Interlaboratory Comparison of Food Folate Concentrations Determined by Microbiological Analysis

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Objective

The goal of this study was to evaluate inter- and intra-laboratory variation in total folate determined in a variety of food matrices by microbiological analysis at several commercial and/or university laboratories.

Samples and Sample Preparation

- Six foods and three commercially available reference materials were chosen to represent matrices varying in concentrations of folate, fat, starch, protein and pH.
- All samples were purchased locally (Blacksburg, VA, USA). Table 1 summarizes the foods, sampling and food preparation methods.
- After preparation, samples (except juices) were immediately frozen in liquid nitrogen and homogenized using a Blixer food processor (Robot Coupe®). Juices were blended thoroughly with a stainless steel whisk.
- Immediately after preparation and homogenization the samples were dispensed among 60 ml glass jars protected from light by foil, blanketed with nitrogen if not homogenized in liquid nitrogen, sealed with Teflon® lined caps and stored at -60 ± 5 °C until they were shipped for analysis.
- Three reference materials (BCR 485: Lyophilized Mixed Vegetables, BCR 487: Lyophilized Pigs Liver and BCR 121: Wholemeal Flour) certified for total folate concentration[1] were purchased from RT Corporation (Laramie, WY). These samples were shipped frozen and were placed in a -60 ± 5 °C freezer upon receipt. Each reference material was thawed and aliquoted in the same manner as the six food samples.

Food	Preparation and Compositing
Cooked Pinto Beans	Dried beans (mature seeds) were washed with tap water for 1 minute followed by distilled-deionized water for 1 minute. 4 lbs of beans were then cooked according to the quick soak directions on the package.
Frozen Meat and Vegetable Pizza	**Two frozen supreme pizzas were cooked according to the package instructions, then cut into ~1-inch pieces.**
Fresh Strawberries	Four packages of strawberries were rinsed for 1 minute with tap water followed by 1 minute with distilled-deionized water. The berries were dried with a lint free towel, then the caps were removed and any rotten or under-ripe berries were discarded. The berries were then cut into ~1-inch pieces.
Frozen Spinach	**Six packages were allowed to thaw on the benchtop for approximately 20 minutes. The partially frozen blocks were then cut into ~1-inch pieces.**
Orange Juice	Two quarts (opaque cardboard containers) of not from concentrate, pulp-free orange juice were purchased. Each quart was shaken for ~1 minute prior to opening.
Dry Macaroni	**The entire contents of one 5 lb box of enriched macaroni was used.**

Table 1

Laboratories, Methods and Analysis Schedule

- Samples were sent to three commercial laboratories and one university, referred to as laboratory A, B, C and D, respectively, for total folate analysis by microbiological assay.
- The samples were shipped frozen on dry ice via overnight delivery on four separate occasions with a separation of approximately one month.
- Each laboratory reported the use of a modified Association of Official Analytical Chemists (AOAC) method to determine total folate. Table 2 gives an overview of the similarities and differences between the standard methods for total folate determination at each laboratory.

Lab	Analyte/ Standard	Extraction Conditions: Buffer	pH	Enzyme	Antioxidant	Microorganism	Detection	QC Material/ Total Folate Acceptable Range
A	Folic acid	0.05 M Potassium phosphate	6.0	Chicken pancreas	0.15 % Ascorbic acid	*Lactobacillus casei* ATCC 7469	Turbidimetric at 680 nm	Infant formula 168 ± 27 µg/100g
B	Folic acid	Sodium phosphate dibasic	7.8	Chicken pancreas	1.0 % Ascorbic acid	*Lactobacillus casei* ATCC 7469	----------	Infant formula 115.1 ± 15.0 µg/100g
C	Folic acid	Sodium phosphate dibasic	6.8	Chicken pancreas	None	*Lactobacillus casei*	Turbidimetric at 600 nm	Cereal 1230 ± 120 µg/100g
D	Folic acid	1.0 M Sodium phosphate	7.8	Pronase (protease), α-amylase, chicken pancreas	Ascorbic acid	*Lactobacillus casei* ATCC 7469	Turbidimetric at 620 nm	Enriched flour 184 ± 14 µg/100g

Table 2

Results

Figure 1 compares each laboratory's mean total folate value according to food matrix. Tukey-Kramer multiple comparisons of laboratories were performed in a one-way ANOVA using the SAS system (version 8.02, SAS Institute, Cary, NC) for each food. Effects were considered significant when $p < 0.05$. Frozen pizza was the only food for which there was no significant difference across all laboratories. Laboratory D reported unexpectedly high values for total folate content in strawberries (69.5 ± 2.5 µg/100 g) and was significantly different from laboratories A, B, and C.

Figure 2 illustrates the European Commission (EC) certified ranges and each folate value from each laboratory (A, B, C, D) for the four shipments of certified reference materials. All of the individual total folate values by each laboratory for BCR 487 lie outside the EC certified ranges. Only two reported values for laboratory A and one value for laboratory B lie within the certified tolerance limits for BCR 485. Two values for laboratory D and one value for laboratories A and B, respectively, lie within the certified tolerance limits for BCR 121.

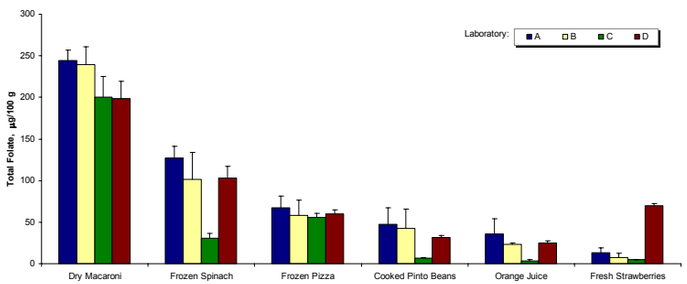
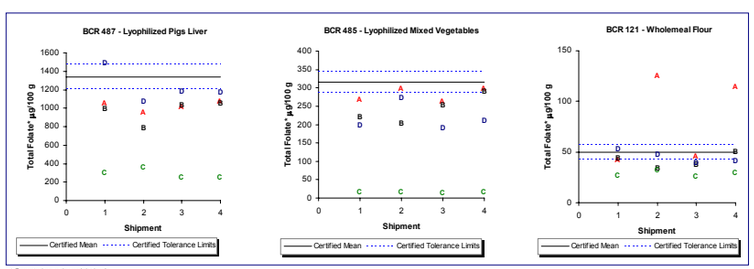


Figure 1



* Reported on a dry weight basis.

Figure 2

Within-laboratory variation of assayed total folate concentration in each food was evaluated using the coefficient of variation (CV). Large intralaboratory variability (CV >10 %) was evident at each laboratory for the majority of foods, and the mean CV at each laboratory also exceeded 10 % (Table 3). Laboratory D had a considerably smaller CV range across all foods with a high CV value of 16.8% for BCR 485. Dry macaroni and frozen pizza are unique since their CV by food (n=16) was 13.1 and 19.2, respectively, while the CV for the other food matrices were all greater than 40 %.

Food	Coefficient of Variation (%)				
	Lab A	Lab B	Lab C	Lab D	By Food
Cooked Pinto Beans	43.5	55.1	6.1	8.0	66.4
Frozen Meat and Vegetable Pizza	19.8	30.6	9.9	9.2	19.2
Fresh Strawberries	45.2	60.6	11.4	3.6	108.6
Frozen Spinach	11.8	32.0	19.0	14.4	45.2
Orange Juice	51.1	8.0	37.9	8.2	61.1
Dry Macaroni	5.1	9.2	12.5	10.7	13.1
BCR 485 (Lyophilized Mixed Vegetables)	6.9	16.2	9.5	16.8	58.3
BCR 487 (Lyophilized Pigs Liver)	5.1	13.1	19.5	14.8	43.9
BCR 121 (Wholemeal Flour)	54.2	18.1	9.6	13.4	58.9
Mean by Laboratory	27.0	27.0	15.0	11.0	

Table 3

Interlaboratory variation in total folate was further analyzed by the SAS system using the General Linear Models Procedure with the model, folate = food laboratory food*laboratory, then evaluating significant differences between individual laboratories using multiple comparisons with the Tukey-Kramer procedure at a p-value < 0.05. The laboratory effect was significantly different when averaged across all food types for total folate. The food*laboratory interaction was significant and analyzed by the simple effects procedure. Figure 3 displays the variability across different laboratories when using the slicing function in SAS by food type for total folate. BCR 485, BCR 487, and spinach were significantly different across laboratories. Figure 4 shows the Tukey-Kramer multiple comparisons of each laboratory's mean total folate value across all food types (significant differences are denoted by different italicized, lowercase letters). High intralaboratory variance for several foods at some laboratories impacts the ability to detect interlaboratory differences in some cases (Table 3).

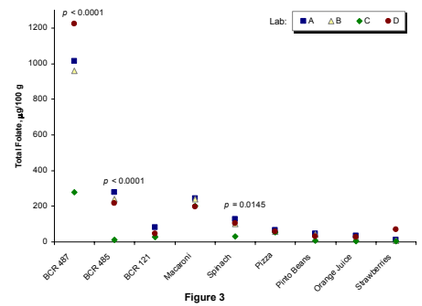


Figure 3

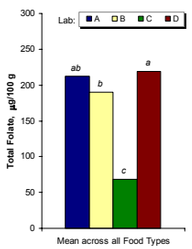


Figure 4

Discussion

Large variations in total folate concentration were observed in the same sample composites at different laboratories using microbiological assay. Dry macaroni and frozen pizza are the only two foods in this study that are fortified with folic acid. Naturally occurring folate in the other foods comprises primarily derivatives of folic acid (e.g. methyl-, formyl-) which are an integral part of the food matrix. Since variability was lower for the folic acid fortified foods, the wide variation in results for the other foods suggests the standard assay is not as reliable for naturally occurring folates. Variation was particularly notable in fruits and vegetables, which contain predominately 5-methyltetrahydrofolate. Additionally, values for the certified reference materials, none of which are fortified with folic acid, fell outside of the certified range at most laboratories (Figure 2).

A likely reason for interlaboratory variability among non-fortified foods is difference in assay parameters that affect the stability and/or extractability of the naturally occurring folates. Laboratory D is unique since it employs a tri-enzyme extraction of the food sample and it has considerably lower intralaboratory variability for most foods compared with laboratories that only use chicken pancreas enzyme. The lack of any antioxidant in laboratory C's standard method may have contributed to folate degradation and to the consistently low values for total folate content from this laboratory. Interestingly, the AOAC microbiological method for total folate[2] uses a folic acid standard to create the calibration curve, which may bias the method when quantitating naturally occurring folates. While the microbiological method should be able to quatitate multiple forms of folate, the test organism may not respond to the same degree for all folate vitamers. The folic acid fortified infant formula used as an internal quality control sample to monitor assay accuracy and precision at the labs (Table 2) would not be expected to reflect errors in folate results for non-fortified foods, which might explain why the results passed internal quality control checks.

Though more research is necessary to determine the causes of the observed discrepancies in folate quantitation in different foods, clearly the use of more definitive analytical methods such as HPLC and LC-MS for quantitation of naturally occurring folate in non-fortified foods should be pursued. Furthermore, caution should be exercised in interpreting microbiologically determined total folate values and multiple replicate analyses of any given sample should be performed to obtain a reliable estimate of the average concentration. Results of the single replicate analyses performed in the typical commercial laboratory analysis scheme appear to be subject to large errors for non-fortified foods.

References

1. European Commission, Community Bureau of Reference – BCR., Finglas, P.M., Scott, K.J., Witthoft, C.M., Van den berg, H., De-Froidmont-Gortz, I. (1998). *The certification of the mass fractions of vitamins in four reference materials: wholemeal flour (CRM 121), milk powder (CRM 421), lyophilized mixed vegetables (CRM 485), and lyophilized pigs liver (CRM 487).* Belgium: Office for official publications of the European communities.
2. *Official Methods of Analysis of AOAC International* (2000) 17th Ed., AOAC International, Gaithersburg, MD, USA, Official Methods 960.46, 992.05.

Acknowledgements

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