File No.11014/02/2021-QA

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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FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Weitare, 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	1				
	It is a Chemical which is corre and eye damage. It is toxic if i tract	inhaled. It is corrosiv	ve to the respiratory			
	Following safety measures ne	eed to be taken duri	ng Handling of			
	Concentrated Nitric Acid:					
	a) Do not breathe dust/f		, , , ,			
	b) Wash face, hands and	any exposed skin un	orouginy arter			
	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 of	-	combustibles.			
	i) Wear respiratory prot					
	2. Hydrogen Peroxide					
	It is Oxidizing, Corrosive					
	Following safety measur	es need to be taken	during Handling of			
	Hydrogen Peroxide:	a ta high concontrat	ions of Hudrogon			
	When handling moderate Peroxide in the workplace	-				
	showers are accessible, a					
	approved Vapor Respira	1 0 00				
Scope	The Scope of this Method is a 10 ppm LOQ Level (with resp a) Limit of Detection 4 m	pplicable for Quanti pect to the Sample) b ng/kg in with respec	y using ICP-MS. tive to the Sample.			
	Limit of Quantification 10 mg		-			
Principle	Weigh 0.25 g (± 0.02 g) Digestion Closed (MDC) Ves Vessel. Heat Milli Q Water at ml Hydrogen Peroxide, Add Vessel tightly. Keep at Roo Vessel rotor in Microwave Di	sel. Transfer to Mic 60 ºC. Add 2.0 ml o 5 ml of Nitric Acio m Temperature fo	crowave Digestion Cool of Hot Milli-Q water, 1.0 d. Close the Microwave r 5 minutes. Keep the			

	Mix well. Ma	ke unto 5	0 ml with M	illi. O Water			
Apparatus/Instruments		•		•		-MS)	
mppulatus/ mstruments						110)	
		-					
Materials and Reagents		Concentrated Nitric Acid (Purity- 69%)					
	2. Hydroger	Hydrogen Peroxide (Purity -30%)					
	3. CRM Used	d : Iron					
	4. Purity of	Argon an	d other gas,	if used must	fulfill the	standard of	
	instrume	nt require	ement				
Preparation of solutions		-		TE STOCK	SOLUTION	<u>I - 1 (100 PPM)</u>	
			27 ml of Sto		0201101	<u> </u>	
	_				umetric Fl	ask containing 2	
		l of Milli (0	
	3. Add 0).5 ml Nit	ric Acid.				
	4. Add M	Ailli Q Wa	ater for Volu	me make-up	to 10 ml.		
	5. Mixed	l by using	g Vortex Sha	ker Mixer.			
			-				
	PREPARATI	ON OF C	ALIBRATIO	N STANDAR	RD SOLUT	IONS	
						ing Calibration	
				oned in below		0	
	Cal.	ISS - 1	VOL. OF	VOL. OF	FINAL	FINAL CONC	
	Standard	Standard (100 ISS – 1 NITRIC VOL. (PPM)					
	Solution	PPM)	(ml)	ACID (ml)	(ml)		
	LS 7	100	2.00	5	100	2.00	
	LS 6	100	1.50	5	100	1.50	
	LS 5 LS 4	100 100	1.00 0.50	5	$\frac{100}{100}$	1.00 0.50	
	LS 4 LS 3	100	0.30	5	100	0.25	
	LS S	100	0.23	5	100	0.10	
	LS 1	100	0.05	5	100	0.05	
		CAL : Calibration					
			Stock Soluti	on			
	VOL: Volu						
	LS : Line		ution				
	NOTE: Use freshly prepared Standard solutions for the analysis.					e analvsis.	
Sample Preparation	PREPARATION OF SAMPLE SOLUTION						
	1. Homo	genize th	e Sample by	Grinding as	finely as p	ossible.	
	2. Weigh 0.25 g (± 0.02 g) Grinded Sample.						
			-	estion Closed	l (MDC) Ve	essel.	
	4. Heat M	Iilli Q Wa	iter at 60 °C.				
	5. Add 2.0 ml of Hot Milli-Q water.						
			-	ide.			
		0 ml Hyd	rogen Perox	ide.			
	6. Add 1. 7. Add 5	0 ml Hyd ml of Niti	rogen Perox ric Acid.		temp for	5 min. to	
	 6. Add 1. 7. Add 5 8. Loosel 	0 ml Hyd ml of Niti y cap the	rogen Perox ric Acid. vessel and l	ide. keep at room	ı temp for	5 min. to	
	 Add 1. Add 5 Loosel predig 	0 ml Hyd ml of Niti y cap the est the sa	rogen Perox ric Acid. vessel and l	keep at room	ı temp for	5 min. to	

	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 m	ıl of Milli Q wat	ær.			
	14. Mix well.					
	15. Transfer	to 50 ml Volum	netric Flask	Κ.		
	16. Volume n	nake-up to 50 r	nl with Mil	li-Q wat	ter.	
Method of analysis	a) Instru	ment :	ICP-MS S	pectro	meter.	
	ь) Condit	tions : As det	ailed in b	elow 1	Table	
	·		-			
	D	1	-		gon (15L /	,
	Plasma co	ndition	-			eed (0.5 rps)
	S/C Temp	oraturo	c) RF pow 2°C	el 1550	walls	
			40 Sec			
	Uptake Ti					
	Delay Tim		40 Sec			
	Stabilize 7		40 Sec			
	Nebulizer	Flow	1.0 ml/Min ORS and KED with Helium Flow:3.8			
	Reaction (Cell	ORS and ml/Min	KED v	vith Heliu	m Flow:3.8
	Numbers	of Replicates	3.0			
	Detector's	parameters	5 mV			
	Mode		Не			
	Recommended mass for 56					
	Iron TMP Revo	lution	100 %			
	TMP Revolution 100 % Working C					
	Auto sam	oler conditions	Mode	Contin	uous	
			Wash	Betwee	en runs	
	c) Microw	ave Digestion I	Program			
		RAMPING	HOLD	TIME	TEMP	POWER
	SL. NO	STAGE	(Minu	ites)	(°C)	(Watt)
	1	01	2	0	180	800
	2	1		160	800	
	3	10		140	800	
	4 COOL DOWN 10 - Iron (ppm) = Instrument Conc. X Make-up Volume					
Calculation with units of	Iron	(ppm) = <u>Instru</u>			-	<u>me</u>
expression			Sample V	veigilt X	1000	
	a) Carry out a regression analysis and calculate Regression					
	coefficient (R2) by analyzing the calibration standards by					
		e data into a lii				-
	-	onse for the rea	-			-

	 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 (%) = (A - B) x 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 N	lame	Samp	e Solutio	n				
	Sample ty		Samp	e					
		Comment —							
	Prep Dilu		1.000						
	Auto Dilu		1.000						
	Total Dilu		1.000	0					
	Operator		Snoot						
	Acq Mode Cal Title	8	Spect	rum					
	Cal Type		Exteri	nal Calibr	ation				
	Last Calib	<u>ו</u>		-2022 18					
	Bkg File	•		2022 10					
	Bkg Mode	e	Count	Substrac	tion exc	cept for	ISTD		
	FQ Blank		_			•			
	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 & Milk Proc						e elements i n.	n Milk	
Approved by	Scientific P	anel on	Methods	of Sampl	ing and	Analys	is		

FOOD SAFETY AND STANDARDS AUTORITY 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	 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 enzalpha-linked polysaccha shorter chains thereof, I amylase, found in humar During handling of Metha a) Skin Contact: Avoid c b) If Inhalation: Avoid i c) Use adequate ventila 	rides, such as starch an Dextrin and Maltose. It as and other mammals. anol, below Safety Meas ontact with skin and ey- ngestion and inhalation	nd glycogen, yielding is the major form of ures to be followed: es.			
	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 Potas	sium 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 °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/Instrumen	
ts	1. LC-MS/MS, system equipped with a quaternary gradient pump, an
	auto sampler (100 μ L maximum loop capacity).
	2. Analytical Balance, -Suitable for weighing samples with accuracy up
	to 0.1 mg.
	 Centrifuge 6000 rpm, holding 50 ml tubes. Micro Pipettes Capable of delivering from 100 -1000 μl, 20 -200 μl
	10 -100 $\mu l.$ of liquids such as Folic Standards, Solvents, Buffers and
	Extracts.
	5. Incubator
	6. Column: T3 1.8 μm, 2.1*100mm7. Sonicator for mixing of solution.
	8. Vortex for preparation of stock solution.
	9. Homogenizer for sample grinding
Materials and	1. Potassium Hydrogen Phosphate, LR Grade
Reagents	2. L-Ascorbic Acid, LR Grade
0	3. α-Amylase
	4. Potassium Hydroxide, LR Grade
	5. Formic Acid, MS Grade
	6. Acetonitrile, MS Grade
	7. CRM Used: Folic Acid
Preparation of	PREPARATION OF MOBILE PHASE
Reagents	a) <u>BUFFER PREPARATION</u>
	1 Accurately weigh 17.4 g of Detacoium Hydrogon Dhoenhate
	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) MOBILE PHASE - A PREPARATION
	1.Transfer 1 ml Formic Acid into 1000 ml Volumetric Flask

	2 Add Milli O Water for Volume make up
	2.Add Milli-Q Water for Volume make up 3.Sonicate to mix & Dissolve well
	4. Filter through 0.45 μm Filter Paper
	4. Filter till ough 0.45 µlli Filter Faper
	c) <u>MOBILE PHASE - B PREPARATION</u>
	1. Transfer 1000 ml Acetonitrile to Mobile Phase Glass Bottle and then
	Sonicate.
Preparation of	A. PREPARATION OF STOCK SOLUTION FOR FOLIC ACID
Standards	<u>(1000 ppm)</u>
Standards	
	1. Accurately weigh 10 mg (\pm 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 ^o C in the light Protected Area.
	B. <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION – 1</u>
	<u>(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. PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 2
	<u>(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. PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 3
	<u>(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. PREPARATION OF STANDARD SOLUTION - 3 (10 ppb)
	1. Pipette out 0.10 ml of Intermediate Standard Solution - 3
	I. I pette out 0.10 ini of interintenduce of and a doration of

	ml of 3. Add I	⁷ Milli Q Wa Milli Q Wate	ter er for Volur	olored Volumet ne make-up to IG STANDARD	10 ml	
	Standard So Solution.	 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 				
				ons as mentior		
	CAL. STD. SOLUTIO N	ISS 3 (1000 ppb)	VOL. OF ISS 3 (ml)	VOL. OF MILLI Q WATER	FINAL VOL. (ml)	FINAL CONC. (ppb)
				(ml)		
	LS 6 LS 5	1000	0.40	9.60	10	40
	LS 5 LS 4	1000 1000	0.20	9.80	10	20 15
	LS 1 LS 3	1000	0.10	9.90	10 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	 Take 1 K Homoger Accurate Transfer Add 0.1 g Phospha Vortex for Maintain Potassiu Keep the for one h Maintain Hydroch Add 0.12 minutes. Place 25 	g of Rice Sa nizer. ely weigh 5 into a 25 n g L-Ascorbi te Buffer in or 5 minute the pH of t m Hydroxic Sample So our at 37 ° the pH of t loric Acid S 25 g of α-am	g (± 0.5 g) of and Amber Co c acid and 1 to the Sample the Sample de Solution lution on an C. the Sample solution. nylase into the Colored Vol	Homogenize the of Homogenize olored Volume 15 ml of 0.1 M I ple Solution betwe	e whole San d Sample. tric Flask. Potassium 1 een 8.0-9.0 er & shake a with 2 N ution and s containing	nple using Hydrogen using 1M at 20 rpm hake for 5

	 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	InstrumentChromatograph	: LC-MS/MS Spo nic Conditions	ectrometer : As detailed in be	low Table	
	Detector	Mass De	tector		
	Column	Τ3 1.8 μ	m, 2.1*100mm		
	Run time	7 min			
	Column Tempera	ture 35 °C			
	Flow rate	0.25 ml/	min		
	Injection Volume	20 µl			
	Mobile Phase A	0.1% Fo	rmic Acid in Wate	r	
	Mobile Phase B	Acetonit	rile		
	Buffer	Potassiu	m Hydrogen Phos	phate	
	Source Temperat	ure 140 °C			
	MRM (Quantifier)	442.2 > 2	295.1		
	MRM (Qualifier)	442.2 > 1	176		
	СЕ	12.00			
	CV	40.00			
	De-solvation 450 °C				
	Source ESI +Ve				
	<u>Gradient Program</u>				
	TIME 0.00	FLOW (ml/Min) 0.25	%A 90	%B 10	

	2.00	0.25	0.0	10			
	2.00	0.25	90 10	10 90			
	5.00	0.25	90	90			
	7.00	0.25	90	10			
	7.00	0.25	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	10			
				11			
Method of Analysis/	Injection Sequ	ience					
Batch Organization	SL.NO	NAME OF IN	IJECTIONS	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 Standa	ard Solution	1			
	TOTAL INJ	ECTIONS	22				
		i					
Calculation with units of expression	(R2) by analy	a regression analysis and calculate Regression coefficient nalyzing the calibration standards by fitting the data into a ression curve, including zero as the response for the reager					
	b) Folic Acid () Folic Acid (Vitamin B9) (ppb) = <u>Instrument Conc. X Make up Volum</u> Sample Weight (g)					
	-	d LOQ are determin vely, for the folic ac					
	at three diff replicates.	e the recovery value using the following equation:					

	С
	where
	A = the concentration of folic acid in the spiked sample (μ g/kg)
	B = the natural content of folic acid in the control sample (μ g/kg)
	C = the spiked concentration of folic acid (μ g/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

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Menapy of Headm and Family Wellaw, Covernment of India	Method for Determination of Cyanocobalamin (Vitamin B12) in Fortified Rice		
Method No.	FSSAI.FR.16.003.2022	Revision No. &	0.0
		Date	
Safety & Precautions	b) Skin Protection: Wea prolonged skin contacc) Keep out reach of Chile	sures to be followed: Vear safety glasses or goggl r appropriate clothing to t.	les. prevent repeated or
	Corrosion/Irritation, serie organ toxicity (single ex During handling of Sodium	is a Laboratory Chemica ous Eye Damage/Eye Irrit posure) and can harm to Acetate, below safety mea any exposed skin thoroughl	ation, Specific target Respiratory system. sures to be followed:
	protection	oves/protective clothing/ fume/gas/mist/vapours/sp	
	 Acetic Acid: It is a Chemic skin, eye and other exp exposure to the Vapors of other respiratory effects darkening of the exposed se During handling of Acetic a) Never add water to the from sources of heat, se 	al which is corrosive that c osed surfaces of the hum of this substance causes ch , erosion of tooth ename skin. Acid, below safety measure nis chemical, and always k parks or flame.	nan body. Long-term nronic bronchitis and el, and cracking and es to be followed: seep acetic acid away
	that isn't well-ventilat c) Wash face, hands and	ory equipment if handling ed. any exposed skin thoroughl oves/protective clothing/	ly after Handling
	 Human Health. During has followed: a) Wash skin thoroughly b) Avoid breathing dust/ c) Do not breathe dust/fu d) IF ON SKIN: Wash with e) Specific measures (see label). f) Wash contaminated cl g) Avoid contact with skii h) Use explosion-proof ee 	fume/gas/mist/vapours/sp nme/gas/mist/vapours/sp n soap and water. ee supplemental first aid othing before reuse. n and eyes. Avoid inhalation	safety measures to be oray. ray. instructions on this

	 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, alphalinked 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 ingention and inhelation
	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 Skip contact, flush skip with plenty of water. Personal contact flush skip with plenty of water.
	 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	respiration. The Scope of this Method includes for Quantification of Cyanocobalamin
Scope	(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 μ g/kg with Respect to the Sample.
	Limit of Quantification is 0.5 μ g/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 °C, 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 μ L 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
	μ
	10 -100 μ l. of liquids such as vitamin B12 Standards, Solvents,

	Buffers and Extracts.	
	5. Incubator	
	6. Column: 2.6μm, C18 Column, 2.1 x 100 mm	
	 Column. 2.0 µm, C10 Column, 2.1 x 100 mm Homogenizer for sample grinding. 	
Materials and Reagents	1. Sodium Acetate, LR Grade.	
indernas and reagents	2. Ammonium Formate, MS Grade	
	3. α-Amylase,	
	4. Acetic Acid, MS Grade.	
	5. Methanol, LR Grade.	
	 Sodium Hydroxide, LR Grade CRM Used : Cyanocobalamine 	
	8. Cartridge Details: C18 60Å 50μm SPE Cartridge, 900mg	
Preparation of Reagents	6. Cartriage Details. Cro con sound in St L Cartriage, Soonig	
.I		
	a) <u>BUFFER PREPARATION</u>	
	1. Weigh accurately 20.5 g of Sodium Acetate.	
	 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) MOBILE PHASE A PREPARATION	
	1. Weigh accurately 1.261 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) MOBILE PHASE B PREPARATION	
	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</u>	
	<u>(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.	
	 Vortex for 2 minutes. 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 STANDARD SOLUTION – 1 (100	
	B) <u>PREPARATION OF INTERMEDIATE STANDARD SOLUTION - 1 (100</u> ppm)	
	h h h h h h h h h h h h h h h h h h h	
	1. Pipette out 1.0 ml of Stock Solution.	

of Milli 3. Add Dilu 4. Vortex fo C) <u>PREPARATI</u> 1. Pipette o 2. Transfer Milli Q W	out 1.0 ml of In to a 10 ml Am Vater.	e make-up t A MEDIATE : atermediate aber Colore	to 10 ml. STANDARD S Standard Solu d Volumetric I	OLUTION - ution – 1.	<u>2(10 ppm)</u>
 4. Vortex for D) PREPARATI 1. Pipette of 2. Transfer 2 ml of M 3. Add Dilu 	out 1.0 ml of In to a 10 ml Am Iilli Q Water. ent for Volum	MEDIATE : Itermediate	<mark>STANDARD S</mark> Standard Solu d Volumetric I	ution – 2.	
 E) PREPARATI ppb) 1. Pipette o 2. Transfer 2 ml of N 3. Add Dilu 	out 1.0 ml of In to a 10 ml Am Ailli Q Water. ent for Volum	termediate iber Colore	Standard Solu d Volumetric I	ution – 3.	
 Transfer Milli Q V Add Dilu 	ON OF STANI out 0.5 ml of In to 10 ml Amb	termediate er Colored ^Y	Standard Solu Volumetric Fla	ution – 4.	ing 2 ml of
 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)

IS6 100 2 8.00 10 20 IS5 100 1 9.00 10 10 IS2 100 0.5 9.50 10 2 IS2 100 0.1 9.90 10 1 IS2 100 0.1 9.90 10 1 IS1 100 0.05 9.95 10 0.5 IS2 100 0.1 9.90 10 1 IS1 100 0.05 9.95 10 0.5 CAL: Calibration ISS: Intermediate Standard Solution VOL: Volume IS: Linearity Solution NOTE: Use freshly prepared Standard solutions for the analysis. 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 Plask. 4. Add 50 mg c-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. 5. Volume make-up 0.25 M Sodium Acetate Buffer. 8. Onicate for 20 minutes. 9. To instere to 50 ml Using 0.25 M Sodium Acetate Buffer. 8. Onicate for 20 minutes. 9. Transfer the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 10. Colect the supernatant layer of the Sample Solution and filter it through 0.45 µm filter							
IS4 100 0.5 9.50 10 5 IS3 100 0.2 9.80 10 2 IS2 100 0.1 9.90 10 1 IS5 Intermediate Standard Solution 9.95 10 0.5 CAL : Calibration 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 c-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. 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 into a 50 ml Phase Extraction Cartridge tube for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution into the 50 ml the Cartridge. 14 % C. 11. Taker 10 ml disposable Syringe Barrel to the top of the Cartridge. 14 % C. 12. Insert 900 mg G18 Solid Phase Extraction Cartridge. 16 % Rinse with 10 ml Water.		LS 6	100	2	8.00	10	20
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. 1. Take 1 kg of Rice Sample. Homogenized Sample. 3. Transfer into a 50 ml Amber Colored Volumetric Flask. 4. Add 50 mg camylase and 20 ml of 0.25 M Sodium Acetate Buffer. 5. Volume make-up to 50 ml using 0.25 M Sodium Acetate Buffer. 5. 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 °C. 11. Collect the supermatant layer of the Sample Solution and filter it through 0.45 µm filter paper. 12. Insert 900 mg C18 Solid Phase Extraction Cartridge tube for shaking vigorously for 2 minutes using Vortex. 13. Attach a 10 ml disposable Syringe Barrel to the top of the Cartridge. 14. Condition the Cartridge. 15. Rinee with 10 ml Vater. 16. Transfer 20 ml of Filtered Sample Solution into the Cartridge. 17. (If Necessary, app		LS 5	100	1	9.00	10	10
IS2 100 0.1 9.90 10 1 IS2 100 0.05 9.95 10 0.5 CAL : Calibration ISS : Intermediate Standard Solution VOL: Volume IS : 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 c-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. 5. Sonicate the Solution for 20 minutes. 7. Volume make-up to 50 ml using 0.25 M Sodium Acetate Buffer. 8. Sonicate the Solution into the 50 ml Centrifuge the for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution into the 50 ml Centrifuge the for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution and filter it through 0.45 µm filter paper. 11. Collect the supernatant layer of the Sample Solution and filter it through 0.45 µm filter paper. 11. Collect the supernatant layer of the Sample Solution into the Cartridge. 12. Inser 400 mg C18 Solid Phase Extraction Cartridge. 13. Ritech a 10 ml disposable Syringe Barrel to the top of the Cartridge. 13. Attach a 10 ml disposable Syringe Barrel to the top of the Cartridge. 16. Transfer 20 ml of Filtered Sample Solution into the Cartridge. 14. Condition the Cartridge wit		LS 4	100	0.5	9.50	10	5
LS1 100 0.05 9.95 10 0.5 CAL : Calibration ISS : Intermediate Standard Solution VOI: 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. Accurately weigh 10 g (± 0.5 g) of Homogenized Sample. 3 Transfer into a 50 ml Amber Colored Volumetric Flask. A do 50 mg colspan="2">Accurately weigh 10 g (± 0.5 g) of Homogenized Sample. 3 Transfer into a 50 ml Amber Colored Volumetric Flask. A Accurately weigh 10 g (± 0.5 g) of Homogenized Sample. 5 Transfer the Sample Solution for 20 minutes. 7 Transfer the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 10. Collect the superratant layer of the Sample Solution and filter it through 0.45 µm filter paper. 10. Collect the superratant layer of the Sample Solution and filter it through 0.45 µm filter paper. 10. Insert "00 minutes. 10. Insert "00 minutes. 10. Solid Phase Extraction Cartridge.		LS 3	100	0.2	9.80	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. Homogenize the Whole Sample using Homogenizer. 2. Accurately weigh 10 g (2 0.5 g) of Homogenized Sample. 3. Transfer into a 50 ml Amber Colored Volumetric Flask. 4. Add 50 mg a-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. 5. Sonicate the Solution for 20 minutes. 6. Sonicate the Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 10. Centrifuge the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. 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. 15. Rinse with 10 ml Vater. 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. Rinse with 10 ml Vater. 19. Rinse the Cartridge by pulling a Vac		LS 2	100	0.1	9.90	10	1
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. 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 °C. 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.		LS 1	100	0.05	9.95	10	0.5
Samples 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. Votrex 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 °C. 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. 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.	Propagation of Tost	ISS : Intermediate Standard Solution VOL: Volume LS : Linearity Solution					
 Accurately weigh 10 g (± 0.5 g) of Homogenized Sample. Transfer into a 50 ml Amber Colored Volumetric Flask. Add 50 mg α-amylase and 20 ml of 0.25 M Sodium Acetate Buffer. Vortex for 5 minutes. Sonicate the Solution for 20 minutes. Volume make-up to 50 ml using 0.25 M Sodium Acetate Buffer. Sonicate for 20 minutes. Transfer the Sample Solution into the 50 ml Centrifuge tube for shaking vigorously for 2 minutes using Vortex. Centrifuge the Sample Solution and filter it through 0.45 µm filter paper. Collect the supernatant layer of the Sample Solution and filter it through 0.45 µm filter paper. Insert 900 mg C18 Solid Phase Extraction Cartridge onto the Stopcock of the Vacuum manifold. Attach a 10 ml disposable Syringe Barrel to the top of the Cartridge. Condition the Cartridge with 20 ml Methanol by allowing Methanol to gravity filter through the Cartridge. (If Necessary, apply enough Vacuum, so that the Sample will drip steadily through the Cartridge). Pass the Sample Solution through the Cartridge. Minse the Cartridge with 5 ml of Water Discard Eluent. Air-Dry the Cartridge by pulling a Vacuum until no more effluent is observed. Close Each Stopcock. Place 5 ml Ria Vial under the Cartridge. Add 4 ml Diluent to the Cartridge. Chromatographic a) Instrument : LC-MS/MS Spectrometer. 	_			pie. Homoge	mze the who	le sample u	sing
Chromatographic a) Instrument : LC-MS/MS Spectrometer.		 3. Transfer 4. Add 50 r 5. Vortex fe 6. Sonicate 7. Volume 1 8. Sonicate 9. Transfer 9. Transfer 9. Sonicate 9. Transfer 9. Sonicate 9. Transfer 9. Sonicate 9. Transfer 9. Sonicate 9. Transfer 9. Centrifug 10. Centrifug 11. Collect th through 12. Insert 90 of the Va 13. Attach a 14. Condition gravity f 15. Rinse wi 16. Transfer 17. (If Necessisteadily feedback 18. Pass the 19. Rinse the 20. Discard 1 21. Air-Dry feedback 22. Close Ea 23. Place 5 r 24. Add 4 m 25. Open Stop 26. Elute the 27. Transfer 	into a 50 ml , ng α -amylase or 5 minutes. the Solution : make-up to 50 for 20 minute the Sample Solution ge the Sample ne supernatar 0.45 μ m filter 00 mg C18 Sol acuum manifo 10 ml disposa n the Cartridg ilter through th 10 ml Wate 20 ml of Filte sary, apply en through the C Sample Solut e Cartridge w Eluent. the Cartridge w Eluent.	Amber Color and 20 ml o for 20 minut 0 ml using 0. es. olution into r 2 minutes o Solution at nt layer of th r paper. id Phase Ext old. able Syringe ge with 20 m the Cartridge ered Sample nough Vacuu artridge). ion through ith 5 ml of W by pulling a der the Cartri e Cartridge. o the Ria Vial Sample Solu	red Volumetri f 0.25 M Sodium f 0.25 M Sodium 25 M Sodium the 50 ml Cen using Vortex. 6000 rpm for e Sample Solu craction Cartr Barrel to the l Methanol by e. Solution into um, so that the the Cartridge Vacuum until ridge.	ic Flask. um Acetate Acetate Bu htrifuge tub 5 minutes a ution and fil idge onto the top of the C y allowing M the Cartrid e Sample wi h.	ffer. e for at 4 ºC. ter it e Stopcock artridge. Iethanol to ge ll drip
Conditions	Chromotographia						
	Conditions	-					
b) Chromatographic Conditions : As detailed in below Table	Conditions	b) Chroma	tographic Co	nditions : A	s detailed in b	pelow Table	

	Detector		Mass Dete	Mass Detector			
	Column		2.6µm, C18	2.6µm, C18 Column, 2.1 x 100 mm			
	Run time		7 min				
		emperature	35°C				
	Flow rate			0.25 ml/min			
	Injection	Volume		20 µl			
	Mobile Ph	ase A	20 mM Am Water	20 mM Ammonium Formate in Water			
	Mobile Ph	ase B	Methanol	Methanol			
	Buffer		Sodium Ac	Sodium Acetate			
	Diluent			Milli Q Water			
		emperature	140°C				
		on Temperature	300°C				
		ANTIFIER)	678.29 > 3				
	MRM (QU	ALIFIER)	678.29 > 6	65.00			
	CE		26 V				
	CV Source		35 V ESI +ve				
	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				
	E 00		10	90			
	5.00	0.25	90	10			
Method of Analysis	5.00 7.00 INJECTION SEQ	0.25 0.25					
Method of Analysis	7.00	0.25 0.25 UENCE	90 90	10			
Method of Analysis	7.00 INJECTION SEQ	0.25 0.25 UENCE	90 90	10 10 NUMBER OF			
Method of Analysis	7.00 INJECTION SEQ SL.NO	0.25 0.25 <u>UENCE</u> . NAME OF INJ	90 90 ECTIONS	1010NUMBER OFINJECTIONS			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1	0.25 0.25 DUENCE NAME OF INJ Blank	90 90 ECTIONS	101010NUMBER OFINJECTIONS2	-		
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1	101010NUMBER OFINJECTIONS26			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3	0.25 0.25 DUENCE Blank Standard Solutio Blank Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2	10 10			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3 4	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2	10 10 10 NUMBER OF INJECTIONS 2 6 2 1			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3 4 5	0.25 0.25 DUENCE Blank Standard Solutio Blank Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3	10 10 10 10 NUMBER OF INJECTIONS 2 6 2 1 1			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3 4 5 6	0.25 0.25 CUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3 n (LS) - 4	10 10 10 10 NUMBER OF INJECTIONS 2 6 2 1 1 1 1			
Method of Analysis	7.00 INJECTION SEQ 1 2 3 4 5 6 7	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3 n (LS) - 3 n (LS) - 4 n (LS) - 5	10 10 10 10 NUMBER OF INJECTIONS 2 6 2 1 1 1 1 1 1 1			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3 4 5 6 7 8	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio Linearity Solutio Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3 n (LS) - 3 n (LS) - 4 n (LS) - 5	10 10 10 10 NUMBER OF INJECTIONS 2 6 2 1 1 1 1 1 1 1 1 1 1			
Method of Analysis	7.00 INJECTION SEQ SL.NO 1 2 3 4 5 6 7 8 8 9	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio Linearity Solutio Linearity Solutio Linearity Solutio Linearity Solutio	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3 n (LS) - 3 n (LS) - 4 n (LS) - 5	10 10 10 10 NUMBER OF INJECTIONS 2 6 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1			
Method of Analysis	7.00 INJECTION SEQ 1 2 3 4 5 6 7 8 9 10	0.25 0.25 DUENCE NAME OF IN Blank Standard Solutio Blank Linearity Solutio Linearity Solutio Linearity Solutio Linearity Solutio Linearity Solutio Blank Blank	90 90 ECTIONS n - 4 (100%) n (LS) - 1 n (LS) - 2 n (LS) - 3 n (LS) - 3 n (LS) - 4 n (LS) - 5	10 10 10 10 10 2 6 2 1 1 1 1 1 1 1 2			

	Total Injections22
Calculation with units of	Cyanocobalamin (Vitamin B12) (ppb) = $C \times V1 \times V3$
expression	W x 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 LOO are between the provided in the S (N of 2 and 10)
	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 (%) = (A - B) x 100 C
	where
	A = the concentration of Vitamin B12 in the spiked sample (μ g/kg) B = the natural content of Vitamin B12 in the control sample (μ g/kg) C = the spiked concentration of Vitamin B12 (μ g/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