**फा. सं. 11014/07/2021 – क्यूए**

**भारतीय खाद्य सुरक्षा और मानक प्राधिकरण**

(खाद्य सुरक्षा और मानक अधिनियम, 2006 के अंतर्गत स्थापित एक वैधानिक प्राधिकरण)

**(गुणवत्ता आश्वासन विभाग)**

एफडीए भवन, कोटला रोड, नई दिल्ली-110002

---

**दिनांक: 22 मई, 2026**

**Order**

**Subject: Revision of Method for Determination of Folic Acid (Vitamin B9) in Fortified Rice Kernel - reg.**

The Method for Determination of Folic Acid (Vitamin B9) in Fortified Rice Kernel (FSSAI.FRK.16.005.2023) has been revised by Scientific Panel on Methods of Sampling and Analysis (SP-09). The revised method is enclosed.

2. Since the process of updation of test methods is dynamic, any changes happening from time to time will be notified separately. Queries/concerns, if any, may be forwarded to email: advisor.qa@fssai.gov.in.

*Encl: as above*

(डॉ. सत्येन कुमार पंडा)
सलाहकार (गुणवत्ता आश्वासन)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories
3. CEO, National Accreditation Board for Testing and Calibration Laboratories (NABL)

| | | | |
|---|---|---|---|
| एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare | **Determination of Folic Acid (Vitamin B9) in Fortified Rice Kernel** | | |
| **Method No.** | FSSAI.FRK.16.005.2023 | **Revision No. & Date** | 1.0 & 22.05.2026 |
| **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)** | **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.<br>**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.<br>**Acetonitrile:** Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Keep away from sources of ignition as it is flammable.<br>**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. | | |
| **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** | 1. Liquid Chromatograph with Tandem Mass Spectrometer (LC-MS/MS), system equipped with a binary gradient pump, an auto sampler<br>2. Analytical Balance -Suitable for weighing samples with accuracy up to 0.0001 g.<br>3. Centrifuge 6000 rpm, capable of accommodating 50 mL tubes.<br>4. Volumetric flasks-Class A 1000 mL<br>5. Amber colored volumetric flask: 100 mL<br>6. Micro Pipettes capable of delivering from 100 -1000 µl, 20 -200 µl 100 µl. of liquids<br>7. Incubator shaker set at 37 $^0$C<br>8. Water bath set at 55 $^0$C<br>9. Column: XB C18 Column, 2.6 µm, 2.1 x 100 mm or equivalent<br>10. Sonicator<br>11. Vortex mixer<br>12. Homogenizer with steel blades | | |
| **Materials and Reagents** | 1. L-Ascorbic Acid, LR Grade<br>2. α-Amylase (TCI, A0447)<br>3. Sodium hydroxide, LR Grade<br>4. Formic Acid, MS Grade<br>5. Acetonitrile, MS Grade<br>6. Sodium acetate (anhydrous) LR Grade<br>7. Hydrochloric Acid, LR Grade<br>8. CRM: Folic Acid (CAS No: 593003) | | |

| | |
|---|---|
| **Preparation of Reagents** | **Sodium acetate buffer (0.1 M)**<br>1. Weigh accurately 8.2 g of anhydrous sodium acetate.<br>2. Transfer it into 1000 mL of volumetric flask.<br>3. Add Type-I water, dissolve and make-up to 1000 mL.<br>4. Sonicate for 15 min to dissolve.<br><br>**Sodium hydroxide (1 M)**<br>Weigh 40 g of NaOH pellets and dissolve in 1000 mL of water. Cool and store<br><br>**Mobile phase A (0.1% Formic acid)**<br>1. Transfer 1 mL Formic Acid into 1000 mL Volumetric Flask.<br>2. Add Type-I water and make up to mark.<br>3. Sonicate to mix<br>4. Filter through 0.45 µm filter<br><br>**Mobile phase B (100% acetonitrile)**<br>Transfer 1000 mL MS grade acetonitrile to solvent reservoir sonicate for 1-2 mins. |
| **Sample Preparation** | 1. Grind 50 g of fortified rice kernels to a fine powder.<br>2. Accurately weigh 1 g (± 0.1 g) of the powder.<br>3. Transfer into a 100 mL Amber colored volumetric flask.<br>4. Add 0.1 g L-Ascorbic acid and 50 mL of 0.1 M sodium acetate buffer.<br>5. Vortex for 5 min.<br>6. Adjust the pH of the solution to between 8.0-9.0 using 1 M NaOH.<br>7. Shake at 20 rpm for 60 min at 37 ºC using an orbital shaker.<br>8. Adjust the pH of the to 7.0 with 2 N HCl.<br>9. Add 0.05 g of α-amylase and shake for 5 minutes.<br>10. Incubate the sample at 55 $^0$C for 30 mins using a water bath.<br>11. Cool the sample to 25 $^0$C.<br>12. Make-up the volume to100 ml with 0.1 M Sodium Acetate.<br>13. Transfer the sample to a centrifuge tube after vigorous vortexing for two min.<br>14. Centrifuge at 6000 rpm for 5 min.<br>15. Filter the supernatant using a 0.45µm Nylon syringe filter.<br>16. Use the filtrate for LC-MS/MS.<br><br>Prepare all samples as described above. |
| **Preparation of Standard** | **A) Preparation of stock solution for folic acid (1000 mg/kg)**<br>1. Accurately weigh 10 mg (± 0.1) of Folic acid standard.<br>2. Transfer to 10 mL amber colored volumetric flask.<br>3. Add 2 mL of 0.1 N NaOH.<br>4. Vortex for 2 min.<br>5. Add Type-I water and make-up to 10 mL.<br>6. Vortex for 2 min.<br>7. Store at -20 $^0$C, protected from light.<br><br>**B) Preparation of intermediate stock solution-1 for folic acid (100 mg/kg)**<br>1. Pipette out 1.0 mL of stock solution. |

2. Transfer to 10 mL amber colored volumetric flask.
3. Add Type-I water and make-up to 10 mL.
4. Vortex for 2 min.

**C) Preparation of intermediate stock solution-2 for folic acid (10 mg/kg)**

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

**D) Preparation of intermediate stock solution-3 for folic acid (1 mg/kg)**

1. Pipette out 1.0 mL of intermediate stock solution-2.
2. Transfer to 10 mL amber colored volumetric flask.
3. Add Type-I 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 Type-I water (mL) | Final volume (mL) | Final conc. (µg/L) |
|---|---|---|---|---|---|
| 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 | 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 |

**Gradient Program**

| 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.***

**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**

Carry out LC-MS/MS analysis and calculate regression coefficient ($R^2$) of the calibration curve.

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

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

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 Quantification (1000 µg/kg)**

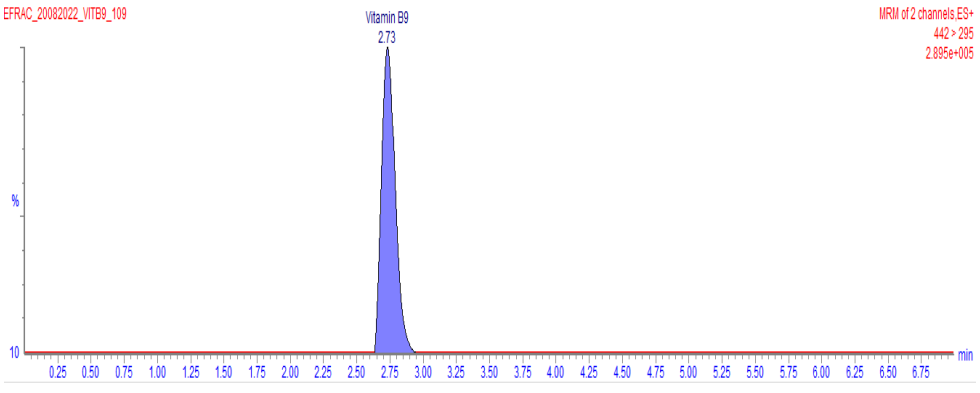
Determine the recovery of folic acid by the external spiking method at 5000 µg/kg) in six replicates. Calculate the recovery value using the following equation:

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

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)

**A representative chromatogram**

Chromatograms



| | |
|---|---|
| **FSSAI Specifications of Vitamin B9 in FRK** | 750 µg to 1250µg/100 g |
| **Reference** | 1. 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.<br>2. 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 |