



**Method for Determination of Folic Acid (Vitamin B9) in Vitamin Mineral Premix for Preparation of Fortified Rice Kernel (FRK)**

**Method No.**

FSSAI.VMP-FRK.16.009.2023

**Revision No. & Date**

0.0

**Scope**

The Scope of this Method is applicable for Quantification of Folic Acid (Vitamin B9) at 200 mg/Kg LOQ Level (With Respect to the Sample) by using HPLC in Premix

- a) Limit of Detection (10 mg/Kg) With Respective to the Standard
- b) Limit of Quantification (20mg/Kg) With Respective to the Standard.
- c) Limit of Quantification (200 mg/Kg) With Respective to the Sample.

**Caution  
(Safety & Precautions)**

**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.

**Ammonium Hydroxide:**

Routes of Exposure: Inhalation, ingestion, skin contact, eye contact

- a. Corrosive. May cause damage to mucous membranes in nose, throat, lungs and bronchial system.
- b. Corrosive. Harmful if swallowed. May produce burns to the lips, oral cavity, upper airway, esophagus and digestive tract.
- c. Corrosive. Causes severe burns.
- d. Corrosive. Causes severe burns. May cause eye damage, impaired sight or blindness.

**Potassium Phosphate Mono Basic:**

- a. Move to fresh air. Get medical attention if symptoms persist.
- b. Wash skin thoroughly with soap and water. Get medical attention if symptoms occur. Wash contaminated clothing before reuse.
- c. Immediately flush with plenty of water for at least 15 minutes. If easy to do, remove contact lenses. Get medical attention if irritation persists after washing.

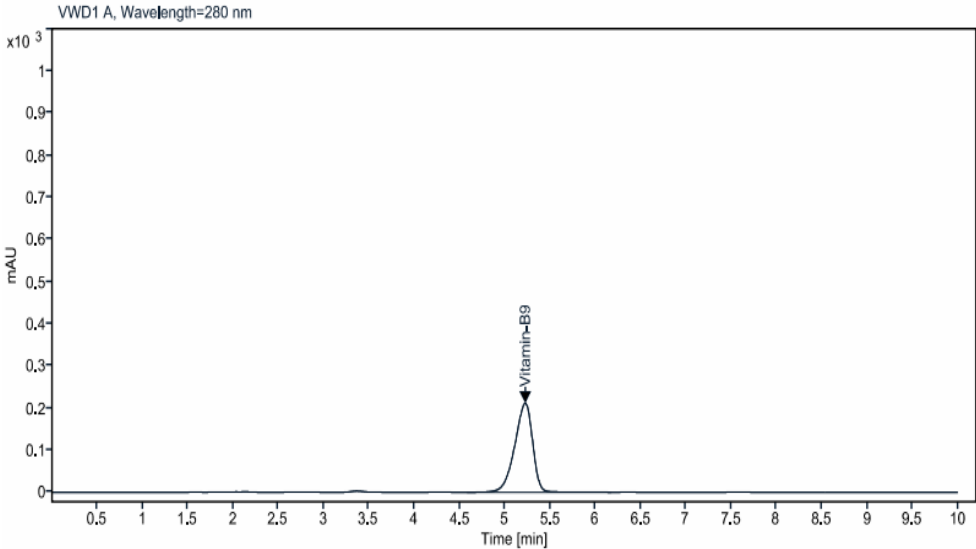
**Tetra methyl Ammonium Hydroxide:**

- a. Rinse thoroughly with plenty of water for at least 15 minutes, lifting lower and upper eyelids. Consult a physician.
- b. Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required.
- c. Remove to fresh air. If not breathing, give artificial respiration. 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. Immediate medical attention is required.

	<p><b>Phosphoric Acid:</b></p> <ol style="list-style-type: none"> <li>Seek medical attention immediately. Move exposed individual to fresh air. Loosen clothing as necessary and position individual in a comfortable position.</li> <li>Remove contaminated clothing and wash before reuse or discard. Rinse skin with for 30 minutes with deluge of water or under a shower. Seek immediate medical attention. Wash affected area with soap and water.</li> <li>Rinse immediately with plenty of water, also under the eyelids, for at least 30 minutes. Remove contact lens(es) if able to do so during rinsing. Seek medical attention immediately. Protect unexposed eye.</li> <li>Seek medical attention immediately. Rinse mouth thoroughly. Do not induce vomiting. Have exposed individual drink sips of water.</li> </ol> <p><b>Methanol:</b></p> <p>It is a Flammable and Toxic Liquid. It creates Hazards to Human Health. During handling of Methanol, below safety measures to be followed:</p> <ol style="list-style-type: none"> <li>Wash skin thoroughly after handling.</li> <li>Avoid breathing dust/fume/gas/mist/vapours/spray.</li> <li>Do not breathe dust/fume/gas/mist/vapours/spray.</li> <li>IF ON SKIN: Wash with soap and water.</li> <li>Specific measures (see supplemental first aid instructions on this label).</li> <li>Wash contaminated clothing before reuse.</li> <li>Avoid contact with skin and eyes. Avoid inhalation of vapour or mist.</li> <li>Use explosion-proof equipment.</li> <li>Keep away from sources of ignition - No smoking</li> </ol>
<b>Principle</b>	The Premix Sample is Extracted by Using Potassium Phosphate Mono Basic & Tetra butyl ammonium Buffer Solution for Quantification of Vitamin B9 (Folic Acid) using HPLC.
<b>Apparatus/Instruments</b>	<ol style="list-style-type: none"> <li>HPLC, Binary gradient pump, an auto Sampler.</li> <li>Analytical Balance, Suitable for weighing samples with accuracy up to 0.1 mg</li> <li>Centrifuge 5000 RPM, holding 50 mL tubes</li> <li>Micro Pipettes (100 -1000 µl, 20 -200 µl 10 -100 µl).</li> <li>HPLC C18 ODS Column: 4.6mm X 250 mm X 5 µm;</li> <li>Sonicator for mixing of solution.</li> <li>Vortex for preparation of stock solution.</li> <li>Homogenizer for sample grinding</li> </ol>
<b>Materials and Reagents</b>	<ol style="list-style-type: none"> <li>Ammonium Hydroxide, LR Grade</li> <li>Phosphoric Acid, LR Grade</li> <li>Monobasic Potassium Phosphate, LR Grade</li> <li>Tetrabutylammonium Hydroxide, LR Grade</li> <li>Methanol, HPLC Grade.</li> <li>CRM: Folic Acid (CAS No: 593003)</li> </ol>
<b>Preparation of Mobile Phase</b>	<p><b><u>PREPARATION OF MOBILE PHASE</u></b></p> <p><b><u>MOBILE PHASE PREPARATION</u></b></p>

	<ol style="list-style-type: none"> <li>1. Accurately weight 2.0 g of monobasic potassium phosphate into a 1000 ml volumetric flask.</li> <li>2. Add 650 mL of Milli-Q Water for Volume make up.</li> <li>3. Add 15 mL of 0.5 M Tetra butyl ammonium hydroxide in methanol.</li> <li>4. Add 7.0 mL of 3 N Phosphoric acid.</li> <li>5. Add 270 mL of methanol</li> <li>6. Cool to room temperature.</li> <li>7. Adjust pH 5.0 with 3 N Phosphoric Acid or 6 N ammonium hydroxide.</li> <li>8. Finally make the volume 1000 ml with Milli-Q Water</li> </ol>
<p><b>Sample Preparation</b></p>	<p><b><u>PREPARATION OF SAMPLE SOLUTION</u></b></p> <ol style="list-style-type: none"> <li>1. Accurately weigh 1 g (<math>\pm 0.1</math> g) of Homogenized Sample.</li> <li>2. Add 0.1 ml of 10 % ammonium hydroxide.</li> <li>3. Transfer into a 10 mL Amber Colored Volumetric Flask.</li> <li>4. Add 5 mL Buffer.</li> <li>5. Vortex for 5 minutes.</li> <li>6. Cool the Sample Solution at Room Temperature.</li> <li>7. Do Volume make-up to 10 ml with mobile phase.</li> <li>8. Vortex for 2 minutes.</li> <li>9. Filter the solution through 0.45<math>\mu</math>m Nylon Syringe Filter.</li> <li>10. Pour the Filtrate into the Vial, and use this for injecting into HPLC.</li> </ol> <p><i><u>Note:</u> If required, dilute the sample for desired concentration.</i></p>
<p><b>Method of Analysis</b> <b>(a) Preparation of Standards</b></p>	<p><b><u>PREPARATION OF STANDARD STOCK SOLUTION</u></b></p> <p><b>a) <u>PREPARATION OF STOCK SOLUTION FOR FOLIC ACID (1000 mg/Kg)</u></b></p> <ol style="list-style-type: none"> <li>1. Accurately weigh 10 mg (<math>\pm 0.1</math> mg) of Folic Acid Standard.</li> <li>2. Add 0.1 ml of 10% Ammonium Hydroxide Solution</li> <li>3. Transfer to 10 mL Amber Colored Volumetric Flask.</li> <li>4. Add Buffer for Volume make-up to 10 mL.</li> <li>5. Vortex for 2 min.</li> <li>6. Store the Solution at -20°C in the light Protected Area.</li> </ol> <p><b>b) <u>PREPARATION OF BRACKETING STANDARD SOLUTION (85 mg/Kg)</u></b></p> <ol style="list-style-type: none"> <li>1. Pipette out 0.85 mL of Standard Stock Solution</li> <li>2. Transfer to 10 mL Amber Colored Volumetric Flask containing 2 mL of Milli Q Water.</li> <li>3. Add Buffer for Volume make-up to 10 mL.</li> <li>4. Vortex for 2 min.</li> </ol> <p><b>c) <u>PREPARATION OF CALIBRATION STANDARD SOLUTIONS</u></b></p> <p>Use Standard Stock Solution for preparing Calibration Standard Solutions as mentioned in below Table.</p>

	CALIBRATION STANDARD SOLUTIONS	SSS (mg/Kg )	VOL. OF SSS (mL)	FINAL MAKE UP VOL. DILUENT (mL)	FINAL CONC. (mg/Kg )																																	
	LS 6	1000	1.50	10	150																																	
	LS 5	1000	1.20	10	120																																	
	LS 4	1000	1.00	10	100																																	
	LS 3	1000	0.85	10	85																																	
	LS 2	1000	0.50	10	50																																	
	LS 1	1000	0.20	10	20																																	
	<p><b>Note:</b> Always make Fresh Preparation of Calibration Standard Solutions.</p> <p>CAL: Calibration  SSS : Standard Stock Solution  VOL: Volume  LS : Linearity Solution</p>																																					
<b>(b)Chromatographic Conditions</b>	<ul style="list-style-type: none"> <li>Instrument : HPLC UV Detector</li> <li>Chromatographic Conditions : As detailed in below Table</li> </ul> <table border="1" data-bbox="544 1003 1541 1346"> <tr> <td>Instrument</td> <td>HPLC</td> </tr> <tr> <td>Detector</td> <td>UV 280 nm</td> </tr> <tr> <td>Column</td> <td>C18 ODS Column: 4.6 mm X 250 mm X 5 µm;</td> </tr> <tr> <td>Run time</td> <td>10 min</td> </tr> <tr> <td>Flow rate</td> <td>1.8 ml/min</td> </tr> <tr> <td>Injection Volume</td> <td>10 µl</td> </tr> <tr> <td>Column Temperature</td> <td>25°C</td> </tr> </table> <p><b>Note:</b> The make, model of Instrument &amp; Column can be changed. However, The Instrument should be able to achieve the desired LOD &amp; LOQ Value &amp; The Column is exactly same in terms of the Composition &amp; Dimensions.</p>					Instrument	HPLC	Detector	UV 280 nm	Column	C18 ODS Column: 4.6 mm X 250 mm X 5 µm;	Run time	10 min	Flow rate	1.8 ml/min	Injection Volume	10 µl	Column Temperature	25°C																			
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<b>Batch Organization</b>	<p><b>Injection Sequence</b></p> <table border="1" data-bbox="544 1518 1556 1984"> <thead> <tr> <th>SL.NO.</th> <th>NAME OF INJECTIONS</th> <th>NUMBER OF INJECTIONS</th> </tr> </thead> <tbody> <tr> <td>1.</td> <td>Blank</td> <td>2</td> </tr> <tr> <td>2.</td> <td>Linearity Solution (LS) - 1</td> <td>1</td> </tr> <tr> <td>3.</td> <td>Linearity Solution (LS) - 2</td> <td>1</td> </tr> <tr> <td>4.</td> <td>Linearity Solution (LS) - 3</td> <td>1</td> </tr> <tr> <td>5.</td> <td>Linearity Solution (LS) - 4</td> <td>1</td> </tr> <tr> <td>6.</td> <td>Linearity Solution (LS) - 5</td> <td>1</td> </tr> <tr> <td>7.</td> <td>Linearity Solution (LS) - 6</td> <td>1</td> </tr> <tr> <td>8.</td> <td>Blank</td> <td>2</td> </tr> <tr> <td>9.</td> <td>Sample Solution</td> <td>1</td> </tr> <tr> <td>10.</td> <td>Blank</td> <td>2</td> </tr> </tbody> </table>					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
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	11.	Bracketing Standard Solution	1
	<b>TOTAL INJECTIONS</b>		<b>14</b>
<b>Calculation with units of Expression</b>	<p>a) Carry out analysis and calculate Regression coefficient (<math>R^2</math>) by analyzing the calibration standards by fitting the data into a linear regression curve.</p> <p>Calculate the Folic Acid Content in Premix using the following equation:</p> <p><b>Folic Acid (Vitamin B9) (mg/Kg) =</b></p> $\frac{\text{Sample Conc. (mg/Kg)} \times \text{Make up Volume (mL)}}{\text{Sample Weight (gm)}}$ <p>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.</p>		
<b>(a) Chromatogram</b>	<p>Chromatogram</p>  <p>VWD1 A, Wavelength=280 nm</p>		
<b>(b) LOD &amp; LOQ</b>	<p>a) Limit of Detection (10mg/Kg) With Respective to the Standard  b) Limit of Quantification (20mg/Kg) With Respective to the Standard  c) Limit of Quantification (200mg/Kg) With Respective to the Sample.  d)</p>		
<b>Inference (Qualitative Analysis)</b>	<p>Method Protocol: PRT/RA/PRM/2023/001, Method Validation Report for Estimation of Folic Acid (Vitamin B9) in Premix using HPLC.</p> <p>United State Pharmacopeia - Folic Acid (Assay)</p>		
<b>Approved by</b>	Scientific Panel on Methods of Sampling and Analysis		