9998 *J. Agric. Food Chem.* **2006,** *54,* 9998–10002

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Difference in Folate Content of Green and Red Sweet Peppers (*Capsicum annuum*) Determined by Liquid Chromatography–Mass Spectrometry

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Folic acid (pteroylmonoglutamic acid) is used in enriched foods; however, very little folic acid occurs naturally in fruits and vegetables. For the U.S. Department of Agriculture's National Food and Nutrient Analysis Program, a number of fruits and vegetables have been assayed for endogenous folates, by a liquid chromatography−mass spectrometry method, to evaluate the accuracy of existing data for total folate determined by standard microbiological analysis. Folate in red and green sweet peppers (*Capsicum annuum*) differed notably (70.2 and 20.7 μg/100 g, respectively) and exceeded existing values determined by microbiological assay (18 and 11 μg/100 g, respectively). 5-Methyltetrahydrofolate was the predominant vitamer, but a significant amount of 5-formyltetrahydrolfolate and some 10-formylfolate were present. These findings may assist in making dietary recommendations or developing research diets related to folate. The data from this study have been used to update the folate values in release 19 of the USDA Nutrient Database for Standard Reference.

KEYWORDS: Folate; red peppers; green peppers; *Capsicum annuum*; LC-MS; 5-methyltetrahydrofolate; food composition; HPLC

INTRODUCTION

Folate is the general term for the B-group vitamin (vitamin B9) sharing the basic 4-[(pteridin-6-ylmethyl)amino]benzoic acid (pteroylglutamic acid) structure (*1*). It is generally accepted that adequate folate intake is important in reducing the occurrence of fetal neural tube defects, and furthermore it may play a significant role in reducing the risk of several human disorders including cardiovascular disease, thromboembolitic processes, colon cancer, and neuropsychiatric disorders (*2*−*6*). Recently, antioxidant activity of folates has also been reported (*7*).

Folic acid (pteroylmonoglutamic acid) is the form of folate used in enriched foods; however, very little folic acid occurs naturally in fruits and vegetables (*8*). The most common naturally occurring food folates are 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolate, 10-formyldihydrofolate, and tetrahydrofolate (*8*), which exist as polyglutamates. Pfeiffer et al. (*9*) reported on the analysis of food folates in cereal grain foods and found that 5-methyltetrahydrofolate (5MTHF) represented approximately 22% of the total folate in white bread but only about 35% of the folate in white rice. Other forms of folate, including folic acid, were found in varying amounts. As a group, fruits and vegetables, especially dark leafy greens, are the richest source of natural folate, and 5MTHF is the major vitamer (*8*, *10*). Although foods enriched with folic acid remain a primary source of dietary folate in the U.S. and other developed countries, in the absence of enriched foods or dietary supplements, as in underdeveloped countries or in very low carbohydrate diets (*11*), foods containing endogenous folate are the sole source of this vitamin. Furthermore, folic acid itself is not metabolically active and must be methylated before it is used by the body in single-carbon metabolism, including DNA methylation and conversion of homocysteine to methionine (*2*). For certain individuals possessing genetic polymorphism in the enzyme tetrahydrofolate reductase (*2*, *12*, *13*), intake of naturally occurring folates may be particularly important, and accurate food composition data for these vitamers is necessary to facilitate research in that area.

The current standard method by which most nutrient database values for folate have been generated is a microbiological assay (*14*−*16*). This assay has been shown to yield unreliable and often inaccurate results for naturally occurring folate in many matrices (*17*, *18*). As part of the USDA's National Food and Nutrient Analysis Program (*19*), a number of fruits and

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10.1021/jf062327a CCC: $33.50
Published on Web 11/29/2006

vegetables have been assayed for 5MTHF by a liquid chromatography−mass spectrometry method (LC-MS) to obtain accurate food composition data and enable evaluation of the accuracy of existing values for total folate determined by microbiological analysis. During this process, red and green sweet peppers (*Capsicum annuum*) were found to differ notably in folate content. The purpose of this paper is to highlight the interesting findings for peppers, for consideration in developing food choice recommendations in dietary practice and further research on dietary folates.

MATERIALS AND METHODS

Reagents and Standards. 5-Methyltetrahydrofolic acid disodium salt, (6*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid calcium salt (5FTHF), tetrahydrofolic acid (THF), 10-formylfolic acid (10FFA), (6*R*,*S*)-5-methyl-5,6-dihydrofolic acid ammonium salt (5MDHF), (6*R*,*S*)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride (5,10-MTHF), 10-methylfolic acid (10-MFA), and 7,8-dihydrofolic acid (7,8-DHF) were purchased from Schirck's Laboratories (Jona, Switzerland).

Reagents and solvents were ACS reagent or HPLC grade. Potassium phosphate, 2-mercaptoethanol, L-ascorbic acid, and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, orthophosphoric acid (∼85%), sodium hydroxide, sodium chloride, and 2-octanol (laboratory grade) were purchased from Fisher Scientific (Pittsburgh, PA). Protease type XIV (*Streptomyces griseus*, 5.7 units/mg) and α-amylase (*Aspergillus oryzae*, 56 units/mg) were also from Sigma. Rat plasma (nonsterile, adult male, with lithium/heparin anticoagulant) was obtained from Harlan Bioproducts (Indianapolis, IN). The plasma was centrifuged at 5500 rpm (7280*g*) for 20 min at 4 °C.

Sample Preparation. Red and green sweet bell peppers (*Capsicum annuum*) were collected from retail outlets in the United States according to a nationally representative statistical sampling plan (*21*). Briefly, four composites of each product were prepared, each comprising a sample of 0.5−1.5 kg of peppers procured from each of 12 retail outlets in four regional sampling areas across the U.S (*21*). For each type of pepper, 350 ± 50 g of peppers from each sample was combined into each of four composites, according to statistical sampling regions, as follows. Peppers were rinsed with distilled deionized water, blotted dry, trimmed of stem, core, and seeds, cut into pieces (approximately 1.5 cm), then immediately frozen and homogenized in liquid nitrogen by use of a 6L industrial food processor (Robot Coupe USA, Jackson, MS). Each composite contained approximately 1−1.2 kg edible portion and was distributed among 60-mL glass jars with Teflon-lined closures and stored at −60 ± 5 °C, in darkness, until analyzed. Note that in addition to the folate analyses described here, these samples were analyzed for proximates, minerals, and vitamins, as well as proanthocyanidins (*23*, *24*), total antioxidant capacity (*25*), and choline (*26*), and the data were used to update values in USDA's National Nutrient Database for Standard Reference (*22*).

Extraction and Analysis of 5-Methyltetrahydrofolate. Folate was extracted and 5MTHF was analyzed as previously reported (*20*). Briefly, samples were homogenized in phosphate buffer (0.1 M, pH 6.0) and treated with a trienzyme procedure involving α-amylase, protease, and rat plasma conjugase, then extracts were purified by anion-exchange solid-phase extraction and diluted to 25 mL with extraction buffer. 5MTHF was quantified in extracts by LC-MS, as described below, with a 6-point external calibration curve of 1 to 80 ng/ mL.

For quality control of 5MTHF analysis, a commercially available reference material, Lyophilized Mixed Vegetables (CRM485) (*27*), supplied by the Institute for Reference Materials and Measurements (Geel, Belgium) and purchased from Resource Technology Corp. (Laramie, WY) and/or a well-characterized canned spinach in-house control material (*20*) was included in each assay batch to monitor precision and accuracy. Each composite sample was assayed in triplicate for 5MTHF, with one replicate being in a separate assay batch to account for any matrix-specific run-to-run analytical variability. The stability of 5MTHF in stored samples was previously demonstrated (*20*).

Table 1. Parent Ion and Product Ion Transition for Folates Analyzed by LC-MS in Multiple Reaction Monitoring Mode

analyte[a]	parent ion (amu)	product ion (amu)	collision energy (eV)
10-FFA	469.90	295.00	21
10-MFA	456.00	309.00	27
5,10-MTHF	456.10	412.10	20
5-FTHF	474.10	327.07	18
5MTHF	460.20	313.13	27
5MDHF	458.10	180.00	18
7,8-DHF	444.00	178.00	37
THF	446.40	299.08	10

[a] 10FFA, 10- formylfolic acid; 10-MFA, 10-methylfolic acid; 5,10-MTHF, (6*R*,*S*)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid; 5FTHF, (6*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid; 5MTHF, 5-methyltetrahydrofolic acid; 5MDHF, (6*R*,*S*)-5-methyl-5,6-dihydrofolic acid; 7,8-DHF, 7,8-dihydrofolic acid; THF, tetrahydrofolic acid

Analysis of Other Folates. Because a substantial difference in 5MTHF content was found in all composites of red versus green peppers, additional analyses were conducted to screen for the presence of other folate vitamers, to determine if the presence of additional forms of folate might explain the observed differences. Due to the potential instability of a number of folate vitamers, these analyses were performed on separate, freshly prepared composites of locally purchased peppers. Approximately 1.2 kg (6 peppers) each of green and red bell peppers were prepared and homogenized as described above (see Sample Preparation), and aliquots were taken for folate extraction while the composite remained frozen in liquid nitrogen. Three 2-g subsamples from each composite were extracted as described above, and each final extract was distributed in 1-mL portions among eight autosampler vials. Seven vials from each extract were frozen at approximately −15 °C, and analysis of 5FTHF and THF in one set, along with two standard mixtures in duplicate, one containing 4 ng/mL and one containing 20 ng/mL each of 5FTHF and THF, was completed by LC-MS within 6 h. The remaining samples (along with duplicate standards) were analyzed by LC-MS as described below, except the autosampler temperature was maintained at 5 °C, using in each case a set of extracts and duplicate standards thawed together at room temperature for approximately 20 min in separate runs for the following components: 7,8-DHF, 10FFA, 10MFA, 5MTHF, 5MDHF, and 5,10-MTHF. Each LC-MS run included standards at known concentrations [4 and 20 ng/mL for all but 5MDHF (40 and 80 ng/mL)], which were used to estimate analyte concentrations in samples. Each of these LC-MS runs was completed in 6−10 h.

LC-MS. All chromatographic separations were performed with a Luna C18 column (150, 2.1 mm, 4 μm dp; Phenomenex, Torrance, CA). In each analysis 20 μL of each solution was injected onto the column by use of a Thermo Survey (San Jose, CA) autosampler 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 as follows: 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 postrun equilibration).

The HPLC column effluent was pumped directly without any split into a Thermo Instrument TSQ triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with ESI source, which was used in positive ion MS−MS mode. The instrument was calibrated with a solution of polytyrosine according to the manufacturer recommendation. Tuning was performed for each analyte of interest by direct infusion of standard solution (1 ng/μL) at a rate of 10 μL/min. **Table 1** shows the tuning parameters for each analyte. Also, **Table 1** shows the parent ion and product ion transition for each analyte when operated with multiple reaction monitoring (MRM) mode.

Statistical Analysis. Analysis of variance was performed with the SAS System for Windows (version 8.02; SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Quality Control. Results for CRM485, Lyophilized Mixed Vegetables, were within the indicative range for 5MTHF on

Table 2. Folate Composition of Sweet Peppers (μg/100 g)

component	green sweet pepper, raw (NDB 11333)[a]	red sweet pepper, raw (NDB 11821)[a]
5-methyltetrahydrofolate (μg/100 g)[b]	9.5 (1.02) A	42 (5.8) A
5-formyltetrahydrofolate (μg/100 g)[c]	11 (2.7) B	28 (3.8) B
10-formylfolate (μg/100 g)[c]	0.2 (0.05)	0.2 (0.2)
total folate,[d] DFE[e] (μg/100 g)	20.7	70.2
total folate per serving[f] (DFE,[e] μg/100 g)	9.5	32.3
RDA[g] per serving (%)	2.4	8.1

[a] Mean values with different capitalized letters within each row differ significantly ($p < 0.001$). NDB, product identification number from the USDA Nutrient Database for Standard Reference (*22*). [b] Mean, with standard deviation in parentheses, for $n = 4$ composites representing four U.S. statistical sampling regions (*21*). [c] Estimated concentration based on analysis of local samples, with standard deviaiton of 3 analytical replicates of one original sample, in parentheses. [d] The following components were assayed but not detected (<0.2 μg/100 g) in all samples: tetrahydrofolic acid, 5-methyldihydrofolic acid, 5,10-methenyl-5,6,7,8-tetrahydrofolic acid, 10-methylfolic acid, and 7,8-dihydrofolic acid. [e] DFE, dietary folate equivalents; 1 DFE = 1 μg of folate from food (not added as folic acid) (*29*). [f] A serving is $^1/_2$ cup (120 mL), sliced; 46 g. [g] RDA, recommended dietary allowance, 400 μg/day for adults in the United States (*29*).

the certificate of analysis [172−256 μg/100 g; Finglas et al. (*27*)], with a mean of 231 μg/100 g (standard deviation 8.0, n = 4). The in-house spinach control values were also within tolerance limits on all runs with a mean of 44.4 μg/100 g (standard deviation 9.1, n = 6).

Folate Composition of Peppers. Table 2 summarizes the 5MTHF content of the national composites of red and green sweet peppers, and the other folates found in the experiment on locally procured peppers. **Figure 1** shows representative chromatograms for 5MTHF, 5FTHF, and 10FFA in red and green peppers. The mean 5MTHF content for the four national samples of each product were 42 and 9.5 μg/100 g for red and green peppers, respectively, and differences were significant ($p < 0.00001$). The 5MTHF content of the locally procured peppers agreed with those for the national composites, with clear differences again observed between red and green peppers. The only other folates found at >0.2 μg/100 g were 5-formyltetrahydrofolate (5FTHF) and 10-formylfolate (**Table 2**). Values for 5FTHF were again higher in red compared to green peppers. The following folates were assayed but not detected (<0.2 μg/100 g) in all samples: tetrahydrofolic acid, 5-methyl-5,6-dihydrofolic acid, 5,10-methenyl-5,6,7,8-tetrahydrofolic acid, 10-methylfolic acid, and 7,8-dihydrofolic acid.

The very same national composites (**Table 2**) had previously been analyzed for "total folate", using a standard microbiological

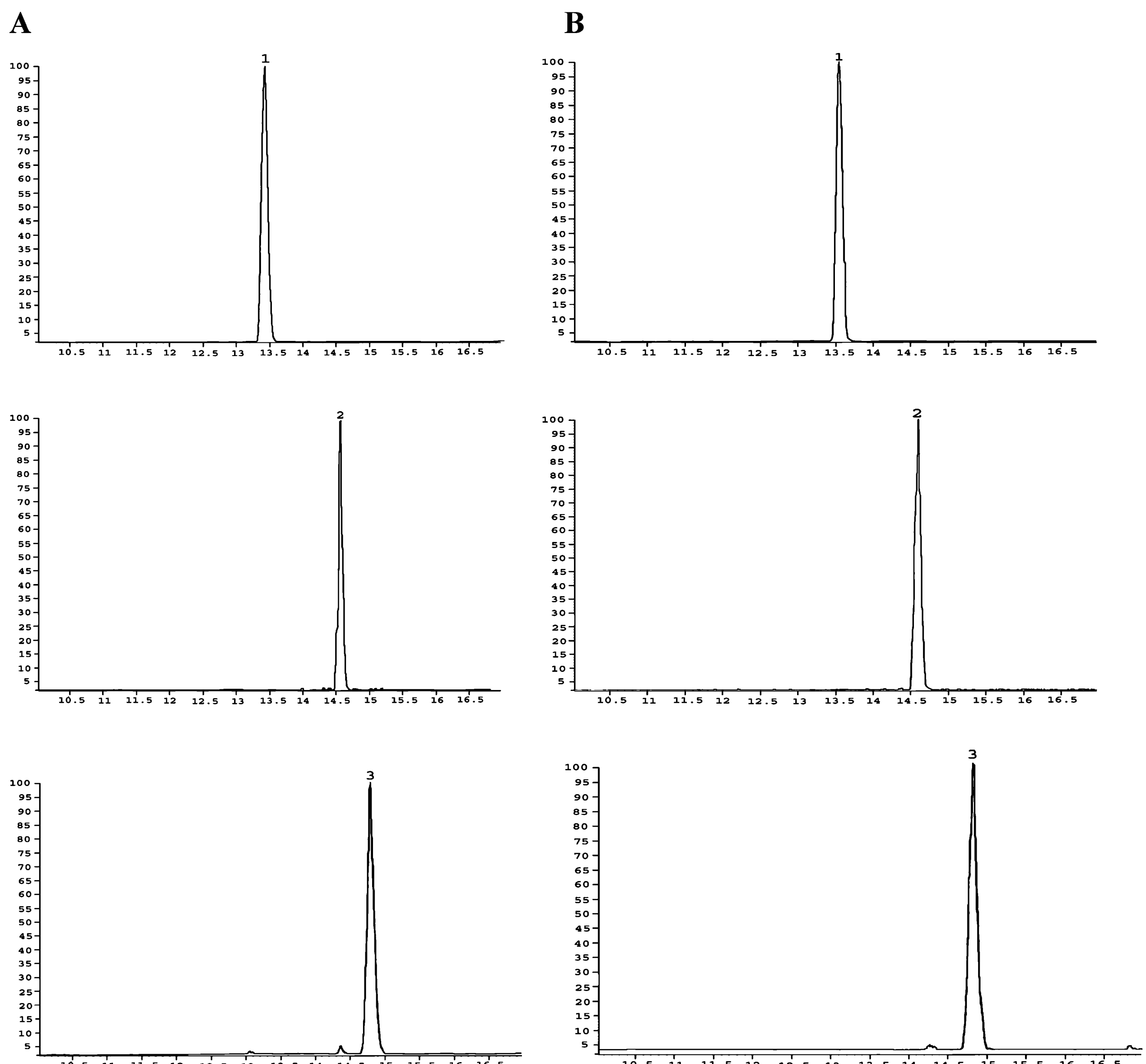

Figure 1. Representative total ion current chromatograms [percent relative abundance of parent ion (**Table 1**) versus retention time], illustrating peak separation for 5-methyltetrahydrofolate (1), 10-formyl folic acid (2), and 5-formyltetrahydrofolate (3) in red peppers (**A**) and green peppers (**B**).

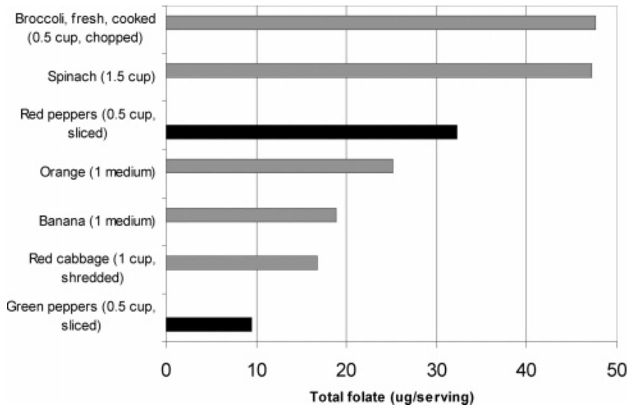

Figure 2. Folate content of peppers determined by LC-MS in this study (black bars) and reported for selected fruits and vegetables by Konings et al. (*8*) (gray bars), based on HPLC analysis after affinity chromatography to isolate folates. Products were raw, except as noted. Typical serving sizes from the USDA Nutrient Database for Standard Reference (*22*) are as follows: tomato, 1 small (91 g); carrot, $^1/_2$ cup (120 mL), chopped (64 g); red and green sweet peppers, $^1/_2$ cup (120 mL), sliced (46 g); red cabbage, 1 cup (240 mL), shredded (70 g); banana, 1 medium (118 g); orange, 1 medium (140 g); spinach, $1^1/_2$ cups (360 mL) (45 g); broccoli, $^1/_2$ cup (120 mL), chopped (70 g); strawberry, $^1/_2$ cup (120 mL), sliced (83 g).

assay (*28*) at a commercial laboratory, to generate the total folate values in release 18 of the USDA National Nutrient Database for Standard Reference (NDB numbers 11821 and 11333 for red and green sweet peppers, respectively) (*22*), yielding values of 18 and 11 μg/100 g for red and green peppers, respectively. Total folate should exceed or equal 5MTHF content, because 5MTHF is but one possible vitamer contributing to total folate. However it is clear that total folate, measured as the specific vitamers, exceeds total folate determined by the nonspecific assay in both cases. Problems with intra- and interlaboratory variability and inaccuracy for naturally occurring folates determined by microbiological assay have been reported previously (*17*).

Contribution to Recommended Dietary Allowance. The recommended dietary allowance (RDA) for folate, in dietary folate equivalents (micrograms of DFE = micrograms of folate from food) is 400 μg/day for individuals 14 years and older in the United States (*29*). Thus, a $^1/_2$ cup (120 mL) serving of sliced green peppers (46 g) provides 2.4% of the RDA, while the same amount of red peppers supplies 8.1% of the RDA. **Figure 2** illustrates how these values compare to those of several other vegetables, as reported by Konings et al. (*8*) based on HPLC analysis after affinity chromatography to isolate folates. Relative to folate contained in typical serving sizes of other fruits and vegetables, red peppers [$^1/_2$ cup (120 mL) sliced, 32 μg] falls between the values reported by Konings et al. (*8*) for oranges (25 μg per medium fruit) and spinach [45 μg per $1^1/_2$ cups (360 mL)], both of which are generally considered rich in folate. Green pepper folate content was comparable to that of carrots and tomatoes, which are not particularly high in folate (<10 μg per serving).

Conclusions. Red sweet peppers are a significantly better source of dietary folate compared to green peppers. These findings may assist in making dietary recommendations or developing research diets related to folate. The values from this study based on the analysis of 5MTHF have been used to update the folate values in release 19 of the USDA Nutrient Database for Standard Reference (*30*). These data also illustrate possible limitations in determination of the folate content of nonenriched foods by microbiological assay, as found in our previous study (*17*), and highlight the importance of having accurate data on food composition for design and interpretation of feeding trials involving this nutrient, and specifically methods for accurately quantifying the range of folate vitamers that naturally occur in foods. While further refinements of methodology for individual folates are needed (*18*), improvements continue [e.g., Rychlik (*31*) and Garbis et al. (*32*)]. Values for naturally occurring folate in nonenriched foods generated by the standard microbiological assay (*15*, *16*), which was developed for enriched cereals and grains, should be reassessed by newer methods. Subsequent publication and updates to the USDA database (*30*) will include expanded/improved folate data for a broader range of foods, including fruits, vegetables, and enriched cereals and grain products.

ABBREVIATIONS AND NOMENCLATURE

The following names for folates were used; refer to IUPAC (*1*) for a full discussion of folate nomenclature: folic acid, pteroylglutamic acid; 5-methyltetrahydrofolic acid, 5-methyl-5,6,7,8-tetrahyropteroylglutamic acid; 5-formyltetrahydrofolic acid, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid; 10-formylfolic acid, 10-formylpteroylglutamic acid; 10-methylfolic acid, 10-methylpteroylglutamic acid; 5-methyldihydrofolic acid, 5-methyl-5,6-dihydropteroylglutamic acid; dihydrofolic acid, 7,8-dihydropteroylglutamic acid; tetrahydrofolic acid, 5,6,7,8-tetrahydropteroylglutamic acid.

ACKNOWLEDGMENT

The detailed work of Amy Rasor and Nancy Conley on coordination of sample preparation and maintenance of sample descriptive information is greatly appreciated.

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Received for review August 12, 2006. Revised manuscript received October 18, 2006. Accepted October 24, 2006. Funding and samples were 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, with support from the National Heart, Lung, and Blood Institute and the National Cancer Institute through interagency agreement Y1-HV-8116 between the National Institutes of Health and the USDA.

JF062327A