Determination of Folate in Cereal-Grain Food Products Using Trienzyme Extraction and Combined Affinity and Reversed-Phase Liquid Chromatography

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A trienzyme treatment (employing rat plasma folate conjugase, α-amylase, and protease), followed by affinity chromatography and reversed-phase HPLC, was evaluated for quantification of folates in unfortified and fortified cereal-grain products. Total folate concentrations determined by HPLC for unfortified white and wheat bread, rice, and spaghetti (21.3 ± 0.69, 29.8 ± 1.94, 10.8±0.57, and 22.3±1.77 mg/100 g of product, respectively) were close to those determined by microbiological assay. In all four products, 5-methyltetrahydrofolate, 10-formyldihydrofolate, 10-formylfolate, 5-formyltetrahydrofolate, and folic acid were identified. The HPLC method allowed good separation and quantification of these folates in unfortified and fortified cereal-grain products within 33 min. Compared to folate conjugase treatment only, trienzyme treatment led to variable increases of measured total folates in white bread (4%), spaghetti (34%), and rice (33%). It is concluded that (a) trienzyme extraction is applicable to both HPLC and microbiological assays and (b) traditional extraction and folate conjugase treatments are not appropriate for analysis of cereal-grain products. This study also provides new information regarding the distribution of naturally occurring folates in this class of foods.

Keywords: *Folic acid; folate; cereal-grain products; fortified foods; trienzyme extraction; HPLC*

INTRODUCTION

The total folate content of foods is usually determined by microbiological assay using *Lactobacillus casei* (Finglas et al., 1993). If the quantification of specific chemical forms of folate is of interest, chromatographic methods, such as ion-exchange or reversed-phase HPLC, can ensure the separation and determination of various folates (Gregory, 1989). Regardless of the analytical procedure, the accuracy of the analysis is highly dependent on the merits of the preparative methods employed. A key preparative phase is the extraction (Gregory et al., 1990). Also important is the enzymatic deconjugation of the polyglutamyl folates, which must be optimized for each type of sample (Engelhardt and Gregory, 1990). Because of the low endogenous folate content of many foods and their complex compositions, food extracts often must be concentrated and purified if chromatographic methods are to be employed for the quantification of folates.

The most common method of extraction is heating a buffered sample homogenate to thermally denature folate-binding proteins and enzymes that may catalyze folate degradation or interconversions (Gregory, 1989). Gregory et al. (1990) showed the need for a second extraction in the analysis of many foods and the superiority of the Wilson and Horne (1984) extraction buffer (mixed Hepes/Ches buffer, 101 mM ascorbate, 200 mM 2-mercaptoethanol, pH 7.85) compared to two buffers of lower pH that contained only ascorbate. These studies provided evidence that folate extraction is enhanced by the higher pH of the Wilson and Horne (1984) buffer, while the stability of folate during extraction is increased by the use of ascorbate and mercaptoethanol as preservatives.

Several researchers have demonstrated that treatment of high-protein products with protease and treatment of food items high in starch and glycogen with α-amylase, in addition to the folate conjugase (pteroylpolyglutamate hydrolase) treatment, provided significantly increased measurable total folate concentration (Yamada, 1979; Cerna and Kas, 1983; Pederson, 1988; DeSouza and Eitenmiller, 1990). In 1990, Martin and his colleagues reported that a trienzyme treatment is necessary to determine folate accurately in certain foods. The method included the digestion of food extracts with α-amylase and a nonspecific protease in sequence with the folate conjugase treatment. However, all of these studies employed a microbiological assay for total folate quantification, and they did not distinguish among the different folate derivatives.

This paper presents an optimized adaptation of the components of several procedures including a modified trienzyme extraction, affinity chromatography, and reversed-phase HPLC with UV diode array detection for quantification of the most abundant folate forms in unfortified and fortified cereal-grain products. HPLC findings are compared to results obtained using a microbiological assay. This study provides further evidence of the benefit of a trienzyme treatment in folate analysis. We have focused on cereal-grain products in this study because of the need for improved methods for use in quality control and in regulatory analysis in the implementation of the folic acid fortification program (Food and Drug Administration, 1996)

MATERIALS AND METHODS

Folate Compounds. 5-Methyltetrahydrofolate (disodium salt), 5-formyltetrahydrofolate (calcium salt), and folic acid were obtained from Sigma. 10-Formylfolic acid, 5,10-meth-

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enyltetrahydrofolate hydrochloride, and pteroyltriglutamate were obtained from Schirck's Laboratories (Jona, Switzerland). Stock standard solutions of folate compounds (except of 5,10-methenyltetrahydrofolate) were prepared by dissolving in deaerated 0.1 M phosphate buffer, pH 7.0, portions of which were taken immediately for checking the concentration spectrophotometrically (Blakley, 1969), followed by the rapid addition of sodium ascorbate to a final concentration of 0.5% (w/v). Standard solutions were flushed with nitrogen and stored in small aliquots at −20 °C for 3 months. 5,10-Methenyltetrahydrofolate was dissolved in 0.01 M HCl, and its concentration was assayed using a molar absorptivity coefficient of 25000 M^{-1} cm^{-1} at 352 nm (Robinson, 1971). When 5,10-methenyltetrahydrofolate was dissolved in 0.05 M Tris/HCl buffer, pH 8.27, rapid conversion to 10-formyltetrahydrofolate occurred, which was spontaneously oxidized in the absence of an antioxidant to 10-formyl-7,8-dihydrofolate within approximately 1.5 h. These observations correspond to those of Baggott et al. (1995). The UV spectra of this preparation obtained in 0.1 M HCl (pH 1: λ_{max} 257, 328 nm and λ_{min} 229, 307 nm) and in 0.1 M Tris/HCl (pH 7.4: λ_{max} 234, 333 nm and λ_{min} 301 nm) showed the same spectral characteristics as reported by those authors. To prevent further oxidation, this preparation was thoroughly flushed with nitrogen and stored in small aliquots at −20 °C for up to 3 months. The purity of this preparation determined with reversed-phase HPLC was 96%, with the remaining 4% further oxidized to 10-formylfolic acid.

Samples. One loaf each of unfortified and fortified white and whole wheat bread, each custom prepared at a fortification level of 140 mg/100 g of flour by the American Institute of Baking (Manhattan, KS) by a sponge and dough procedure, were freeze-dried, ground, and stored frozen until analysis. White long-grain rice, spaghetti, and a folic acid fortified breakfast cereal based on whole grain wheat and brown rice flakes (label claim 400 mg of folate/30 g), all purchased from a local supermarket, were ground and stored frozen until analysis. To prepare folic acid fortified rice, a portion of raw rice was cooked in a rice-cooker according to the instructions on the package, except with folic acid solution added to the cooking water to provide a fortification of 140 mg/100 g of raw rice. The fortified, cooked rice was then freeze-dried, ground, and stored frozen until analysis. Fresh pasta fortified with folic acid (fortification level 200 mg/100 g of flour to account for losses caused by leaching during cooking) was prepared by a local restaurant. This pasta was freeze-dried, ground, and stored frozen until analysis.

Enzymes. *Rat Plasma Folate Conjugase (RPC).* Fresh plasma (100 mL, Pel-Freez, Rogers, AR) was dialyzed for 24 h at 2−4 °C in 2 L of Hepes/Ches buffer, pH 7.85 (50 mM Hepes, 50 mM Ches), containing 2% (w/v) sodium ascorbate, 10 mM 2-mercaptoethanol, and 4 g of acid-washed carbon powder (Fisher Scientific, Pittsburgh, PA). The crude rat plasma folate conjugase preparation was transferred to small tubes and stored at −70 °C for up to 6 months with little loss of activity. Each enzyme preparation was assayed for its activity using 1 μmol/L pteroyltriglutamate or pteroylheptaglutamate as substrate at pH 7.0 in 0.1 M phosphate buffer at 37 °C. Conversion of the substrate to folic acid was assayed after 1 h of incubation with a modification of a reversed-phase gradient HPLC method of Cashmore et al. (1980). The quantity of plasma folate conjugase recommended below provided ample activity for complete hydrolysis of these substrates under conditions of the assay.

α-*Amylase.* α-Amylase, 20 mg/mL in Wilson and Horne (1984) extraction buffer, was obtained from Sigma Chemical Co. (St. Louis, MO; No. A0273).

Protease. Protease, 2 mg/mL in Wilson and Horne (1984) extraction buffer, was also obtained from Sigma (No. P-5147).

To assess the amount of folate contributed by the enzymes to the assayed food extracts, enzyme solutions were prepared in concentrations used for extracting 1 g of a dry food sample, followed by analysis for folate using a microbiological assay (Tamura, 1990). It was found that amylase and, to a lesser extent, rat plasma folate conjugase contributed a small amount of folate (total of 28 ng/g of dry sample). All total folate values for cereal-grain products obtained by HPLC or microbiological assay were corrected for this folate derived from the respective enzyme treatments.

Trienzyme Extraction. All preparative and analytical procedures were performed under gold fluorescent lights (General Electric No. F40G0) to minimize photochemical degradation of folates. Trienzyme extraction was performed as a modification of the procedure of Martin et al. (1990). Ten volumes of Hepes/Ches buffer, pH 7.85 (50 mM Hepes, 50 mM Ches), containing 2% (w/v) sodium ascorbate and 10 mM 2-mercaptoethanol (deaerated by flushing with nitrogen), were added to 2 g of dry sample in a 50 mL Oak Ridge PPCO centrifuge tube (Nalge Co., Rochester, NY). For unfortified cereal-grain products two tubes with 2 g each of dry sample were prepared per sample, whereas for fortified products only a single 2 g sample was used. After mixing on a vortex mixer, the tubes were placed for 10 min in a boiling water bath, cooled rapidly in ice, and then homogenized using a Polytron for 30 s at medium setting (Brinkmann Instruments, Westbury, NY). The Polytron tip was rinsed with extraction buffer. This homogenate was subjected to two types of enzyme treatments: (1) rat plasma folate conjugase only (1, 2, and 4 h at 37 °C) and (2) trienzyme treatment [rat plasma folate conjugase and α-amylase (4 h at 37 °C) followed by protease (1 h at 37 °C]. The following volumes of enzymes were used per tube: 500 μL of rat plasma folate conjugase, 1.0 mL of α-amylase, and 2.0 mL of protease. In certain studies, amylase, protease, or both were omitted to evaluate their respect effects on folate extraction. At the end of all enzyme treatments, tubes were heated in a boiling water bath for 5 min, cooled on ice, and centrifuged (5000*g*, 10 min, 5 °C). The residue was resuspended in 5 mL of extraction buffer and recentrifuged for 10 min. The combined supernatants were filled to an exact volume (e.g. 25 or 50 mL) with extraction buffer, filtered through a Whatman filter paper No. 1, then flushed with nitrogen, and stored at 4 °C until extract purification and analysis. To test the necessity of an overnight incubation with protease, as performed in the procedure developed by Martin et al. (1990), an extract of unfortified white bread was incubated for 4 h at 37 °C with rat plasma folate conjugase and α-amylase, followed by an overnight incubation with protease (at 37 °C).

Extract Purification. An affinity chromatography sorbent with immobilized folate binding protein (FBP), prepared by a minor modification of the method of Selhub et al. (1980) as described by Gregory and Toth (1988), was used to purify and concentrate the sample extracts. FBP isolated by affinity chromatography of bovine milk whey was coupled to beaded agarose (Affigel 10, Bio-Rad Laboratories, Richmond, CA), and 0.7 cm (i.d.) × 20 cm glass columns (Bio-Rad) were filled with 2 mL of the FBP−Affigel matrix (binding capacity of 20 nmol of folate or more). The actual binding capacity was tested periodically (depending on the frequency of usage) by overloading the column with a known amount of folic acid, followed by elution and analysis as described below. All sample application, wash, and elution steps were conducted at ambient temperature at a flow rate of 0.3 mL/min as controlled by a peristaltic pump. The sample extract was applied to a FBP−Affigel column that had been previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The column was washed with 5 mL of 0.025 M potassium phosphate, pH 7.0, containing 1.0 M NaCl, followed by 5 mL of 0.025 M potassium phosphate, pH 7.0. Folate elution was accomplished with 2 mL of 0.02 M trifluoroacetic acid/0.01 M dithiothreitol (discarded, corresponding to the void volume of column plus tubing) and followed by 5 mL of 0.02 M trifluoroacetic acid/0.01 M dithiothreitol, which contained all of the eluted folates. During the elution 30 μL of 1 M piperazine was added to neutralize the eluent, and sodium ascorbate and 2-mercaptoethanol were added as antioxidants to a final concentration of 0.2% (w/v) and 10 mM, respectively. Eluents were flushed with nitrogen gas for 10 s and kept frozen until used for the HPLC analysis (typically within 1−2 days).

HPLC Analysis. A reversed-phase gradient HPLC method was performed as a modification of the procedure of Gregory et al. (1984a). The folates were separated on a Phenomenex

Table 1. Recovery (Percent) of Folates during Trienzyme Extraction and Affinity Chromatography Purification[a,d]

standards	5-CH_3-H_4folate	10-CHO-H_2folate	10-CHO-folate	5-CHO-H_4folate	folic acid
in Hepes/Ches buffer, pH 7.85[b]	94.5 ± 9.9	96.7 ± 6.2	105 ± 6.6	101 ± 6.2	107 ± 13.5
added to white bread[c]	98.6 ± 3.1	84.6 ± 5.4	97.0 ± 6.8	88.6 ± 10.2	107 ± 15.8
added to white rice[c]	94.8 ± 3.4	84.8 ± 5.6	84.0 ± 3.9	85.3 ± 1.2	87.9 ± 6.7
added to pasta[c]	98.7 ± 3.9	96.4 ± 9.9	85.2 ± 5.1	90.4 ± 14.1	88.2 ± 3.7

[a] Heat extraction was for 10 min in capped tubes; trienzyme incubation was for 5 h at 37 °C (4 h of incubation with rat plasma folate conjugase and α-amylase, 1 additional hour with protease); heat denaturation of the enzymes was for 5 min; and other conditions as described in text. [b] Recovery was calculated as percentage of peak area left when compared with the chromatogram of untreated standard mixture. [c] Recovery was calculated by comparing peak areas of spiked samples (measured amount) with the sum of unspiked sample and untreated standard mixture (expected amount). [d] Figures represent means and standard deviation of duplicate determinations, and the amount of each folate compound was 100 ng/sample.

Ultremex C_{18} column (5 mm, 250 × 4.6 mm i.d.). Gradient elution was performed with acetonitrile and 33 mM phosphoric acid, pH 2.3, and a flow rate of 1.0 mL/min. The run time was 33 min and the time between injections 45 min. The gradient was started at 5% (v/v) acetonitrile maintained isocratically for the first 8 min, after which time the acetonitrile concentration was raised linearly to 17.5% (v/v) within 25 min. The injection volume was 1 mL for unfortified products and could be lowered for the analysis of fortified products if folic acid was the only compound of interest. The absorbance of eluted folates was monitored at 280 nm using a diode array detector (LC-235, Perkin-Elmer, Norwalk, CT). Peak identification was based on retention time and comparison of the absorption spectrum acquired for each peak with the spectrum of the corresponding folate standard.

Quantification. Quantification was based on an external standard method in which the peak area was plotted against the molar concentration. Calibration plots using least-squares regression analysis were prepared for 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolic acid, and folic acid in the concentration range of 0.05−0.25 nmol/mL. The quantification of 10-formyldihydrofolate was based on the known starting amount of 5,10-methenyltetrahydrofolate and the measured amount of 10-formylfolic acid in the 10-formyldihydrofolate preparation (after conversion of 5,10-methenyltetrahydrofolate to 10-formyltetrahydrofolate at slightly alkaline pH and oxidation of the latter compound to 10-formyldihydrofolate). The difference between 5,10-methenyltetrahydrofolate and 10-formylfolic acid represents 10-formyldihydrofolate.

Recovery Studies. A standard solution of each of the five folate derivatives found in cereal-grain products was used for spiking to assess the loss of folates during the trienzyme extraction and affinity chromatography. Recovery studies were performed either by carrying the folate standards in Hepes/Ches extraction buffer through the whole procedure or by adding them to white bread, rice, or spaghetti before extraction and carrying them through the trienzyme extraction and affinity chromatography (100 ng of each folate derivative was added per gram of dry sample for recovery studies).

Microbiological Assay. Total folate content of trienzyme treated extracts was also determined by microbiological assay with *Lactobacillus casei* (Tamura, 1990) using folic acid Casei medium (Difco, Detroit, MI). Portions of the extracts were flushed with nitrogen gas and stored at −20 °C until analyzed.

Statistical Analysis. Differences among enzyme treatments (trienzyme versus 4 h rat plasma folate conjugase) were evaluated using unpaired *t*-test (Neter and Wasserman, 1974). Differences were considered to be significant at $P < 0.05$. These analyses were performed using SigmaStat v. 1.0 software (Jandel Corp., San Rafael, CA).

RESULTS AND DISCUSSION

Trienzyme Extraction and Affinity Chromatography Purification. The stability of folate standards carried through trienzyme extraction and affinity chromatography purification as well as recovery of folates added to white bread, rice, or spaghetti was found to be very good (Table 1). Recovery of the five different folates was between 85 and 107%, indicating also that the extraction buffer employed did not lead to chemical interconversion or loss of the analyzed folate derivatives. This emphasizes again the superiority of combined use of ascorbate and 2-mercaptoethanol concerning the stability of folates during the extraction, as has also been reported by Wilson and Horne (1984), Gregory et al. (1990), and Vahteristo et al. (1996). Another important factor is that little or no conversion of 10-formyltetrahydrofolate to 5-formyltetrahydrofolate occurs in the pH 7.85 buffer containing ascorbate and 2-mercaptoethanol (Wilson and Horne, 1984).

This extraction buffer is compatible with the use of rat plasma as source for folate conjugase with no further pH adjustment because the folate conjugase activity in rat plasma exhibits a broad pH profile with an optimum at pH 6.2−7.5 (Horne et al., 1981). Although a 1 h incubation with rat plasma folate conjugase was fully adequate for the complete hydrolysis of synthetic pteroyltriglutamate and pteroylheptaglutamate in buffer solution alone and in extracts from samples of animal tissues, polyglutamyl folates in cereal-grain extracts were not fully hydrolyzed in 1 h under the conditions of this study. We observed increases of measurable folates with increasing time of rat plasma folate conjugase incubation for up to 4 h (Table 2). Although data for 2 h incubations are not presented here, their results were intermediate between those of 1 and 4 h treatments.

The effect of trienzyme treatment compared to the 4 h rat plasma folate conjugase treatment alone was found to be matrix-dependent; that is, the extent of increase provided by the additional amylase and protease treatments depended on the type of food sample. While in white bread trienzyme treatment led to a small and not significant increase of measurable total folates (4%), in spaghetti and rice this treatment caused a significant increase of the total folate content with 34% and 33%, respectively (Table 2). These results confirm and extend previously reported findings regarding the beneficial effect of trienzyme treatment in folate analysis, as discussed below.

Pedersen (1988) reported that folate contents in starch-containing food items were 9.3% higher after a simultaneous treatment with α-amylase and chicken pancreas folate conjugase than those treated with folate conjugase alone. Products used in a study by Martin et al. (1990) represented a variety of foods differing in protein and carbohydrate content. In all 12 foods examined, the trienzyme procedure resulted in higher total folate values with increases ranging from 2.3% for a fortified rice cereal to 50% for canned tuna, giving an average increase of 19% as compared with folate conjugase treatment alone.

Since the procedure of Martin et al. (1990) used an overnight incubation with protease, we evaluated an extract of unfortified white bread that was incubated

Table 2. Effect of Different Enzyme Treatments on Measurable Folate Content (Nanograms per Gram Dry Matter) in Unfortified Cereal-Grain Products As Determined by HPLC[a]

	5-CH_3-H_4folate	10-CHO-H_2folate	10-CHO-folate	5-CHO-H_4folate	folic acid	total folate
white bread						
RPC 1 h[b]	37.0 ± 5.00	54.2 ± 13.9	68.7 ± 0.34	90.8 ± 5.41	10.3 ± 5.94	244 ± 15.5
RPC 4 h[b]	72.8 ± 0.88	89.4 ± 1.00	85.0 ± 1.58	103 ± 4.40	15.3 ± 2.07	341 ± 0.62
trienzyme[c]	85.4 ± 5.58	94.4 ± 2.82	86.5 ± 4.47	97.0 ± 4.85	16.3 ± 2.84	354 ± 10.6
white rice						
RPC 1 h[b]	31.6 ± 5.02	0.00	14.5 ± 8.02	6.92 ± 3.77	9.93 ± 0.14	61.1 ± 15.0
RPC 4 h[b]	46.4 ± 2.55	0.00	31.4 ± 1.50	14.5 ± 1.54	14.8 ± 2.34	102 ± 4.84
trienzyme[c]	48.5 ± 1.42	15.8 ± 2.52	43.0 ± 7.80	17.1 ± 4.69	19.2 ± 1.93	136 ± 5.74[d]
spaghetti						
RPC 1 h[b]	16.6 ± 0.82	10.3 ± 2.04	62.7 ± 6.91	30.2 ± 4.71	13.1 ± 0.18	125 ± 4.40
RPC 4 h[b]	26.2 ± 3.03	9.09 ± 0.31	90.0 ± 24.4	62.4 ± 28.5	11.6 ± 1.89	188 ± 4.86
trienzyme[c]	34.2 ± 3.30	55.2 ± 4.14	105 ± 4.83	59.2 ± 2.90	14.6 ± 5.81	251 ± 17.7[d]

[a] Incubation with rat plasma folate conjugase (RPC) for 1 and 4 h, trienzyme treatment: incubation with RPC and α-amylase for 4 h and with protease for 1 additional hour. [b] Mean and SD of duplicate determination. [c] Mean and SD of four replicates. Samples were analyzed after freeze-drying. Total folate is expressed as folic acid equivalents. [d] Total folate after trienzyme treatment significantly higher than folate after 4 h RPC incubation ($P < 0.05$).

for 16 h with protease after the regular 4 h incubation with rat plasma folate conjugase and α-amylase. This long incubation time caused a 20% decrease of the measured total folate concentration, relative to the 1 h treatment, which is a disadvantage of such a long incubation procedure. Although we have not conducted a full optimization of the length of protease treatment, a 1 h treatment appears to be reasonable.

Purification and concentration of cereal-grain extracts was of critical importance to the success of the final analysis. Affinity chromatography using immobilized folate binding protein gave a very specific and efficient purification. Furthermore, affinity chromatography permitted extracts to be concentrated 10-fold or more, so that even small endogenous folate concentrations present in cereal-grain products could be quantified with sufficient sensitivity using UV detection. Since trienzyme-treated extracts were clearer and less viscous than extracts obtained with rat plasma folate conjugase only, they passed the affinity column more quickly and left less starchy residue remaining on the top of the column. For unknown reasons, the trienzyme treatment often yielded better peak shape of folates during the HPLC separation.

A critical folate derivative with respect to affinity chromatography is 5-formyltetrahydrofolate. It is known to have a lower affinity for the immobilized FBP than the other derivatives (Wagner, 1982). Preliminary studies performed to test the binding and elution behavior of 5-formyltetrahydrofolate showed that no loss occurred if the loading was 10% of the binding capacity of the affinity column. All of the 5-formyltetrahydrofolate was eluted within 5 mL of trifluoroacetic acid/dithiothreitol eluent. In contrast, 10% loss of 5-formyltetrahydrofolate occurred if the loading was 25%, and 20% if the column was loaded with 50% of its binding capacity. This corresponds well with findings of Selhub (1989) and indicates that folate loading should not exceed 25% of the binding capacity to permit recovery of ≥90% of 5-formyltetrahydrofolate. The addition of piperazine to neutralize the acidic effluent of the affinity column is especially important, since 5-formyltetrahydrofolate is unstable at low pH and may interconvert partially to 5,10-methenyltetrahydrofolate (Robinson, 1971).

HPLC Analysis. The adapted gradient HPLC method allowed a good separation of folates within a 33 min run time (Figure 1a). The capacity factors (k') for 5-methyltetrahydrofolate, 10-formyldihydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, and folic acid were 8.2, 8.5, 9.3, 9.7, and 10.7, respectively. Neither of the reducing agents used (ascorbate and 2-mercaptoethanol) interfered with the chromatographic separation of the studied folate forms.

pABG and tetrahydrofolate were also separated from these folates ($k' = 7.0$ for pABG and $k' = 7.3$ for tetrahydrofolate). 5,10-Methenyltetrahydrofolate was not baseline-separated from 5-formyltetrahydrofolate ($k' = 10.0$). In preliminary studies, we found no evidence of 5,10-methenyltetrahydrofolate or tetrahydrofolate in any of the cereal-grain products tested. Thus, the gradient was not adjusted to give a complete separation between 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. Due to the acidic pH of the mobile phase, this chromatographic system is poorly suited to the identification and quantification of 10-formyltetrahydrofolate because of its tendency to form 5,10-methenyltetrahydrofolate rapidly in this acidic mobile phase (i.e. on-column conversion). 5-Formyltetrahydrofolate also undergoes formation of 5,10-methenyltetrahydrofolate in acidic media, although at a much slower rate (Robinson 1971). Thus, this acidic mobile phase is fully suitable for separation and quantification of 5-formyltetrahydrofolate, as shown previously (Gregory et al., 1984a). In additional preliminary studies, we evaluated a modification of the ion-pair HPLC procedures developed by Horne and Wilson (1984), Varela-Moreiras et al. (1991), and Seyoum and Selhub (1993) using a neutral pH mobile phase, which we recently applied to the measurement of folates in animal tissues (Scott and Gregory, 1996). By this ion-pair HPLC method, no 10-formyltetrahydrofolate was detected in the cereal-grain products analyzed in this study. Because the ion-pair HPLC method is somewhat lengthier than the method described here, its use for cereal-grain products is not recommended.

The detection limits (signal-to-noise ≥3) in this HPLC system were estimated to be 2−4 pmol per injection for 5-methyltetrahydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, and folic acid and 7 pmol for 10-formyldihydrofolate. The response of the UV detector was linear in the normal working condition range. The retention times were very repeatable (CV < 2%, $n = 4$ injections).

Using UV diode array detection, the endogenous folates in cereal-grain products were identified and quantified with adequate sensitivity and great specificity (Figure 1b). The spectra of the eluted folates in the analyzed cereal-grain products matched the spectra of corresponding folate standards reasonably well. Ap-

Table 3. Total Folate Content in Unfortified Cereal-Grain Products Determined by HPLC and Microbiological Assay; Percentage of Each Folate Derivative Relative to the Total Folate Content[a]

	total folate[b] (mg/100 g)		% of each folate derivative relative to the total folate content				
unfortified cereals	microbiol assay	HPLC assay	5-CH_3-H_4folate	10-CHO-H_2folate	10-CHO-folate	5-CHO-H_4folate	folic acid
white bread[c]	21.1 ± 0.19	21.3 ± 0.69	22.5	24.9	22.8	25.5	4.31
wheat bread[d]	29.6 ± 0.40	29.8 ± 1.94	11.0	19.4	33.2	28.9	7.49
white rice[c]	11.2 ± 0.56	10.8 ± 0.57	34.9	10.9	30.3	11.9	13.3
spaghetti[c]	27.5 ± 2.24	22.3 ± 1.77	12.7	20.6	39.1	22.2	5.43

[a] Trienzyme-treated samples. [b] Total folate content corrected for folate contribution by enzymes (28 ng/g dry cereal). All data are expressed on a wet-weight basis (i.e. with no prior dehydration). [c] Mean and SD of four replicates. [d] Mean and SD of three replicates.

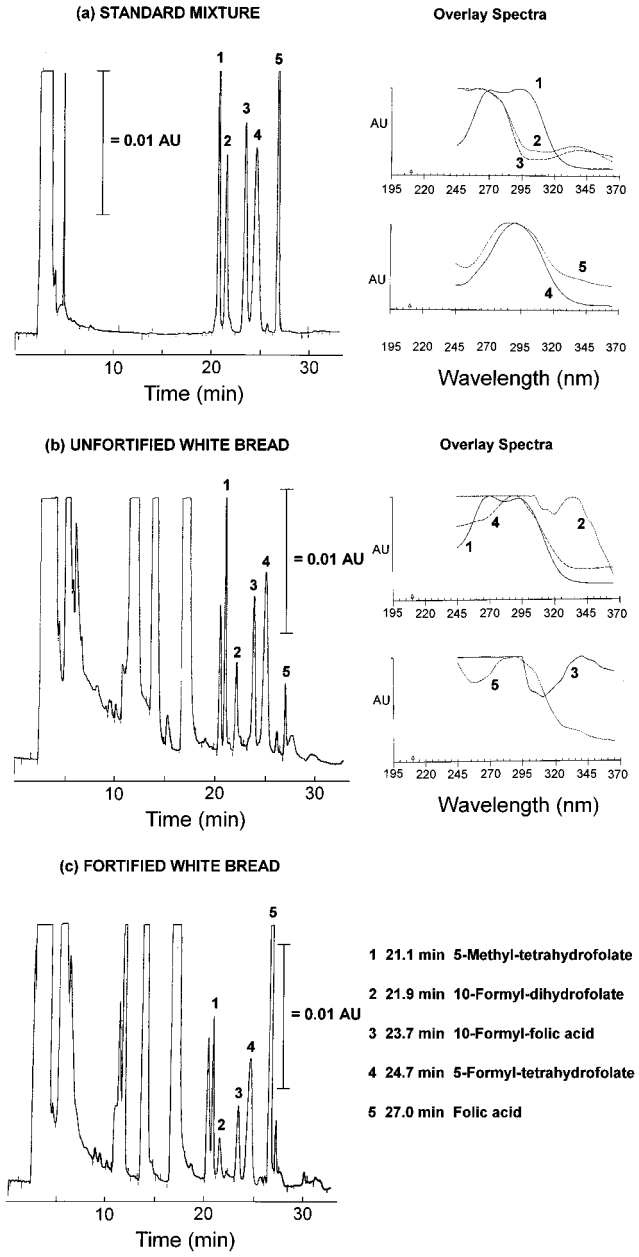

Figure 1. Reversed-phase HPLC separation of the main folate forms found in cereal-grain products and respective UV absorption spectra: (a) standard mixture (0.2 nmol of each folate injected); (b) unfortified white bread (folate amounts from 0.8 g of dry bread); (c) fortified white bread (folate amounts from 0.4 g of dry bread). A Phenomenex Ultremex C_{18} column (5 mm, 250 × 4.6 mm i.d.) and a mobile phase of 33 mM phosphoric acid, pH 2.3, with increasing acetonitrile concentration were used. The gradient was started at 5% (v/v) acetonitrile maintained isocratically for the first 8 min, after which time the acetonitrile concentration was raised linearly to 17.5% (v/v) within 25 min. UV absorption monitoring was at 280 nm.

parent spectral differences between parts a and b of Figure 1 are attributable largely to the difficulty in adjusting the spectra to comparable absorbance ranges for comparison between samples and standards using with the software of the Perkin-Elmer LC-235 diode array detector. As shown for fortified white bread (Figure 1c), this method is suitable for simultaneous identification and quantification of endogenous folates and folic acid derived from fortification. It should be noted that certain non-folate components of foods and biological materials are retained on this affinity chromatography matrix. These compounds, along with reagent peaks, account for the peaks not attributed to folates in this analysis (Figure 1b,c).

Stover and Schirch (1992) provided strong evidence for the existence of 5,10-hydroxymethylenetetrahydrofolate as a prominent intermediate in the nonenzymatic equilibrium between 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. In view of the detection of 5-formyltetrahydrofolate in these cereal-grain products, the concurrent presence of 5,10-hydroxymethylenetetrahydrofolate is a distinct possibility. However, the close correlation between the results of microbiological and HPLC assays as discussed below (Table 3) suggests that this derivative, if present, exists at negligible concentration relative to other folates.

Folate Contents in Unfortified and Fortified Cereal-Grain Products. Total folate contents determined by HPLC for unfortified white and wheat bread, rice, and spaghetti showed excellent correlation with the folate contents determined with microbiological assay (Table 3). These values also were in agreement with previous publications. Previously published data indicate an average folate content of 20−54 mg/100 g for a variety of breads (Ranhotra et al., 1985a), and an average of 19−26 mg/100 g for selected dry pasta products (Ranhotra et al., 1985b). The USDA Food Composition Handbook 8-18 (1991) lists average folate contents of 34 and 50 mg/100 g for white and whole wheat bread, respectively. In Handbook 8-20 (1989) average folate contents of white, long-grain rice and dry spaghetti are listed as 8 and 18 mg/100 g, respectively.

All of these previously reported data have been acquired using microbiological assay. Mueller (1993) determined folate contents of grains, cereal products, and bakery products by means of reversed-phase HPLC. He found folate contents in the range of 14.8−37.1 mg/100 g for a variety of breads. For whole wheat bread he reported a total folate content of 26.0 mg/100 g, with the following molar distribution of folate derivatives: 43.1% 5-formyltetrahydrofolate, 25.4% 10-formylfolic acid, 15.0% 5-methyltetrahydrofolate, 14.2% folic acid, and 2.3% tetrahydrofolate.

We detected and identified in all analyzed cereal-grain products the same five folate derivatives: 5-methyltetrahydrofolate, 10-formyldihydrofolate, 10-formyl-

Table 4. Total Folate Content in Fortified Cereal-Grain Products Determined by HPLC and Microbiological Assay[a]

	total folate[b] (mg/100 g)	
fortified cereal	microbiol assay	HPLC assay
white bread[c]	87.9 ± 4.71	89.1 ± 3.93
wheat bread[c]	86.4 ± 6.29	94.3 ± 1.96
white rice[c]	114 ± 3.98	120 ± 9.00
pasta[c]	87.1 ± 8.47	84.7 ± 0.70
breakfast cereal[d]	1570 ± 185	1550 ± 171

[a] Trienzyme-treated samples. All data are expressed on a wet-weight basis (i.e. with no prior dehydration). [b] Total folate content corrected for folate contribution by enzymes (28 ng/g dry cereal). [c] Mean and SD of two replicates. [d] Mean and SD of three replicates.

folic acid, 5-formyltetrahydrofolate, and folic acid, with the first four contributing 85−95% of the total folate content (Table 3). Compared to Mueller's (1993) results, we found a higher amount of formylfolates, because of the presence of 10-formyldihydrofolate, a form that Mueller (1993) did not attempt to monitor. This folate derivative as well as 10-formylfolic acid is an oxidation product of 10-formyltetrahydrofolate. Whether the oxidation occurred in the grain before or after harvest or during processing cannot be concluded. The findings concerning the good stability and recovery of the tested folate derivatives suggest that no significant interconversions or oxidations occur during the analysis. This appears to be the first report of 10-formyldihydrofolate in foods. We have shown that 10-formylfolic acid exhibits folate activity in rats and chicks (Gregory et al., 1984b). Recently, Baggott et al. (1995) reported that 10-formyldihydrofolate functions as a cofactor in the 5-aminoimidazole-4-carboxamide transformylase reaction in mammalian and avian cells. Thus, it appears that both of these uncommon folates would contribute to the overall folate activity of cereal-grain products.

Total folate contents determined by HPLC for fortified white and wheat bread, rice, pasta, and breakfast cereal show also very good agreement with the microbiological assay (Table 4). This suggests that this method of sample preparation and HPLC analysis may be useful either as a routine assay method or as a reference method. In addition, these findings support the accuracy of the microbiological assay method when proper sample preparation is used. Traditional methods of extraction and folate conjugase treatment are not appropriate, however.

In conclusion, an efficient extraction and purification method providing adequate prevention of folate degradation and interconversion is presented. The chromatographic system shows effective and rapid separation and a sensitive and specific identification and quantification of different folate derivatives found in cereal-grain foods. This study provides further evidence of the benefit of a trienzyme treatment in folate analysis and its essentiality in the determination of folate in cereal-grain products by either HPLC or microbiological assay methods.

ABBREVIATIONS USED

FBP, folate-binding protein; folate conjugase, pteroylpolyglutamate hydrolase; pABG, *p*-aminobenzoylglutamate; H_4folate, tetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate; 10-CHO-H_4folate, 10-formyltetrahydrofolate; 10-CHO-H_2folate, 10-formyldihydrofolate.

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