**Determination of Folic acid**

Folic acid is quantified by Reversed-Phase Liquid Chromatography (RP-HPLC) with UV detection.

**Apparatus**

1. HPLC system equipped with a quaternary gradient pump, an autosampler (100 μL maximum loop capacity) and a diode array detector with 60 mm path length for the highest sensitivity and 10 mm path flow cell and column oven
2. Solid-phase extraction vacuum manifold apparatus
3. Shaker incubator: 37°C
4. Water-bath 70°C
5. Vortex mixer
6. Analytical balance -Suitable for weighing samples with accuracy up to 0.1 mg
7. Centrifuge tubes. Autoclavable 50 mL polypropylene amber-colored centrifuge tubes
8. Centrifuge-5000 rpm, holding 50 mL tubes
9. Filter paper Whatman 597.5 filters (185 mm).
10. Screw-capped amber glass bottle-100 mL
11. Measuring cylinder: 10 and 50 mL
12. Amber-colored volumetric flasks- 10 and 100 mL.
13. Micropipettes-Capable of delivering 0.5-10, 2-20, 5-50, 10-100, 20-200, 200-1000 μL, and 1-5 mL of liquids such as vitamin standards, solvents, buffers, and extracts.

**Chemicals**

1. Folic acid analytical standard
2. Acetonitrile-HPLC grade
3. Water: HPLC grade ≥18 megohm in resistivity
4. Absolute ethanol (99.8%)
5. Sodium hydroxide pellets
6. L-ascorbic acid
7. Sodium phosphate monobasic (NaH2PO4)
8. Sodium phosphate dibasic heptahydrate (Na2HPO4·7H2O)
9. Trifluoroacetic acid (HPLC grade)
10. Pancreatin (CAS No. 8049-47-6) (4 × USP specifications)
11. Folic acid Immunoaffinity kit.

**Preparation of reagents**

**0.1 M phosphate buffer:** Dissolve 9.36 g of monobasic sodium phosphate(anhydrous) and 32.74 g sodium phosphate dibasic heptahydrate in 2 Ldistilled water. When fully dissolved, adjust the pH of the buffer to 7.0 with a few drops of orthophosphoric acid.

**10% L-Ascorbic acid solution:** Dissolve 10 g L-ascorbic acid in 100 mL distilled water in a volumetric flask. Mix well, and store in an amber glassware. Prepare fresh every week.

**Elution solution:** 30% acetonitrile containing 0.2% TFA. Add 70 mL water containing 200 μL TFA to 30 mL acetonitrile.

**Folic acid diluent:** Prepare 15% acetonitrile in water with 0.1% TFA in a 100 mL volumetric flask. Add 85 mL water containing 100 μL TFA to 15 mL acetonitrile.

**1 M NaOH solution:** Dissolve 400 mg sodium hydroxide pellets in 10 mL water in a volumetric flask.

**1 M HCl solution:** Dilute 880 μL HCl (approximately 37%) to 10 mL with water in a volumetric flask.

**Preparation of standards**

* 1. Prepare all the standards in dark in low-actinic volumetric glassware and store at 2-8 °C in tightly stoppered volumetric flasks.
  2. Prepare the stock solution (200 mg/L) by dissolving 20 mg (accurately weighed) folic acid with 200 μL 1 M sodium hydroxide in a 15 mL tube.
  3. To this add 10 mL distilled water, and adjust the solution pH to 6.0 with 1 M HCl (by adding approximately 150 μL 1 M HCl).
  4. Mix thoroughly on a vortex mixer for 30 s and transfer to a 100 mL amber volumetric flask. Add 10 mL distilled water and 20 mL ethanol.
  5. The final volume is made up to 100 mL mark with distilled water (200 mg/L) in amber colored volumetric flask.
  6. Prepare stock solution fresh every week. Store in the dark at 2-8°C.
  7. The calibration standards of 800, 400, 200, 100, and 50 μg/L concentrations are prepared daily by serial dilutions from the stock solutions using 15% acetonitrile in water containing 0.1% TFA).

**Preparation of test samples**

1. All glassware used for analysis must be made of low-actinic glass.
2. Weigh (5 g) of the sample directly into a 100 mL screw-capped amber colored glass bottle, add 50 mL 0.1 M phosphate buffer (pH 7.0), mix thoroughly (~15 min).
3. Add 1 g pancreatin (4 × USP specifications**)** and allow to dissolve (~5 min). Add 6 mL, 10% L-ascorbic acid solution. Mix well on a vortex mixer for 5 min. Incubate in a shaking incubator at 37°C for 2h.
4. Then incubate at 70°C in a water bath for 20 min for inactivate enzyme.
5. Cool to room temperature (25 ±2 C).
6. Transfer the contents to a 100 mL amber-colored volumetric flask, and make up to the mark with 0.1 M phosphate buffer.
7. Transfer the sample to 2 × 50 mL centrifuge tubes
8. Centrifuge at 5000 rpm (~5500 × g) for 10 min.
9. Filter the supernatant through a Whatman S&S 597.5 filter.
10. Use 15 mL aliquots of filtrate for immunoaffinity cleanup

**Immunoaffinity Chromatography**

1. Bring the immunoaffinity cartridges to room temperature (25 ±2 0C) before use.
2. Place the cartridges vertically in a vacuum manifold. Pass 15 mL of the filtrate through the cartridge at a flow rate of 2-3 mL/min.
3. A steady pressure is maintained for the optimal interaction of folic acid with the antibody in the immunoaffinity cartridges.
4. With the help of a glass syringe barrel, pass 10 mL of distilled water pass through the immunoaffinity cartridge.
5. Any remaining traces of water are removed from the cartridge under vacuum.
6. Place an amber-colored vial (2 mL) directly beneath the column. Elute Folic acid with 1 mL elution solution (30% acetonitrile: 70% water containing 0.2% TFA) at a flow rate of one drop per second or by gravity.
7. Add 1 mL distilled water and repeat the same elution procedure.
8. The elute is then analyzed by HPLC after appropriate dilution.

**Chromatography Analysis**

1. The HPLC system (e.g. Agilent 1260 Infinity II Prime) should consist of a quaternary gradient pump, an autosampler (100 μL maximum loop capacity) and a diode array detector with 60 mm path length for the highest sensitivity and 10 mm path flow cell.
2. C18 column (e.g. Poroshell SB-C18 column, 3.0 × 100 mm, 2.7 μm or equivalent). The column oven temperature is maintained at 30°C.
3. Flow rate 0.6 mL/min and detection at 280 nm
4. Injection volume: 10 μL
5. Mobile phase A (0.1% TFA in water) and B (acetonitrile)

**Calculation**

1. Carry out a regression analysis and calculate Regression coefficient (R2) by analyzing the calibration standards (800, 400, 200,100, and 50 μg/L) by fitting the data into a linear regression curve, including zero as the response for the reagent blank.
2. Calculate the folic acid content by using the following equation:

**Folic acid IC x Makeup volume x Dilutions x Standard purity**

**Folic acid (mg/kg) =**

**Sample weight (g) x 100**

Where, makeup volume = 100 mL; dilutions = 0.133; sample weight = approximately 5 g; and folic acid IC = folic acid concentration in sample measured against a calibration curve.

**Reference**  Mahato A., Vyas S., Chatterjee N. (2020). HPLC-UV Estimation of Folic Acid using Enzymatic Extraction and Immunoaffinity Chromatography Enrichment: An Inter laboratory Validation Study. Journal of AOAC International 103 (1): 73-77. DOI: https://doi.org/10.5740/jaoacint.19-0207