	Method for Determination of Folic Acid (Vitamin B9) in Fortified Rice		
Method No.		**Revision No. & Date**	
Safety and Precautions	1) **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 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) Skin Contact: Avoid contact with skin and eyes. b) If Inhalation: Avoid ingestion and inhalation. c) Use adequate ventilation to keep Airborne Concentrations low.		

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	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 Potassium 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.

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	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
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 ^{0}C. Do Volume make-up to 25 ml with 0.1 M Potassium Hydrogen Phosphate Buffer. Shake Vigorously and centrifuge at 6000rpm. Filter through 0.45 µm membrane into an amber LC Vial for UHPLC MS/MS Analysis.
Apparatus/Instruments	1. LC-MS/MS, system equipped with a quaternary gradient pump, an auto sampler (100 µL maximum loop capacity) and a LCMSMS. 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 Folic Standards, Solvents, Buffers and Extracts. 5. Incubator 6. Column: ACQUITY UPLC HSS T3 1.8 µm, 2.1*100mm
Materials and Reagents	1. Potassium Hydrogen Phosphate, LR Grade 2. L-Ascorbic Acid, LR Grade 3. α-Amylase (TCI, A0447) 4. Potassium Hydroxide, LR Grade 5. Formic Acid, MS Grade 6. Acetonitrile, MS Grade 7. CRM Used: Folic Acid (CAS No: 593003)

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Preparation of Reagents	**PREPARATION OF MOBILE PHASE** **a) <u>BUFFER PREPARATION</u>** 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) <u>MOBILE PHASE - A PREPARATION</u>** 1. Transfer 1 mL Formic Acid into 1000 mL Volumetric Flask 2. Add Milli-Q Water for Volume make up 3. Sonicate to mix & Dissolve well 4. Filter through 0.45 µm Filter Paper **c) <u>MOBILE PHASE - B PREPARATION</u>** 1. Transfer 1000 mL Acetonitrile to Mobile Phase Glass Bottle and then Sonicate.
Preparation of Standards	**<u>PREPARATION OF STOCK SOLUTION FOR FOLIC ACID (1000 PPM)</u>** 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 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 ^{0}C in the light Protected Area. **<u>PREPARATION OF INTERMEDIATE STOCK SOLUTION – 1 (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. **<u>PREPARATION OF INTERMEDIATE STOCK SOLUTION - 2 (10 PPM)</u>** **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 Milli Q Water for Volume make-up to 10 mL 4. Vortex for 2 minutes.

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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 Milli Q Water for Volume make-up to 10 mL
4. Vortex for 2 minutes.

PREPARATION OF STANDARD STOCK SOLUTION - 3 (100%)

1. Pipette out 0.10 mL of Intermediate Stock Solution - 3
2. Transfer to 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

PREPARATION OF BRACKETING STANDARD SOLUTION

1. Pipette out 0.10 mL of Intermediate Stock Solution - 3
2. Transfer to 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

PREPARATION OF CALIBRATION STANDARD SOLUTION

1. Use Intermediate Stock Solution - 3 for preparing Calibration Standard Solutions as mentioned in below Table.

CAL. STD. SOLUTION	ISS 3 (1000 PPB)	VOL. OF ISS 3 (mL)	VOL. OF MILLI Q WATER (mL)	FINAL VOL. (mL)	FINAL CONC. (PPB)
LS 6	1000	0.40	9.60	10	40
LS 5	1000	0.20	9.80	10	20
LS 4	1000	0.15	9.85	10	15
LS 3	1000	0.10	9.90	10	10
LS 2	1000	0.05	9.95	10	5
LS 1	1000	0.02	9.98	10	2

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

NOTE: Use freshly prepared Standard solutions for the analysis.

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<table>
<tr>
<td>Preparation of Test Samples</td>
<td>
1. Take 1 Kg of Rice Sample and Homogenize the whole Sample using Homogenizer.
2. Accurately weigh 5 g (± 0.5 g) of Homogenized Sample.
3. Transfer into a 25 mL Amber Colored Volumetric Flask.
4. Add 0.1 g L-Ascorbic acid and 15 mL of 0.1 M Potassium Hydrogen Phosphate Buffer into the Sample
5. Vortex for 5 minutes.
6. Maintain the pH of the Sample Solution between 8.0-9.0 using 1M Potassium Hydroxide Solution (KOH).
7. Keep the Sample Solution on an Orbital Shaker & shake at 20 rpm for one hour at 37 ºC.
8. Maintain the pH of the Sample Solution to 7.0 with 2 N Hydrochloric Acid Solution.
9. Add 0.125 g of α-amylase into the Sample Solution and shake for 5 minutes.
10. Place 25 mL Amber Colored Volumetric Flask containing Sample Solution on the Water Bath at 55 ^{0}C for 30 minutes.
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.
</td>
</tr>
<tr>
<td>Chromatographic Conditions</td>
<td>
• Instrument : LC-MS/MS Spectrometer.
• Make & Model : Waters & TQ Detector.
• Chromatographic Conditions : As detailed in below Table

<table>
<tr><td>Instrument</td><td>WATERS TQD</td></tr>
<tr><td>Detector</td><td>Mass Detector</td></tr>
<tr><td>Column</td><td>ACQUITY UPLC HSS T3 1.8 µm, 2.1*100mm</td></tr>
<tr><td>Run time</td><td>7 min</td></tr>
<tr><td>Column Temperature</td><td>35 °C</td></tr>
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Flow rate	0.25 mL/min
Injection Volume	20 µl
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	Acetonitrile
Buffer	Potassium Hydrogen Phosphate
Source Temperature	140 ^{0}C
MRM (Quantifier)	442.2 > 295.1
MRM (Qualifier)	442.2 > 176
CE	12.00
CV	40.00
De-solvation Temperature	450 ^{0}C
Source	ESI +Ve

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/ Batch Organization

Injection Sequence

SL.NO	NAME OF INJECTIONS	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

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	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

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) Folic Acid (Vitamin B9) (PPB) = $\frac{\text{Instrument Conc. X Make up Volume}}{\text{Sample Weight (g)}}$

c) The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the folic acid signal in the matrix.

d) Determine the recovery of folic acid by the external spiking method at three different spike levels (10, 25, 50 and 100 µg/kg) in six replicates.

e) Calculate the recovery value using the following equation:

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

where

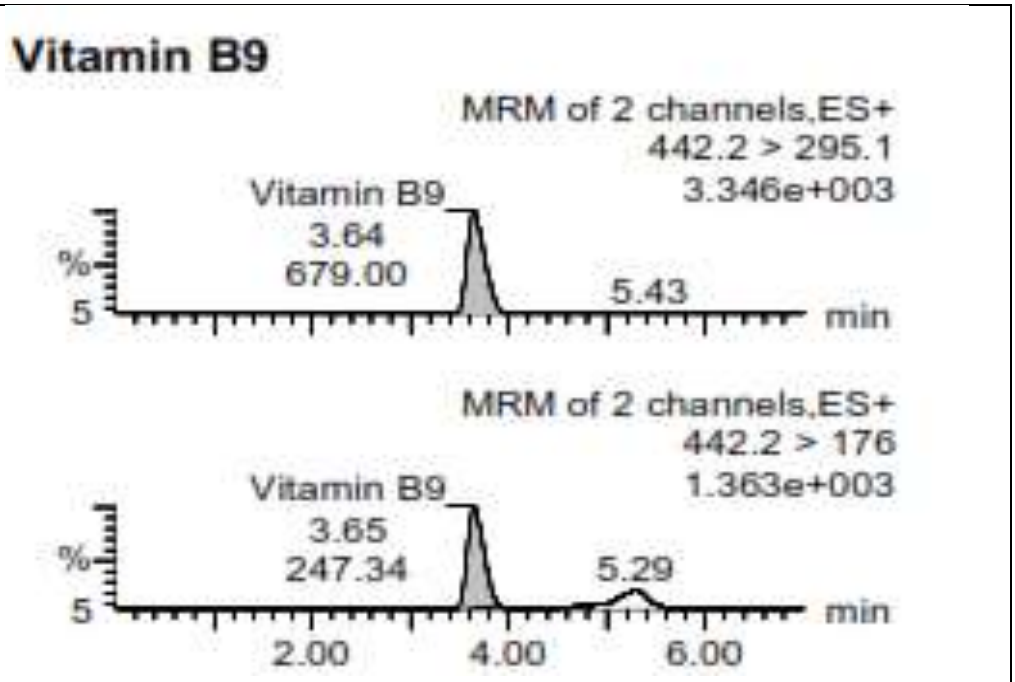
A = the concentration of folic acid in the spiked sample (ug/Kg)

B = the natural content of folic acid in the control sample (ug/Kg)

C = the spiked concentration of folic acid (ug/Kg)

Results

Chromatograms

	
LOD & LOQ	a) Limit of Detection (5 ppb) With Respective to the Sample. b) Limit of Quantification (10 ppb) With Respective to the Sample.
Reference	Method Protocol: PRT/RA/FTR/2022/003, Method Validation Protocol for Estimation of Folic Acid (Vitamin B9) in Fortified Rice 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. PRT/RA/FTR/2022/003
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

nd volume need spaces 12 g not 12g, 100 mL not 100mL
so needs space 10 h not 10h, 15 min not 15min
atures need spaces
etween value and degree sign: **37 ˚C**, not 37˚ C or 37˚C
ut the degree sign for angles goes with the number: 90º angle
gal forces need spaces
n both sides of the "×“ (remember not x)
0,000 × g, not 10,000g or 10,000xg
laces for spaces"
round equals sign: **n = 3**, not n=3

- also around >, <, ~, etc

around plus/minus: 29 ± 7, not 29±7
ages is the only exception
% 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"
space is required: 10 mM or 6 M, never 10mM or 6M
merals to express numbers 10 and above.
rds to express numbers below 10.
merals when you have 3 or more numbers in a series, even if each of the numbers is below 10.
umbers begin a sentence, you must write them out in words.
ns 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

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