INFANT FORMULA AND ADULT NUTRITIONALS

A Rapid Method for the Determination of Biotin and Folic Acid in Liquid Milk, Milk Powders, Infant Formula, and Milk-Based Nutritional Products by Liquid Chromatography–Tandem Mass Spectrometry

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Background: **Biotin and folate are B-group vitamins that play a critical role in numerous metabolic reactions, and they are supplemented to infant and adult nutritional formulas as free biotin and folic acid.** ***Objective:*** **We describe a rapid method for the analysis of biotin and folic acid that is applicable to liquid milk, milk powders, infant formula, and milk-based nutritional products.** ***Methods:*** **Samples are autoclaved, centrifuged, filtered, and analyzed by HPLC–MS/MS, with quantitation accomplished by the internal standard technique.** ***Results:*** **The method was shown to be accurate, with acceptable spike recovery (biotin: 96.5–108.2%; folic acid: 92.6–104.4%), and no bias ($\alpha = 0.05$) against either a certified reference material (biotin: $P = 0.70$; folic acid: $P = 0.23$) or established analytical method (biotin: $P = 0.10$; folic acid: $P = 0.48$) was found. Acceptable precision was confirmed with repeatability relative standard deviation (RSD_r) and Horwitz ratio (HorRat) values (biotin: $RSD_r = 0.5–5.6\%$, $HorRat_r = 0.1–0.6$; folic acid: $RSD_r = 2.0–3.1\%$, $HorRat_r = 0.3–0.5$). Method detection limit and ruggedness experiments further demonstrated the suitability of this method for routine compliance testing.** ***Conclusions:*** **This rapid method is intended for use in high-throughput laboratories as part of the routine product compliance release testing of biotin and folic acid in the manufacturing of infant formulas and adult nutritional products.**

Biotin is a water-soluble vitamin that participates in numerous metabolic reactions because of its critical role as a cofactor for multiple carboxylase enzymes. Of the eight possible stereoisomers, only *d*-biotin is biologically active and nutritionally significant, and other isomers are not found in nature. Biotin is widely distributed in food products in the free form, particularly in fruits and vegetables, whereas a significant quantity of the biotin found in meat and cereal is bound with protein. In bovine milk, biotin is found primarily in the free form, with minor quantities being protein bound (1, 2).

Folate is a collective term for a water-soluble vitamin that plays a key role in providing single carbon units in both amino acid metabolism and nucleotide biosynthesis. Green vegetables, legumes, liver, and egg yolk are rich dietary sources of folates. Folates consist of many different chemical forms, which exhibit different bioactivity and bioavailability. Endogenous folate is present predominantly as 5-methyltetrahydrofolic acid bound to folate-binding protein, with a number of other forms varying in pteridine ring oxidation state, one-carbon substituent, and polyglutamate chain length (3). Folic acid is a synthetic form of folate and, because of its stability, is the form that is predominantly used for the supplementation of most foods, including infant formula (4, 5).

A number of analytical techniques have been applied to the analysis of biotin and folate in milk-based foods. Analyte extraction that employs a varied range of enzymatic and/or acid hydrolysis steps is a critical component of the methods that measure total vitamin content, whereas the extraction of free biotin and folic acid is less challenging.

Microbiological assays are sensitive and specific and remain important for the analysis of biotin and folates in food. These methods report a single aggregate value for each vitamin, although they are highly manipulative, offer poor precision, and may be both susceptible to variations in vitamer response and prone to interferences (6–8).

Various immunoassay techniques and avidin or streptavidin binding methods offer sensitive and rapid strategies for the routine food compliance testing of biotin in food matrices (9–11). An automated biosensor immunoassay method for the routine testing of biotin and folic acid in bovine milk and infant formulas has been described (12).

HPLC methods have proliferated, and many such methods for both biotin and folates or supplemental folic acid in infant formulas have been reported. As biotin lacks a selective UV chromophore, detection has commonly been achieved by postcolumn derivatization with fluorescence (Fl; 13–16) or MS (17, 18). HPLC methods allow for the determination of multiple different folate vitamers, although it remains a challenge to ensure the complete extraction and deconjugation of polyglutamates to monoglutamate forms (19). In contrast, the measurement of supplemental folic acid is relatively straightforward (20).

There are two AOAC *Official Methods*^{SM} for the analysis of biotin in infant formulas, an HPLC–Fl method (AOAC Method **2016.11**; 21) and an HPLC–UV method utilizing immunoaffinity solid-phase extraction (AOAC Method **2016.02**; 22), and three AOAC *Official Methods* for the analysis of folate in infant formulas, a microbiological method (AOAC Method **992.05**; 23)

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and two UHPLC–MS/MS methods (AOAC Method **2011.06**; 24; AOAC Method **2013.13**; 25).

The purpose of this study was to develop a rapid method for the simultaneous analysis of biotin and folic acid in milk powders and infant formulas. LC–MS/MS offers a number of advantages over other methods, including accuracy afforded from the use of a stable-isotope-labeled internal standard and specificity from multiple reaction monitoring (MRM). Method performance was evaluated by using a wide range of infant formulas, including the *Stakeholder Panel on Infant Formula and Adult Nutritionals* (SPIFAN) kit, and was validated in accordance with single-laboratory validation procedures endorsed by the AOAC Expert Review Panel (26).

Methods

Apparatus

(**a**) *Micropipettes*.—Eppendorf Research plus, 200 and 1000 µL (Hauppauge, NY) or equivalent.

(**b**) *Centrifuge*.—Heraeus Multifuge X3 centrifuge (Thermo Fisher, Waltham, MA) or equivalent.

(**c**) *Syringes*.—A 3 mL Luer-lock (Hapool, Shandong, China) or equivalent.

(**d**) *Syringe filters*.—MCE, 0.22 µm pore size × 25 mm id (Carrigtwohill, Cork, Ireland) or equivalent.

(**e**) *Centrifuge tubes*.—Polypropylene, 50 mL (ThermoFisher) or equivalent.

(**f**) *Vortex mixer*.—Genius 3 (IKA, Wilmington, NC) or equivalent.

(**g**) *pH meter*.—Orion SA520 (ThermoFisher, Waltham, MA) or equivalent.

(**h**) *Analytical balance*.—Mettler-Toledo (Columbus, OH) AE 260 analytical delta range (±0.1 mg) or equivalent, calibrated with National Institute of Standards and Technology (NIST; Gaithersburg, MD) traceable calibration weights.

(**i**) *HPLC column*.—Kinetex core-shell C_{18} 50 mm × 2.1 mm, 2.6 µm (Phenomenex, Torrance, CA) or equivalent.

(**j**) *HPLC system*.—Nexera X2 UHPLC system consisting of two LC-30AD pumps, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A control module, and DGU-20A5R degasser unit (Shimadzu, Kyoto, Japan), or equivalent.

(**k**) *Mass spectrometer*.—6500 QTrap triple quadrupole detector with Analyst software version 1.6 (Sciex, Foster City, CA) or equivalent.

(**l**) *Graduated cylinders*.—100 and 1000 mL.

(**m**) *Volumetric flasks*.—25 and 50 mL.

(**n**) *Reagent bottles*.—250, 500, and 1000 mL.

(**o**) *HPLC injection vials*.—Amber 1 mL with Teflon coated caps.

Reagents

(**a**) *Formic acid (CHOOH)*.—LC–MS grade or equivalent.

(**b**) *Acetic acid (CH_3COOH)*.—Reagent grade or equivalent.

(**c**) *Ammonium hydroxide (NH_4OH)*.—Reagent grade or equivalent.

(**d**) *Ammonium acetate (NH_4CH_3OO)*.—Reagent grade or equivalent.

(**e**) *Methanol (CH_3OH)*.—LC–MS grade or equivalent.

(**f**) *Acetonitrile (CH_3CN)*.—LC–MS grade or equivalent.

(**g**) *Water (H_2O)*.—HPLC grade or equivalent.

(**h**) *Potassium dihydrogen phosphate (KH_2PO_4)*.—Reagent grade or equivalent.

(**i**) *Biotin*.—Purity ≥99.0% (Sigma-Aldrich, St. Louis, MO) or equivalent.

(**j**) *Folic acid*.—Purity ≥99.0% (Sigma-Aldrich) or equivalent.

(**k**) *$^2H_4$-Biotin*.—Purity ≥95.0% and isotope ≥95.0% (Cambridge Isotope Laboratories, Tewksbury, MA) or equivalent.

(**l**) *$^{13}C_5$-Folic acid*.—Purity ≥95.0% and isotope ≥95.0% (Cambridge Isotope Laboratories) or equivalent.

Solutions

(**a**) *Dilute ammonia solution (about 3.6 mM; pH = 10.4)*.—Dilute 60 µL of ammonium hydroxide in 250 mL of water.

(**b**) *Ammonium acetate buffer (50 mM; pH = 4.0)*.—Dissolve 2.32 g of ammonium acetate in 500 mL of water, adjust pH to 4.0 with acetic acid, and make to 600 mL with water.

(**c**) *Potassium hydroxide solution (50% m/v)*.—Dilute 50 g of potassium hydroxide in water.

(**d**) *Phosphate buffer (59 mM; pH = 7.0)*.—Dilute 0.68 g of potassium dihydrogen phosphate in 95 mL of water, adjust to pH = 7.0 with potassium hydroxide solution, and make to 100 mL with water.

(**e**) *Ascorbate solution (10% m/v)*.—Add 6 g of sodium ascorbate to 60 mL of water.

(**f**) *Mobile phase A (formic acid, 0.1% v/v)*.—Dilute 1 mL formic acid to 1 L of water.

(**g**) *Mobile phase B (methanol, 100% v/v).*

Standards

Biotin and folic acid are poorly soluble at neutral pH; therefore, separate $^2H_4$-biotin and $^{13}C_5$-folic acid stable-isotope-labeled stock standards (100 µg/mL) were prepared by dissolving 5 mg in 50 mL of dilute ammonia solution. Aliquots (about 1.3 mL) of these standards were transferred to cryogenic vials and stored at –80°C. A stable-isotope-labeled internal standard solution was prepared by diluting 1 mL of each stable-isotope-labeled stock standard in 25 mL of water. Biotin was dried at 102°C for 2 h, cooled, and then used immediately. Separate biotin and folic acid nonlabeled stock standards (200 µg/mL) were made by dissolving 20 mg in 100 mL of dilute ammonia solution. A folic acid purity standard was prepared fresh each run by diluting 1 mL of folic acid stock standard to 25 mL with phosphate buffer solution. The accurate concentration of this solution was determined by UV absorbance measurement ($E^{1\%}_{1cm}$: 611 dL/g.cm at 282 nm; 27). A mixed biotin and folic acid working standard was prepared by diluting 1 mL of each nonlabeled stock standard in 50 mL of water. Calibration standards were made by pipetting 500 µL of stable-isotope-labeled internal standard and 125, 250, 500, 1000, or 2000 µL of mixed biotin and folic acid working standard into separate 50 mL volumetric flasks and diluting to volume with water.

Samples

Method performance was evaluated using a wide range of biotin and folic acid supplemented infant formulas, including the NIST 1849a Certified Reference Material (CRM), a partially hydrolyzed soy-based infant formula powder, an infant elemental powder, a high-protein adult nutritional ready-to-feed liquid, a milk-based infant formula ready-to-feed liquid, a soy-based infant formula, a milk-based infant formula powder, a high-fat adult nutritional ready-to-feed liquid, a milk-based child formula powder, a partially hydrolyzed milk-based infant formula powder, a milk-based toddler formula powder, a fructooligosaccharide and galactooligosaccharide fortified milk-based infant formula powder, and a low-fat adult nutritional powder. Products not supplemented with biotin and folic acid were used for the recovery study, including a high-protein adult nutritional ready-to-feed liquid, a high-fat adult nutritional ready-to-feed liquid, a milk-based infant formula ready-to-feed liquid, an infant elemental powder, and a milk-based child formula powder.

Sample Preparation

Approximately 2 g of powder, or 20 mL of liquid, was weighed accurately into a 50 mL disposal centrifuge tube, made to 25 mL with water, and mixed on a vortex mixer for 30 s. Then, 3 mL of ascorbate solution, 500 µL of stable-isotope-labeled internal standard, and 20 mL of acetate buffer were added to the sample, and it was mixed on a vortex mixer for 30 s. The centrifuge tube was placed in a horizontal shaker for 5 min and then autoclaved at 121°C for 30 min. After removing the tube from the autoclave and cooling to room temperature in a cold water bath, it was mixed on a vortex mixer and placed in a centrifuge at 2700 × *g* for 5 min, and the extract was filtered through a syringe filter into an HPLC vial.

LC–MS Analysis

(**a**) *HPLC system*.—Nexera X2 UHPLC system.

(**b**) *HPLC column*.—Kinetex core-shell C_{18} 50 mm × 2.1 mm, 2.6 µm.

(**c**) *Column temperature*.—40°C.

(**d**) *Chiller temperature*.—15°C.

(**e**) *Injection volume*.—5 µL.

(**f**) *Binary gradient*.—The settings are in Table 1.

(**g**) *Detection*.—Electrospray ionization in positive mode with MRM. The settings are presented in Tables 2 and 3.

Calculations

For each vitamin, a linear calibration plot was constructed plotting the ratio of peak areas against the ratio of concentrations. The concentrations of biotin and folic acid in powder samples and liquid samples in micrograms/hectograms or micrograms/deciliters, respectively, is given by the following equations:

$$\text{Vitamin}\,(\mu g/hg) = \frac{A_{NV}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{M_S} \times \frac{100}{1000}$$

$$\text{Vitamin}\,(\mu g/dL) = \frac{A_{NV}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_S} \times \frac{100}{1000}$$

where Vitamin is the concentration of biotin or folic acid in sample (micrograms/hectogram), A_{NV} is the peak area of biotin

Table 1. Chromatographic gradients[a]

		Mobile phase composition	
Time, min	Flow rate, mL/min	A[b], %	B[c], %
0	0.8	95	5
2.75	0.8	75	25
3.25	0.8	0	100
3.75	0.8	0	100
4.0	0.8	95	5
7.0	0.8	95	5

[a] Oven temperature = 40°C.

[b] Mobile phase A = Formic acid (0.1%, v/v).

[c] Mobile phase B = Methanol.

Table 2. MS compound parameters

Analyte	Precursor ion, *m/z*	Product ions, *m/z*	DP[a], V	EP[b], V	CE[c], V	CXP[d], V	Dwell time, ms
NLB_7[e]	245.0	227.1[f]	31	10	19	22	100
		123.0[g]			37	14	50
$SILB_7$[h]	249.0	231.1[i]			19	22	100
NLB_9[j]	442.0	295.1[f]	26	10	21	16	100
		176.0[g]			53	22	50
$SILB_9$[k]	447.0	295.1[i]			21	16	100

[a] DP = Declustering potential.

[b] EP = Entrance potential.

[c] CE = Collision energy.

[d] CXP = Collision cell exit potential.

[e] NLB_7 = Biotin.

[f] Quantifier ion.

[g] Qualifier ion.

[h] $SILB_7$ = $^{2}H_4$-biotin.

[i] Internal standard.

[j] NLB9 = Folic acid.

[k] $SILB_9$ = $^{13}C_5$-folic acid.

Table 3. MS instrument settings

Instrument parameter	Value
Ionization mode	ESI$^+$
Curtain gas	30 psi
Nebulizer gas GS1	40 psi
Heater gas GS2	40 psi
Collision gas	N_2
Source temperature	250°C
Ion spray voltage	5500 V

or folic acid in the sample, A_{IS} is the peak area of $^2H_4$-biotin or $^{13}C_5$-folic acid in the sample, L is slope of the calibration curve, C_{IS} is the concentration of $^2H_4$-biotin or $^{13}C_5$-folic acid in the internal standard (nanogram/milliliter), V_{IS} is the volume of the internal standard spiked to sample (milliliters), M_S is the mass of the sample (grams), V_S is the volume of the sample (grams), 1000 is the concentration conversion factor (nanograms/gram to micrograms/gram), and 100 is the mass conversion factor (micrograms/gram to micrograms/hectogram).

Results and Discussion

Method Optimization

Simplified extraction techniques can be used for the rapid, routine product compliance release analysis of biotin and folic acid in infant formulas because the free vitamers are the predominant forms that are present. Biotin is stable over a wide pH range, provided it is protected from UV light exposure and is a relatively heat-stable vitamin. Folic acid is a fully oxidized form of folate and is more stable than naturally occurring folates at both ambient and elevated temperatures. Ascorbate is added to samples to prevent oxidative loss of folic acid or biotin throughout sample preparation. Autoclaving is a common extraction technique used in the analysis of biotin and folic acid to denature milk proteins, inactivate endogenous enzymes, and release any complexed vitamin (27).

Optimization of the ion source parameters was performed by direct infusion of biotin and folic acid standards. Product ion scans were used to identify major fragment ions, and the most intense MRM transitions were selected for quantitation, with the second most intense ion selected as the qualifier ion. Unambiguous identification of biotin and folic acid was assured by selecting a specific elution time window for monitoring characteristic quantifier and qualifier ions, with performance criteria for the ion ratios set at ±25%. To simplify laboratory throughput, the chromatographic elution system used the same mobile phases as the routine analysis of vitamin D (28), with a higher fraction of aqueous mobile phase used at the start of the gradient (Figure 1).

Method Validation

Detector linearity was performed by the analysis of eight biotin and folic acid standard solutions covering an analyte range from 4 to 4000 ng/mL. Linearity was evaluated by least-squares regression analysis of peak area ratio versus concentration ratio, with acceptable values of 0.997 and 1.000 obtained for biotin and folic acid, respectively. Residual plots were assessed as a further test of linearity, with a deviation from the line of best fit of ≤2.5%.

Recovery was determined by spiking a set of unfortified samples from the SPIFAN kit (milk-based ready-to-feed infant formula, high-protein ready-to-feed adult nutritional, high-fat ready-to-feed adult nutritional, elemental infant formula

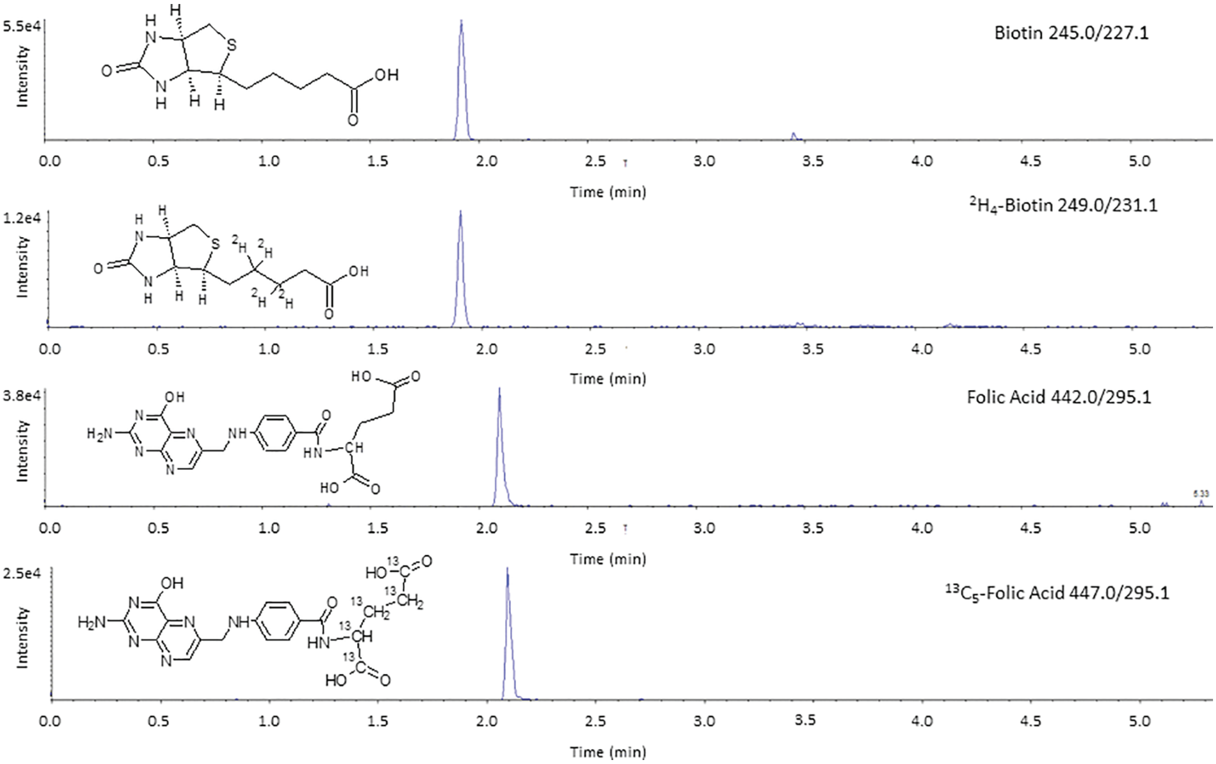

Figure 1. MRM chromatograms of quantifier ions of biotin, folic acid, $^2H_4$-biotin, and $^{13}C_5$-folic acid.

powder, and milk-based infant formula powder). Powder samples were spiked with standard solutions to 0, 50, 100, and 150% of nominal 40 μg/hg (biotin) and 100 μg/hg (folic acid). Ready-to-feed samples were spiked with standard solutions to 0, 50, 100, and 150% of nominal 10 μg/dL (biotin) and 25 μg/dL (folic acid). Recovery for biotin ranged from 96.5 to 108.2%, with a mean recovery of 100.3%. Recovery for folic acid ranged from 92.6 to 104.4%, with a mean recovery of 99.5%.

Bias against a CRM was evaluated by replicate analyses ($n = 10$) of NIST 1849a, with differences between the measured value and the certified value determined with the mean and SD of the differences, and the *t*-test statistic and *P* values were calculated. No statistical bias was found ($\alpha = 0.05$) between the measured results and the certified values (biotin: $P = 0.70$; folic acid: $P = 0.23$).

The method was compared with an automated surface plasmon resonance-based immunoassay method currently used for the routine product release analysis of biotin and folic acid (12), which was previously validated against reference microbiological methods (29). A total of 43 samples, including a wide range of infant and growing-up formulas (biotin: 23–208 μg/hg; folic acid: 9–234 μg/hg), were tested over 5 different days, and a paired *t*-test was used to evaluate bias. The bias between the measured results obtained by LC–MS/MS and the automated immunoassay method (mean bias biotin: 1.5 μg/hg; folic acid: 0.9 μg/hg) was negligible and not statistically significant ($\alpha = 0.05$, biotin: $P = 0.10$; folic acid: $P = 0.48$). Bland–Altman plots illustrate the level of agreement between the analytical methods for the determination of biotin and folic acid in infant formulas (Figures 2 and 3).

Method precision was evaluated by the analysis of duplicate pairs of an instant skim milk powder, an instant whole milk powder, a whole milk powder, a buttermilk powder, a whey protein concentrate powder, a range of infant formulas, and an ultra-high temperature-treated liquid milk. Acceptable precision was demonstrated with a repeatability of 0.5–5.6% RSD (HorRat: 0.1–0.6) and an intermediate precision of 0.7–8.5% RSD for biotin as well as a repeatability of 2.0–3.1% RSD (HorRat: 0.3–0.5) and an intermediate precision of 2.7–6.3% RSD for folic acid. In products not supplemented with vitamins, the presence of natural levels of biotin and the absence of synthetic folic acid were confirmed.

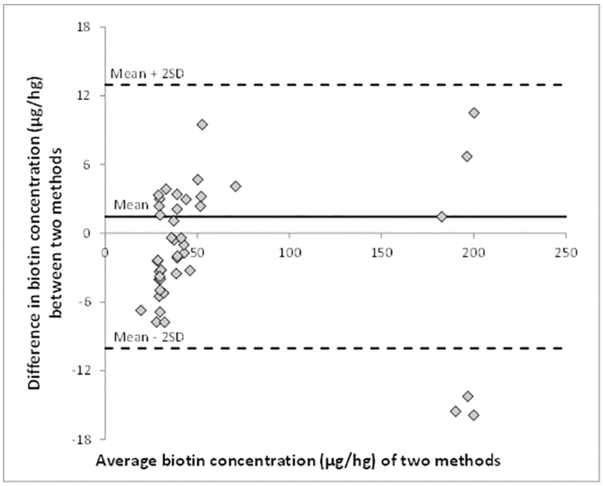

Figure 2. Bland–Altman plot for biotin, comparing LC–MS/MS and automated immunoassay methods.

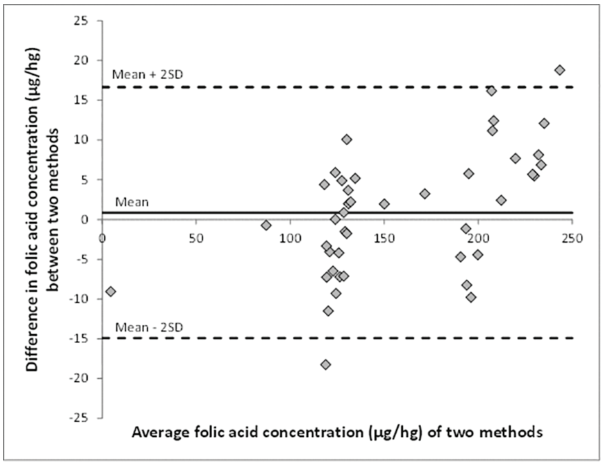

Figure 3. Bland–Altman plot for folic acid, comparing LC–MS/MS and automated immunoassay methods.

The method detection limit (MDL) is the smallest amount of an analyte that can be quantitated with reliability, which is estimated from the precision of the complete method applied to a sample containing low levels of analyte (30). The MDL for biotin was estimated by replicate analysis ($n = 8$) of a skim milk powder sample containing endogenous levels. Because folic acid is a synthetic form of folate, the MDL was assessed by thoroughly mixing a skim milk powder with a fortified infant formula (4:1, m/m) prior to analysis. The measured values for MDL were 3.6 and 9.1 μg/hg for biotin and folic acid, respectively, signifying that the described method is suitable for application to fortified infant formulas, as these typically contain biotin and folic acid at concentrations that are an order of magnitude, or more, higher.

The robustness of the method was assessed by conducting a Plackett–Burman trial (31, 32) in the manner described previously (33). The seven factors assessed were pH of acetate buffer (3.9 and 4.1), volume of acetate buffer (23 and 27 mL), volume of ascorbic acid (2 and 4 mL), shaker time (3 and 7 min), autoclave time (20 and 40 min), cool temperature (15 and 25°C), and a dummy factor. The method was found to be robust for the method parameters evaluated, and the results obtained were normally distributed, with variances conforming to that expected by chance (Figures 4 and 5). As with similar methods

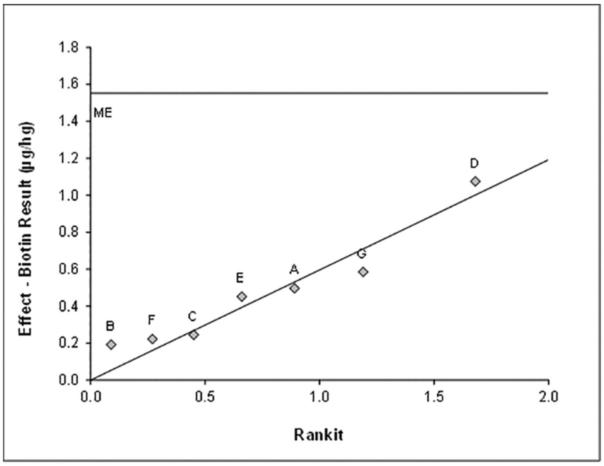

Figure 4. Half-normal plot of biotin ruggedness experiment.

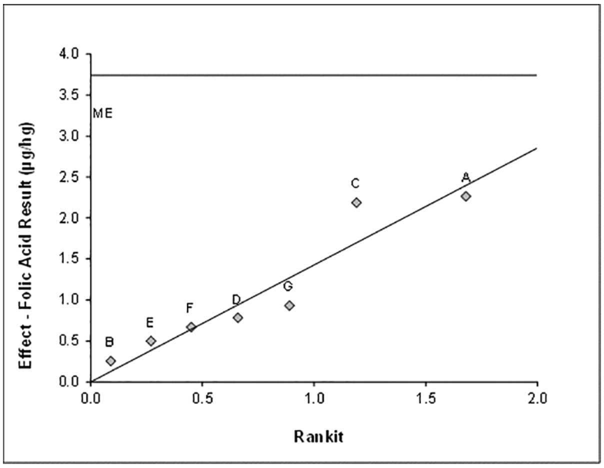

Figure 5. Half-normal plot of folic acid ruggedness experiment.

exploiting stable-isotope-labeled internal standard quantitation, critical method parameters included accurate measurement of sample weight and the ratio of internal standard in samples and calibration standards. Both parameters were tightly controlled by the use of appropriately calibrated pipettes and balances.

The method described can yield a single result in approximately 3 h, and the procedure is capable of significant sample throughput, with more than 40 samples per day completed by a single analyst.

Conclusions

A rapid method that is intended for use in high-throughput laboratories as part of routine product compliance release testing of biotin and folic acid in the manufacturing of infant formulas and adult nutritional products is described. This method was subjected to single-laboratory validation and was found to be accurate, precise, and fit for purpose.

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