Comparison of Food Folate Concentrations Assayed by HPLC after Tri-Enzyme Extraction and Solid-Phase Extraction versus Affinity Chromatography

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Objective

To compare the concentration of 5-methyltetrahydrofolate (5MTHF) determined in a variety of foods at two different laboratories using similar HPLC methods but different sample preparation techniques, including affinity chromatography versus solid-phase extraction sample clean-up procedures.

Abstract

High-performance liquid chromatography (HPLC) provides a means of quantifying folate in foods with more specificity than the standard microbiological assay. Among laboratories using HPLC, differences exist in methods of sample extraction and clean-up. A key variable is the use of affinity chromatography or anion-exchange solid-phase extraction (SPE) for purification of extracts prior to HPLC. Affinity chromatography offers greater specificity, but SPE is less expensive and employs commercially available cartridges, making it attractive for routine applications. In this study, we compared the concentration of 5-methyltetrahydrofolate (5MTHF) assayed in a range of fresh produce samples in laboratories using different HPLC and sample preparation techniques.

For most foods, 5MTHF values were in excellent agreement (±6 %) and well within expected normal limits of analytical variability. For example, mean concentrations of 52 and 55 µg/100 g and 17 and 16 µg/100g were obtained for canned spinach and fresh potatoes, respectively, assayed by the two approaches. However, for some foods differences were evident. 5MTHF in broccoli assayed using affinity chromatography for sample clean-up was nearly half the concentration determined using SPE (28 vs. 53 µg/100 g), despite diode array spectra being consistent with 5MTHF. Our results suggest that SPE can be used reliably for most fresh produce samples, but some foods require more specific purification of the sample extract or mass spectrometry in conjunction with HPLC.

Samples and Sample Preparation

- Thirteen foods matrices were chosen to represent foods matrices with varying levels of folate concentration.
- Samples were purchased locally (Blacksburg, VA, USA), except russet potatoes, broccoli and bananas were sampled according to a statistical probability plan at various outlets in the U.S. for the National Food and Nutrient Analysis Program (NFNAP)[1].
- The foods were trimmed of inedible parts, cooked where specified, cut into pieces, quickly frozen in liquid nitrogen, then homogenized using a Blixer® food processor (Robot Coupe, USA, Ridgeland, MS). Canned spinach was homogenized without liquid nitrogen. Table 1 provides sample preparation details on each food matrix.
- Immediately after homogenization the samples were dispensed among 30 ml or 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 either analyzed or shipped for analysis.
- Samples were shipped overnight on dry ice to Netherlands Food and Consumer Product Safety Authority/ Inspectorate for Health Protection and Veterinary Public Health (VWA).

Food	Preparation and Compositing
Steamed Asparagus	~2 lbs of asparagus was rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The asparagus was steamed using distilled-deionized water, and the hard ends were removed as waste.
Steamed Cauliflower	**~3 lbs of cauliflower (2 heads) were rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The flouretes were then cut from the main stalk ~1 inch from where they branched off. The cauliflower was steamed using distilled-deionized water.**
Broccoli	Obtained from three outlets in the U.S. as part of the NFNAP sampling plan. The broccoli was rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water, then dried with a lint free wipe. The broccoli fluorettes were removed ~1 inch from the main stalk.
Brussels Sprouts	~3 lbs of brussels spouts were rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The brussels spouts were steamed using distilled-deionized water, and the hard ends were removed as waste.
Collard Greens	~4 lbs of greens were rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The leaves were dried with a lint free wipe and the hard stems were removed.
Meat and Vegetable Pizza	4 prepared supreme pizzas were purchased from two local (Blacksburg, VA, USA) vendors.
Russet Potatoes	The potatoes were scrubbed by hand for ~1 minute with tap water followed by ~2 minutes with distilled-deionized water, then dried with a lint free wipe. The potatoes were baked for 50 minutes on an oven rack.
Frozen Blackberries	Two entire bags (1 lb) of frozen blackberries were used.
Green Peppers	~3 lbs of peppers were rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The peppers were then dried with a lint free wipe and the top, seeds and bottom were removed.
Celery	~4 lbs of celery was rinsed for ~1 minute with tap water followed by ~1 minute with distilled-deionized water. The celery was then dried with a lint free wipe and the leafy top and white bottoms of the stalk were removed.
Canned Pinto Beans	3 cans of pinto beans were rinsed for ~1minute with tap water followed by ~1 minute with distilled-deionized water. The beans were spread out and dried with a lint free wipe until there was a minimal amount of water remaining.
Bananas	Removed ~1 inch from each end, removed peel, and chopped into ~1-inch pieces.
Canned Spinach (control composite)	Canned spinach with no salt added, liquids and solids were used.

Table 1

Methods

- The Food Analysis Laboratory Control Center (FALCC) at Virginia Tech assayed samples using HPLC with fluorescence detection after a tri-enzyme extraction and sample clean-up using strong anion exchange solid-phase extraction[2,3] (Table 2).
- The Netherlands Food and Consumer Product Safety Authority/ Inspectorate for Health Protection and Veterinary Public Health (VWA) assayed samples by HPLC with fluorescence detection after a tri-enzyme extraction but used affinity chromatography for sample clean-up[4] (Table 2).
- The analyses at FALCC and VWA were conducted on replicates of the same sample composites.

Method Component	FALCC	VWA
Sample Size Range	2-6 g sample was taken based on sample density and estimated folate content.	2-6 g sample was taken based on sample density and estimated folate content.
Sample Extraction	**0.1 M phosphate buffer with 10 mM ascorbic acid, 10 mM 2-mercaptoethanol, pH 6.0; homogenize sample in buffer with tissue homogenizer.**	**50 mM CHES- 50 mM HEPES buffer, containing 10 mM ascorbic acid and 10 mM 2-mercaptoethanol, pH 7.85; homogenize sample in buffer with Ultra Turrax apparatus.**
Tri-Enzyme Treatment	▪ Add 20 mg of α-amylase from *Asporigillus oryzae* (Sigma), degas sample 15 min at 37 °C followed by 45 min incubation at 37 °C. ▪ Add 1 mg of protease type XIV: Bacterial from *Steptomycesgriseus* (Sigma), incubate in shaking water bath for 3 hours at 37 °C. ▪ Deactivate enzymes by placing samples in boiling water bath for 15 minutes and then quickly cooling in an ice bath. ▪ Add 100 µl of rat plasma (Harlan Bioproducts) and incubate in shaking water bath for 14-16 hrs. at 37 °C.	▪ Adjust to pH 7.0 ▪ Take a portion of 2-8 gram extract (depending on folate content) and add 0.5 ml rat plasma, 50 µl Fungamyl (α-amylase, NOVO Nordisk) and 50 µl Flavourzyme (aminopeptidase, NOVO Nordisk). ▪ Mix and incubate in shaking water bath for 4 hours at 37 °C. ▪ Deactivate enzymes by placing samples in boiling water bath for 5 minutes and then quickly cooling in an ice bath. Centrifuge for 20 minutes at 5000 g.
Sample Clean-up	**▪ Used a strong-anion exchange solid-phase extraction column. ▪ Rinsed with folate extraction buffer listed above, loaded sample, and rinsed again with folate extraction buffer. ▪ Eluted sample with 1 M sodium chloride in 0.1 M phosphate buffer with 10 mM 2-mercaptoethanol, 25 % acetonitrile and 10 mM ascorbic acid.**	**▪ Transfer samples into columns filled with Folate Binding Protein (Scripps) coupled to Affi-Gel10 (Bio-Rad). ▪ Rinse columns with phosphate buffer 0.025 M, pH 7.0 containing 1 M NaCl and phosphate buffer 0.025 M, pH 7.0. ▪ Elute folates with 0.02 M trifluoroacetic acid-0.02 M dithioerytritol in tube containing 10 mM 2-mercaptoethanol and 10 mM ascorbic acid.**
HPLC Analysis	▪ Used Perkin Elmer (PE) Autosampler (ISS200), PE Binary Pump (250), with PE diode array detector (235C) (wavelengths 280 and 350 nm) and Shimadzu Fluorescence detector (RF-10AXL) (Excitation 290 nm, Emission 350 nm). ▪ Mobile Phase was a 30 mM potassium phosphate buffer, pH 2.2/acetonitrile using a gradient program. 5MTHF was quantitated using a calibration curve made from an external standard and run in duplicate with every assay batch.	▪ Used secondary gradient pump, autosampler adjustable to 2-4 °C, column oven adjustable to 20 °C, fluorescence detector (Excitation 280 nm, Emission 359 nm), diode array detector (Waters). ▪ Mobile Phase was a 33 mM phosphate buffer pH 2.1/acetonitrile using a gradient program. 5MTHF was quantitated using a calibration curve made from an external standard and run in duplicate with every assay batch.

Table 2

Each laboratory includes a commercially available reference material[5] (BCR 485: Lyophilized Mixed Vegetables) with each assay batch for quality control purposes. This reference material contains sweetcorn, tomatoes and carrots and has an indicative value for 5MTHF. The chart below shows the values received at both FALCC and VWA over the past two year period (Figure 1).

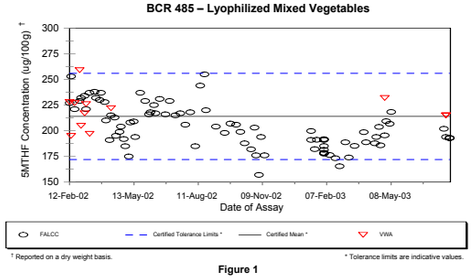

Figure 1

Results

The results were evaluated by relative comparison due to the low number of replicate analysis at each laboratory. In cases where more than one sample replicate was run at FALCC, ±2 × (the within laboratory standard deviation) is illustrated on the graphs in Figure 2 as an estimate of variation that can be expected within the FALCC laboratory. Canned spinach was used as the control composite at FALCC for each assay to monitor intralaboratory variability, therefore the mean for a total of 88 replicates was reported in Figure 2. Values of 5MTHF content in bananas were not plotted since FALCC reported <5 µg/100 g and VWA reported 0 µg/100 g.

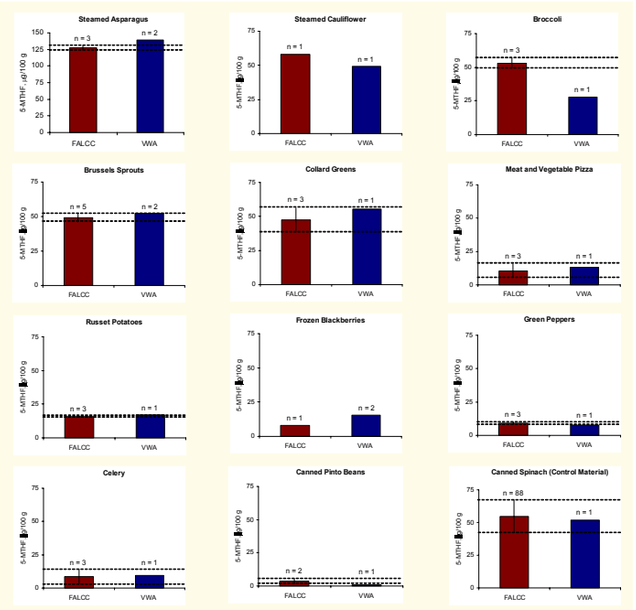

Figure 2

Discussion

5MTHF values from the two laboratories were in relative agreement within the limits of variability except for steamed asparagus (139.4 µg/100 g; 9.3 % difference) and broccoli (28.0 µg/100 g; 47.4 % difference). A second composite of broccoli was analyzed by the HPLC method at FALCC, and by LC-MS at the USDA Food Composition Laboratory (FCL, Beltsville, MD).[6] The 5MTHF values obtained by LC-MS (38.5 ± 1.8 µg/100 g) were close to the values found by FALCC (42.2 ± 7.6 µg/100 g) for this composite, therefore it may be possible to have interfering compounds present in different samplings of broccoli. Additional sampling and analysis would need to be completed to determine the source of the differences in the initial sampling of broccoli. A recent study using sample clean-up by SPE, HPLC-MS-MS, and deuterated isotopomers as internal standards to quantify folate vitamers also reported unexpectedly low folate content in broccoli[7].

Additional replicates of all the sample matrices must be analyzed at both labs and also by LC-MS to determine the statistical significance and reason for any apparent differences in this preliminary comparison. However, results from the two methods seem to be in excellent agreement except for isolated foods such as broccoli.

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Acknowledgements

This study was conducted as part of cooperative agreement #Y1-HV-8116-11 between the USDA Nutrient Data Laboratory and Virginia Polytechnic Institute and State University.