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Capillary Electrophoresis and High-Performance Liquid Chromatography Determination of Polyglutamyl 5-Methyltetrahydrofolate Forms in Citrus Products

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The 5-methyltetrahydrofolate (5mTHF) polyglutamates in citrus products were analyzed by capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). Folate species were purified from citrus products and concentrated from 2- to 100-fold using combined folate-affinity chromatography and C_{18} extraction. Seven polyglutamyl 5mTHFs were found in most not-from-concentrate (NFC) orange juices (OJ) in total amounts of ~1 nmol/mL, with varying distributions of individual polyglutamates. Folate amounts and distributions were also measured in orange fractions, single-strength OJ from concentrate, NFC grapefruit juice, and citrus peel molasses. Models containing ascorbic acid had folate thermal degradation rates one-seventh that of models without ascorbic acid. Pasteurization studies demonstrated that folate loss was <2% for commercial OJ pasteurization conditions (i.e., 93 °C for 5 s, 88 °C for 15 s, and 82 °C for 30 s). Both methods were precise, reproducible, and potentially faster than traditional analytical procedures requiring enzymatic deconjugation and microbial assays.

KEYWORDS: Capillary electrophoresis; citrus byproducts; 5-methyltetrahydrofolate; high-performance liquid chromatography; orange juice

INTRODUCTION

Orange juice (OJ) is a significant source of folate, which is mainly in the form of polyglutamyl 5-methyl tetrahydrofolates (5mTHF) (*1*). Folate in OJ can account for up to 20% of the recommended dietary allowance per serving. This important vitamin degrades with heat, O_2, light, and extreme pH. Traditional methods of analysis in foods have relied on microbiology assays or high-performance liquid chromatography (HPLC) requiring a conjugase enzyme treatment. The enzyme treatment may contribute to analytical variability, and several hours may be needed to completely hydrolyze folates in citrus products to their monoglutamate forms. Consequently, there is a need for faster and more reproducible methods for analysis of the various forms of folate in citrus juices.

In recent years, capillary electrophoresis (CE) has shown promise as an alternative to some HPLC analyses. Its advantages in speed, separation, peak definition, and small sample requirements make it a better technique than HPLC for certain assays. There have been few studies analyzing polyglutamyl folates with CE (*2*, *3*) and no studies to date analyzing them in citrus products.

Direct polyglutamyl folate analysis in citrus avoids conjugase treatment and may improve precision. Folate affinity chromatography (FAC) combined with HPLC was shown to be an effective technique employed by various investigators to monitor and quantify polyglutamyl folates in certain biological media and foods (*4*−*9*). Early polyglutamyl analysis of folates in OJ used microbiological assays (*10*) and chromatography with DEAE and Sephadex resins (*11*). Polyglutamyl distributions in OJ have been reported to be mostly monoglutamyl forms (*10*) or pentaglutamyl forms (*11*).

There have been few reports on the stability of citrus juice folate under common pasteurization conditions or of folate concentrations in citrus byproducts. Folates in citrus products (especially juice) should be relatively stable, because ascorbic acid (Asc) has been shown to be a powerful protective agent (*12*). However, quantitating folates within citrus products using traditional methods has been too cumbersome for routine kinetic studies or byproduct analysis.

The objectives of this experiment were to determine polyglutamyl folates in citrus products using CE with photodiode array (PDA) detection and HPLC with fluorescence (F) detection and also to determine folate degradation kinetics considering citrus juice processing variables.

MATERIALS AND METHODS

The following experiments were conducted under conditions to protect folate from unintended degradation. Folate-containing samples were handled as follows: sample storage at −80 °C for no more than

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3 days, shielding from light, frequent deaeration with N_2 gas, and cooling in an ice bath during use. Commercial not-from-concentrate (NFC) OJ and NFC grapefruit juice (GJ) samples were stored at 0−5 °C for no more than 5 days after purchase. After samples were opened, they were flushed with nitrogen, resealed, and stored at 0 °C.

Limits of Detection (LOD). The LOD were determined for the PDA and F detectors used in these experiments with 5mTHF monoglutamate standard solutions (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate buffer, pH 7.0, containing 50 mM mercaptoethanol (*6*). PDA limits were determined by applying standards to a Beckman-Coulter CE instrument with a PDA detector (model 605168-AA, Beckman Coulter, Inc., Fullerton, CA). The samples were analyzed under the following parameters: 60 min run time, 55 mM sodium tetraborate buffer with 5% acetonitrile, 18 kV voltage, fused silica column (50 cm × 50 μm), 25 °C, 20 s injection at 0.5 psi, λ_{max} of 290 nm absorbance, and λ_{scan} of 190−400 nm.

LOD for HPLC analysis with F detection were determined using an LDC Analytical HPLC instrument (Constametric 3200, Riviera Beach, FL) with a Spectrasystem F detector (FL3000, Thermo Separation Products, Inc., San Jose, CA). The samples were analyzed under the following parameters: 25−35 min run time; 33 mM phosphoric acid mobile phase with 4% (v/v) acetonitrile; 10 μL injection; 1 mL/min flow rate; Perkin-Elmer HS3-C18 column (3.3 cm × 4.6 mm); 3 μm particle size; excitation, 295 nm; and emission, 356 nm (*5*, *6*).

Purification and Preconcentration. Folate affinity chromatography (FAC) columns were previously prepared by immobilizing folate-binding protein (FBP) (*8*) on agarose gel (Affigel 10, Bio-Rad Laboratories, Richmond, CA) as previously described (*13*). The gel (2 mL) was poured into 0.7 cm (i.d.) × 20 cm glass columns (Bio-Rad) and was conditioned with 5 mL of 0.025 M potassium phosphate (pH 7.0). Binding capacities were determined periodically (~70 nmol of folate/column) by overloading columns with known amounts of a folic acid standard solution (*5*, *6*).

C_{18} solid-phase extraction (SPE) cartridges (200 mg Sep-Pak, WAT054945, Waters, Taunton, MA) conditioned with 95% ethanol (5 mL) and water (10 mL) were used to concentrate samples further for PDA analysis. A 20% (v/v) ethanol solution containing either 50 mM mercaptoethanol or 1% (w/v) L-Asc, 99+% ACS reagent (Sigma-Aldrich), was used as the C_{18} eluting mobile phase. Void volumes, capacities, and folate recoveries were determined using 5mTHF standard solutions.

CE-PDA Method. For the CE-PDA method, commercial samples of NFC OJ (50 mL) were centrifuged (10000*g* for 10 min at 0 °C) and adjusted to pH ~7. Clarified supernatants were applied through Whatman no. 1 filter paper to a FAC column equilibrated with 0.025 M potassium phosphate (pH 7.0, 5 mL). The column was washed with 5 mL of 0.025 M potassium phosphate containing 1 M sodium chloride (pH 7.0) followed by 5 mL of 0.025 M potassium phosphate (pH 7.0). Trifluoroacetic acid (TFA) (0.02 M, 2 mL) with 0.01 M TFA/DTT was applied and discarded to account for the void volume. Another 5 mL of TFA/DTT was applied and collected in a 5 mL volumetric flask that contained 50 mM piperazine to neutralize the pH as well as 1% Asc (w/v) and 50 mM mercaptoethanol to protect the eluted folates (*6*). Samples achieved a 10-fold volume reduction (from 50 to 5 mL) that theoretically would concentrate the folates 10-fold.

The 5 mL fraction containing the concentrated folate was adjusted to pH 3.5 using concentrated phosphoric acid (A242-500, Fisher Scientific, Fair Lawn, NJ) to protonate the folates. The sample was applied to a conditioned 200 mg C_{18} SPE cartridge (Waters). The column was then washed with 2 mL of deionized water. A 0.4 mL void volume of 20% ethanol containing 50 mM mercaptoethanol (or 1% w/v Asc) was applied. Upon elution, 0.5 mL of 20% ethanol containing 50 mM mercaptoethanol (or 1% w/v Asc) was applied and collected for analysis. This subsequent 10-fold volume reduction accounted for a theoretical 100-fold folate concentration from the original sample. These samples were analyzed by a CE-PDA using the parameters described above.

HPLC-F Method. For the HPLC-F method, NFC OJ (10 mL) was centrifuged (10000*g* for 10 min at 0 °C) and adjusted to pH ~7. The clarified sample was applied to FAC columns under the conditions previously described. The resulting 5 mL fraction (2-fold concentration) was analyzed with HPLC-F parameters previously described.

Quantitation and Tentative Identification. The folates of citrus products and citrus fractions were represented in CE and HPLC methods by series of peaks for the homologous series of polyglutamyl species. Each peak was quantitatively integrated. Each folate homologue peak was quantitatively integrated for both the CE and HPLC analyses. Peak concentrations were determined by comparison to 5mTHF monoglutamate standards. Total folate was reported as a combined total of all of the homologue peaks. The loss of folate for each method was accounted for by multiplying the total by the appropriate factor as determined from recovery studies.

Polyglutamyl folates were tentatively identified by their ultraviolet−visible (UV−vis) spectra (λ_{max} = 190−400 nm), F excitation and emission λ parameters (295 and 356 nm, respectively) (*5*, *6*), and relative retention times of 5mTHF polyglutamates extracted from human red blood cells on the basis of published procedures (*5*). The predominance of 5mTHF pentaglutamate as the major folate form in human red blood cells, as confirmed previously by the use of a synthetic 5mTHF pentaglutamate standard (5), aided in the assignment of chain lengths in the series of polyglutamyl folate species.

To further establish that all of the peaks represented folate homologues, folate samples that had been purified and concentrated from OJ were treated with a folate hydrolyase (1 mg/mL of γ-carboxypeptidase G at pH 7.3 at 30 °C, Sigma Chemical Co., St. Louis, MO) in triplicate (3.2 units/mL). Samples were periodically monitored with HPLC-F until fully hydrolyzed to the analogous pteroic acid form, 5-methyltetrahydropteroic acid. After complete hydrolysis, the 5-methyltetrahydropteroic acid peak concentration was determined and compared with the combined concentrations of all the folate peaks before treatment. A Student's *t* test was used to compare average folate concentration from the treated samples with average concentration from untreated samples ($\alpha = 0.05$, df = 4).

Method Studies. *Precision.* Three independent trials of the same OJ sample were analyzed with HPLC and CE methods. The total folate amounts were averaged for each method, and standard errors of the mean were calculated.

Folate Recovery. For each method, three of the same NFC OJ samples were spiked with a known amount of 5mTHF monoglutamate standard and compared to an unspiked sample analyzed in triplicate. The concentration of the stock 5mTHF solution added to "spiked" samples and used in standard curves was determined spectrophotometrically (*5*). Percent recovery values were determined by dividing actual spiked concentrations by the theoretical spiked concentration and multiplying by 100. Actual spiked concentrations were determined after sample preparation and analysis. During the course of all analyses, representative samples were spiked to permit adjustment for folate loss. Losses were accounted for in final total folate amounts reported in the data.

Method Comparison. NFC OJ samples were analyzed by both methods in three independent trials. The results were averaged, and a Student's *t* test ($\alpha = 0.05$, df = 4) was done to determine any significant differences between the two methods.

Method Applications and Orange Component Analysis. Although both methods were used for several citrus products, the HPLC-F method was the main working method used throughout these experiments due to its speed over the CE-PDA method. NFC OJ, NFC GJ, single-strength OJ from concentrate (SSFC), and citrus peel molasses (CPM) adjusted to 12 °Brix were analyzed for folate content. Orange components (juice, juice pulp, peel, seeds, membranes, and core) were also investigated for their folate amounts per total wet weight in two separate trials using whole Valencia oranges. Folate extractions from these fractions were adapted from published procedures (*5*, *6*).

Kinetic Studies. *Citrus Juice Models.* In three independent trials, model solutions mimicking citrus juice were prepared (12% sucrose, 0.6% citric acid in deionized water adjusted to pH 3.5 with 0.1 N HCl). One model solution type contained Asc ($model_{asc}$) in amounts normally found in OJ (40 mg/100 mL), and the other type contained no Asc ($model_{no\ asc}$). Model solutions were placed in test tubes (1 cm × 7.5 cm) in 4.5 mL aliquots. The samples were heated to 88 °C in a water bath, and temperatures were monitored using a thermocouple submerged

within an extra sample. Once the set-point temperature was reached, a 0.5 mL aliquot of known 5mTHF standard was added to each tube to give the following final concentrations: $model_{asc} = 0.16\ \mu M$, $model_{no\ asc} = 0.45\ \mu M$. Timing began once the aliquot was added. Reactions were heated for various times ($model_{asc}$, 0−20 min; $model_{no\ asc}$, 0−9 min) and then immediately quenched within an ice bath. Samples were then analyzed with HPLC-F methods. The natural logs of each concentration were plotted versus time using linear regression analysis. The resulting slopes represented the apparent rate constants.

Orange Juice Pasteurization. Approximately 64 L of OJ from Valencia oranges was extracted with a commercial citrus juice extractor (model 63, FMC Food Tech Citrus Machinery Division, Lakeland, FL) and extra pulp removed by finishing with a 20-mesh screen. The juice, contained within a tank, was continually stirred as it was fed into a tubular pasteurizer (model 25, Microthermics, Inc., Raleigh, NC). Juice was pasteurized at 82, 88, and 93 °C for 0, 5, 15, 30, and 60 s in the pasteurizer hold tube. Duplicate samples were collected and analyzed by HPLC-F. Concentrations of 5mTHF were determined, and ln(concn) versus time was plotted to determine apparent rate constants. A one-way ANOVA test ($\alpha = 0.05$, df = 11) was used to determine any significant differences among the average apparent rate constants. The apparent rate constants were used to predict theoretical folate loss under commercial pasteurization conditions and compared with observed values.

Heat Evaporation. Valencia OJ was extracted and finished and then concentrated in a pilot-scale continuous high-temperature−short-time five-stage evaporator (Cook Machinery, Dunedin, FL). Before evaporation, raw Valencia OJ (12.6 °Brix) was analyzed using HPLC-F methods. The concentrated product was reconstituted to 12.6 °Brix and analyzed in triplicate with HPLC-F methods, and the folate concentrations of the samples were compared.

RESULTS AND DISCUSSION

Analytical Methods. *LOD.* The upper F LOD (0.619 μM) in HPLC analysis was 40 times greater than the lower limit (0.0155 μM). This range allowed adequate detection and quantification of all significant polyglutamyl 5mTHFs in OJ after the 2-fold concentration during sample preparation. PDA detection by CE was found to be 200 times less sensitive than the F detection used in HPLC, with a lower LOD of 3.09 μM. The upper LOD was not determined because it was >150 times the folate amount normally found in OJ. A 100-fold concentration was adequate to detect all polyglutamyl folates within OJ.

Method Precision. Both methods demonstrated adequate precision for OJ, with standard errors of the mean of 1% (for CE-PDA) and 2.2% (for HPLC-F). The precisions of the injection techniques were not determined. Better precision of the CE-PDA method was observed throughout the study, which was attributed to the instrument's autosampler reducing injection variability relative to manual HPLC-F injections.

Method Speed. Although both methods were considerably faster than traditional methods, the HPLC-F method (~60 min) was almost twice as fast as the CE-PDA method (~120 min). However, the most time-consuming parts of the CE-PDA method were the purification and concentration steps, which were necessary because of the lower PDA sensitivity. Faster CE methods may be obtained with use of F detection.

Folate Recovery. Folate recovery from an OJ medium was considered to be adequate. Greater recovery was observed with the HPLC-F method (90%) than with the CE PDA method (83%) (**Table 1**). This was expected, as more folate may have been lost from the SPE step. Recoveries using other products (i.e., GJ, CPM, certain extraction buffers, etc.) were checked and found to be similar to the OJ values. Similar recoveries were expected because all media contained protective agents and few interfering components.

Table 1. Percent Folate Recovery Determined by Theoretical and Actual Concentrations of 5mTHF Spiked in Orange Juice ($n = 3$) Using CE-PDA and HPLC-F Methods

method	theor spiked concn (nmol/mL)	actual or recovered spiked concn (nmol/mL)	folate recovery (%)
CE-PDA	0.51 ± 0.013	0.42 ± 0.014	83
HPLC-F	0.50 ± 0.015	0.45 ± 0.011	90

Method Comparison. Although both methods appeared to have different recoveries, folate amounts determined before adjustment for losses were not significantly different. When folate loss was accounted for, the folate concentrations were still not significantly different (Student's t test, df = 4, $\alpha = 0.05$). This indicates that folate recoveries in both methods and the methods themselves were comparable to each other.

Quantitation and Tentative Identification. *Quantitation.* For both detection methods, all folate peaks were integrated. Concentrations were determined from monoglutamyl 5mTHF standard curves and then added for a total folate amount. Folate concentrations of each polyglutamyl folate from the same sample were similar by both CE-PDA and HPLC-F [within 1−3% relative standard deviation (RSD), $n = 3$]. This demonstrates that similar folate concentrations were determined by two independent detection modes. Also, this validates the assumption that glutamic acid moieties make little contribution to spectral characteristics. Once the individual peak concentrations were added, the following equation was employed to account for folate loss:

$$[1 + ((100 - \text{recovery\%})/100)] \times (\text{total folate determined})$$

It should be noted that our analysis involved only the detection and measurement of the homologous series of 5mTHF mono- and polyglutamyl forms. Previous studies of OJ folate by conventional chromatography (*11*) and by HPLC (*14*−*16*) showed no evidence of other folate forms at significant concentrations. Two recent papers have reported that folic acid (i.e., pteroylglutamic acid) is present in low and variable proportions in OJ (*17*, *18*). The biochemical or chemical basis behind these unexpected findings of folic acid in juice is unknown because folic acid is not a known product of the oxidation of 5mTHF and folic acid is generally absent in biological materials such as fruit. The total of all detected folate forms before enzyme treatment was not significantly different from quantitations after enzyme treatment (Student's t test, df = 4, $\alpha = 0.05$). It should be noted that carboxypeptidase G series enzymes are different from traditional folate conjugases (e.g., rat plasma and hog kidney preparations), because carboxypeptidase G enzymes hydrolyze all folates to their pteroic acid form instead of the monoglutamyl folate. In this study, the resulting 5-methyltetrahydropteroic acid concentrations were not significantly different from folate homologue concentrations before treatment. Because all folates were subjected to the same reaction, this further validates that the homologue peaks indeed represent 5mTHF species.

Tentative Identification. Conclusive identification of each folate homologue was not done in these investigations. Future work using LC−mass spectrometry (MS) or nuclear magnetic resonance spectroscopy would be required to verify identities of each polyglutamyl folate.

Polyglutamyl 5mTHF extracted from human red blood cells used as one form of identification showed a characteristic distribution, with 5mTHF pentaglutamate predominating, similar

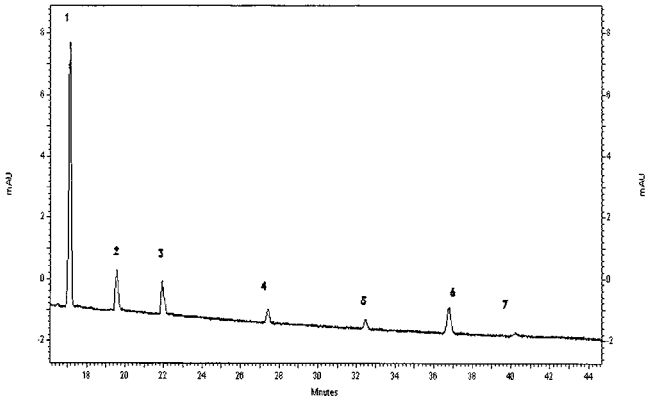

Figure 1. CE electropherogram of a 100-fold folate concentrated NFC OJ sample in a 20% ethanol solution with 50 mM mercaptoethanol showing a polyglutamyl 5mTHF distribution of predominantly 5mTHF monoglutamate. Peak labels are the various polyglutamyl forms from 1 to 7. The lack of other early-eluting UV-absorbing peaks suggests the absence of folic acid.

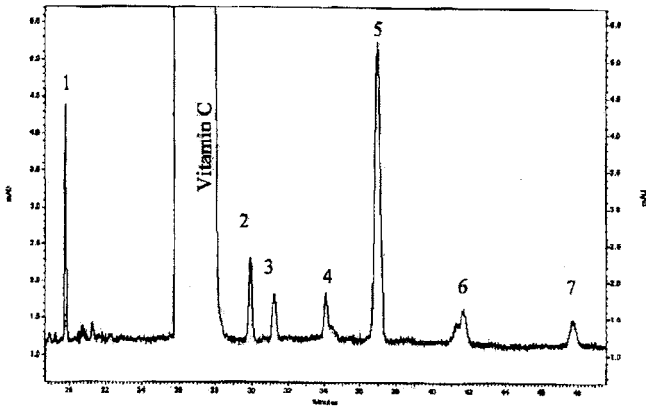

Figure 2. CE electropherogram of a 100-fold folate concentrated NFC OJ sample in a 20% ethanol solution containing 1% ascorbic acid showing a polyglutamyl 5mTHF distribution of predominantly 5mTHF pentaglutamate. Peak labels are the various polyglutamyl forms from 1 to 7.

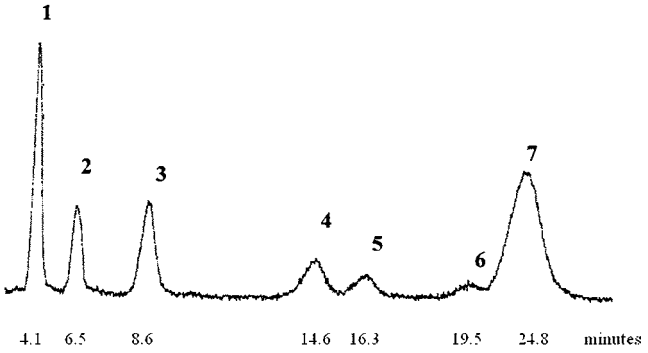

Figure 3. HPLC chromatogram of a 2-fold folate concentrated NFC OJ sample in a TFA/DTT solution showing a polyglutamyl 5mTHF distribution of predominantly 5mTHF heptaglutamate. Peak labels are the various polyglutamyl forms from 1 to 7.

to previous reports (*5*). From this distribution each 5mTHF homologue was tentatively identified by its relative retention time.

From PDA spectra, 5mTHF forms had spectra very similar to the monoglutamyl commercial 5mTHF standard spectrum in the program library, showing fits of 0.85 and higher to the monoglutamyl commercial standards. Typically, OJ samples contained larger amounts of monoglutamyl 5mTHF with fits >0.90. Lower fits (0.85−0.90) were observed with less concentrated homologues, as background interferences and/or imprecision reduced confidence in the spectra readings.

Fluorescence emission and excitation parameters were also adequate for tentative identifications of folate homologues. Excitation and emission spectra were determined to validate the maximum excitation and emission λ values. All peaks showed a maximum excitation λ of 295 nm. Emission spectra showed maximum λ values of 356 nm on only the largest peaks, whereas the smallest peaks showed maximum λ values at 325 nm. However, when the same samples were monitored for F at 325 nm, no peaks were observed. It is thought that the less concentrated peaks may have had a higher incidence of background interference (i.e., Raman scattering) that attributed to this result.

Citrus Products. *NFC OJ.* NFC OJ folate amounts ranged between 0.6 and 1.4 nmol/mL throughout the study, indicating that folate amount may vary with individual fruit as well as maturity, cultivar, growth conditions, processing, etc. The literature has shown similar variability in folate amount (*1*, *10*, *11*, *14*−*16*). It was noted that most NFC OJ samples analyzed within this study contained ∼1 nmol/mL, which was similar to previous findings (*1*, *14*−*16*). A recent study of citrus juice folates using HPLC-MS reported values in the lower range (0.6 nmol/mL) of our results (17). As stated above, these authors also reported finding folic acid in citrus juices. In addition, Konings et al. (*18*) reported that folic acid was a minor constituent (∼10%) of OJ folate; curiously, it was not reported as a constituent of oranges.

The 5mTHF polyglutamyl distribution also varied within this study. Most distributions showed a dominance of the monoglutamyl form (**Figure 1**), which coincides with certain previous findings (*10*). Other distributions, however, showed a dominance of the pentaglutamyl form (**Figure 2**), which agrees with a previous paper (*11*). The present study also found a distribution with a dominance of the heptaglutamyl form (**Figure 3**), which had not been previously reported. The variability in distribution seen both in this study and within the literature may be attributed to variability in cultivars, maturities, growth conditions, etc.

Table 2. Folate Amounts and Percents of Total Wet Weight in Various Components of Valencia Oranges ($n = 2$)

fraction	wt in orange (g)	wt analyzed (g)	5mTHF concn (nmol/g)	total folate within fruit (%)
juice	94.6	5.0	1.2	64
floating pulp	11.0	5.0	0.42	2
peel	37.0	5.0	0.88	18
rag and core	30.5	5.0	0.86	15
seeds	5.0	5.0	0.39	1
total	178.1			100

Orange Components. Interestingly, the 5mTHF polyglutamyl distribution was similar for all fractions of raw Valencia oranges, with folates mostly existing in the monoglutamyl form. The juice fraction contained the majority of the folate based on the total wet fruit weight (**Table 2**). Because the pulp also contained some folates, it seems likely that pulp may contribute to total folate amount in added-pulp juice products. The peel, rag, and core contained significant folate based on total wet weights. This disagrees with Streiff's observations (*10*); however, the *Lactobacillus casei* assay and amounts were not specifically described from that study. Components within the peel may have inhibited or altered *L. casei* growth, as it has been shown that peel contains certain terpenes with antimicrobial properties (*19*).

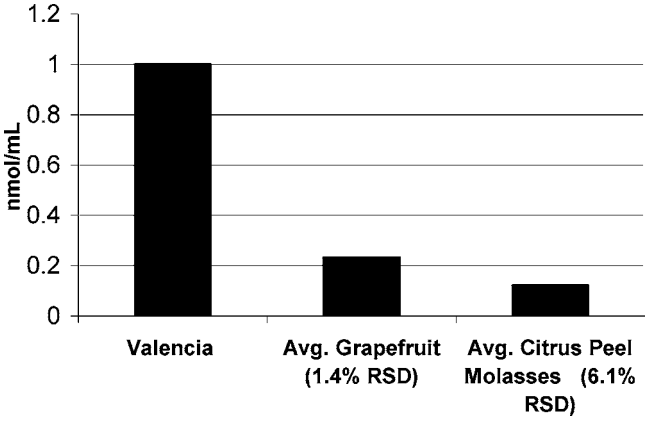

Figure 4. Concentrations of 5mTHF (nmol/mL) in commercial NFC GJ and diluted citrus peel molasses compared to amounts normally found in Valencia NFC OJ. RSD = $s/\bar{x}$.

Like juice, the peel, rag, and core (processing residue) consist mostly of water (*19*); therefore, it seems likely that water-soluble folates may also be distributed within these fractions. In contrast, the percentage of seeds in fruit is low, and they contain the least water, thus, accounting for ~1% of the total folate of an orange. These investigations provide only an estimate of folate amounts within each component. Because there have been few previous studies, more work is required to confirm these findings.

NFC GJ. The NFC GJ folate results confirm previous findings (*1*, *10*, *15*, *16*). The folate was found to be ~0.2 nmol/mL, ~80% less than a typical NFC OJ sample (~1 nmol/mL) (**Figure 4**). It would be expected that variability in NFC GJ folate amount would be similar to that observed with NFC OJ; however, only three commercial GJ samples were analyzed. A commercial GJ sample was assumed to offer adequate sampling of many different grapefruits, cultivars, and maturities; however, this was not certain. The distributions showed folates existed in mostly the 5mTHF pentaglutamyl form. To date, no other reports of polyglutamyl distributions in GJ have been reported.

CPM. CPM adjusted to 12 °Brix showed ~90% less folate (~0.1 nmol/mL) than amounts normally found in NFC OJ (**Figure 4**). The difference seems to be reasonable because there was less folate in the peel residue than in juice (**Table 2**). It is also possible that folates were not completely extracted from the peel during commercial molasses manufacture. Furthermore, oxidative or hydrolytic losses are likely in the presence of CaO (slaked lime), which is used commercially to release the water and soluble components from the peel. These factors combined with the thermal evaporation process for molasses manufacture may largely diminish folate amounts in the CPM. The studies were limited in that only one commercial molasses sample was analyzed in triplicate. The polyglutamyl distributions of molasses showed larger proportions of hexa- and heptaglutamyl followed by the monoglutamyl folate forms.

Kinetic Studies. *Citrus Juice Models.* The results of the studies of citrus juice models demonstrate that Asc protected 5mTHF from degradation by ~7-fold (**Table 3**). The protective effect of Asc has been shown in the literature; however, several of the studies investigated degradation in buffer systems or model food systems under conditions of composition different from those actually present in citrus juice (*12*, *20*−*23*). Certain investigators showed slightly better protection with Asc than was observed in this study; however, their models were at neutral pH with different constituents (*12*, *21*). It has been shown that 5mTHF stability is affected by pH and the presence of various other food constituents (*20*−*24*).

Table 3. Average Apparent Rate Constants (k) at 88 °C of Citrus Juice Model Solutions with and without Vitamin C (40 mg/100 mL)

model	apparent $k_{88°C}$ (min^{-1})	n	RSD[a] (%)
without vitamin C	0.196	3	7.1
with vitamin C	0.0307	3	4.1

[a] RSD = relative standard deviation = $s/\bar{x}$.

Table 4. Calculated and Observed Folate Losses of NFC Valencia Orange Juice Pasteurized at Three Commercial Conditions

pasteurization conditions	apparent k[a] (min^{-1})	folate concn (nmol/mL)		loss of initial folate (%)
		raw juice	pasteurized	
	Calculated Losses			
82 °C, 30 s	0.031	1.28	1.26	1.56
88 °C, 15 s	0.038	1.28	1.27	0.78
93 °C, 5 s	0.048	1.28	1.27	0.78
	Observed Losses			
82 °C, 30 s	0.031	1.28	1.26	1.56
88 °C, 15 s	0.038	1.28	1.25	2.34
93 °C, 5 s	0.048	1.28	1.26	1.56

[a] Apparent rate constant calculated as the slope $\ln(C_{final}) = -kt + \ln(C_{initial})$.

Models with and without Asc were expected to be pseudo-first-order because O_2 was unlimited, as seen in previous studies (*12*, *23*, *25*). In a pseudo-first-order process, the kinetics of a bimolecular reaction follow first-order kinetics because one of the reactants (i.e., folate) is limiting. Under such conditions, O_2 concentration is in sufficient excess that it has little or no effect on the rate of folate degradation. However, under reduced O_2 content, reaction rates would be expected to significantly decrease and follow either first- or second-order kinetics (*12*, *21*−*24*). From three independent trials with both models, estimates of average apparent rate constants were determined at 88 °C ($k_{no\ vit\ C}$ = 0.196 min^{-1} and $k_{vit\ C}$ = 0.0307 min^{-1}); however, the studies were carried to only a single half-life with models without vitamin C and to less than one half-life for models with vitamin C. This was mainly a result of the stability of 5mTHF in the presence of vitamin C, requiring long time periods for folate degradation.

NFC OJ Pasteurization. The model studies demonstrated the degree of 5mTHF degradation under certain processing variables. These findings were extended with pasteurization studies of Valencia NFC OJ at various temperatures (82, 88, and 93 °C) in a continuous pasteurizer (**Table 4**). Degradation rates of OJ folate in unlimited O_2 environments appeared to follow pseudo-first-order kinetics, presumably because oxygen is in sufficiently large excess that the rate is governed by the folate concentration. Actual rate constants (k) could not be determined for each temperature because 5mTHF degradation was not taken through the typically recommended three half-lives. Accurate determination of rate constants was impractical because of the long processing times that would be required as a result of the relatively high stability of 5mTHF in OJ. Furthermore, most citrus juice pasteurizers are designed with hold times of <2 min. Apparent rate constants were determined for each temperature and found to be significantly different (one-way ANOVA, $\alpha = 0.05$, df = 11). The apparent $k_{88°C}$ in OJ (0.038 min^{-1}) was similar to the apparent $k_{88°C}$ of the model with vitamin C (0.0307 min^{-1}). Minor differences may be attributed to chemical composition, efficiency of heating, and quenching and amount of O_2 dissolved in the juice. Better comparisons with a broader range of processing times were not within the scope of these experiments.

High-temperature−short-time pasteurization typically yields less loss of vitamins, flavor components, and other labile constituents of thermally processed foods (*25*−*28*). Calculated folate losses in this experiment followed this pattern with maximum losses of <2% (**Table 4**), whereas observed losses were ∼2% or less. In view of the observed stability, these data provide evidence that few differences exist in folate loss among the various pasteurization conditions. The stability of 5mTHF in OJ is mostly attributed to the protective properties of Asc to scavenge free radicals and O_2 as well as reduce oxidized folate forms (*29*). These results confirm the citrus juice model results and observations seen by other investigators (*12*, *20*, *30*). A recent study of temperature and pressure effects, including 5mTHF kinetics in OJ, concluded that 5mTHF was stable over 30 min up to 120 °C (*31*). Although microbes and enzymes are deactivated in juice at 93 °C for 5 s, a 30-s hold time at 93 °C is commonly used in the industry for NFC OJ (*19*, *32*). Even under these conditions, minimal folate loss is expected (∼2.4%).

Polyglutamyl homologues were found to degrade uniformly. This was expected, as the glutamic acid moieties were not believed to contribute significantly to stability (or lability). Apparent rate constants or percent losses were not calculated for each homologue, because differences after heat treatments were too small.

It should be noted that these values might overestimate the folate losses encountered in commercial processing because the pilot-scale pasteurizer and model studies were conducted without attempting to minimize juice exposure to air. Industrial pasteurization processes usually deaerate NFC OJ to 0.5−1 ppm of O_2 (*19*). However, this amount still is in excess of the folate concentrations found in OJ; therefore, further work is required in this regard to determine any differences in OJ folate degradation rate and behavior.

Heat Evaporation. SSFC from a pilot evaporator was found to have a much larger loss of initial folate (>60% loss) than pasteurized OJ samples (folate concentration: fresh OJ, 1.0 nmol/mL, 4.1% RSD, $n = 3$; SSFC, 0.36 nmol/mL, 9.6% RSD, $n = 3$). Although juice in an evaporator is at high temperatures for long periods of time (>65 °C for 2−5 min) (*32*), this large loss was not expected. Further work is required with more samples to evaluate this large apparent loss of folates during the evaporation process, including specific determinations of effects of temperature and time in each stage of the evaporator.

Polyglutamyl homologues were again observed to degrade at rates similar to those of the monoglutamate form. These folate losses were not due to evaporation because all folate forms are large ionic, nonvolatile molecules, which enable them to remain within the OJ at temperatures and vacuums used during evaporation.

Conclusions. The CE-PDA and HPLC-F methods have been shown to be efficient and reproducible with folate recoveries between 82 and 90%. However, the HPLC-F method was found to be more practical than the CE-PDA method because of overall shorter total analysis time. This research offers the first account of polyglutamyl folate determination in citrus products using CE and may serve as a base for future applications of the CE approach.

These results confirmed previous observations that NFC OJ contained ∼1 nmol/mL of folate, and the methods used had the additional advantage of separating up to seven polyglutamyl forms of 5mTHF. Orange byproduct fractions were also found to contain polyglutamyl folates, with the juice accounting for the most folate, followed by the peel, rag and core, pulp, and seeds. It also appeared that the polyglutamyl distributions from sample to sample may have varied with cultivar and/or maturity. SSFC, NFC GJ, and 12 °Brix CPM, respectively, contained 60, 80, and 90% less folate than NFC OJ. NFC OJ was the most folate-abundant commercial citrus product of those tested.

Results of kinetic studies provided evidence that vitamin C protects 5mTHF from degradation 7-fold within model solutions and within Valencia NFC OJ. Loss of initial folate from fresh Valencia OJ was minor (<2%) with most commercial pasteurization conditions and greater with heat evaporation conditions. Individual polyglutamyl folates were observed to degrade similarly during heat treatments, which confirmed that glutamic acid residues have no apparent effect on stability.

ABBREVIATIONS USED

5mTHF, 5-methyltetrahydrofolate; ANOVA, analysis of variance; Asc, ascorbic acid; CE, capillary electrophoresis; CPM, citrus peel molasses; DTT, dithiothreitol; F, fluorescence; FAC, folate affinity chromatography; FBP, folate-binding protein; GJ, grapefruit juice; HPLC, high-performance liquid chromatography; LOD, limits of detection; MS, mass spectrometry; NFC, not-from-concentrate; OJ, orange juice; PDA, photodiode array; RSD, relative standard deviation; SPE, solid-phase extraction; SSFC, single-strength orange juice from concentrate; TFA, trifluoroacetic acid; UV−vis, ultraviolet visible.

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