STABILITY OF 5-METHYLTETRAHYDROFOLATE IN FRESH FROZEN FRUITS AND VEGETABLES DETERMINED BY HPLC ANALYSIS.

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Objective

- The goal of the study was to determine how long 5MTHF was stable in fresh, frozen, homogenized produce in order to validate the sample preparation and analysis protocol used for determination of folate in fresh produce sampled for the National Food and Nutrient Analysis Program (NFNAP).

Samples and Sample Preparation

- Seven fruits and vegetables were chosen to give a broad representation of the types of produce analyzed in the NFNAP and were purchased locally (Blacksburg, VA). Other fresh produce was sampled according to a statistical probability plan at various outlets in the U.S.
- The fruits and vegetables were trimmed of inedible parts, cut into pieces, quickly frozen in liquid nitrogen, then homogenized using a Blixer® food processor (Robot Coupe, USA, Ridgeland, MS).
- The homogenized material was kept frozen in liquid nitrogen and dispensed among 2oz glass samples jars with Teflon® lined caps.
- Stored samples were kept at -60(±5)°C in darkness until the day of analysis.
- Samples were analyzed in triplicate immediately after homogenization, and then after storage for 2, 7 and 30days. Followed by analysis at approximately three month intervals for up to 1 year.

Analytical Method

Extraction from Sample Matrix

- Frozen samples were thawed in a 25±2°C water bath for 20 minutes immediately before analysis. Fresh material was analyzed immediately after homogenization.
- The amount of sample used for the assay was adjusted based on estimated folate content, to achieve a 5MTHF concentration of 80-200 ng/ml in the final dilution.
- The sample was further homogenized by adding 10ml extraction buffer (0.1M potassium phosphate, 10mM ascorbic acid, 10mM 2-mercaptoethanol, pH 6.0) and blending at high speed for 2 minutes with an Omni-Mixer® tissue homogenizer.
- The pH of the sample was adjusted to 6.0 using 4M NaOH when necessary.

Tri-enzyme Treatment

- α-amylase from *Aspergillus oryzae* (Sigma 56units/mg), (0.5ml of 40mg/ml) was added to each sample followed by a 1 hour incubation at 37°C water bath. For the first 15 minutes of the incubation the samples were degassed with argon.
- Protease from *Streptomyces griseus* (Sigma 5.7units/mg), (1ml of 1mg/ml) was added to each sample followed by a 3 hour incubation at 37°C.
- Samples were placed in a boiling water bath for 15 minutes to inactivate the enzymes.
- 0.1ml of rat plasma conjugase (Harlan Bioproducts) were added to each sample, followed by incubation at 37°C for 14-18 hours.
- Samples were placed in a boiling water bath for 15 minutes to inactivate the conjugase.

Recovery of Sample Extract

- The samples were centrifuged at 5500rpm (7280G) for 20 minutes. The supernatant was then decanted and the pellet was resuspended in 8ml of extraction buffer.
- Centrifugation was repeated and supernatants were combined.
- For each sample, the combined supernatants were filtered through a Büchner funnel using Whatman 43® ashless filter paper.

Solid-Phase Extraction

- Extract-Clean® strong anion exchange SPE cartridges (Alltech, Deerfield, IL) were activated with 15ml of extraction buffer.
- Sample extract was loaded onto the column.
- Column was washed with 15ml extraction buffer.
- 5MTHF was eluted from the column using 1M NaCl in 0.1M potassium phosphate buffer with 10mM 2-mercaptoethanol, 10mM ascorbic acid and 25% acetonitrile ("SPE elution solvent").
- Acetonitrile was evaporated from the sample by bubbling argon through it for 30 minutes at 50°C.

Adjustment of Final Sample Concentration

- Final dilution volume (10-50ml) was determined based on estimated total folate content of the sample to yield final 5MTHF concentration within working HPLC calibration range (10-200 ng/ml).
- The sample was quantitatively transferred to the appropriate sized volumetric flask, diluted to volume with extraction buffer and mixed.
- 1ml of the diluted extract was transferred to an amber HPLC autosampler vial.

HPLC Analysis

- Samples were analyzed by HPLC using an Adsorbosphere™ HS C18 150mm x 4.6mm 3u column (Alltech, Deerfield, IL), Phosphate buffer/Acetonitrile mobile phase, gradient elution from 100% phosphate buffer (pH 2.2) to 30% acetonitrile with a flow rate of 0.8ml/min.
- Six levels of 5MTHF standards ranging from ~10ng/ml to ~200ng/ml were run in duplicate with each batch of samples.
- Data were recorded on a fluorescence detector with an emission wavelength of 290nm and an excitation wavelength of 350nm, also on a diode array detector at 280nm and 350nm.

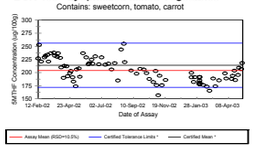
Reporting Data

- Identity of 5MTHF peak in each sample was found to be either consistent or inconsistent by comparing the spectral scan of the sample to that of a standard of similar concentration.
- Fluorescence detector chromatograms were used for all quantitation.
- Quantitation of each peak required having >75% peak resolution and being within ±0.1 minute of the retention time of the standard.

Method Validation and Quality Control

- Analysis of BCR 485 reference material (lyophilized mixed vegetables) (RT-Corp, Laramie, WY) provided indicative values for 5MTHF. (Fig 1)
- Inter-assay precision was monitored with an in-house quality control composite of canned spinach, which was analyzed with each batch of samples. The stability of this composite was also monitored and a decrease in 5MTHF was observed after one year of storage. (Fig 2)
- Selected composites were additionally analyzed at the USDA Food Composition Laboratory by HPLC-mass spectrometry using an isotope dilution of $^{13}C_5$-glutamyl-5-MTHF. (Pawlosky & Flanagan 2001; Pawlosky etal., 2001) Good correlation was found between the results from the two laboratories with the exception of broccoli. (Table 1)
- Homogeneity of each composite was verified through moisture analysis which showed no significant difference (p>0.3) between sub-samples of at least 2 grams (the minimum aliquot used for 5MTHF analysis).
- The practical limit of quantitation (LOQ) was ~3µg/100g and was dependent on the sample matrix and folate content. (i.e. small amounts of interfering compounds could inhibit quantitation for samples with low levels of folate, and large interference can inhibit quantitation at any folate level).

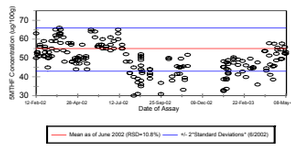
BCR 485 - Lyophilized Mixed Vegetables
Contains: sweetcorn, tomato, carrot



Mean	204
SD	21.4
RSD	10.5

Figure 1

Canned Spinach Control Composite



Mean	55
SD	5.9
RSD	10.8

Figure 2

Composite Matrix	Average 5MTHF content µg/100g	Average 5MTHF content by HPLC-MS µg/100g
Spinach II	87	89
Broccoli II	41	35
Russet Potatoes II	10	10
Strawberry II	20	24
Strawberry III	15	22

Table 1

Stability of 5MTHF

- 5MTHF concentration in a given composite at the time it was prepared ($5MTHF_0$) was compared to 5MTHF concentration after the maximum storage time ($5MTHF_f$). Confidence intervals were calculated for the difference between 5MTHF in the test and control composites at each of time zero and final storage time ($\Delta 5MTHF_0$ and $\Delta 5MTHF_f$, respectively), as ±1.96 times the estimated standard error (SE). SE was a pooled estimate of variance calculated from the between- and within-assay variance. Because between-assay analytical variance for the test composites could not be separated from actual change in 5MTHF concentration which was the subject of study, data for the first 39 assays of the canned spinach control material were used to estimate analytical variance.

Spinach

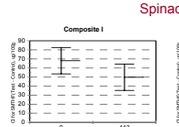


Figure 3

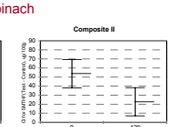


Figure 4

Navel Oranges

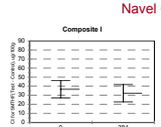


Figure 5

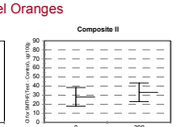


Figure 6

Russet Potatoes

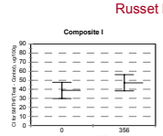


Figure 7

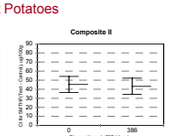
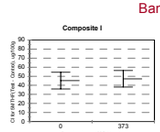


Figure 8

Bananas



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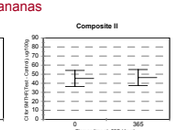
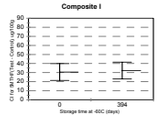


Figure 10

Strawberries



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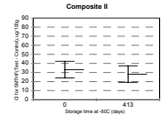

Figure 12

Broccoli

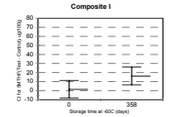

Figure 13

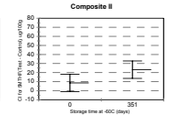

Figure 14

Red Delicious Apples

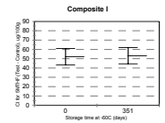

Figure 15

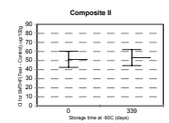

Figure 16

Data from National Food Nutrient Analysis Program Samples and Other Local Produce Samples

- Samples were analyzed from the NFNAP within one year of the compositing date. All NFNAP composites were made from samples obtained from three outlets in the same region of the country. There are four possible regions which consisted of the following states; Pennsylvania, New York and New Jersey; Missouri, Tennessee and Arkansas; Texas and Illinois; California, Oregon and Washington. In cases where all four regions were analyzed the values are averaged and reported as national. Selected fruits and vegetables were obtained locally (Blacksburg, VA) to determine baseline data for the sample matrix.

Sample Matrix	Composite Type	Average 5MTHF µg/100g	n
Asparagus, Cooked	Local	114	1
Asparagus, Cooked	Regional	117	2
Bananas	Regional	<10	3
Beets	Local	46	3
Bok Choy	Local	46	3
Broccoli	Regional	45	3
Brussels Sprouts	Local	50	2
Celery	Local	9	3
Clementines	National	12	12
Collards	Local	48	3
Dates	Regional	<10	1
Green Cabbage	Local	17	3
Green Leaf Lettuce	Local	36	6
Green Peppers	Local	9	3
Pinto Beans, Cooked	Regional	<10	2
Pinto Beans, Dried	Regional	<10	2
Prunes	Regional	<10	1
Red Cabbage	Local	32	3
Red Potatoes	Regional	14	1
Romaine Lettuce	Local	35	6
Strawberries	Regional	17	3
Sweet Onion	Regional	8	1
Swiss Chard	Local	61	6

Table 2

Conclusions

- A validated assay for quantitation of 5-methyltetrahydrofolate (5MTHF) in fresh fruits and vegetables was developed.
- 5MTHF was characterized in a wide range of fresh produce types, with content varying from <10µg/100g to >100µg/100g.
- Within the limits of assay precision, 5MTHF was stable in the fruits and vegetables tested over one year in storage (-60°C/nitrogen/darkness).
- The results validate the sample preparation and analysis protocol used for determination of folate in fresh fruits and vegetables in the National Foods Nutrient Analysis Program.

Acknowledgments

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Pawlosky, R. J. & Flanagan, V. P. (2001). A quantitative stable-isotope LC-MS method for determination of folic acid in fortified foods. *Journal of Agricultural and Food Chemistry, 49*(3), 1282-1286.