

Determination of Folic Acid (Vitamin B9) in Fortified Rice Kernel

Method No.	FSSAI.FRK.16.005.2023	Revision No. & Date	0.0
Scope	This method is only applicable for quantitative analysis of Folic acid (Vitamin B9) in fortified rice kernels using LC-MS/MS.		
Caution (Safety & Precautions)	<p>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.</p> <p>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 fume hood</p> <p>Acetonitrile: Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Keep away from sources of ignition as it is flammable.</p> <p>Hydrochloric acid: Handle with extreme care. Concentrated HCl is corrosive. Avoid breathing vapors and avoid contact with skin and eyes. Handle only inside a fume hood.</p>		
Principle	Extraction of folic acid using acetate buffer in the presence of α -amylase and then quantitative analysis using reverse phase liquid chromatography followed by tandem mass spectrometry (LC-MS/MS)..		
Apparatus/Instruments	<ol style="list-style-type: none"> 1. Liquid Chromatograph with Tandem Mass Spectrometer (LC-MS/MS), system equipped with a binary gradient pump, an auto sampler 2. Analytical Balance, -Suitable for weighing samples with accuracy up to 0.0001 g. 3. Centrifuge 6000 rpm, capable of accomodating 50 mL tubes. 4. Volumetric flasks-Class A 1000 mL 5. Amber colored volumetric flask: 100 mL 6. Micro Pipettes capable of delivering from 100 -1000 μl, 20 -200 μl 100 μl. of liquids 7. Incubator shaker set at 37 $^{\circ}$C 8. Water bath set at 55 $^{\circ}$C 9. Column: Kinetex, XB C18 Column, 2.6 μm, 2.1 x 100 mm or equivalent 10. Sonicator 11. Vortex mixer 12. Homogenizer with steel blades 		
Materials and Reagents	<ol style="list-style-type: none"> 1. L-Ascorbic Acid, LR Grade 2. α-Amylase (TCI, A0447) 3. Sodium hydroxide, LR Grade 4. Formic Acid, MS Grade 5. Acetonitrile, MS Grade 6. Sodium acetate (anhydrous) LR Grade 7. Hydrochloric Acid, LR Grade 8. CRM: Folic Acid (CAS No: 593003) 		
Preparation of Reagents	<p>Sodium acetate buffer (0.1 M)</p> <ol style="list-style-type: none"> 1. Weigh accurately 8.2 g of anhydrous sodium acetate. 		

	<ol style="list-style-type: none"> 2. Transfer it into 1000 mL of volumetric flask. 2. Add Milli Q Water dissolve and make-up to 1000 mL. 3. Sonicate for 15 min to dissolve. <p>Sodium hydroxide (1 M) Weigh 40 g of NaOH pellets and dissolve in 1000 mL of water. Cool and store</p> <p>Mobile phase A (0.1% Formic acid)</p> <ol style="list-style-type: none"> 1. Transfer 1 mL Formic Acid into 1000 mL Volumetric Flask. 2. Add Milli-Q Water and make up to mark. 3. Sonicate to mix 4. Filter through 0.45 µm filter <p>Mobile phase B (100% acetonitrile) Transfer 1000 mL MS grade acetonitrile to solvent reservoir sonicate for 1-2 mins.</p>
Sample Preparation	<ol style="list-style-type: none"> 1. Grind 50 g of fortified rice kernels to a fine powder. 2. Accurately weigh 1 g (± 0.1 g) of the powder. 3. Transfer into a 100 mL Amber colored volumetric flask. 4. Add 0.1 g L-Ascorbic acid and 50 mL of 0.1 M sodium acetate buffer 5. Vortex for 5 min. 6. Adjust the pH of the solution to between 8.0-9.0 using 1 M NaOH. 7. Shake at 20 rpm for 60 min at 37 °C using an orbital shaker. 8. Adjust the pH of the to 7.0 with 2 N HCl. 9. Add 0.05 g of α-amylase and shake for 5 minutes. 10. Incubate the sample at 55 °C for 30 mins using a water bath. 11. Cool the sample to 25 °C. 12. Make-up the volume to 100 ml with 0.1 M Sodium Acetate. 13. Transfer the sample to a centrifuge tube after vigorous vortexing for two min. 14. Centrifuge at 6000 rpm for 5 min. 15. Filter the supernatant using a 0.45µm Nylon syringe filter. 16. Use the filtrate for LC-MS/MS. <p>Prepare all samples as described above</p>
Preparation of Standard	<p>Preparation of stock solution for folic acid (1000 mg/kg)</p> <ol style="list-style-type: none"> 1. Accurately weigh 10 mg (± 0.1) of Folic acid standard 2. Transfer to 10 mL amber colored volumetric flask 3. Add 2 mL of 0.1 N NaOH 4. Vortex for 2 min. 5. Add Milli Q Water and make-up to 10 mL 6. Vortex for 2 min 7. Store at -20 °C in protected from light. <p>B) Preparation of intermediate stock solution-1 for folic acid (100 mg/kg)</p> <ol style="list-style-type: none"> 8. Pipette out 1.0 mL of stock solution. 9. Transfer to 10 mL amber colored volumetric flask 10. Add Milli Q Water and make-up to 10 mL 11. Vortex for 2 min <p>C) Preparation of intermediate stock solution-2 for folic acid (10 mg/kg)</p> <ol style="list-style-type: none"> 1. Pipette out 1.0 mL of intermediate stock solution-1. 2. Transfer to 10 mL amber colored volumetric flask 3. Add Milli Q Water and make-up to 10 mL 4. Vortex for 2 min <p>D) Preparation of intermediate stock solution-3 for folic acid (1 mg/kg)</p>

1. Pipette out 1.0 mL of intermediate stock solution-2.
2. Transfer to 10 mL amber colored volumetric flask
3. Add Milli Q Water and make-up to 10 mL
4. Vortex for 2 min

Preparation of calibration standards

Use Intermediate Stock Solution(ISS) – 3 (1 mg/kg) for preparing calibration standards as described in below Table.

Cal. standard solutions	ISS 3 (µg/kg)	Vol. of iss 3 (mL)	Vol. of Milliq water (mL)	Final volume (mL)	Final conc. (µg/kg)
LS7	1000	2.00	8.00	10	200
LS6	1000	1.50	8.50	10	150
LS5	1000	1.00	9.00	10	100
LS4	1000	0.75	9.25	10	75
LS3	1000	0.50	9.50	10	50
LS2	1000	0.25	9.75	10	25
LS1	1000	0.10	9.90	10	10

NOTE: Prepare Calibration Standards fresh everyday

Chromatographic Conditions	Instrument : LC-MS/MS			
	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	0.1 % Formic acid in water		
	Mobile Phase B	Acetonitrile		
	Water	Milli Q Water		
	Source Temperature	140°C		
	Desolvation Temperature	300°C		
	MRM (Quantifier)	442 > 295		
	MRM (Qualifier)	442 > 176		
	CE	26 V		
	CV	35 V		
	Source	ESI + VE		
	<u>Gradient Program</u>			
	Time (min)	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
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				
(C) Sequence of injection	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) Carry out LC-MS/MS analysis and calculate regression coefficient (R²) of the calibration curve.

Calculate the Folic acid content in Fortified Rice Kernel using the following equation:

$$\text{Folic acid } \frac{\mu\text{g}}{\text{kg}} = \frac{C \times \text{Makeup volume}}{\text{Sample weight (g)}}$$

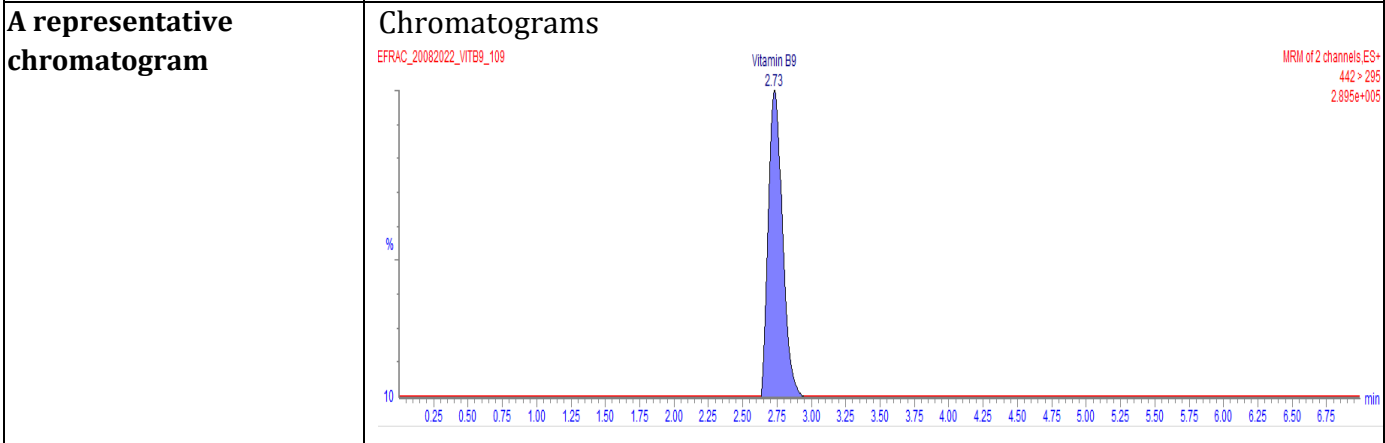
Wherein
C= Concentration obtained from instrument software

The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the folic acid signal in the matrix.
Limit of Detection (10 µg/kg)
Limit of Quantification (25 µg/kg)

Determine the recovery of folic acid by the external spiking method at 5000 µg/kg in six replicates. Calculate the recovery value using the following equation:

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

where
A = the concentration of folic acid in the spiked sample (µg/kg)
B = the folic acid content in the control sample (µg/kg)
C = the spiked concentration of folic acid (µg/kg)
C = Spiked concentration of Vitamin B9 (µg/Kg)



Reference	Method Protocol: PRT/RA/FRK/2022/005, Method Validation Report for Estimation of Folic Acid (Vitamin B9) in Fortified Rice Kernel using LC-MS/MS. 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