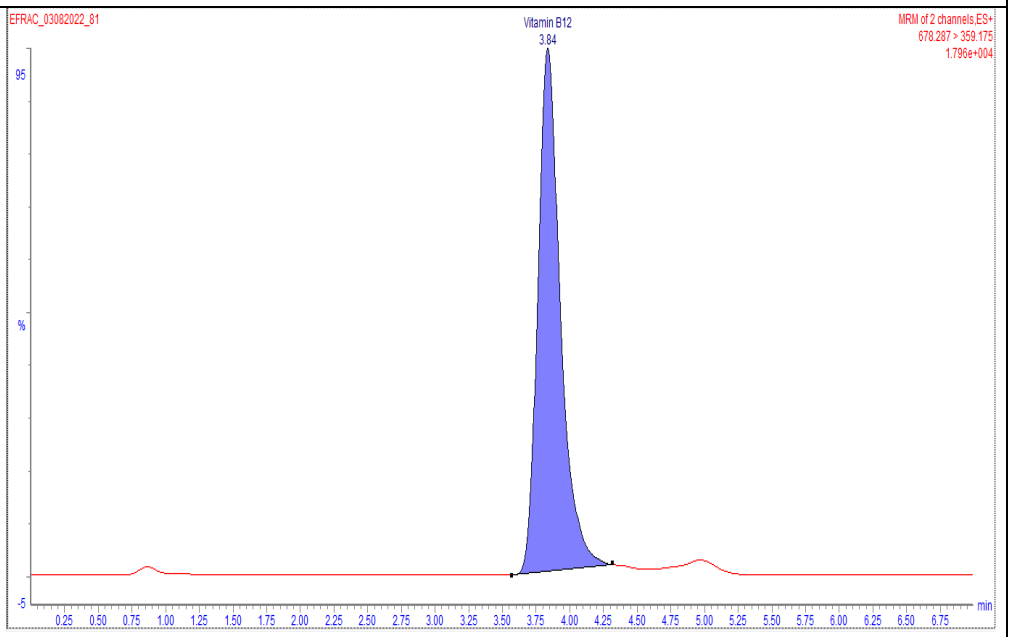
Where

C= concentration cyanocobalamine 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