Determination of Folate in Infant Formula and Adult/Pediatric Nutritional Formula by Optical Biosensor Assay: First Action 2011.05

Harvey Indyk
Fonterra Co-operative Group Ltd, Waitoa, New Zealand

Dawn Dowell
AOAC INTERNATIONAL, 481 N. Frederick Ave, Gaithersburg, MD 20877

After a review of data from a single-laboratory validation (SLV) study published in the International Dairy Journal 21, 783–789 (2011), a method for folate in infant formula and adult/pediatric nutritional formula was submitted for consideration of adoption by AOAC as an automated assay that is rapid and simple. The method uses an optical biosensor assay to quantify total folate content in milk and milk-based pediatric and adult nutritional products. The assay uses folate binding protein and a functionalized sensor surface. The SLV showed an instrumental LOD of 0.1 ng/mL (equivalent to 2.5 μg/100 g for a typical infant formula). The method detection limit was 6.5 μg/100 g with a repeatability of 3.48% and an intermediate reproducibility of 4.63% RSD.

Folate, a water-soluble B vitamin, is essential for good health during periods of rapid cell division. Deficiencies in maternal folate during pregnancy are associated with neural tube defects, the most common of which are spina bifida and anencephaly. Folate is also necessary for the conversion of homocysteine, and folate deficiency is associated with cardiovascular risk (1). Because of the importance of dietary folate, it is imperative to have analytical methods for the quantification of folates in infant formula and adult/pediatric nutritional formula. This method is being submitted as a suitable alternative to conventional microbiological assay methods and HPLC techniques, which, while successful, are relatively complex and time-consuming.

The method, which was single-laboratory validated (SLV; 2), received First Action status as an AOAC Official Method™ under the AOAC Board of Directors-approved alternative pathway (3). Under this pathway, each method is reviewed by an Expert Review Panel (ERP), and if the method adequately meets standard method performance requirements, it is approved First Action.

AOAC Official Method 2011.05
Folate in Infant Formula and Adult/Pediatric Nutritional Formula
Optical Biosensor Assay
First Action 2011
(Applicable to the determination of folate content in infant formula and adult/pediatric nutritional formula by using optical biosensor assay.)

Caution: The method requires the use of chemicals that are irritants, corrosive, and toxic. Consult Material Safety Data Sheets for all substances that are hazardous. Follow all required laboratory safety precautions and wear the proper personal protective equipment. Extraction using toxic substances should be completed under ventilation.

A. Principle
An automated surface plasmon resonance (SPR) biosensor system with exchangeable carboxymethyl-dextran-functionalized chip utilizes folate binding protein (FBP) for the estimation of endogenous reduced folates and supplemental folic acid. Folates are extracted in a reducing buffer and subjected to enzymatic and heat treatments. The extracts are mixed with FBP, and binding to surface-immobilized folic acid is inhibited in an inverse dose-response manner. Detection of FBP binding to the chip is achieved via an SPR optical platform. The resulting binding responses are used to interpolate the concentration of folate in unknown samples by establishing a calibration curve using a four-parameter logistic regression.

B. Apparatus
(a) SPR biosensor instrument.—Capable of using a CM5 sensor chip (Biacore Q, GE Healthcare, Uppsala, Sweden, or equivalent).
(b) Balance.—4 dp.
(c) Automatic pipets.—10–100 μL, 100–1000 μL, and 1–10 mL.
(d) Volumetric flasks.—5, 10, 50, 100, and 1000 mL.
(e) Centrifuge.—6000 × g.
(f) Centrifuge tubes.—10 and 50 mL, disposable.
(g) Sephadex G25 column.—30 × 3 cm.
(h) CM5 sensor chip.
(i) Ultrasonic bath.

(j) Water baths.—Set at 100 and 37°C.

(k) Microtiter plates.—96-well, polystyrene and adhesive microtiter plate lids compatible with SPR instrument.

(l) Graduated microtubes.—1.5–2.0 mL, polypropylene.

(m) UV spectrophotometer.

(n) Mixing devices.—Vortex mixer.

(o) Filters.—0.45 and 0.22 μm.

(p) pH meter.—With pH and temperature probe. Capable of two-decimal place reading.

C. Reagents

Note: Refer to adequate manuals or safety data sheets approved by local authorities and ensure that safety guidelines are applied before using chemicals.

(a) 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC).—0.4 M (GE Healthcare). (Caution: Corrosive and irritant.)

(b) N-hydroxysuccinimide (NHS).—0.1 M.

(c) Ethanolamine-HCL.—1 M, pH 8.5.

(d) HBS-EP buffer.—10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4 (GE Healthcare).

(e) Borate buffer.—Dissolve 1.90 g Na2B4O7·10H2O in water, adjust to pH 8.5 with 2 M HCl, and bring up to 100 mL.

(f) Folic acid.—Sigma-Aldrich (St. Louis, MO), or equivalent.

(g) Ethylenediamine.—Sigma-Aldrich, or equivalent.

(h) Sodium ascorbate.

(i) β-mercaptoethanol.—See caution statement above.

(j) Purified bovine FBP.—Obtain after affinity chromatographic isolation from whey (4). An alternative commercial source is Scripps Labs (San Diego, CA).

(k) Chicken pancreas conjugase purification.—Dissolve 1 g crude chicken pancreas extract in 10 mL phosphate buffer (0.02 M, pH 7.2) containing NaCl (0.15 M) and ascorbic acid (0.001 M). Centrifuge at 4°C (6000 × g, 10 min). Filter supernatant through glass wool. Transfer solution to a Sephadex G25 column (30 × 3 cm) prepared following the standard hydration protocol in buffer; solution may be turbid. Perform elution with buffer and collect the red-colored band. Dilute to 150 mL with buffer. Dispense aliquots (15 mL) into plastic vials. Store at –18°C.

Note: Procedure produces purified enzyme at approximately 6 mg/mL.

(b) Extraction buffer.—Prepare extraction buffer daily. Dissolve sodium ascorbate (1.0%, w/v) and β-mercaptoethanol (0.1%, w/v) in HBS-EP buffer and use for sample extraction and preparation of calibration standards (2).

(c) Biosensor surface preparation.—Convert folic acid to the hydroxysuccinimidyld derivative and couple to amino groups on the surface of the CM5 sensor chip by the following procedure:

Using an external immobilization procedure at room temperature, activate a CM5 sensor chip with 0.05 M NHS and 0.2 M EDC (20 min). Follow by adding 200 mM ethylenediamine in 50 mM borate buffer, pH 8.5 (20 min). Block remaining binding sites with 1.0 M ethanolamine, pH 8.5 (15 min). Mix equal volumes of 0.1 M NHS and 0.4 M EDC, and dilute 4:1 with 6 mM folic acid in 50 mM borate buffer, pH 8.5. Incubate (20 min), dilute 1:1 (v/v) with 50 mM borate buffer, pH 8.5, and couple to the ethylenediamine-functionalized sensor surface (30 min). Following each step, rinse the sensor surface with water and dry under nitrogen.

(d) Storage.—Store prepared biosensor chips at 4°C over desiccant in a sealed container.

E. FBP Preparation

(a) Stock FBP solution.—Prepare at 1.0 mg/mL in HBS-EP buffer. Aliquot and store at –18°C.

(b) Assay FBP standards.—Intermediate (10 μg/mL) and assay (1000 ng/mL) solutions are prepared daily in HBS-EP buffer.

F. Calibration Standard Preparation

(a) Stock standard (100 μg/mL).—Dry approximately 60 mg USP folic acid over phosphorus pentoxide in the dark until constant weight. Accurately weigh 50 mg of the dry folic acid into a 100 mL beaker. Disperse the folic acid in 50 mL water, add 0.1 M sodium hydroxide dropwise until the solution just clears, followed by 1% (v/v) acetic acid dropwise until pH is between 6.9 and 7.2. Wash quantitatively into a 500 mL volumetric flask with water, make to 500 mL, and mix well. Aliquot and store at –18°C.

(b) Intermediate standards (10 μg/mL and 100 ng/mL).—Prepare daily in extraction buffer.

(c) Calibration standards (80, 33.3, 11.1, 5.55, 3.7, 1.85, 1.23, and 0.41 ng/mL).—Prepare daily in extraction buffer.

G. Preparation of Test Samples

Perform extraction and analysis using low-level, yellow incandescent light. Dissolve 0.1 g sample in approximately 3 mL extraction buffer. Sonicate 15 min. Heat sample at 100°C for 3 min and cool to room temperature. Add 0.5 mL purified chicken pancreas conjugase solution. Dilute to 5.0 mL with extraction buffer. Incubate 2 h at 37°C. Heat sample at 100°C for 15 min. Cool to ambient temperature. Remove 1.0 mL aliquot and centrifuge (4500 × g, 15 min). Remove supernatant and filter through a combined 0.45 and 0.22 μm membrane filter. Dilute filtrate in extraction buffer to obtain a final folate concentration of 2–6 ng/mL based on expected content.

Note: Dilute nonsupplemented milk products typically from two- to five-fold and supplemented nutritional powders typically from five- to 50-fold with extraction buffer.

H. SPR Analysis Conditions

SPR biosensor assay.—Equilibrate reagents and folic acid-immobilized sensor chips to ambient temperature prior to use. Dispense calibration standards and sample extracts (150 μL) into the appropriate wells of 96-well microtiter plate and cover with adhesive foil (light protected).

Equilibrate the sensor chip and microfluidics system with HBS-EP buffer. Position FBP (1000 ng/mL) and regeneration reagent (75 mM sodium hydroxide) in regeneration rack appropriately.

Run assay under the following conditions: injection time, 480 s; flow rate, 20 μL/min at 25°C. Perform each analysis cycle under automated instrument control: (1) Mix FBP (1000 ng/mL) with calibrants and sample extracts (1 + 1, v/v). (2) Inject 160 μL over sensor surface. (3) Remove bound FBP by injecting 78 μL regeneration solution (75 mM sodium hydroxide) at flow rate of 50 μL/min.
Acquire binding response 30 s after injection relative to initial baseline (10 s before injection).

I. Data Handling

Review all sensograms for any abnormalities, e.g., air spikes, drifting response.

Check for completeness of regeneration of each sample by ensuring that regenerated baseline has returned to initial baseline response.

J. Determination of Folate Standard Curve

Establish a folic acid calibration curve based on a 4-parameter logistic regression and calculate folate content of unknown samples by interpolation:

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\text{Response} = R_{hi} - \left( \frac{R_{hi} - R_{lo}}{1 + \left( \frac{\text{Concentration}}{A_1} \right)^{A_2}} \right)
\]

where response = FBP relative binding response; \( R_{hi} = \) response at infinite folic acid concentration; \( R_{lo} = \) response at zero folic acid concentration; \( A_1 = \text{IC}_{50}; A_2 = \text{slope factor}; \) and concentration = folate concentration in extract (ng/mL).

Concentration of folate in sample (μg/100 g):

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\text{Folate, } \mu \text{g/100 g} = \frac{\text{folate (ng/mL)} \times \text{DF} \times V \times 100/W \times 1/1000}{\text{where DF = dilution factor, V = extract volume, and W = sample mass (g).}}
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Reference: *J. AOAC Int.* 95, in press(2012)

Results and Discussion

The results of the SLV indicate that this method is a suitable routine alternative to methods currently used to determine folate content in milk, and milk-based pediatric and adult nutritional products (2). According to previously published data, the performance of the method shows an instrumental LOD of 0.1 ng/mL (equivalent to 2.5 μg/100 g for a typical infant formula). The method detection limit is 6.5 μg/100 g with a repeatability and intermediate reproducibility of 3.48 and 4.63% RSD, respectively. Further details regarding the development and validation of the method are as described in Indyk (5).

The method was adopted by an AOAC ERP using the alternative pathway to First Action status. This method process has been approved by the AOAC Board of Directors. Methods approved under this process will remain First Action for approximately 2 years to allow time to collect additional information and to allow laboratories to use the method and submit any comments regarding the method.

References

(1) Centers for Disease Control and Prevention (January 3, 2011) http://www.cdc.gov/Features/FolicAcid/