FOOD COMPOSITION AND ADDITIVES

Comparison of Total Folate Concentrations in Foods Determined by Microbiological Assay at Several Experienced U.S. Commercial Laboratories

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Analysis of total folate concentration measured by microbiological assay in a variety of foods submitted in a routine manner to experienced laboratories that regularly perform folate analysis on fee-for-service basis was evaluated. Homogenates of fresh strawberries, frozen spinach, orange juice, frozen meat and vegetable pizza, dry macaroni, and dried pinto beans were prepared and stored under conditions previously determined to maintain stability of folate content. An aliquot of each composite and of 3 certified reference materials were sent on each of 4 occasions to 4 laboratories. Results for macaroni and pizza, the only folic acid-fortified foods, had considerably lower between-laboratory variation (CV_B) with CV_B of 9–11% versus >45% for other foods. Mean total folate ranged from 14 to 279 μg/100 g for a mixed vegetable reference material, from 5 to 70 μg/100 g for strawberries, and from 28 to 81 μg/100 g for wholemeal flour. Only 1 laboratory reported using a tri-enzyme extraction, and all laboratories used folic acid fortified foods as internal control materials. Users of commercial total folate analysis should understand the uncertainty in values determined by microbiological assay, particularly for foods containing primarily naturally occurring folate, which may not be apparent when replicate samples are not submitted for analysis.

Folate is the general term for the various chemical forms of a water-soluble B-group vitamin. Most naturally occurring folates in foods exist as reduced tetrahydropteroylglutamates (1), often in polyglutamyl form, which include tetrahydrofolate (H_4 folate), 5-methyltetrahydrofolate (5-CH_3-H_4 folate), 5-formyltetrahydrofolate (5-CHO-H_4 folate), and 10-formyltetrahydrofolate (10-CHO-H_4 folate). Folic acid (pteroylmonoglutamic acid) is the synthetic form used in supplements and fortification of foods, such as enriched flour and other enriched cereal-grain products. The Daily Value on the Nutrition Facts panel for packaged products refers to total folate content (2). The latest Dietary Reference Intakes (DRI) for folate are reported as dietary folate equivalents (DFE), which take into account the greater bioavailabilty of synthetic folic acid compared to naturally occurring food folate; DFE are calculated by multiplying micrograms of folic acid by a factor of 1.7 and adding micrograms of food folate (3). The Dietary Reference Intakes Guiding Principles for Nutrition Labeling and Fortification recommends that, in the future, the DFE calculation be used to estimate the Daily Value (4). Increasingly, it has been observed that folate may play a significant role in reducing the risk of several human disorders, including neural tube defects, adverse pregnancy outcomes, cardiovascular disease, macrocytic anemia, thromboembolitic processes, and neuropsychiatric disorders (5). The expanding role of folate nutrition has major health implications; therefore, precise and accurate data on the total folate content of foods are essential.

The two general approaches currently used for analysis of food folate are a microbiological assay and high-performance liquid chromatography (LC). The microbiological assay involves quantifying the growth response of a specific microorganism to the mixture of folates that is present (6, 7) and does not distinguish different forms of folate. In contrast, LC methods specifically quantify individual vitamers (8–11), but there are difficulties with measuring all folates because of problems with sample cleanup, interfering chromatographic peaks, and lack of stability during extraction (6, 7, 12). Although the microbiological assay was developed and validated for folic acid-fortified foods, it has been applied to the determination of total naturally occurring folates in foods (6, 13), especially at U.S. commercial laboratories.

Recently the AOAC and American Association of Cereal Chemists (AACC) conducted collaborative studies of a microbiological method that emphasizes tri-enzyme extraction to determine total folate in enriched cereal

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Table 1. Summary of some modified parameters of the AOAC microbiological method reported by experienced laboratories

Lab	Calibrant	Extraction conditions				Microorganism	Detection	Internal control material (tolerance range, μg/100 g)	Pre-assay storage temperature, °C
		Buffer	pH	Enzyme(s)[a]	Ascorbic acid, %, w/v of buffer				
A	Folic acid	0.05M Potassium phosphate	6.0	Chicken pancreas (Difco[b] or Sigma[c])	0.15	*Lactobacillus rhamnosus* (ATCC 7469)	Turbidimetric at 680 nm	Infant formula (168 ± 27)	–10–30
B	Folic acid	0.1M Sodium phosphate	7.8	Chicken pancreas (Difco[b])	1.0	*Lactobacillus rhamnosus* (ATCC 7469)	Turbidimetric at 660 nm	Infant formula (115 ± 15)	–18
C	Folic acid	0.1M Sodium phosphate	6.8	Chicken pancreas (Difco[b])	1.0	*Lactobacillus rhamnosus* (ATCC 7469)	Turbidimetric at 600 nm	Cereal (1230 ± 240)	–20
D	Folic acid	1.0M Sodium phosphate	7.8	Pronase (Calbiochem)[d], α-amylase (Fluka)[e], chicken pancreas (Difco[b])	0.7–1.0	*Lactobacillus rhamnosus* (ATCC 7469)	Turbidimetric at 620 nm	Enriched flour (184 ± 14)	–50

[a] Laboratories did not report enzyme activity.
[b] Difco, Detroit, MI.
[c] Sigma Chemical Co., St. Louis, MO.
[d] Calbiochem, La Jolla, CA.
[e] Fluka, Buchs, Switzerland.

products (14, 15). This method has also been recommended for the analysis of intrinsic folates in nonenriched foods, to replace AOAC Official Method **992.05** [Folic Acid (Pteroylglutamic Acid) in Infant Formula], which was developed and validated for determination of free folic acid in infant formula (16, 17). Various U.S. commercial food analysis laboratories offer "modifications" of this method as the standard procedure for analysis of total folate.

The USDA National Food and Analysis Program (NFNAP) began in 1998 and is an ongoing project with the goal of updating and increasing the reliability of food composition data in the USDA National Nutrient Database for Standard Reference (18). Food samples are collected according to a national probability sampling plan, prepared and composited at a central processing facility, and sent to experienced contract laboratories for analysis of a broad range of nutrients. The quality of folate data was a particular concern because of the lack of an official method for folate in its naturally existing forms. Preliminary data suggested wide variation in total folate content of different food samples, too large to be explained by agricultural variability, reported by different laboratories. For example, the total folate content of fresh strawberries reported by 3 different facilities was 62, 8, and 7 μg/100 g (USDA Nutrient Data Laboratory, unpublished data). From additional studies it was apparent that these differences were not due to instability of folate in the homogenized strawberry composites (19). We therefore sought to evaluate performance by experienced laboratories that regularly perform folate analysis for food composition tables and nutritional labeling by submitting a variety of food samples and standard reference materials in the manner typical of a client of commercial laboratories.

Materials and Methods

Sample Preparation

Six commonly consumed foods and three commercially available reference materials were chosen to represent different matrixes with a significant concentration of folate, varying in content of fortified folic acid and naturally occurring folates, as well as having differences in pH. Fresh strawberries, frozen spinach, orange juice, frozen meat and vegetable pizza, dry macaroni, and dried pinto beans were purchased locally (Blacksburg, VA) at a large supermarket. Standardized protocols for handling and processing food samples were used to minimize folate loss or degradation.

Dried pinto beans (*Phaseolus vulgaris*; 1.97 kg) were thoroughly washed and cleaned of debris, soaked for 1 h in distilled, deionized water according to package directions, and then cooked slowly at the boiling point for 2 h. Two frozen meat and vegetable pizzas (1.41 kg) were cooked according to the package instructions. Four packages of fresh strawberries (*Fragaria X ananassa*; 2.26 kg) were rinsed with tap water for 1 min followed by a 1 min rinse with distilled, deionized water, trimmed of stems, leaves, and damaged or moldy areas, and dried with a clean lint-free cloth. Six packages of frozen spinach (*Spinacia oleracea*; 1.71 kg) were partially thawed

Table 2. Total folate in selected foods and certified reference materials determined by microbiological method and reported as mean ± standard deviation (n = 4)

Food product	Total folate (μg/100 g)[a]			
	Lab A	Lab B	Lab C	Lab D
Cooked pinto beans	47 ± 21 (34–78)	42 ± 23 (28–76)	7.0 ± 0.4 (6.6–7.8)	31 ± 3 (30–35)
Frozen meat and vegetable pizza	68 ± 13 (51–83)	58 ± 18 (44–84)	55 ± 5.5 (49–62)	60 ± 5 (55–67)
Fresh strawberries	13 ± 6 (9.5–20)[b]	7.8 ± 4.7 (4.9–13)[b]	4.7 ± 0.5 (4.2–5.2)[b]	70 ± 3 (67–73)
Frozen spinach	127 ± 15 (113–148)	101 ± 32 (57–131)	31 ± 6 (26–40)	103 ± 15 (85–121)
Orange juice	36 ± 18 (25–63)	23 ± 2 (22–26)	3.7 ± 1.4 (2.6–5.3)[b]	25 ± 2 (23–27)
Dry macaroni	244 ± 13 (227–254)	239 ± 22 (216–262)	200 ± 25 (164–218)	198 ± 21 (178–228)
BCR 121 wholemeal flour[c,d]	81 ± 44 (36–108)	41 ± 7 (29–44)	28 ± 3 (22–27)	45 ± 6 (34–45)
BCR 485 mixed vegetables[c,e]	279 ± 19 (255–291)	240 ± 39 (196–282)	14 ± 1 (12–15)	217 ± 36 (186–264)
BCR 487 pig's liver[c,f]	1015 ± 52 (910–1020)	959 ± 25 (746–1009)	278 ± 54 (227–337)	1222 ± 181 (1024–1428)

[a] Ranges are reported in parentheses.
[b] n = 3 due to one observation below the limit of quantitation.
[c] Values for BCR reference materials are reported on a dry matter basis.
[d] Certified total folate, 50 ± 7 g/100 g (20).
[e] Certified total folate, 315 ± 28 g/100 g (20).
[f] Certified total folate, 1340 ± 130 g/100 g (20).

prior to homogenization. Not-from-concentrate, pulp-free orange juice (3.98 kg) was shaken in its original packaging for ca 1 min before opening and then poured into a stainless steel bowl. Enriched dry elbow macaroni (1.37 kg) from 1 package was not further prepared before homogenization.

Immediately after preparation the food for each composite (except orange juice) was cut into pieces approximately 2.5 cm in size, frozen in liquid nitrogen, and homogenized with an industrial food processor (Blixer BX6V; Robot Coupe, Ridgeland, MS). Orange juice was manually mixed with a stainless steel wire whisk for 1 min. Homogenized samples were dispensed in 20–25 g aliquots into 30 mL glass jars with polytetrafluoroethylene (PTFE)-lined closures (Qorpak, Bridgeville, PA) using a standardized protocol, which included intermittent stirring and care being taken during the process to avoid sedimentation. The samples, containing residual nitrogen gas, were sealed with the PTFE-lined closures, surrounded with aluminum foil, and stored at –60° ± 5°C protected from light. Homogenization and dispensing procedures have been validated at this laboratory to yield homogeneous aliquots (19).

Three certified reference materials used for the quality control of analytical measurements of total folate content, supplied by the Institute for Reference Materials and Measurements (Retieseweg, Belgium), were purchased from Resource Technology Corporation (Laramie, WY). The materials were shipped frozen by overnight express and stored at –60° ± 5°C upon receipt. BCR 121 wholemeal flour is a commercially produced whole wheat flour packaged in sachets under nitrogen, and vacuum-sealed (20). BCR 485 mixed vegetables is a mixture of sweet corn, carrot, and canned tomatoes (10 + 1 + 1 by weight), blended and then lyophilized, milled, and packaged into sachets under nitrogen followed by vacuum-sealing (20). BCR 487 pig's liver is a milled, lyophilized powder prepared from fresh pig's liver and filled into bottles under nitrogen and sealed (20). To maintain blinding of the identity of these samples as reference materials, each package of reference material was thawed and dispensed into 30 mL glass jars, with attention to maintaining stability and homogeneity. The jars were sealed under argon, surrounded with aluminum foil, and stored at –60° ± 5°C protected from light. The moisture content of each reference material was measured in the dispensed samples after thawing and then drying at 103° ± 2°C and 635 mm Hg vacuum for 2 h. The mean residual moisture contents of BCR 121, BCR 485, and BCR 487 were determined to be 13.0, 2.2, and 3.7%, respectively, and these values were used to calculate the total folate concentrations in reference materials on a dry weight basis for the purpose of comparison to certified values.

Laboratories

Samples were shipped to 3 commercial laboratories (referred to as A, B, and C) and 1 university laboratory (D), all experienced in and routinely offering analysis of total folate in foods using a microbiological method. One subsample from each composite and reference material was sent to each laboratory on 4 occasions separated by approximately 1 month. The samples were shipped frozen, on dry ice, via overnight express delivery. Each laboratory was provided with the generic name of each food sample to allow the selection of optimal assay conditions and was instructed to analyze each sample for total folate concentration following

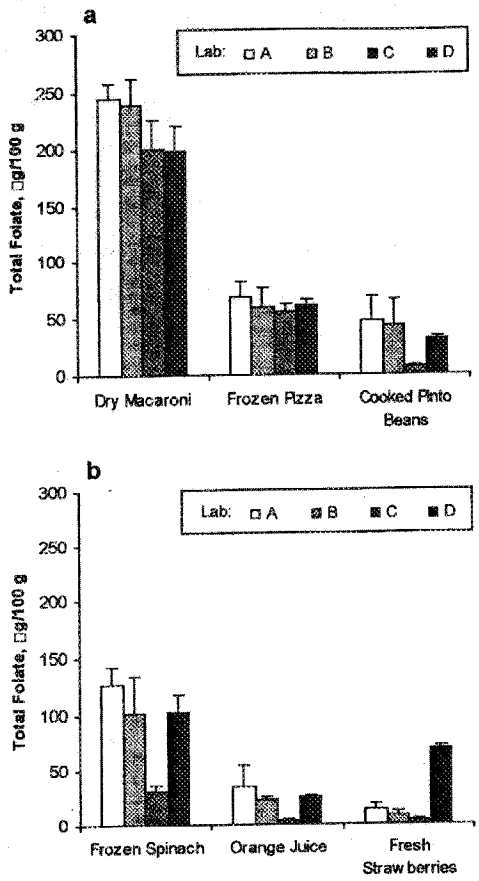

Figure 1. Mean ± standard deviation of total folate concentration by microbiological assay in (a) high-starch and (b) fruit and vegetable matrixes.

usual internal protocols. After all samples had been assayed, documentation of sample handling, the analytical procedure, and internal quality control procedures was requested from each facility. Samples from the same food composites analyzed at the 4 laboratories performing microbiological analyses were also analyzed for 5-methyltetrahydrofolate (5-CH_3-H_4 folate) content using an LC method at this laboratory, as described previously (19). This method was validated to measure 5-CH_3-H_4 folate in fruit and vegetable matrixes but does not quantify all folate vitamers and, therefore, could not be used to verify total folate content. The 5-CH_3-H_4 folate value was used to identify cases in which the microbiologically determined total folate content was clearly underestimated.

Statistical Analysis

Statistical analyses were performed using the SAS system (version 8.02, SAS Institute, Cary, NC). Mean total folate values were calculated from the 4 replicates of each food composite by laboratory, except means for orange juice at laboratory C and for strawberries at laboratories A, B, and C were calculated from 3 values because one observation at each of these laboratories fell below the laboratory's reported limit of quantitation. The coefficient of variation (CV) was used to evaluate the variability within laboratory (CV_W) and variability among laboratories (CV_B) of total folate concentration of each food matrix. Tukey-Kramer multiple comparisons were performed in a one-way ANOVA using the SAS system. Effects were considered significant at $p < 0.05$.

Results and Discussion

Sample Handling and Analytical Parameters

Table 1 summarizes selected sample handling and analytical parameters reported by each laboratory. Each facility reported the use of a microbiological method, specified as a modification of the AOAC official method for determination of folic acid (16, 21). Either infant formula, cereal, or enriched flour was reported as the internal quality control material. Pre-assay storage temperature of food samples varied from −10° to −50°C, and laboratories reported analyzing samples anywhere from within 1 week to 9 weeks after receipt, with laboratory D analyzing the first sample shipment of frozen spinach 13 weeks after receipt. The 4 shipments of each type of food composite at each laboratory were assayed in separate batches, with the exception of

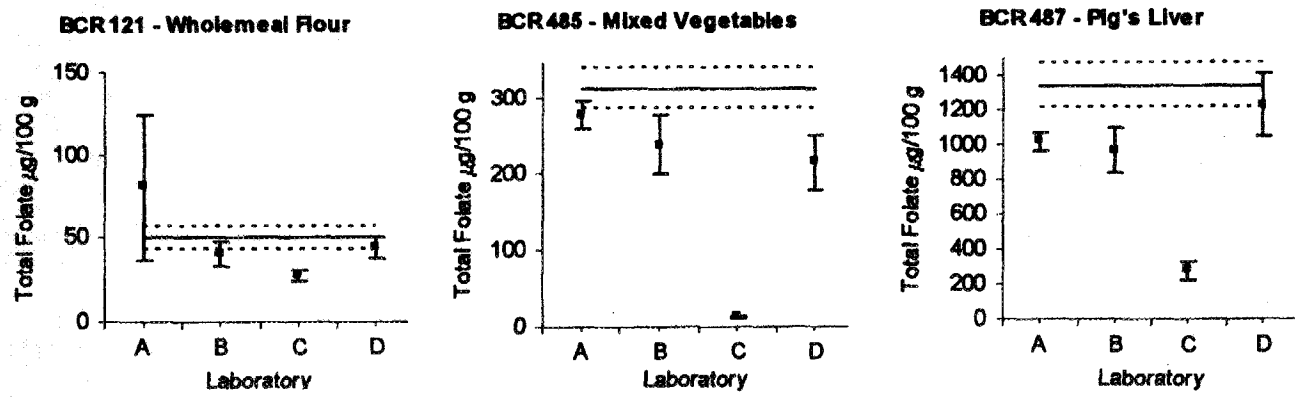

Figure 2. Mean ± standard deviation of total folate concentration in 3 certified reference materials on a dry matter basis measured by microbiological assay. Certified means (——) and certified tolerance limits (-----) have been defined (20).

Table 3. Within-laboratory (CV_W) and between-laboratory (CV_B) variation of total folate concentration determined by microbiological assay in selected foods and certified reference materials

Food product	CV_W, %[a]				CV_B, %[a,b]
	Lab A	Lab B	Lab C	Lab D	
Cooked pinto beans	44	55	6	8	56
Frozen meat and vegetable pizza	20	31	10	9	9
Fresh strawberries	45	61	11	4	129
Frozen spinach	12	32	19	14	46
Orange juice	51	8	38	8	61
Dry macaroni	5	9	13	11	11
BCR 121 wholemeal flour[c]	54	18	10	13	47
BCR 485 mixed vegetables[c]	7	16	9	17	63
BCR 487 pig's liver[c]	5	13	19	15	47
Mean CV_W, %	27	27	15	11	

[a] Coefficient of variation (CV) is expressed as percentage.
[b] Mean CV_B, % was calculated using the mean total folate value at each laboratory for each food product.
[c] *See* reference 20.

2 samples each of frozen spinach and BCR 487 pig's liver at laboratory D.

Repeatability and Reproducibility

The mean total folate concentrations measured by each laboratory using microbiological assay in several foods and certified reference materials are summarized in Table 2 and Figures 1 and 2. Laboratory C reported remarkably lower total folate values than other laboratories for spinach, pinto beans, orange juice, and strawberries. Within- (CV_W) and between-laboratory (CV_B) variabilities of total folate concentration for all of the food products are shown in Table 3. High within-laboratory variability was evident at each laboratory for the majority of foods, with mean CV_W of 27, 27, 15, and 11% at the 4 laboratories, and CV_B values ranging from 46 to 129% for pinto beans, strawberries, orange juice, spinach, wholemeal flour (BCR 121), pig's liver (BCR 487), and lyophilized mixed vegetables (BCR 485). Table 3 clearly shows that values for macaroni and pizza had considerably lower between-laboratory variation with respective CV_B values of 11 and 9%, whereas the variation for all other foods, including certified reference materials, was very high with CV_B values of >45%. Dry macaroni, which contains nearly all folate primarily as added folic acid, was the only food in this study with a CV_W of <15% at all 4 laboratories. Frozen pizza was the only food for which there was no statistically significant difference in mean total folate concentration across all 4 laboratories (Figure 1a). Laboratory D had a considerably narrower CV_W range (4–17%) and mean (11%) across all food matrixes relative to the other laboratories.

As illustrated in Figure 2, none of the mean total folate values for the 3 BCR reference materials were within the certified tolerance limits at laboratories A, B, C, or D for BCR 485 (lyophilized mixed vegetables). For BCR 121 (wholemeal flour) and BCR 487 (lyophilized pig's liver), only laboratory D mean total folate values were within the tolerance limits.

Total folate concentrations determined by laboratory C were remarkably lower for BCR 485 and BCR 487 than results from the other sites, consistent with that facility's lower values for other foods (Table 2).

5-Methyltetrahydrofolate Content of Fruit and Vegetable Samples

Konings et al. (22) determined by LC analysis that 5-CH_3-H_4 folate is the most abundant form of folate in nonenriched foods and found that 62% of all vitamers in the groups of vegetables and fruit, bread, milk products, potatoes, and meat products were 5-CH_3-H_4 folate. Similarly, Müller (23) estimated about 70% of the total folate in most fruits and vegetables to be 5-CH_3-H_4 folate based on LC analysis. The 5-CH_3-H_4 folate concentrations of the fruit and vegetable samples in this study are summarized in Table 4. Therefore, it was apparent that laboratory C's total folate values for unfortified foods failed to detect at least a significant portion of 5-CH_3-H_4 folate. Because the total folate concentration cannot be less than the concentration of 5-CH_3-H_4 folate, total folate reported by laboratory C is clearly underestimated for several foods. The values for spinach, orange juice, and strawberries were greater than 2 standard deviations below the mean 5-CH_3-H_4 folate concentration determined by LC. As shown in Figure 2, laboratory C also reported 13.5 ± 1.3 μg/100 g total folate for BCR 485 mixed vegetables, which is >15-fold lower than the assayed 5-CH_3-H_4 folate content of 253.2 ± 1.5 μg/100 g and

Table 4. Mean ± standard deviation of 5-CH_3-H_4 folate concentration in fruit and vegetable samples and certified reference material determined by LC

Food product	5-CH_3-H_4 folate, g/100 g	5-CH_3-H_4 folate, CV_W, %[a]
Frozen spinach	69.5 ± 3.6	5.2
Orange juice	26.6 ± 1.2	4.3
Fresh strawberries	43.6 ± 3.7	8.4
BCR 485 mixed vegetables[b]	253.2 ± 11.5	4.5

[a] Within-laboratory coefficient of variation, expressed as percentage.
[b] 5-CH_3-H_4 folate value for BCR 485 reported on a dry matter basis. Indicative value for 5-CH_3-H_4 folate, 214 ± 42 μg/100 g (20).

the indicative value of 214 ± 42 μg/100 g (20). These results suggest that the consistently lower total folate values from laboratory C for the remaining foods (except dry macaroni and pizza) are of questionable accuracy as well.

5-CH_3-H_4 folate is reported to be the predominant form of folate (>95%) in several types of berries by LC, including strawberries (24). The total folate content of strawberries reported by laboratories B and C (7.8 ± 4.7 μg/100 g and 4.7 ± 0.5 μg/100 g, respectively) was considerably lower than the mean 5-CH_3-H_4 folate concentration of 43.6 ± 3.7 μg/100 g determined by LC (Figure 1b). Laboratory D's total folate value of 69.5 ± 2.5 μg/100 g was in line with reported literature values of 36 and 65 μg/100 g total folate in strawberries determined by LC detecting H_4 folate, 5-CH_3-H_4 folate, and 5-CHO-H_4 folate (22, 25).

Comparison with Other Studies

The consensus certified values for total folate concentrations in the BCR reference materials (BCR 121 wholemeal flour, BCR 485 mixed vegetables, BCR 487 pig's liver) were determined by microbiological assay using a single-enzyme treatment, with the source of the conjugase (hog kidney, human plasma, chicken pancreas) varying across laboratories (20), yielding values for CV_W and CV_B of 10 and 23%, respectively, with 20 laboratories participating. In a more recent study, Finglas et al. (7) reported a mean within- and between- laboratory variation of 9 and 18%, respectively, among BCR 121 wholemeal flour, BCR 421 milk powder, BCR 485 mixed vegetables, and BCR 487 pig's liver for total folate by microbiological assay. Note that assessment of the accuracy of the certified total folate values is not possible because the concentrations in the BCR reference materials are based on consensus using a microbiological assay, not definitive analysis, and thus are subject to any inaccuracy inherent in the analytical approach itself.

The design of the present study differed from the controlled interlaboratory comparison and certification protocols, insofar as analyses were obtained by submitting samples to laboratories for food folate determination in the manner typical of a client of commercial food analysis laboratories. The data show considerably higher within- and between-laboratory variations of 16 and 52%, respectively, for total folate among BCR 121, BCR 485, and BCR 487, and were considerably affected by the values reported by laboratory C (Figure 2). The findings are also consistent with a recent study by Puwastien et al. (26) who conducted an international study of total folate analysis using soybean flour, fish powder, and breakfast cereal, yielding interlaboratory coefficients of variation of 24–35% among the foods assayed at 26 laboratories. We conclude that interlaboratory variability in designated collaborative studies may not be applicable to the conditions under which samples are regularly assayed by commercial laboratories.

Possible Sources of Variability

Several factors may explain the extraordinarily high within- and between-laboratory variability in total folate values for the non-enriched foods observed in this study. First, more rigorous extraction conditions might be necessary to release naturally occurring folates from their bound state, with optimal conditions varying among particular food matrixes. For example, several researchers have suggested that "tri-enzyme extraction" results in a more accurate evaluation of total food folate in various cereal-grain, high-protein, vegetable, and dairy products (15, 27–29). In addition to folate conjugase, the tri-enzyme treatment uses α-amylase for extraction of folate trapped in carbohydrate matrixes and protease for extraction of folate bound in protein matrixes. Tamura (30) reported that the optimal conditions of the tri-enzyme treatment, including pH and incubation time, may vary depending on the specific food matrix. In this study, all the laboratories except laboratory D used a single-enzyme extraction of food samples with chicken pancreas; laboratory D used a tri-enzyme extraction and reported total folate values with the greatest reproducibility (CV_W, 11.0%) when averaged across all food types. Interestingly, total folate values for dry macaroni and pizza, both high-starch foods containing predominantly fortified folic acid, were not substantially different among the 4 laboratories, only 1 of which used a tri-enzyme extraction. Paradoxically, the lowest mean total folate concentration in macaroni was reported by the facility using tri-enzyme extraction (Table 2). Therefore, other assay variables, such as those listed in Table 1, seem to have a significant effect on assay performance across many types of foods.

Additionally, the complete conversion of folate to mono- or diglutamate form using conjugase is essential for accurate measurement of total folates because *L. rhamnosus* cannot utilize polyglutamates with more than 3 glutamic acid residues (31). Naturally occurring folates often exist in polyglutamate form, so incomplete deconjugation of folates could explain high within- and between-laboratory variability and a lack of accuracy of total folate concentration that is dependent on the specific food matrix. Insufficient deconjugation of the natural folate polyglutamate chains is one potential explanation for the relatively low total folate

Table 5. Growth response of *Lactobacillus rhamnosus* to several folate vitamers relative to folic acid as reported in the literature

	Growth response (%, relative to folic acid)		
Folate vitamer	Goli (39)	Reingold (40)	Martin (38)
H_2 folate	29	75	26
H_4 folate	15	44	15
5-CH_3-H_4 folate	83	87	44
5-CHO-H_4 folate	115	55	58
10-CHO-H_4 folate	95	—	—

concentrations reported by laboratory C for all foods with the exception of macaroni and pizza (Table 2). The macaroni and pizza products contain the majority of their total folate as added folic acid in monoglutamate form and, therefore, the lack of a sufficient deconjugation treatment would not affect the accuracy of these results. No information on conjugase activity was supplied by any of the laboratories during this study.

All laboratories reported use of a folic acid standard to create the calibration curve, which may also bias the method when quantifying naturally occurring folates. Although it should theoretically be possible to detect multiple forms of folate with the microbiological assay, there is evidence that the test organism may not respond to the same degree for all folate vitamers, though it has been generally assumed that growth response is the same for the various folate monoglutamate derivatives (32, 33). *L. rhamnosus* has remained the preferred test microorganism for many years because it exhibits a growth response to most metabolic forms of folate found in biological systems (29). However, the ability of *L. rhamnosus* to respond equally on a molar basis to natural monoglutamate forms of folate is questionable. Phillips and Wright (34) demonstrated that the growth response of *L. rhamnosus* to 5-CH_3-H_4 folate was poor relative to folic acid when the folate concentration was 0–1 ng/10 mL assay and the initial pH was 6.8. Wright and Phillips (35) also found that at levels below 2 ng/mL assay the reduced growth response of *L. rhamnosus* to 5-CH_3-H_4 folate was due to a decrease in the log-phase growth rate relative to an equal amount of folic acid. Furthermore, a European Commission intercomparison study found that total folate was underestimated by microbiological assay using *L. rhamnosus* if pH 6.7 was used for assay growth in samples with a high proportion of methyl-folate present, such as Brussels sprouts and mixed vegetables (36). Newman and Tsai (37) also reported differences in growth response between folic acid, 5-CH_3-H_4 folate, and 5-CHO-H_4 folate. Table 5 shows several additional studies of the growth response of *L. rhamnosus* to dihydrofolate (H_2 folate), H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, and 10-CHO-H_4 folate relative to folic acid (38–40). Because the growth response of *L. rhamnosus* for different folates is variable, the microbiological assay using this microorganism would be expected to yield inaccurate results for food samples containing a mixture of natural folates. The degree of error, either an under- or overestimation of total folate, would likely be dependent on the individual folate forms present in each unique food matrix.

Another potential cause of reported total folate inaccuracy is the assumption that the test microorganism has an absolute requirement for folate vitamers. Any stimulation or inhibition of *L. rhamnosus* growth by compounds other than folates in sample extracts would invalidate the assay for determining folate levels in foods. Thymidine, amino acids, purines, and pyrimidines were reported substances that may affect the growth of *L. rhamnosus* when there is no folic acid present (41). There has been little concern about the influence of nonfolate compounds on the bacterial growth response, even though this supposition has not been refuted (41, 42).

Many other factors may contribute to method variability such as incubation time, sample storage and preparation conditions, incubation temperature, pH, growth medium, sterilization procedures, and inoculation volumes (41), and these parameters differed among facilities (Table 1). The microbiological assay requires an additional incubation time for growth of *L. rhamnosus* compared to LC methods, and some oxidative and thermal degradation of labile folates, especially H_4 folate, could occur during this period. Folic acid and natural folate vitamers have been shown to degrade considerably in the presence of oxygen upon heating (43, 44). The use of ascorbic acid as an antioxidant at different levels in folate extraction buffers was another factor that may have resulted in increased variability among laboratories. Vahteristo et al. (10) reported that H_4 folate, 5-CH_3-H_4 folate, 5-CHO-H_4 folate, and folic acid had greater stability with a combination of 0.5% ascorbic acid and 20mM mercaptoethanol than with 1.0% ascorbic acid alone at pH 4.9. It is uncertain whether all laboratories performed pH adjustment of the extraction buffer for each type of food sample, which may impact folate stability or the growth rate of *L. rhamnosus*.

It is important to note that the results with the lowest variability among laboratories in this study were for folic acid-enriched foods (macaroni and pizza, which included enriched flour), as might be expected since the microbiological methods were originally developed and validated for the measurement of added folic acid. Gregory et al. (45) indicated good agreement between results of *L. rhamnosus* assays and LC analysis for breakfast cereal and infant formula products, whereas significant differences were observed for cabbage. De Souza and Eitenmiller (28) reported fairly good agreement in total folate values between microbiological and radioassay methods for infant formula, but poor agreement for several baby foods. This result may be expected because the main form of folate in infant formula and cereal is added folic acid, which is suitable for the microbiological assay as it was validated, whereas a mixture of natural folates is present in cabbage and baby foods.

Another collaborative study using AACC method 86-47 reported the between-laboratory variability (CV_B) of 16 fortified cereal-grain samples ranging from 2 to 22%, but found considerably higher variability (28–53%) for 4 unfortified samples (14).

The internal quality control materials for each laboratory, presented in Table 1, are inadequate for monitoring total folate content in different food matrixes. All controls are folic acid-fortified foods and would not be expected to reflect errors in total folate content for foods containing natural folates. The effects of different assay conditions on total folate determination may depend on characteristics of the specific food matrix; thus, if control materials assayed in parallel do not share these same characteristics, incorrect values may go undetected.

Implications for Users of Commercial Folate Analysis

The results of this study suggest that data from commercial analysis of total folate in unfortified foods should be viewed with caution in terms of accuracy and precision. Contrary to what typical clients may believe, some of the largest and most popular commercial laboratories do not strictly follow the new AOAC tri-enzyme methods for folate analysis. In 2003, the total folate method with tri-enzyme extraction was recommended (46), yet only 1 out of 4 laboratories was using that procedure when this study was conducted. An international interlaboratory study of food folate published in 2004 also showed that of 26 participants (primarily university and government laboratories) tri-enzyme extraction was used in only 9 (26).

All laboratories reported using a modification of the AOAC official method. Method details are not customarily provided by commercial laboratories and, when requested in this study, they were incomplete, not specific, and difficult to obtain. Consequently, differences among procedures all referred to as "total folate by microbiological assay" are not readily apparent to clients. Furthermore, validation studies are apparently not conducted on a matrix-specific basis, and control samples are often enriched cereal products even when test samples are nonenriched foods. Results from the typical single replicate analysis performed in the commercial laboratory scheme may be subject to gross error, and the large uncertainty in test sample results is not apparent from data for internal control samples, which are fortified foods. Clients who are not trained analytical chemists are particularly susceptible to false assumptions about the accuracy and precision of values reported by commercial laboratories, and trust the laboratory to conduct quality control analysis and to use methods with demonstrated validity. Users of commercial folate analysis can increase the quality of results by determining that the lab is using the AACC/AOAC microbiological method with tri-enzyme extraction (46, 47), submitting samples for replicate analyses and including matrix-matched control samples in every assay batch. However, total folate values determined by microbiological methods nonetheless do not provide the specificity of individual forms of folate required to calculate DFE, the unit of measurement used for the folate DRI (3, 4). The overall accuracy and precision of results will remain questionable in the absence of matrix-specific assay validation.

Total folate values in U.S. food composition tables (48) are primarily generated by microbiological assay using a tri-enzyme extraction as part of the analytical procedure (27). In the past, food composition tables have been criticized for including inaccurate (low) folate values based on data generated without the tri-enzyme extraction (3). The present study suggests that other sample handling and/or assay variables besides the tri-enzyme extraction, such as those known to have differed among laboratories in this study (Table 1), have a significant impact on the determination of total folate in foods and deserve attention. The effect of these conditions has not been systematically investigated or optimized for individual food matrixes, nor are these parameters routinely disclosed by commercial laboratories.

The results of this study indicate a need for AOAC partners to work together to review the state of folate methodology used at commercial laboratories, especially as applied to determination of intrinsic food folates. Accurate quantitation of naturally occurring folates is extremely vital in countries that do not enrich their food supplies with folic acid. Even in countries with folate-enriched foods, individuals who are following low-carbohydrate diets are at risk of being folate deficient if the diet is also low in fruits and vegetables and other sources of naturally occurring folate. LC (8–11) and LC/MS (49–51) methods hold promise for development as a standard method for intrinsic food folates but would still require rigorous standardization, validation, and monitoring to be successfully applied at production scale analytical laboratories.

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