Folic Acid Content of Ready-to-Eat Cereals Determined by Liquid Chromatography-Mass Spectrometry: Comparison to Product Label and to Values Determined by Microbiological Assay

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**ABSTRACT**

Twelve popular ready-to-eat breakfast cereals fortified with folic acid were sampled in the United States in 2006, and the data have been incorporated into the USDA National Nutrient Database for Standard Reference. Cereals were collected from three statistically selected retail outlets in each of four primary census regions, and four composites of each product were prepared using random groupings of three locations each. Folic acid was determined using a validated LC-MS method, with 13C-folic acid as an internal standard, after trienzyme treatment and solid phase extraction. A cereal reference material (AACC VMA399) was analyzed as a control. Selected samples were also assayed using the standard microbiological method, with and without trienzyme extraction, to generate an estimate of endogenous folate. On average, as shown on the label, folate content was underestimated. In seven cereals, folate was within 5% of the declared value; in four cereals, it was 5–20% higher; and in two cereals, it was 20% greater, representing −75 to +69 μg/serving (mean 17%) of the label value, equivalent to −19% to +17% of the 400 μg/daily value. The microbiologically determined folic acid was higher than LC-MS by 10–67% (mean 40%). Therefore, use of label values might underestimate folate intake from some breakfast cereals.

The importance of folate in prevention of neural tube defects is well recognized, and there is evidence for, and continued study of, its role in reducing the risk of cardiovascular disease, colon cancer, and neuropsychiatric disorders (CDC 1992; Luckcock 2000; Kim 2003; Stover 2004; Regland 2005; Musket and Kemperman 2006). Data supporting the importance of folate in preventing neural tube defects led to the 1996 decision by the FDA for fortification of grain products with 1.4 μg of folic acid/g, which became effective on 1 January 1998 (FDA 1996) (see review by Bailey et al 2003). Fola tes are a group of compounds sharing the 4-{[(2-amino-3,4-dihydro-4-oxopteridin-6-yl)methyl]amino} benzoic acid molecule conjugated with one or more L-glutamate units (Cornish-Bowden 1987; Moss 2006). Folic acid (pteroylglutamatic acid) is used in fortification, but naturally-occurring folate exists primarily as 5-methyltetrahydrofolate and formyl folates, with very little folic acid (Konings et al 2001). In the United States, folic acid is added to refined cereals and grain products, as well as dietary supplements, and these products are major contributors to total folate intake.

The current daily value (DV) established by the Food and Drug Administration for nutrition labeling of adult foods is 400 μg (FDA 1996). Likewise, a recommended dietary allowance (RDA) of 400 μg of dietary folate equivalents (DFE) was established by the National Academy of Sciences Institute of Medicine for adult males and nonpregnant females (NAS 1998). Ready-to-eat (RTE) cereals in the United States are typically fortified to contain a labeled content of 25–100% of DV for folic acid (100–400 μg) per serving. According to the USDA Food Surveys Research Group (Beltsville, MD), based on the What We Eat in America—National Health & Nutrition Examination Survey (USDA 2006a–2005–2006 data, the per capita average daily consumption of RTE cereals in the United States is estimated at 13 g for males and females ≥2 years of age and contributes ≥22% of the U.S. total daily folate intake (personal communication, Alanna Moshfegh). Approximately 27% of the respondents ≥2 years of age reported eating RTE cereals on a given day. For people who ate RTE cereal, mean per capita daily intake was 49 g (≈1.5 cups of a typical cereal), which provided ≈53% of total daily folate intake.

Under the current labeling regulations established in compliance with the Nutrition Labeling and Education Act (FDA 2008a), nutrients added for fortification purposes must be present in an amount at least equal to the declared amount. Under the provisions of 21CFR172.5 (FDA 2008b), there are no specific guidelines for the amount of overage that can be added for many fortification nutrients, including folic acid. The amount of vitamin added over the label claim amount depends upon the stability of the vitamin, the specific product, the processing system, shelf life requirements, packaging, and analytical error in determining the vitamin concentration. Overages vary from ≥15% for the more stable vitamins to 50% for the less stable vitamins. Folic acid is considered to have good heat stability, but it is sensitive to light, oxygen, and metals (Caldwell et al 2000).

Fortificants, including minerals and vitamins, may be added to cereal before processing or during puffing, flaking, or extrusion. This method provides uniform distribution of the added nutritional components. However, this method can result in unacceptable nutritional deterioration and flavor changes due to high temperatures and pressures encountered during processing. Typically, incorporation before processing requires overapplication of fortificants or limitation to heat-stable nutrients. Most cereals are exposed to multiple coating processes: phase 1 coatings typically include vitamin enrichment; phase 2 may include slurries with sugars, honey, and flavoring ingredients. Dry components such as nuts, fruit pieces, cinnamon, and sugar may be added as “tack-on” coatings. Vitamins added as coatings may be sprayed onto the product as it travels down a conveyor belt or may be incorporated through use of a coating drum. Either method presents challenges in achieving a uniformity of spray coverage and therefore relatively even dispersal of the vitamin mix throughout the cereal. Because the coating zone is relatively small compared to the total volume of cereal, it is possible for some areas to become supersaturated with vitamins while the rest receive little or no coating (Burns et al 2000). As discussed by Rader et al (2000), addition of

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fortification nutrients by manufacturers at a level higher than that labeled is likely to ensure that the actual content complies with (meets or exceeds) the declared level. It is therefore quite possible that folic acid in a given box or production lot of cereal would deviate from the label, even if the lowest amount in any one package matches the declared content.

For the purpose of diet design for clinical feeding trials or intake estimation from food consumption surveys (e.g., Cho et al 2002), the diet or food content of folic acid is typically calculated using values from nutrient databases, primarily the USDA National Nutrient Database for Standard Reference (SR) (USDA 2006b). Accurate data on folic acid in fortified foods is required to support these studies. Imprecise, biased, or inaccurate food composition database values might lead to large errors in estimated intake of folic acid from RTE cereals that could critically affect conclusions from controlled feeding trials and epidemiological studies that relate folate intake to assumed health effects. Also, reliance on label values by the general public could lead to false assumptions about folate intake from particular products.

As part of the USDA National Food and Nutrient Analysis Program (NRFAP) (Haytowitz et al 2008), popular RTE cereals were sampled in 2006 according to a statistically-based nationwide sampling plan. The purpose of the NRFAP is to provide ongoing updating and expansion of data in SR that reflect changes in the food supply. Also, one cornerstone of the NRFAP is the use of validated analytical methods and quality control procedures. Folic acid was a key nutrient assayed, and the data were incorporated into SR beginning with release 20 (USDA 2006b).

The current standard method for analysis of folic acid in foods is a microbiological assay (Devries et al 2005). This approach measures total folate based on the growth response of a microorganism (usually Lactobacillus casei or L. rhhamnosus) after a trienzyme extraction (amylase, protease, and conjugase) (Arcot and Shrestha 2005). Rader et al (2000) previously analyzed folate in selected RTE cereals and compared the results to label values, but those products were sampled in 1998-1999 and were assayed by a standard microbiological method (Rader et al 1998; Devries et al 2001; Devries et al 2005). The deficiency of the microbiological method has been recognized since 1995 (Life Sciences Research Office 1995); more recently liquid chromatography/mass spectrometry (LC-MS and LC-MS-MS) methods have been developed that chemically quantify specific folate vitamers (Pfeiffer et al 1997; Finglas et al 1999; Konings et al 2001; Freisleben et al 2003; Rychlik 2003, 2004). LC-MS is preferred for definitive quantitation of folic acid (Konings 2006). All cereal samples for the NRFAP were analyzed using LC-MS, and a subset was assayed using the microbiological method.

The goal of this study was to compare the folic acid content of nationwide samples of popular RTE cereals in the United States determined using a validated LC-MS method to the labeled content, as well as to the concentration determined using the standard microbiological assay.

MATERIALS AND METHODS

Overview

Nationwide samples of popular vitamin-fortified RTE cereals were procured according to statistical sampling plans. Composites were prepared and LC-MS analysis was conducted at Virginia Polytechnic Institute and State University. Subsamples of the composites were shipped to the University of Georgia and subjected to microbiological assay. Quality control measures included assay of control samples in each run, analytical replicates, as well as method validation performed by each laboratory.

Samples

Cereals were selected for analysis based on four criteria: 1) fortification with vitamin D, a primary criterion because the products were also used in a study on vitamin D; 2) cereal consumption figures, by specific brand name, from the 2001 dietary component of NHANES, What We Eat In America (USDA 2002); 3) market share information by brand and by best sellers within brand, based on 2005 unit sales (A.C. Nielsen 2005); 4) varied selection of cereal types, particularly with respect to grain ingredients.

The products, which were major name brands and fortified with 25–50% DV (100–200 μg) per serving according to product labels, were sampled during June-July 2006 and included toasted oat shapes with marshmallows, crisp cinnamon-flavored wheat and rice squares, corn and oat puffs, oat rings, multigrain apple-flavored rings, crisp toasted rice, multigrain fruit-flavored rings, bran flakes with raisins, frosted corn flakes, multigrain flakes with oat clusters, wheat and barley nuggets, and sweetened puffed wheat. The statistical plan called for each product to be sampled at a selected retail outlet (Perry et al 2003) in each of 12 cities in the United States (Paradise, CA; Fremont, CA; Denver, CO; Waterbury, CT; Lake Worth, FL; Anderson, IN; Port Huron, MI; Belton, MI; Hillsborough, NC; College Point, NY; Sapulpa, OK). Ten of the cereals were available in all 12 locations. The corn and oat puffs product was available in 11 locations, and the sweetened puffed wheat product was found in only four of the locations. The 12 sampling locations (abbreviated AL1, CA1, CA2, CO2, CT1, FL1, IN1, MI1, MO1, NC1, NY1, OK1) were put in four random groups containing three samples/locations each (AL1/CA1/MI1, CA2/NC1/OK1, CO2/CT1/IN1, and FL1/MO1/NY1), and a composite of these samples was prepared for each brand/type of cereal.

Samples were shipped to Virginia Tech, inspected for integrity of packaging on receipt, and stored at room temperature (22–25°C) before compositing. Composites were prepared within four weeks of sample procurement and before expiration dates indicated on package labels. Labeled folic acid content was recorded from the packages.

Composite Preparation

For each composite, the entire contents of one package from each outlet were included. The samples were combined in a 6-L stainless steel industrial food processor (Robot Coupe 6L Blixer; Robot Coupe USA, Jackson, MS) and ground to a fine powder, with a total of ~1 min of grinding time in 30-sec intervals after an initial 10-sec pulse. Subsamples (12–15 g) were dispensed among 60-ml straight-side glass jars with Teflon-lined lids, sealed under nitrogen, surrounded with aluminum foil, and stored at ~60°C until analyzed.

A control composite of a multigrain ring cereal labeled to contain 100% DV folic acid was also prepared in the same manner using 17 boxes (7,785 g) and distributing subsamples among 960 jars. A cereal reference material (VMA399) with a certified value of 1,160–1,620 μg/100 g for folic acid was obtained from the AACC International (St. Paul, MN).

Subsamples, including blind control samples, were shipped frozen, overnight, on dry ice, to the University of Georgia (Athens, GA) and maintained at ~55 ± 2°C until analyzed.

Determination of Folic Acid by LC-MS

Reagents and standards. All reagents and standards were as described previously (Phillips et al 2006). Additionally, 13C-folic acid (≥98% purity) was purchased from Merck Eprova AG (Schaffhausen, Switzerland); folic acid (≥98% purity) was obtained from Sigma-Aldrich (St. Louis, MO). The purity (98%) reported in the certificate of analysis supplied by the manufacturer was used to calculate the concentration of prepared folic acid standards.

Extraction. Samples were extracted as reported in Phillips et al (2005), except 13C-folic acid was included as an internal standard (IS). Briefly, 0.5 ± 0.2 g of thawed (20 min at 30 ± 1°C) ground cereal composite, estimated to contain 200–1,400 μg of folic acid, was homogenized in phosphate buffer (0.1M, pH 6.0) then treated sequentially with α-amylase, protease, and rat plasma conjugase,
followed by anion-exchange solid-phase extraction to isolate folic acid. Extracts were diluted (100 to 250-fold) with the extraction buffer using volumetric flasks to place the folic acid concentration at 10–100 ng/mL (optimal range of the calibration curve). The IS was added to the sample extract after solid-phase extraction and before dilution, with the amount of IS adjusted depending on the final targeted dilution, to yield an IS concentration of ≥20 ng/mL. Extracts were stored at –60°C until LC-MS analysis.

LC-MS analysis. Sample extracts were subjected to chromatographic separation performed with an HPLC system (1100 series, Agilent Technologies, Santa Clara, CA) with a Luna C18 column (150 mm length, 2.1 mm diameter, 4 μm dp) (Phenomenex, Torrance, CA). Sample solution (20 μL) was injected onto the column using an autosampler (Thermo Survey, San Jose, CA) maintained at 10°C. Mobile phase A consisted of 1% aqueous formic acid and mobile phase B consisted 1% (v/v) formic acid in acetonitrile. The mobile phase was delivered to the HPLC column at a flow rate of 0.2 mL/min. The gradient elution program was time 0: 97/3% A/B; time 4 min: 95/5% A/B; time 10 min: 70/30% A/B; time 17 min: 0/100% A/B; time 18 min: 0/100% A/B; time 22 min: 97/3% A/B; 5 min post run. The HPLC column effluent was pumped directly without any split into a triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an ESI source, which was used in positive single ion-monitoring (SIM) mode (442 folic acid and 447 13C folic acid). The instrument was calibrated with a solution of polytrosin according to the manufacturer’s recommendation, with tuning performed for folic acid by direct infusion of standard solution (1 ng/μL) at a rate of 10 μL/min. MS parameters for detection of folic acids were spray voltage 4,100V, sheath gas pressure 49 arbitrary units, auxiliary gas pressure 13 arbitrary units, capillary temperature 300°C. Preliminary results showed a detection limit of 500–800 pg/mL; limit of quantification was 1–2 ng/mL, corresponding to 1–2 and 2–4 μg of folic acid/100 g of cereal, respectively, for a 0.5-g subsample assayed.

Folic acid calibration standards of 10–100 ng/mL of folic acid (0.5-5 analytic/IS ratio) were run in duplicate with each set of samples, with one set of standards at the beginning and one set at the end of the run. A linear calibration curve was constructed to determine folic acid in samples based on the sample/IS ratio.

Quality control. Eleven of the 48 cereal samples, as well as the control composite and reference material (VMA399), were assayed in replicate (n = 2 or 3 for samples, n = 7 for VMA399). For eight of these cases, at least one replicate was run in a separate analytical batch to allow some assessment of between-assay variability and an approximation of a reasonable estimate of uncertainty in assayed mean values among samples.

Spike recovery studies were performed on three cereals (corn and oat puffs, crisp toasted rice, and the multigrain cereal control composite). Each composite was assayed in triplicate with and without addition of 1.5–5 μg of folic acid (in extraction buffer) to the analytical portion of sample (0.5 g) before extraction. Recovery was calculated as the assayed folic acid in the spiked sample minus the expected amount, which was calculated from the mean assayed concentration of folic acid in the three unspiked samples plus the amount of folic acid added. The three cereal spike studies were performed in separate analytical runs. Recovery data were analyzed and if the average recovery was <100 ± 5%, with a relative standard deviation (RSD) of <2% across all nine spiked samples, then the assayed folic acid content in each sample in all runs was adjusted for recovery (% recovery/100).

Microbiological analysis (MA) of folic acid. Folic acid was determined by a differential assay designed to measure folic acid independently from total food folate (Chun et al 2006). The method is a modification of AOAC Method 2004.05 (Devries et al 2001, 2005; AOAC 2005) and Approved Method 86-47 (AACC International 2000) for total folate in fortified and nonfortified cereals. Elimination of the protease and conjugase digestion steps from the traditional trienzyme digestion for total folate recovers folic acid and not the naturally occurring conjugated food folates. Microbiological assay of the extracts was completed by standard microassay techniques using Lactobacillus casei (ssp. rhamnosus) ATCC 7469 and 96-well microtiter plates (Tamura 1990; Chen and Eitenmiller 2007).

Analyses of 12 composites, the cereal control composite, and VMA399 cereal reference material were conducted using microbiological assay at the University of Georgia using aliquots of the same random grouping composites. Method validation included recovery studies and analysis of NIST SRM 1846 infant formula (NIST, Gaithersburg, MD), which has a reference concentration of 101–157 μg/100 g for folic acid, as reported previously (Chun et al 2006). An inhouse control material (enriched flour) was also assayed for quality control.

Data Analyses

The data from the control material and from the specific composites that were analyzed in replicate were evaluated for equality of their mean values using a standard F-test based on the appropriate degrees of freedom (df). All analyses were performed with statistical software (v.9.1.3; SAS Institute, Cary, NC). Based on these tests, uncertainty for the assayed value for each product (or specific composite, if the case of composites assayed by both MA and LC-MS) was estimated.

The use of random composites, as described above, allowed the derivation of both an estimate of the standard error of the mean (an estimate of the standard deviation of the estimated mean) and an estimate of the serving-to-serving standard deviation (an estimate of standard deviation of the individual measurements). This is achieved by multiplying the standard error by the square root of the sample size (the number of composites × the number of samples in each composite). Under the assumption of perfect mixing of composites, these relationships follow easily from equations established by Hansen et al (1953).

RESULTS AND DISCUSSION

Quality Control

LC-MS. R2 values for all calibration curves were >0.999. Figure 1 shows representative chromatograms for folic acid and 13C-folic acid in a 20 ng/mL standard, a typical cereal composite, and the VMA399 cereal reference material.

Recovery. The mean recovery of added folic acid from spiked samples assayed by LC-MS was 92.4% (90.2–93.9%) with a 1.5% RSD and did not differ significantly among the three cereals tested. Therefore, the assayed folic acid concentration was divided by 0.924 for all samples to correct for recovery.

Control samples. The mean folic acid concentration assayed by LC-MS in VMA399 cereal reference material was 1.219 μg/100 g with an RSD of 5.2% (1.139–1.324 μg/100 g), which agrees with the certificate of analysis value within the tolerance limits specified (1.160–1.620 μg/100 g). The between-assay range as percent of the mean for the seven cereal composites analyzed in replicate in two or three separate runs (1.8–15.3%; average 8.2%) was similar to the RSD for the reference material. While as expected, the within-assay range for replicates (n = 2 or 3) was lower (1.2–4.9%; average 3.0%) (Table I). The expected between-assay RSD values were calculated as in Horwitz et al (1980) using the mean assayed analyte concentrations for the composites analyzed in more than one run (VMA399, 5.2% RSD, n = 7; frosted corn flakes, 12.4 RSD, n = 3). HORIZAR (ratio (actual/expected RSD) was <2 for both for VMA399 (0.71) and the frosted corn flakes (1.4), indicating acceptable repeatability.

Analytical uncertainty. The variance of the control values obtained in separate assays compared to those for replicate analyses of a given cereal composite within an assay were not distinguishable (P = 0.474). However, smaller variance from measurements
made on a given composite within the same assay compared to the larger variance between assays was statistically significant \((P = 0.0003)\), as would be expected. These results suggest some disparity in analytical precision depending on the particular sample, perhaps due to differences in homogeneity or extractability of folic acid from specific products, as well as fundamental variables within the assay performance. Also, the variance of between-assay measurements made on the same and different composites was not distinguishable \((P = 0.328)\), at least with these sample sizes. It seems reasonable to use these measurements to estimate analytical uncertainty of results for composites that were run individually. We chose to use the highest \%RSD determined for any of the composites that were assayed in replicate, across all products, to estimate the SD for all other composites. This was achieved by multiplying the \%RSD for the replicate composite by a measured value.

The variance of measurements made on four independent composites of a given cereal was larger than the variance of different day measurements made on the control material \((P = 0.037)\), supporting the importance of obtaining multiple samples of a product to obtain an accurate estimate of the mean and range of folic acid concentration in a given cereal. This analysis was conducted with a relatively small number of samples. A follow-up study with more samples would be desirable to support these conclusions.

The random group compositing method used in this study has several advantages compared to other methods of combining individual samples into composites for nutrient analysis. First, regardless of how the composites are formed, so long as each one contains an equal number of individual food samples, the sample mean of the composite nutrient analyses provides an estimate of the population nutrient mean for the food and the standard error can be computed. Second, unless the random group method is used to form the composites, it is not possible to estimate the serving-to-serving standard deviation of the nutrient content of the food. Third, when the random group compositing method is used, an estimate can be obtained of the serving-to-serving standard deviation of the nutrient content of the food for an average serving by multiplying the estimated standard error of the mean by the square root of the sample size (equivalent to multiplying SD of the composites by the size of the random groups). For the four cereal composites containing three samples each, the SE of the mean is multiplied by the square root of 12 \((\approx 3.46410)\) to get a rough estimate of the serving-to-serving standard deviation, or equally, the SD of the composite measurement could be multiplied by the square root of 3 \((\approx 1.73205)\).

Control results for microbiological assay. For the MA, results previously reported for NIST SRM 1846 infant formula (Chun et al 2006) were 136.6 ± 6.7 μg/100 g \((n = 11)\) and agreed well with
the reference concentration of 129 ± 28 μg/100 g (also determined by microbiological analysis [NIST 2007]). Results for the flour control showed good precision across analytical batches (mean 179 μg/100 g, 1.4 %RSD, n = 3).

Comparison of Folic Acid in Cereals Determined by LC-MS to Labeled Content

Table II summarizes the folic acid content of the cereals determined by LC-MS. Figure 1 illustrates the mean assayed versus labeled folic acid content of the products. The folic acid content of seven cereals was within 5% of the declared value. In four cereals, folic acid content was 5–20% higher, and in two cereals, it was >20% greater. Based on serving sizes, these differences represented −75 to +69 μg/serving of the 400 μg/day DV (~19% to +17%). Overall, validated LC-MS data indicate that for some RTE cereals, mean folic acid content will differ from labeled content.

Figure 1 illustrates the mean and confidence intervals (CI) for each product. The narrow CI values (except for wheat and barley nuggets) and ranges for the four composites of each cereal (Table II) suggest that the deviation from label value is consistent within a product. Depending on the precision needed in a particular application in which folic acid intake is to be estimated, the average might provide a reasonable estimate. However, the potential exists for significant deviation in a particular lot in the marketplace. For example, the control composite (multigrain rings with a fortification level of 100% DV) was prepared from a single lot and had 81% of the labeled folate content. Therefore, in controlled feeding trials in which it is likely that a single lot of cereal would be consumed as a folic acid source, it is critical to measure folic acid in that specific sample to accurately estimate intake of the study participants.

Comparison of LC-MS results to microbiological assay (MA). A comparison of the folic acid content of the 12 cereal composites, control cereal, and VMA399 reference material that were assayed by LC-MS and MA is shown in Fig. 2. Again, the most conservative estimate was used to determine the uncertainty indicated by the bar range around the LC-MS values. For all products except the reference material, folic acid determined by MA was higher than by LC-MS. It is not possible to determine why the value for VMA399 was lower by MA than by LC-MS. Overall, the data show MA gives higher values. Because MA results can be quite variable (for example, >13% within-laboratory inter-assay RSD was observed for enriched macaroni in a previous study [Koontz et al 2005]), the most likely explanation for the relatively

### Table I

<table>
<thead>
<tr>
<th>Cereal</th>
<th>Composite</th>
<th>Between-Assay</th>
<th>Within-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Oat rings</td>
<td>CO2/CT1/IN1</td>
<td>652</td>
<td>47</td>
</tr>
<tr>
<td>Crisp cinnamon-flavored wheat &amp; rice sq</td>
<td>AL1/CA1/MII</td>
<td>348</td>
<td>43*</td>
</tr>
<tr>
<td>Toasted oat shapes with marshmallows</td>
<td>FL1/MO1/NY1</td>
<td>244</td>
<td>12</td>
</tr>
<tr>
<td>Bran flakes with raisins</td>
<td>CA2/NC1/OK1</td>
<td>199</td>
<td>6.5</td>
</tr>
<tr>
<td>Bran flakes with raisins</td>
<td>FL1/MO1/NY1</td>
<td>1,122</td>
<td>8.2</td>
</tr>
<tr>
<td>Crisp toasted rice</td>
<td>FL1/MI1</td>
<td>CO2/CT1/IN1</td>
<td>1,219</td>
</tr>
</tbody>
</table>

* Number of replicates (n); range = difference between highest and lowest values; range % = range as percent of the mean, except for values marked with * (standard deviations and relative standard deviations when n > 2). In between-assay results range = difference between assays calculated using average within-assay value (assay n = 1 for a given composite). Range SD, standard deviation; RSD, relative standard deviation.

b Composite samples from three randomly grouped outlets from 12 statistically representative regions with indicated sampling codes (letters refer to U.S. state). CA2/NC1/OK1 composites and control were also analyzed by microbiological assay (see Fig. 2).

c Reference material (AACC International, St. Paul, MN) with certified folic acid concentration 1,160–1,620 μg/100 g.

### Table II

<table>
<thead>
<tr>
<th>Cereal</th>
<th>Description</th>
<th>% DV per serving</th>
<th>Serving (cup)</th>
<th>Folate μg/100g</th>
<th>Mean</th>
<th>SE</th>
<th>RSD (%)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Oat rings</td>
<td>50%</td>
<td>1.00 (30 g)</td>
<td>667</td>
<td>620</td>
<td>15.5</td>
<td>5.0</td>
<td>589–659</td>
</tr>
<tr>
<td>B</td>
<td>Crisp cinnamon-flavored wheat &amp; rice squares</td>
<td>25%</td>
<td>0.75 (30 g)</td>
<td>333</td>
<td>357</td>
<td>9.4</td>
<td>5.2</td>
<td>324–379</td>
</tr>
<tr>
<td>C</td>
<td>Corn and oat puffs</td>
<td>50%</td>
<td>1.25 (30 g)</td>
<td>667</td>
<td>768</td>
<td>19.5</td>
<td>5.1</td>
<td>723–813</td>
</tr>
<tr>
<td>D</td>
<td>Toasted oat shapes w/marshmallows</td>
<td>50%</td>
<td>0.75 (30 g)</td>
<td>741</td>
<td>726</td>
<td>12.0</td>
<td>3.3</td>
<td>701–757</td>
</tr>
<tr>
<td>E</td>
<td>Multigrain apple-flavored rings</td>
<td>25%</td>
<td>1.00 (33 g)</td>
<td>303</td>
<td>377</td>
<td>9.7</td>
<td>5.1</td>
<td>349–393</td>
</tr>
<tr>
<td>F</td>
<td>Multigrain fruit-flavored rings</td>
<td>25%</td>
<td>1.00 (32 g)</td>
<td>313</td>
<td>362</td>
<td>19.3</td>
<td>10.6</td>
<td>327–414</td>
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<td>G</td>
<td>Frosted corn flakes</td>
<td>25%</td>
<td>0.75 (31 g)</td>
<td>323</td>
<td>362</td>
<td>7.9</td>
<td>4.3</td>
<td>348–384</td>
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<tr>
<td>H</td>
<td>Bran flakes with raisins</td>
<td>25%</td>
<td>1.00 (59 g)</td>
<td>169</td>
<td>229</td>
<td>13.9</td>
<td>12.1</td>
<td>196–257</td>
</tr>
<tr>
<td>I</td>
<td>Crisp toasted rice</td>
<td>25%</td>
<td>1.25 (33 g)</td>
<td>303</td>
<td>535</td>
<td>23.4</td>
<td>8.7</td>
<td>469–577</td>
</tr>
<tr>
<td>J</td>
<td>Sweetened puffed wheat</td>
<td>25%</td>
<td>0.75 (27 g)</td>
<td>370</td>
<td>421</td>
<td>22.9</td>
<td>10.9</td>
<td>379–478</td>
</tr>
<tr>
<td>K</td>
<td>Wheat and barley nuggets</td>
<td>50%</td>
<td>0.50 (58 g)</td>
<td>345</td>
<td>446</td>
<td>28.5</td>
<td>12.8</td>
<td>382–495</td>
</tr>
<tr>
<td>L</td>
<td>Multigrain flakes and oat clusters</td>
<td>50%</td>
<td>0.75 (30 g)</td>
<td>667</td>
<td>798</td>
<td>12.1</td>
<td>3.0</td>
<td>769–824</td>
</tr>
<tr>
<td>Controlb</td>
<td>Multigrain rings</td>
<td>100%</td>
<td>1.00 (29 g)</td>
<td>1,379</td>
<td>1,119</td>
<td>–</td>
<td>2.4</td>
<td>1,088–1,136</td>
</tr>
</tbody>
</table>

* DV, daily value (as established for nutrition labeling) (FDA 1996); SE, standard error (n = 4); RSD, relative standard deviation.

b Local sample, single lot.

c AACC International (St. Paul, MN): certified range for folic acid 1,160–1,620 μg/100 g.
lower MA value for VMA399 is that analytical uncertainty contributed to the result for VMA399 being lower than the LC-MS mean \( (n = 7) \).

Folic acid concentration measured by microbiological vs. LC-MS chemical analysis was 37–533 \( \mu g/100\) g (12–160 \( \mu g/100\) g) higher in the composites that were analyzed by both methods (Fig. 3), representing a 10% (frosted corn flakes) to 67% (bran flakes with raisins, multigrain flakes, and oat clusters) difference. Because food folate (i.e., polyglutamyl folates occurring endogenously in the grain) represented just 1–8% (4–45 \( \mu g/100\) g) of total folate (Fig. 3), endogenous folate was not enough to explain the lower values for LC-MS determined compared to label values for folic acid (Fig. 2). Furthermore, the total folate only increases the MA value for folate. Chun et al (2006) discussed nonspecific responses of \( L.\) casei (ssp. \( rhamnous\)) in detail. The level of such response is usually <10 \( \mu g/100\) g. Higher measured response without conjugase treatment in a raw food product would indicate that native conjugase enzymes were allowed to react before inactivation. Chun et al (2006) showed that mean response for non-conjugase-treated, unenriched, white wheat flour was 8 \( \mu g/100\) g \( (n = 24)\), representing 5% of the mean folic acid content of enriched, white flour (158 \( \mu g/100\) g, \( n = 46\)). Clearly, such response would be negligible in the highly fortified cereals assayed in this study.

The goal was to accurately measure folate levels in RTE cereals using the chemically specific, validated, LC-MS method and compare those levels to the labeled values. It should be noted that because the LC-MS and MA analyses were conducted in different laboratories, with each laboratory preparing an extract that was subjected to folic acid measurement, it is not possible to definitively attribute differences to the detection method (MA or LC-MS) versus the specific extraction method or its implementation. Also, there were no specific measures of uncertainty obtained for the MA values. However, the extraction technique in each case was validated at the laboratory performing it, and across all samples the MA values exceeded those from LC-MS, so it is likely the majority of the discrepancy is explained by the detection method.

The standard approach of trienzyme extraction was used before LC-MS. The microbiological analysis was done with and without deconjugation to obtain a measure of endogenous folate and give an estimate of added folic acid. The protease was eliminated altogether because it was validated for this matrix to not affect recovery of folate (as discussed in Chun et al [2006]). The actual folate content includes added and endogenous folate and would include all forms, not just folic acid (e.g., 5-methyltetrahydrofolate and formyl folates). As expected, and as indicated by the microbiological results for total folate with and without deconjugation during extraction (Fig. 2), naturally occurring folate was negligible compared to the fortified amount. These data support that there was no important underestimation of total folate by the LC-MS analysis of folic acid, in which only folic acid and no other forms of folate was measured. Because LC-MS analysis of multiple forms of folate increases the cost, and given that the microbiologically determined folate was much higher than that determined by LC-MS, whether or not endogenous folates were included, it is reasonable to conclude that folic acid determined by LC-MS is a more accurate measure of folate content of fortified RTE cereals.

Comparison of results to previous studies. Rader et al (2000) and Whittaker et al (2001) reported MA-determined total folate for RTE cereal samples obtained in 1998-1999, where 23 out of 27 total cereals analyzed had total folate >120% of the label value. For 10 of those, the excess was ≥25% of the DV (≥100 µg), compared to the current study in which only 2 of 12 cereals had >120% of label value, as determined by the specific LC-MS method, with excesses of 39 and 69 µg/100 g (Fig. 2). The overages by MA assay in Rader et al (2000) were similar to those in the present study for folic acid determined by MA. The additional measurement of endogenous food folate showed that it did not contribute >1–8% of the total folate in any product (Fig. 3). It should be noted that the exact products in Rader et al (2000) were unspecified and each type of cereal reported included different brands sampled from a single lot of each, while those in the present work represented multiple composites across different lots and statistical sampling locations for specific products. Therefore, while the values from the two studies cannot be compared definitively, the results suggest that the higher contents determined in the current work by MA compared to LC-MS overestimate total folate.

Other researchers also have compared LC-MS to MA of folic acid in RTE cereals, however differences in samples and methodology make direct comparison difficult. Ossey et al (1998) studied locally procured (Nebraska) samples of fortified RTE cereals using HPLC analysis with UV detection and a standard MA, with both assays performed on samples subjected to only \( \alpha\)-amylase treatment. They reported excellent correlation between the MA and HPLC results, but the absolute differences in µg/100 g was 80–240 (indicating a strong correlation, but not agreement), similar to the findings for several products in this study (Fig. 2). Be-

![Fig. 2. Comparison of folic acid in selected cereal composites (Table I) determined by LC-MS and microbiological assay (MA). Products, left to right, in order of increasing content of folic acid determined by LC-MS. Error bars are ±2 standard deviation. * Control, ** reference material (AACC International, St. Paul, MN). Certified folic acid concentration: 1,160–1,620 \( \mu g/100\) g.](image1)

![Fig. 3. Comparison of assayed folic acid content to product label values as determined by LC-MS. Error bars indicate estimated 95% confidence interval (CI) among four regional nationwide composites (Table I). Labeled serving size in parentheses for each product (1 cup = 240 mL). Letter codes refer to cereals listed in Table I. * Within 5% of label value (i.e., upper 95% CI limit is >95% than label or lower 95% CI is <105% of label), ** 105–120% of label value (i.e., lower 95% CI limit), *** >120% of label (i.e., lower 95% CI limit is >120% of label).](image2)
cause food folate comprised only 4–45 μg/100 g in the present work (Fig. 3), the results confirm there is a fundamental discrepancy in folic acid measured by MA versus HPLC methodology, which measures the specific chemical species; therefore, the latter is preferred for reliable determination of added folic acid.

CONCLUSIONS

Because many dietitians and researchers rely on the USDA National Nutrient Database for Standard Reference (USDA 2006) to estimate nutrient intake, the work of the USDA FNAP to update values for folic acid in RTE cereals using state-of-the-art measurements, appropriate sampling plans, and analytical quality control, provides critical support for research and dietary recommendations. Accurate estimation of the folate intake depends on accurate data for the major food sources of folate, including RTE cereals. Furthermore, in controlled diet studies incorporating RTE cereals, analysis of specific samples of products consumed is necessary for precise and accurate estimate of actual folate intake because label values can significantly underestimate or overestimate folate content in many cases, and there can be lot-to-lot variability within a product. LC-MS determined that folic acid, though different from the label value in some cases, more closely matched the declared folate content compared to microbiologically assayed folate, which consistently returned higher values.

It is important for all food composition researchers, dietitians, and analysts to understand that methods for folate analysis of foods have a relatively high analytical uncertainty compared to other nutrient analyses such as total fat, individual fatty acids, and nitrogen (for protein estimation), even when performed using a carefully validated method at a highly experienced laboratory. Therefore, it is critical to include quality control samples in each run and enough sample replicates to obtain an estimate of uncertainty in all measured values. As always, the selection of an appropriate analytical method, validation of method performance in the analysts’ hands, and inclusion of quality control measures influence the reliability of results.

ACKNOWLEDGMENTS

Funding was provided by the USDA Nutrient Data Laboratory as part of the National Food and Nutrient Analysis Program, through specific cooperative agreement #Y1-HV-8116-11 between the United States Department of Agriculture (USDA) Nutrient Data Laboratory and Virginia Polytechnic Institute and State University and #1235-52000-051-16 between the USDA and the University of Georgia, with support from the National Institutes of Health through interagency agreement #Y1-HV-8116 between the National Institutes of Health, under coordination of the National Cancer Institute, and the USDA. The detailed work of Amy Rason and Nancy Conley on coordination of sample preparation and sample descriptive information is greatly appreciated.

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[Received April 7, 2009. Accepted July 8, 2009.]