Reversed-Phase HPLC/EC Determination of Folate in Citrus Juice by Direct Injection with Column Switching†

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An automated HPLC method for the determination of 5-methyltetrahydrofolate (5-MeTHF) in citrus juice was developed. The method utilized a two-position, 10-port valve that enabled direct injection of filtered citrus juice into an HPLC system. Sample cleanup occurred on a precolumn, followed by backflush of the analyte to the analytical column. Separation was by reversed-phase "ion-pair" HPLC with selective amperometric detection on a glassy carbon electrode (E = +200 mV vs Ag;AgCl). Treatment of juice samples with a folate polyglutamate hydrolase (conjugase) enzyme prior to analysis yielded higher results than those obtained by microbiological assay using *Lactobacillus casei* without enzyme treatment, thus providing additional evidence for the existence of 5-MeTHF polyglutamates in citrus juices. The total analysis time was less than 15 min per sample. Folate levels in over 100 commercial juices analyzed ranged from 0.229 to 0.401 μg mL^{-1} (10–18% U.S. RDA) and from 0.078 to 0.178 μg mL^{-1} (4–8% U.S. RDA) for orange and grapefruit, respectively. Replicate analyses (N = 14) of a single unpasteurized juice averaged 0.247 μg mL^{-1} with a coefficient of variation of 2.8%.

INTRODUCTION

Naturally occurring folic acid compounds and their derivatives, collectively referred to as folate or folacin, are nutritionally essential due to their role as coenzymes in the biosynthesis of nucleic acids, amino acids, and proteins. Folic acid deficiency is known to be a cause of megaloblastic bone marrow and macrocytic anemia and is either directly or indirectly responsible for the defective synthesis of DNA (Hawkes and Villota, 1989).

Citrus juice, in particular orange juice, has been reported to be a good source of dietary folate (Hill and Attaway, 1971; Streiff, 1971), the principal forms being reduced 5-methyltetrahydrofolate (5-MeTHF) and its polyglutamate derivatives (Gregory et al., 1984). As with most naturally occurring folate compounds, 5-MeTHF is sensitive to heat and light and can readily undergo air oxidation. However, the presence of mild reducing agents such as ascorbate does exert a protective effect.

With increasingly more attention given to the nutritional value of processed foods, it is important to have accurate analytical methods that can be used routinely to monitor nutritional status. Traditional methods of analysis for folate have been limited due to the fact that most samples contain only trace levels of these compounds. Also, the relative instability of many folate derivatives has led to wide variability in analytical results.

In a recent paper (White, 1990), an HPLC method for the determination of 5-MeTHF monoglutamate in citrus juices was reported. The method utilized solid-phase extraction (SPE) for sample cleanup, followed by reversed-phase separation, and selective amperometric detection on a glassy carbon electrode (E = +200 mV vs Ag;AgCl). Treatment of the juice samples with a folate polyglutamate hydrolase (conjugase) enzyme (Engelhardt and Gregory, 1990) prior to analysis caused an increase in the apparent level of the monoglutamate and yielded results in reasonable agreement with those obtained by microbiological assay using *Lactobacillus casei* (Herbert, 1966). The objectives of this work were to apply and evaluate a column-switching technique that enables direct injection of filtered citrus juice into the HPLC system. The two-position, 10-port valve directs the injected sample to a short precolumn where sample cleanup occurs. This is followed by a backflush of the analyte to the analytical column. The automated method reduces sample handling, thus minimizing preparation time and possible oxidative losses of folate.

MATERIALS AND METHODS

Materials. 5-Methyltetrahydrofolic acid (5-MeTHF, barium salt) for use as a standard was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. An estimated 80% purity was calculated from the absorbance at 290 nm of a freshly prepared standard solution (18.3 mg L^{-1}) and a published molar absorptivity of 2.9 × 10^4 (Larabee et al., 1961). L-Ascorbic acid and tetrabutylammonium dihydrogen phosphate (TBAP, 1.0 M solution) were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Glacial acetic acid, concentrated sodium hydroxide, and metaphosphoric acid were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Concentrated (85%) phosphoric acid was obtained from Mallinckrodt (Paris, KY).

Methods. *Preparation of Standard and Samples.* A primary stock solution of 5-MeTHF was prepared by dissolving approximately 12.5 mg of the salt in 100 mL of 0.05 M phosphate/acetate buffer (adjusted to pH 7.2 with concentrated NaOH) containing 4.0 g of ascorbic acid and 5.0 g of metaphosphoric acid. This was kept frozen at −20 °C until needed. Secondary stock solutions of approximately 1.25 μg mL^{-1} folate and 0.4 mg mL^{-1} ascorbate were prepared fresh daily by dilution of the primary stock with HPLC mobile phase A. Commercial frozen concentrated orange juice (FCOJ), single-strength orange and grapefruit juices (SSOJ and SSGJ), and reconstituted orange and grapefruit juices from concentrate (OJFC and GJFC) were obtained from grocery store shelves. FCOJ samples were diluted 2.5 times (by weight) with deionized water to approximately 11.8 °Brix. Ten-milliliter portions of each juice were centrifuged at 10 000 rpm and 2 °C for 15 min. One milliliter each of the clarified

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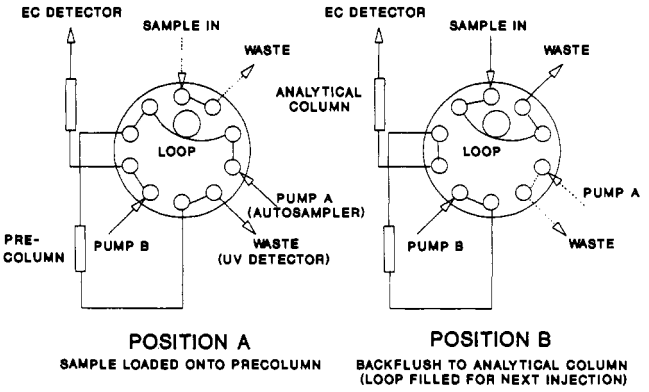

Figure 1. Ten-port valve configured for loading to a precolumn with backflush to the analytical column.

juice samples was pipetted into separate 1.5-mL microcentrifuge tubes. The pH was adjusted to about 5.0 by using 1.0 M NaOH, followed by the addition of 0.2 mL of a conjugase enzyme suspension. The enzyme suspension had been previously extracted from "fresh frozen" hog kidney according to the procedure outlined by Gregory et al. (1984); it was estimated to contain approximately 0.33 mg of protein mL^{-1}. This volume of enzyme (per milliliter of juice) was found to be optimum for maximum yield of the monoglutamate after incubation. The samples were incubated in a water bath at 37 °C for 90 min, cooled rapidly on ice, and then centrifuged again at 10 000 rpm for 5 min. The samples were finally filtered through 0.45-μm nylon Acrodisc syringe-type filters (Gelman Scientific, Ann Arbor, MI) and transferred to amber autosampler vials of 300-μL capacity for injection. Samples were kept cold and shielded from light when not on the autosampler tray.

HPLC Methods. Mobile phase A was 10% methanol in phosphate/acetate buffer (pH 5.0) containing 0.005 M TBAP. It was prepared by mixing 100 mL of HPLC grade methanol (Fisher) with sufficient 0.05 M phosphate/acetate buffer (adjusted to pH 5.0 with concentrated NaOH) to give 1 L. Five milliliters of 1.0 M TBAP was added prior to the final dilution. Mobile phase B (30% methanol) was prepared in a similar manner except that 300 mL of methanol was used. Both solvents were filtered through a 0.45-μm filter and degassed by sparging with helium. The HPLC system consisted of Waters (Milford, MA) Model 510 and Model 6000 LC pumps (pump A and B, respectively), a Model 721 system controller, and a Model 710B WISP autosampler. The injection volume was 20 μL and the flow rate 1.0 mL min^{-1}. A two-position, 10-port stainless steel valve with electric actuator (Valco, Houston, TX) was used to direct the injected sample to a precolumn and to allow backflushing to the analytical column. The valve was equipped with a 20-μL sample loop for manual injections. For the initial experiments, a UV detector was employed to detect unretained ascorbate as it eluted from the precolumn. The UV detector was a Waters Model 441 with fixed-wavelength (254 nm) and a sensitivity setting of 2.0 AU full scale. The EC detector was an EG&G Princeton Applied Research Model 400 (Princeton, NJ) equpped with a glassy carbon electrode operated at +200 mV (vs Ag;AgCl, 3 M NaCl) with the sensitivity at 10 nA full scale. The precolumn was a Waters Nova-Pak C18 (3.9 × 75 mm) with 4-μm packing. The analytical column was a Zorbax ODS (Du Pont, Wilmington, DE) (4.6 mm × 25 cm) with 5-μm packing. Separations were performed at ambient temperature. Typically, a 5-min equilibration period was employed between samples, yielding a total analysis time of about 15 min per sample. Data were acquired with a 20-bit A/D converter (CSI Model 160S, Autochrom Inc., Milford, MA) at a rate of no less than 2 Hz. Peak height calculation was performed by using APEX chromatography software (Autochrom, Inc.) with the aid of a 286 AT-style computer (CompuAdd Corp., Austin, TX). Data were corrected for the purity of the standard and dilution which accompanied addition of enzyme.

RESULTS AND DISCUSSION

Figure 1 illustrates the switching valve rearrangement for sample cleanup and backflush. Injection by autosampler was accomplished with the valve initially in position A. The injected sample was carried by mobile phase A onto a short precolumn. The optimum switching time was taken to be the point at which the ascorbate peak (detected by UV absorbance at 254 nm) just returned to baseline, about 1.25 min under our conditions (see Figure 2). Switching time depends, of course, on the void volume of the precolumn and the length and diameter of tubing used. Switching the valve to position B allowed mobile phase B to backflush the analyte from the precolumn onto the analytical column and eventually to the EC detector. Manual injection was conveniently performed by filling the sample loop while the valve was in position B. Switching to position A injected the sample, and the procedure was then carried out in the same manner as described for autosampler injection.

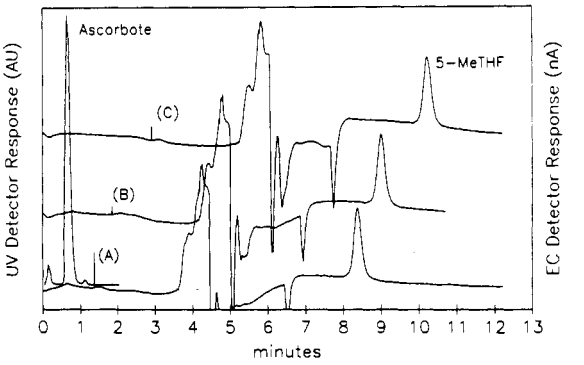

Figure 2. Ascorbate (0.4 mg mL^{-1}) by UV detection and standard 5-MeTHF (1.25 μg mL^{-1}) by HPLC/EC. Column switching times are indicated by baseline markers: (A) 1.25 min; (B) 1.90 min; (C) 3.00 min. Chromatographic conditions are described in the text.

It was determined earlier (White, 1990) that the presence of ascorbate in citrus juice was the main obstacle to selective detection of 5-MeTHF when amperometric detection at +200 mV vs Ag;AgCl was used. It was therefore necessary to reduce the level of ascorbate in the samples by using a solid-phase extraction procedure. The 10-port switching valve enables this extraction to be carried out automatically. Figure 2 is an overlay of typical chromatograms obtained by using the switching technique. The large band that elutes about 2.5 min after column switching is probably residual ascorbate and perhaps other unretained compounds electroactive at +200 mV. This peak is followed by some disturbance which eventually subsides before elution of 5-MeTHF. Peaks were usually symmetrical in shape, and peak height was used for the calculations. A series of standard dilutions over the range $x = 0.25$–1.25 μg mL^{-1} resulted in a linear detector response, $y = 0.003 + 0.996x$ ($R^2 = 0.9992$).

At pH 5, 5-MeTHF is essentially anionic and the use of an ion-pairing reagent (TBAP) in mobile phase A improved its retention on the C18 precolumn (Rebello, 1987). A small percentage of methanol (10%) facilitated the washing of less tightly bound, and possibly interfering, compounds (such as ascorbate) through the precolumn while 5-MeTHF was retained. Increasing the methanol content to 30% in mobile phase B provided sufficient solvent strength to elute 5-MeTHF. Ion-pairing reagent (TBAP) was also added to mobile phase B to preserve the integrity of the ion pairs; otherwise, deterioration of the peak shape of 5-MeTHF was noted. Complete retention of 5-MeTHF on the precolumn is illustrated in Figure 2. A delay in switching time (position A to B) for up to 3 min had little effect on the height of the 5-MeTHF peak; no significant difference in the calculated results was noted.

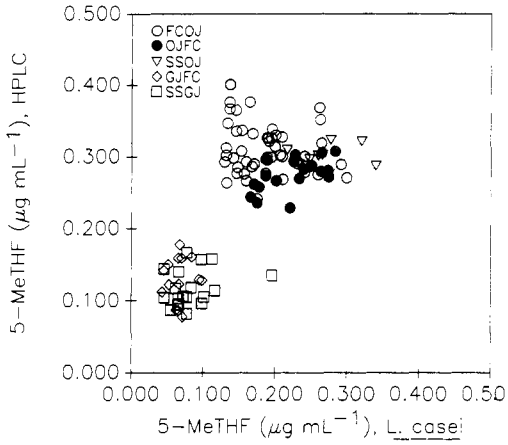

Figure 3. 5-MeTHF in orange and grapefruit juices. Results are from the HPLC method vs microbiological assay (*L. casei*).

Table I. 5-MeTHF (Micrograms per Milliliter) in Commercial Orange Juices: Results from Microbiological Assay with *L. casei* and the HPLC Method

	OJFC (N = 18)		SSOJ (N = 9)		FCOJ (N = 50)[a]	
	micro	HPLC	micro	HPLC	micro	HPLC
mean	0.224	0.275	0.267	0.302	0.187	0.313
max	0.284	0.308	0.340	0.324	0.300	0.401
min	0.166	0.229	0.217	0.283	0.130	0.241

[a] Data normalized to 11.8 °Brix.

Table II. 5-MeTHF (Micrograms per Milliliter) in Commercial Grapefruit Juices: Results from Micobiological Assay with *L. casei* and the HPLC Method

	GJFC (N = 14)		SSGJ (N = 19)	
	micro	HPLC	micro	HPLC
mean	0.068	0.130	0.084	0.117
max	0.099	0.178	0.196	0.167
min	0.043	0.078	0.046	0.082

Over 100 commercial juices were analyzed according to this method, and results were compared with those obtained from an independent analysis by microbiological assay using *L. casei* (FDOC, 1990). These data are plotted in Figure 3 and summarized in Tables I and II. Although there was some agreement between methods, the HPLC results were generally higher. There are two reasons why higher results are to be expected when the HPLC method is used. First, no conjugase enzyme treatment of juice samples was employed prior to the microbiological assay. Tamura (1972) has shown that the response of *L. casei* to folates is dependent on the polyglutamyl chain length, with poorer response elicited for folate compounds with greater than three glutamic acid residues. The higher levels of 5-MeTHF monoglutamate following the enzyme treatment, confirmed by the HPLC analyses, lend support to earlier evidence that polyglutamate forms are present in citrus juices (Stokstad et al., 1977). Figure 4 illustrates the dramatic increase in 5-MeTHF monoglutamate as a result of enzyme treatment of a typical orange juice sample. Second, calibration of the microassay method was performed by using the oxidized folic acid monoglutamate (pteroylglutamic acid) rather than the reduced 5-methyl-substituted derivative. Earlier evidence suggests (Phillips and Wright, 1982) that *L. casei* responds better to the former, a factor which would undoubtedly led to underestimation of the level of 5-MeTHF in the juice samples.

The U.S. Recommended Dietary Allowance (U.S. RDA) for daily folate intake is declared to be 400 μg for adults (*Federal Register*, 1984). For a food to be declared a significant source of a nutrient, that nutrient must be present in the food at a level at least 10% of the U.S. RDA for a reasonably sized serving (*Federal Register*, 1973). The standard size serving for orange juice is 6 fl oz or about 177 mL. From the data summarized in Table I, it can be seen that all of the orange juice samples exceed the 10% requirement of 40 μg/6 fl oz (0.226 μg mL^{-1}); results by HPLC analysis indicate a range of 10–18% of the U.S. RDA for a standard serving. As for grapefruit juice, the means of the HPLC results indicate that, on average, a 6 fl oz serving of GJFC or SSGJ can provide 5% of the U.S. RDA (see Table II). It should be noted that in a more recent report on Recommended Dietary Allowances (FNB, 1989) it is suggested that the current U.S. RDA of 400 μg might be an overestimation and that 200 μg is probably sufficient for an adult male.

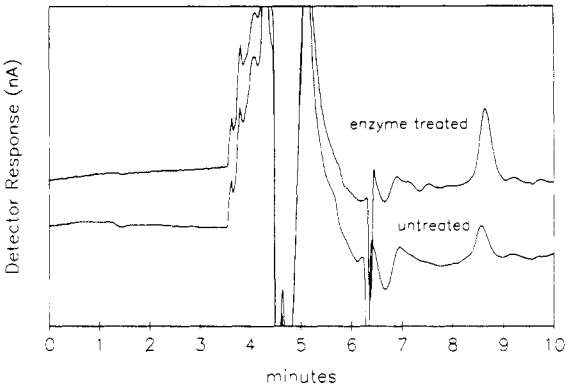

Figure 4. Effect of conjugase enzyme treatment on the level of 5-MeTHF monoglutamate in orange juice.

To estimate the reproducibility of the HPLC method, repetitive analyses (N = 14) of a single unpasteurized juice sample (9.8 °Brix) were carried out. Results ranged from 0.237 to 0.265 μg mL^{-1}, with a mean of 0.247 μg mL^{-1} and a standard deviation of 0.007 μg mL^{-1}. This corresponds to a coefficient of variation of about 2.8%. Thus, it appears that the rather wide range of 5-MeTHF measured in the juices is due to actual variability in folate content and is not an artifact of the method. There was no evidence of 5-MeTHF loss from samples when prepared as described under Methods, even after refrigerator storage for up to 3 days. Further work remains to be done to ascertain the effects of processing on the folate content of citrus juices.

CONCLUSION

An automated HPLC method for the determination of citrus juice folate has been developed. The major advantage over earlier methods is the capability of direct injection of filtered juice into the HPLC system. The method obviates the need for manual solid-phase separation of 5-MeTHF from ascorbic acid prior to HPLC. Allowing the 5-MeTHF to remain in the citrus juice matrix up to the time of injection reduces the possibility of oxidative losses as a result of sample preparation. The total time from injection to elution of the 5-MeTHF peak is about 8 min. Treatment of juices with a conjugase enzyme prior to analysis is recommended for quantitating total folate. The method should prove useful for monitoring the folate content of processed citrus juices.

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