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Determination of 5-Methyltetrahydrofolic Acid and Folic Acid in Citrus Juices Using Stable Isotope Dilution−Mass Spectrometry

PAUL M. THOMAS, VINCENT P. FLANAGAN, AND ROBERT J. PAWLOSKY*

Food Composition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

A stable isotope liquid chromatography−mass spectrometry (LC-MS) method was developed for the quantitative determination of 5-methyltetrahydrofolic acid (5-MTHFA) and folic acid in a variety of commercial citrus juices. Folates were extracted from juices, and the polyglutamyl side chain of 5-MTHFA was cleaved to the monoglutamate form using rat plasma conjugase. The folates were purified on a Bond-Elut column and analyzed by LC-MS with electrospray ionization. The analytes were quantified using the $^{13}C_5$ analogues of 5-MTHFA and folic acid as internal standards. The relative standard error of the method was 3.35% based on replicate analyses ($n = 4$). This method was then applied to the determination of 5-MTHFA and folic acid in a variety of citrus juices obtained from local supermarkets. It was observed that although both "store" brands and "national" brands of fresh (nonfrozen) juices contained similar concentrations of 5-MTHFA, the "store" brands of fresh juices had on average >5-fold the amount of folic acid compared to the "national" brands. In addition, the "total" folate concentrations were generally below values listed on the food label.

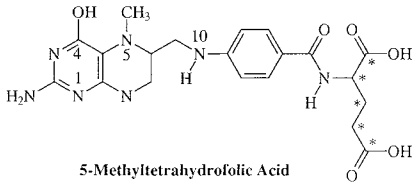
KEYWORDS: 5-Methyltetrahydrofolic acid; folic acid; mass spectrometry; electrospray ionization; isotope dilution; citrus

INTRODUCTION

Folates are classified as a group of water-soluble vitamins based on the glutamyl derivatives of 4-[(pteridin-6-ylmethyl)-amino]benzoic acid (**Figure 1**). These vitamin cofactors found in quantity in legumes, spinach, and citrus fruits (such as orange and grapefruit juices) are essential for the synthesis of purines and pyrimidines and in the production of methionine from homocysteine (*1*). Folates are required in the human diet and the current adult Daily Recommended Intake in the United States is 400 μg (*2*). A deficiency in dietary folates may give rise to megaloblastic anemia, a blood disorder, characterized by enlarged and misshapen blood cells formed during erythropoesis (*3*).

In January 1998, a folate food fortification program took effect in the United States whereby grain-based foodstuffs intended for human consumption were fortified with folic acid at a level of 140 μg 100 g^{-1} (*3*). Citrus juices, which are readily available throughout the year, contain high levels of naturally occurring folates mainly in the form of L-5-methyltetrahydrofolic acid (5-MTHFA) and therefore are an important source of the nutrient in the diet (*4*).

Several analytical methods have been developed for the determination of folates in biological matrices. A microbiological assay (*Lactobacillus casei*), commonly used for determining "total" folate concentrations in foods, lacks specificity and may not be equally responsive to the different folate vitamers (*5*). More specific high-performance liquid chromatography (HPLC) methods have been developed with electrochemical detection (ECD), UV, and/or fluorescence detection to identify different forms of folates in foods (*6*−*8*). Recently, highly specific HPLC−electrospray ionization mass spectrometry (ESI-MS) methods have been developed for the determination of folates



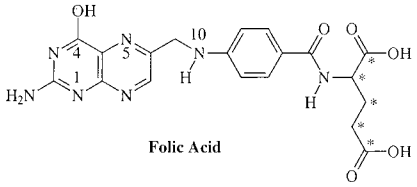


Figure 1. Chemical structures of 5-methyltetrahydrofolic acid and folic acid. The vitamers consist of a pterine ring coupled to *p*-aminobenzoic acid that is linked to glutamic acid. Asterisks denote labeled positions on $^{13}C_5$ analogues that were used as internal standards.

* Address correspondence to this author at the Laboratory of Membrane Biochemistry and Biophysics, Room 114, 12420 Parklawn Dr., Rockville, MD 20852 [telephone (301) 443-9029; fax (301) 594-0035; e-mail bpawl@mail.nih.gov].

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in foods (*9*, *10*) and plasma (*11*, *12*). The recent availability of $^{13}C_5$-labeled folates has facilitated the development of highly specific quantitative methods for the determination of folates occurring in various background matrices using mass spectrometry detection.

A recent report issued by the Institute of Medicine of the National Academy of Sciences recommended that the individual folate vitamers be listed in separate categories when nutrient database tables of foods are compiled (*13*). This suggestion was based, in part, on evidence that the synthetic fortificant, folic acid, when consumed with foods has greater bioavailability than do the naturally occurring folates. As a consequence, folic acid is to be weighted more heavily in determining the Dietary Folate Equivalent (DFE) compared with 5-MTHFA (*13*). Therefore, quantitative methods for 5-MTHFA and folic acid were developed using stable isotope dilution HPLC-ESI/MS to determine the concentrations of folic acid and 5-MTHFA in citrus juices. The $^{13}C_5$ isotopes of 5-MTHFA and folic acid (each compound was labeled at each of the five carbons of glutamic acid, see **Figure 1**) were used as internal standards for the quantification of folates in a variety of commercially available citrus juices.

MATERIALS AND METHODS

Chemicals and Reagents. Monoglutamylfolic acid and lyophilized rat plasma were purchased from Sigma (St. Louis, MO). The lyophilized plasma was reconstituted with distilled water according to the supplier's recommendations. 5-MTHFA, [$^{13}C_5$]glutamyl-5-MTHFA, and [$^{13}C_5$]-glutamylfolic acid were obtained from Merck Eprova AG (Schaffhausen, Switzerland). The ^{13}C atoms occupied the five carbons of the glutamic acid portion of each molecule. The folic acid standards were dissolved in a 0.1 N solution of sodium hydroxide, and the 5-MTHFA standards were dissolved in ACN/H_2O/MeOH 26:60:14 + 0.1% formic acid. All standards were stored in a potassium phosphate buffer containing mercaptoethanol (10 mM) and ascorbic acid (10 mM) and kept frozen at −60 °C. All solvents were of HPLC grade and were obtained from commercial suppliers and used without further purification.

Sample Preparation. Four milliliters of commercially available citrus juice were added to 20 mL of 0.1 M dibasic potassium phosphate containing 10 mM each mercaptoethanol and ascorbic acid with 10 mg L^{-1} sodium azide (NaN_3), and the suspension was adjusted to pH 6.0 with 5% (w/w) potassium hydroxide. Prior to pH adjustment, the $^{13}C_5$-labeled 5-MTHFA and folic acid internal standards were added to the suspension in amounts commensurate with the concentration of the analyte. The sample was then homogenized using a Sorval Omni-Mixer (Newtown, CT) for 30 s at room temperature at maximum velocity. Prior to homogenization 50 μL of 2-octanol was added to prevent frothing and 100 μL of the reconstituted lyophilized rat plasma was added to cleave the polyglutamyl side chain to the monoglutamyl form. In a separate experiment, a subset of samples was analyzed without conjugase treatment to determine the endogenous concentration of monoglutamyl 5-MTHFA. The sample was purged with nitrogen (1 h) and then heated for 16 h at 37 °C. The sample was then centrifuged at 40000*g* (20 min) at 4 °C and adjusted to pH 3.0 with trifluoroacetic acid (TFA), and a 0.5 mL aliquot was taken for solid-phase extraction.

Standard Method of Addition of 5-Methyltetrahydrofolic Acid to Orange Juice. To determine the linear quantitative range of the procedure, a standard method of addition of 5-MTHFA experiment was carried out in orange juice. Orange juice (12 mL) was added to phosphate buffer (60 mL), and 2.88 μg of [$^{13}C_5$]-5-MTHFA (240 ng mL^{-1} of juice) was added. Six aliquots (12 mL each) were taken and were amended with 5-MTHFA (range = 60−300 ng mL^{-1} of orange juice). All samples were processed as described above.

Solid-Phase Extraction. A 0.5 mL aliquot of the supernatant of each sample was diluted with 0.5 mL of 0.03 M dibasic potassium phosphate with 0.1% each ascorbic acid and mercaptoethanol adjusted to pH 3.5 with TFA. The sample was loaded onto a 100 mg Bond-Elut Ph column (Varian, Walnut Creek, CA) that had previously been washed with methanol (1 mL) and 0.03 M phosphate buffer (1 mL). The column was then washed with 0.03 M phosphate buffer (1 mL) and 0.1% formic acid (1 mL) to remove traces of salts. The analytes were eluted with 500 μL of ACN/H_2O/MeOH 26:60:14 + 0.1% formic acid. After elution, 200 μL of 0.1% formic acid was added.

LC-ESI/MS Instrument and Conditions. Samples (40 μL) were injected onto a 150 × 4.6 mm Luna C-18 HPLC column (5 μ) (Phenomenex, Torrance, CA) using a binary pumped HPLC (Agilent Co. HP1100, Palo Alto, CA) coupled to an ion trap mass spectrometer (Finnigan LCQ Classic, San Jose, CA) fitted with an ESI source. The solvent system was a gradient of solvent A (0.1% formic acid) and solvent B (ACN/H_2O/MeOH 26:60:14 + 0.1% formic acid). The following gradient was applied: isocratic, 0.175 mL min^{-1}, 30% B, 0−9 min; linear, 0.3 mL min^{-1}, 100% B, 9−14 min; isocratic, 0.3 mL min^{-1}, 100% B, 14−25 min; linear, 0.175 mL min^{-1}, 30% B, 25−30 min; isocratic, 0.175 mL min^{-1}, 30% B, 30−45 min. Under these conditions, 5-MTHFA eluted in ∼10 min and folic acid in ∼21 min. The ESI was operated in positive ion mode for 5-MTHFA using selective ion monitoring (at *m*/*z* 460.2 and 465.2 for [^{12}C]-5-MTHFA and [$^{13}C_5$]-5-MTHFA, respectively). The ESI was switched to negative ion mode for folic acid using selective ion monitoring (at 440.2 and 445.2 for [^{12}C]folic acid and [$^{13}C_5$]folic acid, respectively). The spray voltage was set to 4.5 kV, and the capillary temperature was adjusted to 200 °C. The sheath gas was set to 80% of its maximum flow rate.

Confirmation of 5-MTHFA and Folic Acid Using Tandem Mass Spectrometry. Both 5-MTHFA and folic acid in citrus juices were confirmed using tandem mass spectrometry. The molecular cation $[M + H]^+$ of 5-MTHFA (*m*/*z* 460.2) and molecular anion $[M - H]^-$ of folic acid (*m*/*z* 440.2) were subjected to helium collision-induced dissociation (CID) using 26 and 31% of the maximum energy of the end cap electrode (5 V) of the ion trap, respectively. Using an isolation mass width of 1.2 Da, first-generation product ions were collected in full scan mode, and these spectra were compared to those of the authentic compounds.

RESULTS AND DISCUSSION

Citrus products contain relatively high amounts of 5-MTHFA compared with many other foods and therefore contribute an important dietary source of naturally occurring folates for some segments of the American population and in countries that do not fortify their foods with folic acid. A specific and quantitative assay was developed for the determination of 5-MTHFA and folic acid in a variety of citrus juice products using HPLC-ESI/MS. Previous analyses of folates from citrus juices reported the presence of 5-MTHFA, but folic acid was not included in those determinations (*8*).

Both the mono- and polyglutamyl forms of 5-MTHFA were found in citrus juices (*8*). We observed that a relatively high amount (60%) of the 5-MTHFA was present as the monoglutamyl form compared to the polyglutamyl derivatives in orange juice. **Figure 2** illustrates a typical LC-MS chromatogram of 5-MTHFA and folic acid from orange juice after treatment with conjugase. Both 5-MTHFA and folic acid coeluted on the column with their respective $^{13}C_5$-labeled analogues. Under these chromatographic conditions, 5-MTHFA eluted on the column prior to the folic acid peak.

The precision of the stable isotope method was determined by amending orange juice samples with 5-MTHFA and then spiking with [$^{13}C_5$]-5-MTHFA. The mean concentration (n = 4) was 354.5 ng mL^{-1} with a relative standard error of 3.35%. The accuracy of the LC-MS method was determined using a standard method of addition of 5-MTHFA to orange juice (range = 60−300 ng mL^{-1}). Using least-squares approximation, the best-fit regression line to the experimental values was $y = 0.0015x + 0.4156$, $R^2 = 0.955$. This range bracketed the concentration range of 5-MTHFA (64−212 ng mL^{-1}) found in

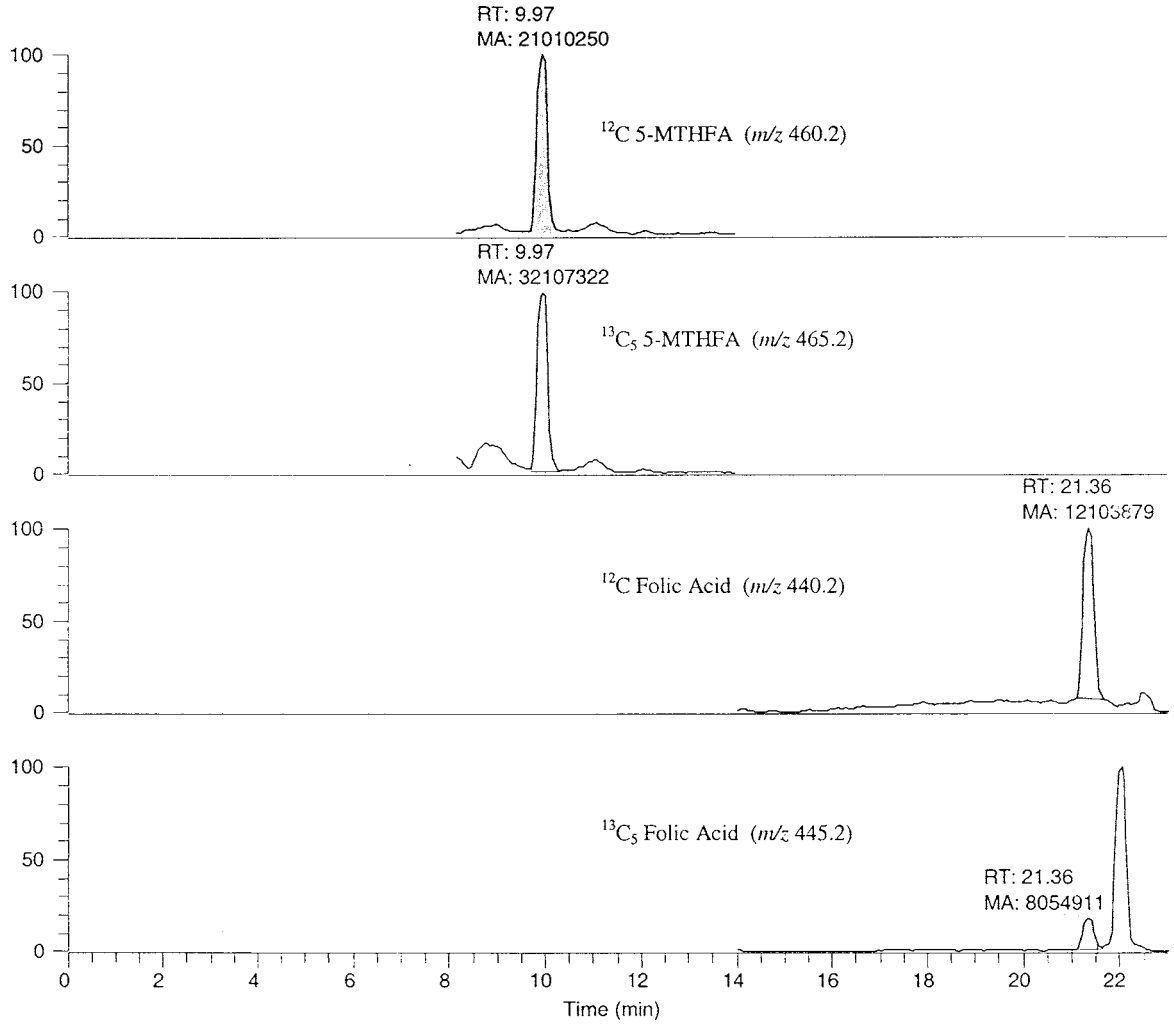

Figure 2. Selected ion chromatograms of molecular cations of labeled and unlabeled 5-methyltetrahydrofolic acid at *m/z* 460.2 and 465.2, respectively, and the molecular anions of labeled and unlabeled folic acid at *m/z* 440.2 and 445.2, respectively, using LC-MS with electrospray ionization. The chromatograms are produced from an analysis of an extract of a store brand of fresh orange juice.

Table 1. Determinations of 5-Methyltetrahydrofolic Acid and Folic Acid from Citrus Juice Products Using Stable Isotope Dilution LC-MS

	sample	5-MTHFA[a]	FA[b]	total[c]	product label	% difference
national brand of orange juice, fresh	NFC1	21.2[d]	1.5	22.7	25.0	–9.2
	NFC2	16.1	0.79	16.9	25.0	–32.4
	NFC3	15.6	0.75	16.4	25.0	–34.4
	NFC4	17.9	1.42	19.5	25.0	–22.0
	NFC5	12.9	2.79	15.6	25.0	–37.6
national brand of orange juice, frozen	FCC1	9.1	1.88	11.0	25.0	–56.0
	FCC2	13.5	1.17	14.7	25.0	–41.0
	FCC3	11.1	1.5	12.6	25.0	–49.6
store brand of orange juice, fresh	NFG1	17.5	12.9	30.4	N/A	
	NFG2	17.5	12.7	30.2	N/A	
	NFG3	12.9	8.3	21.2	16.7	+27.0
	NFG4	10.0	11.6	21.7	N/A	
	NFG5	13.3	3.2	16.5	N/A	
store brand of orange juice, frozen	FCG1	17.9	4.4	22.3	N/A	
	FCG2	17.9	4.3	22.2	N/A	
grapefruit juice, fresh	GR1	9.1	6.1	15.2	3.3	+361
	GR2	6.5	2.7	9.2	10.0	–8.1
orange grapefruit blend, fresh	BL1	18.1	5.3	23.5	25.0	–6.0

[a] 5-Methyltetrahydrofolic acid. [b] Folic acid. [c] 5-MTHFA + FA. [d] All values listed are in μg 100 g^{-1} of citrus juice.

samples. Folic acid was quantified in the juice samples using a similar procedure with [$^{13}C_5$]folic acid as the internal standard and adjusting instrument settings to negative ion conditions.

The presence of 5-MTHFA and folic acid in orange juice was confirmed on the basis of the tandem mass spectral analyses of the parent ions of the analytes. The spectra that were obtained are consistent with previously reported spectra (*9*, *11*) and confirm the presence of both 5-MTHFA and folic acid in the samples. To our knowledge, folic acid has not been reported as a major constituent of citrus products previously. However, in

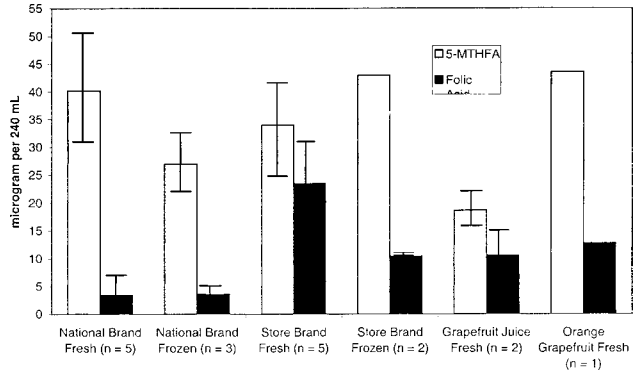

Figure 3. Summary of 5-methyltetrahydrofolic acid and folic acid concentrations found in a variety of juice products. 5-MTHFA is given in the first column and folic acid in the second column for each category. The ordinate indicates the amount of folate in 240 mL (1 cup) of juice. Store brand fresh juices contained the highest concentrations of folic acid compared to national brands. Vertical lines indicate the range of values observed.

this study folic acid was detected in all samples, and relatively large amounts were found in many of the "store" brand juice varieties compared to the "national" brands (**Figure 3**). The amount of folic acid and 5-MTHFA in the samples ranged from 8 to 129 ng mL^{-1} and from 65 to 212 ng mL^{-1}, respectively. Although the concentration of 5-MTHFA was generally greater than that of folic acid in orange juices, grapefruit juice contained the least amount of 5-MTHFA and relatively large quantities of folic acid (**Table 1**).

Previously, White et al. reported 5-MTHFA in several orange juice varieties using HPLC analyses with electrochemical detection (ECD) (*8*). Those values were somewhat higher than the determinations reported here. This may be due, in part, to variations in the juices that were analyzed or to differences in the analytical methodologies that were employed. We found that the sum of the concentrations of folic acid and 5-MTHFA was, on average, ~73% of the folate concentration listed on the food label (**Table 1**). The national brands of frozen orange juices had lower combined concentrations of folates compared to the fresh juices and were only equal to ~50% of that listed on the container label (**Table 1**). It was presumed that minor amounts of other folates (e.g., 5-formyltetrahydrofolic acid) not determined by the LC-MS method may account for some of the differences observed between the food label and LC-MS values. However, the discrepancy that was observed in concentrations of folates in frozen products may be due to juice processing procedures, which may contribute to a reduction of 5-MTHFA. The highest combined concentrations of 5-MTHFA and folic acid were found in the store brands of fresh juices, in which the concentration of folic acid was >5-fold that found in national brands. Grapefruit juices also contained relatively large amounts of folic acid and moderate concentrations of 5-MTHFA (**Table 1**).

We adapted a stable isotope LC-MS procedure for the determination of 5-MTHFA and folic acid to citrus products. Citrus juices provide an important source of naturally occurring folates in the diet, and specific information concerning the concentrations of the vitamer forms in foods is necessary for accurate compiling of nutrient data for food databases and for determining the DFE from nutrient intake information (*13*). In this preliminary investigation the concentrations of the different folates were found to vary across different product lines and were generally lower than values listed on the food labels. Furthermore, folic acid was found in high concentrations in store brand varieties of juices compared with national brands. Additional research is required to determine if juice processing, seasonal or geographical variation in crop production, or different orange varieties contribute to the variation observed in the folate concentration of commercial citrus products.

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