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A Method for the Analysis of Natural and Synthetic Folate in Foods

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The essentiality of dietary folates for human beings has been known for many years. Over the shorter term, biological activities associated with several human maladies and the attenuation of biomarkers for several chronic diseases also have been assigned to folates. In the United States, these observations have led to the addition of folic acid to several foods and food ingredients (food fortification) and to dietary recommendations that assign biological activity to each of the forms of folate in the food supply. There currently is unavailable a robust, instrumental procedure that will distinguish between naturally occurring food folates and synthetic folic acid as part of the routine analysis of foods. The procedure proposed in this publication is unique in that it uses "off-the-shelf" supplies and instrumentation, to the extent possible, and was developed with "normal" corporate work schedules in mind. This method takes advantage of the tri-enzyme food digestion and folate deconjugation steps but was optimized with a commercially available rat plasma as the source of conjugase. A high-capacity styrene−divinylbenzene-based solid-phase extraction column was identified, and conditions were developed for quantitative recovery of 5-methyltetrahydrofolate and folic acid (FA) with it. The various forms of food folates are separated on a C-18 high-performance liquid chromatography (HPLC) column which is resistant to degradation at low pH. As a result, the mobile phase was simplified to a gradient of low-pH phosphate buffer (pH 2.2) and acetonitrile. Although FA does not exhibit fluorescence, a UV-induced photolysis system was added, which is controlled by the HPLC system, so that an appropriate segment of the HPLC column effluent is subjected to photolytic conditions and, thereby, FA can be measured as a fluorescent product. The application of the system was verified by analyzing several certified reference materials and foods and comparing results with certified values and/or total folate values as determined by microbiological assay.

KEYWORDS: Folic acid; 5-methyltetrahydrofolate; HPLC; DAD; fluorescence; food analysis

INTRODUCTION

Compounds that have folate activity are essential nutrients for human beings. During the past decade or so, this activity also has been associated with the attenuation of biomarkers for cardiovascular disease and other human health maladies. For many years, the method of choice for determination of folate activity in foods has been microbiological, based on the growth of various microorganisms (*1*−*3*.) These methods give a measure of total folate activity. With recent developments in U.S. dietary recommendations for folate intake (*4*) and fortification of the U.S. food supply (*5*, *6*), it has become important to have available a robust analytical method for the measurement of folate activity in foods. Such a method must distinguish between natural ("food") folate and synthetic folate in foods during routine analysis. High-performance liquid chromatography (HPLC)-based analysis is one choice for such a determination.

Many HPLC methods have been developed for analyzing folates (*7*−*29*). Most methods use C-18 columns and reversed-phase conditions in combination with ion-pair or ion-suppression techniques (*30*). Addition of ion-pairing reagent complicates the mobile phase and may interfere with the absorption/fluorescence spectrum of the eluting analyte, thus making positive identification difficult. In the past, ion-suppression conditions for folate molecules (pH 2.2) gradually degraded the silica of columns, which changed their performance characteristics and nullified the use of "automatic peak picking" routines on computer-controlled HPLC systems. Recent advances in HPLC column technology have resulted in packing material that is resistant to degradation at these low pH values.

Detection and identification of folate molecules which elute from HPLC columns have most commonly been accomplished with UV absorbance, fluorescence, or electrochemical detectors

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(instrumental difficulties and cost have hindered the use of the various mass spectrometric methods until recently.) A common difficulty with these analyses is the measurement of synthetic folic acid. It does not fluoresce; therefore, another detection method must be used, usually UV absorbance. Because UV absorbance is simultaneously less selective and less sensitive, folic acid is more difficult to measure than the other major folate vitamer in foods, 5-methyltetrahydrofolic acid, which is fluorescent. Methods have been devised which convert folic acid into a compound suitable for fluorescence detection (*27*, *29*, *31*). However, these methods involve postcolumn chemical reaction, which require the addition of pump(s), mixing tees, and tubing to the HPLC, thereby substantially complicating the system and its maintenance.

Folic acid also is known to undergo photolysis in ultraviolet light, and the photolysis product is *fluorescent (32*−*34*); thus, "in-line" photolysis (*35*−*37*) could be an efficient method for fluorescence analysis of folic acid. Methodology for an in-line photochemical reactor for photolytic conversion of HPLC analytes has been developed (*35*−*37*) and has the advantage of being a system that is static and less complicated than one with additional pumps, mixing tees, and tubing.

Folates in foods are present in relatively low concentrations, and their determination frequently requires preliminary cleanup procedures. The first step in these processes is the treatment of a food−buffer slurry with a series of enzymes that degrade the matrix carbohydrates and proteins and deconjugate the terminal glutamyl peptides of folates to mono- or diglutamyl derivatives. The procedure that is most widely used to accomplish these steps is the tri-enzyme method described by Eitenmiller's group (*38*, *39*). The resultant folates require concentration and removal of some contaminants for accurate determination with instrumental techniques. The method of choice in recent years has involved the use of a solid-phase extraction (SPE) column utilizing affinity columns (*40*), strong anion exchange bonded to silica (*41*), or phenyl bonded to silica (*42*). Each of these systems has advantages, but they also have disadvantages. Commercially available strong anion-exchange styrene−divinylbenzene SPE columns appear to combine the benefits of several of the previously used columns with few of the detracting attributes.

This paper outlines a robust procedure, using "off-the-shelf" components, for the separation and quantification of folic acid and 5-methyltetrahydrofolic acid, the most prominent naturally occurring folate, of foods. We have introduced an SPE column, mentioned above, into the cleanup procedures for food folates analysis and have taken advantage of "new" columns mentioned above to achieve the separation of natural and synthetic folates in foods. Both folates are quantified with sensitive and selective fluorescence detection. The only instrumentation modification required is the simple addition of a static photolysis system (low-wavelength UV lamp) immediately before the fluorescent detector of the HPLC.

MATERIALS AND METHODS

Instrumentation. A Hewlett-Packard (HP, Wilmington, DE) 1050 modular HPLC with a quaternary pump, programmable autosampler, in-line degasser, diode array detector (DAD), and programmable fluorescence detector (FLD) was used for HPLC. The HPLC was controlled by a personal computer running HP ChemStation software. A Beckman (Mountain View, CA) DU7 recording UV/vis spectrophotometer was used for absorbance measurements. An Orion Research (Beverly, MA) model 350 pH meter was used for pH measurements. The in-line photochemical reactor consisted of a 10-W short-wavelength mercury lamp from Atlantic Ultraviolet Corp. (Hauppage, NY), model D12, around which was wrapped 15.24 m (50 feet) of 0.254 mm (0.010 in.) i.d. PTFE tubing of 1.5875 mm (1/16 in.) o.d. from Alltech Associates (Deerfield, IL). The lamp was fitted with a relay (24-V coil) that turned the lamp voltage (110 V ac) on and off, and which was controlled by a 24-V signal from one of the programmable external ports of the HPLC.

Materials. Folic acid (FA), tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (MTHF), 5-formyltetrahydrofolic acid (leucovorin, LV), pterin-6-carboxylic acid, 3′,5′-dichlorofolic acid (DCFA), lyophilized rat plasma, lyophilized human plasma, *Aspergillus oryzae* α-amylase, *Streptomyces griseus* protease, and hog kidney acetone powder were obtained from Sigma (St. Louis, MO). FA and MTHF were also obtained from B. Schircks Laboratory (Jona, Switzerland), as were THF, 5,10-methenyltetrahydrofolic acid, and pteroyltriglutamic acid. Potassium hydrogen phosphate, potassium dihydrogen phosphate, phosphoric acid, and mercaptoethanol were obtained from Baker (Easton, PA). Sodium azide, HPLC-grade acetonitrile, and ascorbic acid were obtained from Fisher (Fairlawn, NJ). Solid-phase extraction (SPE) columns and HPLC columns were obtained from Alltech Associates (Deerfield, IL). The SPE column used contained 500 mg of strong anion exchanger (SAX) with a polystyrene−*p*-divinylbenzene matrix in a 20-mL, 9-mm flared column (Alltech catalog no. 309750.) The HPLC column was 15 cm × 4.6 mm, packed with Adsorbosphere C18 HS 3μ (Adsorbosphere C18 UHS 5μ columns were used for some analyses). Orange juice concentrate, enriched refined wheat flour, frozen spinach, and nonfat dried milk were purchased at local supermarkets. SRM 1846 Infant Formula was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). BCR 121 Wholemeal Flour, BCR 421 Dried Milk, and BCR 485 Freeze-dried Mixed Vegetables were obtained from the European Commission, Institute for Reference Materials and Measurement (Brussels, Belgium.)

Buffers and Solutions. All manipulations were performed under fluorescent lamps fitted with polycarbonate shields that have a UV cutoff at 370 nm. The extraction buffer was 0.1 M potassium phosphate, pH 6.0, with 10 mM mercaptoethanol, 10 mM ascorbic acid, and 10 mg/L sodium azide. The SPE elution buffer contained 0.1 M phosphate, pH 6.0, and 1 M sodium chloride containing 250 mL/L of acetonitrile. The HPLC buffer was 30 mM potassium phosphate, pH 2.2, with 10 mg/L sodium azide. The amylase solution was prepared by dissolving 1 g of *A. oryzae* α-amylase in 25 mL of extraction buffer and filtering. The protease solution was prepared by dissolving 25 mg of *S. griseus* protease in 25 mL of extraction buffer and filtering. The conjugase solution was prepared by reconstituting lyophilized rat plasma with extraction buffer.

Standards. Folic acid standards were prepared by slurrying 10−20 mg of accurately weighed folic acid in 25 mL of extraction buffer, adding sufficient 5% NaOH to dissolve the folic acid, and then diluting to 100 mL with extraction buffer. The resulting solution was stored at −60 °C. Working standards were prepared by dilution with extraction buffer of 50, 100, 200, or 500 μL of standard solution to 25 mL. 5-Methyltetrahydrofolate standards were prepared by dissolving 10−20 mg of accurately weighed MTHF and diluting to 100 mL with extraction buffer. Working standards were prepared by appropriate dilutions with extraction buffer as with folic acid. LV standards were prepared as for MTHF standards. The purity of the folate vitamers used was determined by substituting water for the extraction buffer above and measuring the absorbance at the wavelength maxima of the vitamers at appropriate dilutions. Concentrations were calculated from absorbance and extinction coefficients (*43*−*45*). The working standards were placed in 2-mL × 11-mm-diameter sample vials, blanketed with nitrogen, sealed, and stored at −60 °C. DCFA standards were prepared as for FA, but the purity was not determined.

Extraction. In all cases, samples were taken from thoroughly mixed and, if frozen, completely thawed materials. Ten grams of material was slurried with 50 mL of extraction buffer. Two drops of 2-octanol were added. For spinach, this slurry was mixed for 2 min in an OmniMix mixer (Omni International, Waterbury, CT). For SRM 1846 Infant Formula, 10 g of material was suspended in 50 mL of hexane and stirred for 30 min. None of the other materials had enough lipid to cause difficulties in the extraction. The resultant suspension was filtered and the solid phase washed with additional hexane. It was then

suspended in 50 mL of extraction buffer and treated as the other materials. For orange juice, the pH of this mixture (initially 4.8) was adjusted to 6.0 with 5% sodium hydroxide solution. In all cases, 1 mL of amylase solution was added, and the solution was degassed with a gentle stream of nitrogen and incubated at 37 °C for 1 h. Subsequently, 1 mL of protease solution was added to the mixture, nitrogen was gently blown across the surface of the mixture, and it was incubated for 3 h at 37 °C. The mixture was then placed in a boiling water bath and heated to 90 °C for 15 min. It was then cooled to room temperature, and 0.2 mL of reconstituted rat plasma was added. The mixture was blanketed with nitrogen, stoppered, and incubated overnight at 37 °C. The mixture was again heated to 90 °C in a boiling water bath for 15 min and then cooled to room temperature. The mixture was centrifuged in a Beckmann (Mountain View, CA) J2 centrifuge at 40000*g* for 20 min. For all but the SRM 1846 Infant Formula, the supernatant was decanted and made up to 100 mL with extraction buffer. In some cases, the pellet was resuspended in extraction buffer, stirred for 15 min, and recentrifuged. This second extract was analyzed separately. The supernatant was made up to 100 mL with extraction buffer. For SRM 1846, the floating pellet was carefully removed and washed with extraction buffer, and the wash was combined with the supernatant decanted from the insoluble material and made up to 100 mL with extraction buffer.

SPE Treatment. An SPE column was prepared by swelling with extraction buffer and then conditioned by allowing 15 mL of extraction buffer to flow through by gravity. Twenty milliliters of supernatant from the extraction step above was added and allowed to flow through by gravity. Some SPE treatments used the total supernatant from the extraction of 2 g of food with 15 mL of extraction buffer, in which case the total supernatant was passed through the column. The column was washed with 15 mL of extraction buffer. The folates were eluted with 6 mL of SPE elution buffer. A gentle stream of nitrogen was bubbled through the eluate for 30 min at 50 °C to remove most of the acetonitrile. The residue was made up to 5 mL with extraction buffer.

HPLC Analysis. A flow rate of 1 mL/min was used, resulting in pressures of 100−200 bar. The detectors were arranged sequentially with the DAD first, followed by the photochemical reactor, and then the FLD. Two channels were used in the DAD: channel A at 280 nm, and channel B at 350 nm. The fluorescence detector was set for excitation at 290 nm and emission at 355 nm with a photomultiplier gain of 10. A gradient program was used for analyte separation. The program consisted of 5 min with 99% A (pH 2.2 phosphate buffer) and 1% B (acetonitrile) and then a gradient of 20 min to 80% A, 20% B, followed by a step gradient to 60% A for 5 min. Regeneration consisted of 5 min of 99% A, 1% B. Later analyses used an in-line photochemical reactor as described above. The UV lamp of the reactor was turned on after the MTHF had passed through the fluorescence cell but before the LV started to elute from the DAD cell and turned off after the FA photolysis product had eluted from the fluorescence cell. At the time the reactor lamp was turned on, the characteristics of the fluorescence detector were changed to 230 nm excitation and 440 nm emission, with a gain of 11, and when the lamp was turned off the fluorescence parameters were returned to their original values. One hundred microliter injections onto the HPLC column were used. Calculations were performed using external standards bracketing the expected concentrations of MTHF and FA. The calibration curves for both MTHF and FA for both fluorescence and UV detection were linear from concentration values corresponding to 3.5 to 350 μg/100 g. Recovery of added MTHF and FA was determined in selected cases by adding standards to the extract before enzyme treatments and carrying the spiked samples through the rest of the analysis.

Whenever possible, the DAD spectrum of the analyte being measured was compared against the spectrum of an authentic sample. In many cases, the spectrum obtained from experimental samples was too weak for identification or obscured by background materials. In these cases, authenticity was inferred if the experimental spectrum had no features incompatible with an authentic spectrum. In the case of FA, an additional point of identification is the ratio of the integrated absorbance at 280 nm to that at 350 nm. In all cases, the peaks identified as FA had a ratio which agreed with authentic samples.

Microbiological Analyses. Foods analyzed were sent to Covance Laboratories (Madison, WI) for microbiological analyses. These analyses were performed by AOAC Official Method 960.46H "Tri-Enzyme analysis for total folate".

SPE Recovery. Recoveries from the SPE columns were determined by subjecting standard solutions of FA, MTHF, THF, LV, and DCFA to SPE treatment and determining the recoveries at the various stages of SPE development. The recovery of MTHF and FA from the SPE column was determined by preparing a mixed standard containing 500 μg of MTHF and 1000 μg of FA per milliliter in extraction buffer. Two milliliters of this standard was applied to a prepared SPE column and allowed to flow through by gravity. The eluent was collected. Two 2-mL portions of extraction buffer were applied to the column and allowed to flow through by gravity. The eluent was collected and made up to 5 mL. Two 2-mL portions of elution buffer were applied, and the eluent was collected. The combined eluents were treated as above and made to 5 mL with extraction buffer. Two additional 2-mL portions of elution buffer were applied and treated as before. MTHF and FA were measured in the column effluents and compared with results from 2 mL of mixed standard made up to 5 mL. The recovery of LV was determined by a similar procedure, starting with a standard solution of 1000 mg of LV per milliliter. The LV and its transformation product, 5,10-methenyltetrahydrofolic acid, were measured in the effluent. From the known extinction coefficients of the two substances, the recovery was calculated by comparison with suitably diluted standard. The recovery of THF was determined as for MTHF, except that all buffers and eluents were thoroughly degassed with nitrogen and maintained under a blanket of nitrogen during manipulations. The recovery of DCFA was determined as for FA.

Conjugase Evaluations. Various conjugases (human plasma, rat plasma, and hog kidney acetone powder) were evaluated by incubating the materials with pteroyltriglutamic acid and measuring the time course of hydrolysis of the tripeptide to folic acid. Folic acid and pteroyltriglutamic acid were cleanly separated under the chromatographic conditions used. The incubations were performed at various pH values at 37 °C.

Fluorescence Excitation and Emission Spectra. To measure fluorescence excitation and emission spectra, the flow of the HPLC system was stopped when the analyte was starting to enter the fluorescence flow cell. The residual pressure caused the peak to enter the cell. With the flow stopped, the excitation and emission spectra were measured, and the data were transferred to external files, imported into Excel, and plotted.

RESULTS AND DISCUSSION

Overview. Our purpose in this study was the development of an analytical method for folates, both natural and synthetic, in foods. Since we intend to offer this method as a routine analysis method, we decided that the chemicals and instrumentation used should be commercially available whenever possible. This criterion guided some of the choices that we made below, especially with respect to the choice of conjugase and SPE column. In no case were folate vitamers other than MTHF and FA positively identified. Peaks corresponding in retention time to other vitamers (a) had a different UV spectrum, (b) had a different fluorescence behavior, or (c) were not present in sufficient quantity to identify by UV spectrum.

Folate Extraction. Many buffer systems have been employed to extract folates from foods. The criteria that we used were to minimize the number of steps in the extraction (e.g., pH changes), to develop a procedure compatible with a "normal" 8-h work day, and to minimize chemical modification of the folates during extraction. Our choice of buffer was determined by the pH optimum of the enzymes that we utilized, especially that of the conjugase. The extraction was conducted under reducing conditions, provided by the presence of both ascorbic acid and mercaptoethanol, and with air displaced by nitrogen sparging.

Table 1. Conversion of Polyglutamylfolic Acid to Monoglutamyl Folic Acid by Reconstituted Rat Plasma

pH	conversion (%)
7	72.3
6	99.4
5	85.6
4	81.6

Enzyme Pretreatment. Most of the recent methods for the analysis of folates in foods have used the tri-enzyme method, first proposed by Eitenmiller (*38*, *39*), for pretreatment of foods to be analyzed for folate (*46*−*48*). We have not independently evaluated the efficacy of enzyme pretreatment but have utilized existing methodology. Blank runs of the amylase and protease enzymes did not show identifiable amounts of folic acid or MTHF.

Conjugase Evaluations. Several commercially available conjugases were tested for completeness of deconjugation. Isolated hog kidney conjugase (*29*), however, was not tested because a commercial source could not be identified. The most commonly used conjugase, chicken pancreas conjugase, is an endopeptidase, and its action largely produces the dipeptide rather than the desired monopeptide. The conjugases that we chose to evaluate were lyophilized human plasma, lyophilized rat plasma, and hog kidney acetone powder. These were evaluated at pH 6 at 37 °C by their action on pteroyltriglutamic acid. The results after overnight incubation were as follows: rat plasma, 99.4%; human plasma, 81.1%; and hog kidney, 25.6% of theoretical conversion to folic acid. As a result, rat plasma was chosen for further evaluation. For reconstituted rat plasma, the conversion at various pH's is reported in **Table 1**. These results indicate that deconjugation overnight with rat plasma conjugase at pH 6 and 37 °C produced quantitative yields from the test material. The overnight incubation was arbitrary because the length of time involved in the other enzyme treatments (amylase and protease) would not allow the entire procedure to be completed in a single-shift day.

SPE Treatment. A number of different SPE columns have been used to clean up food extracts for subsequent folate analysis. Affinity columns using folate-binding protein (FBP) from various sources have been popular; however, completely assembled, "ready to use" columns are not commercially available. In addition, the use of FBP adds significantly to the cost of the analysis over nonbiological SPE columns. The affinity columns also have limited capacity and react unpredictably to overloading with either folate or other substances which it binds. Similarly, silica-based strong anion exchange (SAX) columns are attractive but have a limited capacity. In light of these factors, we settled on a styrene−divinylbenzene-based SPE system because of its large capacity and robust matrix. The presence of the aromatic backbone of the resin appears to add an additional binding component for folates. Initial experiments used 1 N HCl in water with 500 mL/L acetonitrile as SPE elution buffer; however, later experiments showed equivalent recoveries with the neutral elution buffer described above. The alternative eluting buffer gives a milder elution environment with less chance of modifying or destroying the folate vitamers. The results of recovery experiments with pure vitamer solutions were 95−103% for MTHF, 95−98% for FA, 75−85% for THF, 80−90% for LV, and 50−60% for DCFA. The DCFA represented an attempt to identify an internal standard for folate analysis. Since it is very strongly retained by the chromatographic column and its recovery from the SPE column is unsatisfactory, no further work was done characterizing it or other compounds as internal standards.

Chromatographic System. Modern chromatographic systems for separating folates have used either ion-pairing reagents or ion-suppression techniques. The former uses reagents such as tetrabutylammonium phosphate in the chromatographic buffer to allow retention of ionic species on reversed-phase C18 columns. The technique requires that the analyte be ionized so that ion-exchange ionic association with the ion-pairing reagent can occur. For folates, this requires a neutral or basic pH. This has the unfortunate consequence of eliminating the fluorescence of 5-MTHF and the other reduced folates, and thereby negating the use of fluorescent detection. Although it is possible to adjust the pH of the column effluent with a stream of acidic buffer, that adds further complications to the system.

Ion suppression uses the pH of the buffer to ensure the analyte is not charged, so that it can be directly adsorbed by the lipophilic column modifier. For folates, this requires an acidic pH, so that 5-MTHF is directly fluorescent in the effluent. Using this approach and employing the HPLC columns and mobile-phase program outlined above, we obtained baseline resolution of MTHF, LV, and FA standards and near-baseline resolution of MTHF and FA in extracts of foods.

Figure 1 illustrates typical chromatograms of synthetic standards obtained with both 280 nm UV detection (a) and fluorescence detection (b). The baseline of the chromatogram obtained using fluorescence detection is noticeably quieter than that of the chromatogram obtained using UV detection. These chromatograms are typical of a large number of standardization runs and illustrate the superior selectivity of fluorescence detection compared to UV detection for folates.

Figure 2 shows a chromatogram of BCR 421 Dried Milk powder obtained with 280 nm UV detection (a) and fluorescence detection (b) The fluorescence chromatogram has far fewer peaks, with cleaner separation of FA from other peaks, compared with the chromatogram obtained by UV detection. For MTHF, the fluorescence detection method shows a closely spaced doublet of peaks, the first of which is MTHF. Although this separation is far from optimal, MTHF in this sample is at a level below the level of positive identification, and the result gives an estimate of the MTHF level which agrees with the level determined by isotope dilution MS (R. F. Doherty, unpublished data). In general, only a single peak is observed in the fluorescence chromatogram near the retention time of MTHF.

The results of spiking experiments with FA and MTHF of selected food matrices showed recoveries of 90−95% for FA and 95−105% for MTHF. In addition, a second extraction of centrifugation pellets showed less than 5% of either FA or MTHF left in the pellet after centrifugation.

Photochemical Reactor. The photochemical reactor consisted of a length of 0.010-in.-i.d., 1/16-in.-o.d. Teflon tubing. Preliminary experiments were performed with 30-gauge thick-walled tubing. The reactor was initially placed between the column exit and the DAD to investigate the action of UV light on the various vitamers. MTHF was not detectable by the DAD after photolysis. The degradation product of FA, however, was detectable by both DAD and FLD. To investigate the nature of the degradation product of FA, flow was stopped just as the product was entering the FLD flow cell. The broadband emission spectrum was obtained while scanning the excitation wavelength. The excitation wavelength of 230 nm resulted in maximum emission at 440 nm (**Figure 3a**). A solution of 6-carboxypterin was subjected to the same chromatographic

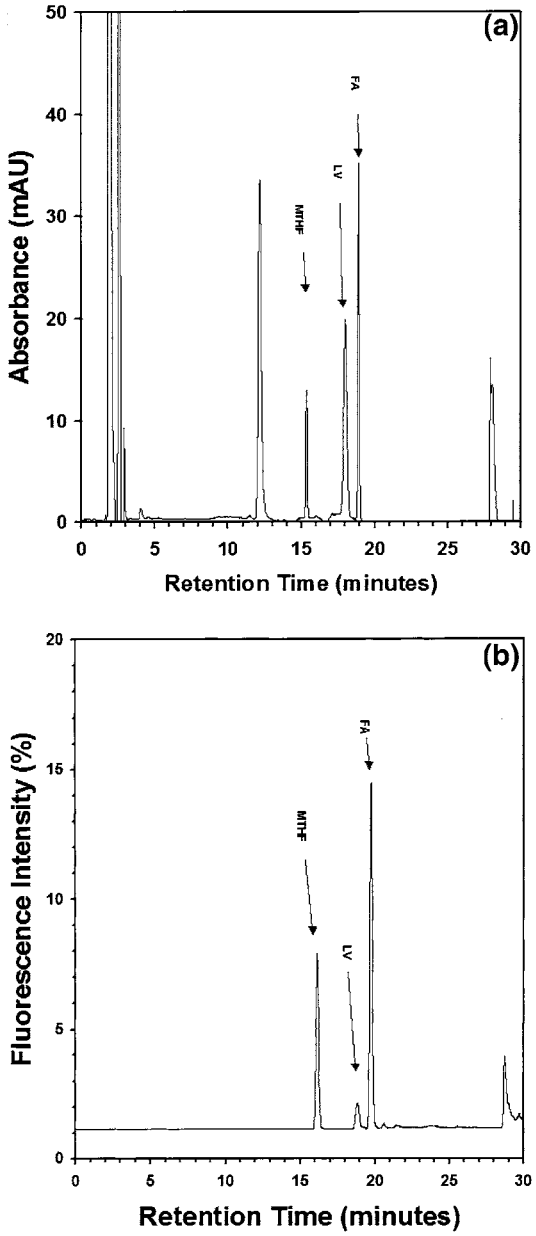

Figure 1. Chromatograms of synthetic standard (a) at 280 nm and (b) with FLD.

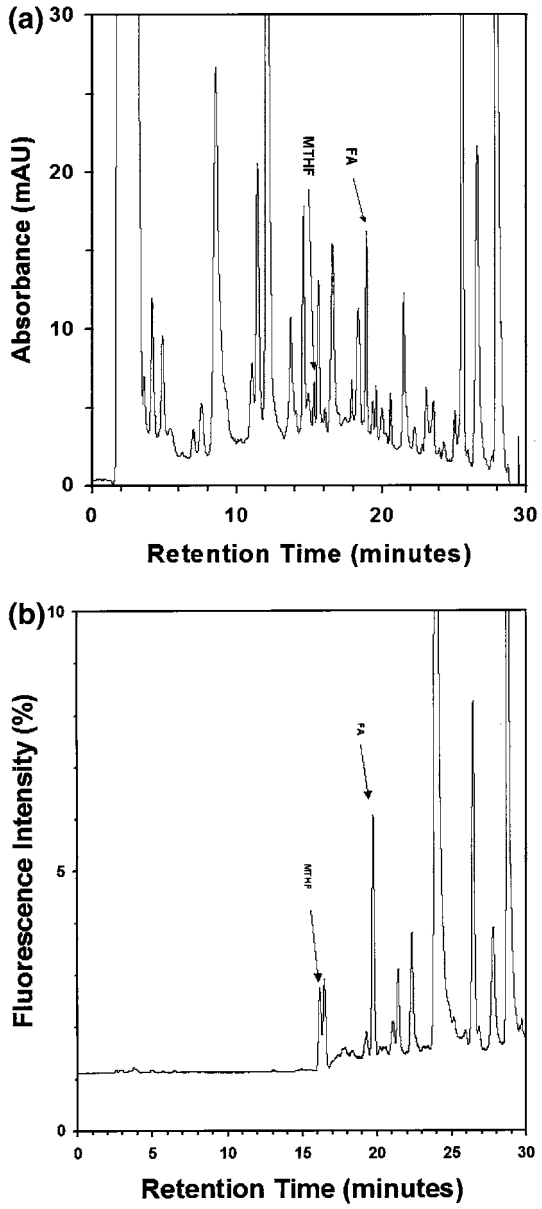

Figure 2. Chromatograms of BCR 421 Non-Fat Dried Milk (a) at 280 nm and (b) with FLD.

conditions (without photolysis) and found to have the same DAD spectrum as the degradation product of FA. The fluorescence excitation spectrum (emission at 440 nm) of this compound is shown in **Figure 3b**. This spectrum is very similar to the photolysis product of FA (**Figure 3a**). On this basis, the degradation product of FA is tentatively identified as 6-carboxypterin.

One literature report has shown a dependence of the photolysis of FA on oxygen (*34*), although this photolysis was conducted with long-wavelength mercury radiation and at a higher pH than we used. To test whether the photolytic reaction we observed was oxygen dependent, we enclosed the photochemical reactor in a glovebag and flushed the bag with nitrogen. This procedure should prevent any oxygen from diffusing through the walls of the Teflon tubing. Since the chromatographic mobile phase was automatically degassed before the column, the photolysis, if the photolytic reaction required the presence of oxygen, should not have taken place. Results from this experiment employing pure FA were identical with results obtained when the reactor was in the open laboratory air. This indicates that the photolysis does not depend on the presence of oxygen.

We also tested for completeness of the photolysis reaction. To accomplish this, we varied the length of tubing of the photolytic reactor and hence the time the reactant was exposed to UV light. We measured the outcome of these experiments by comparing the ratio of the integrated fluorescence area to the integrated 280 nm absorbance area (DAD in-line prior to the reactor). The length of the tubing was varied from 25 to 100 ft, corresponding to residence times in the coil of 30 s to 2 min. The results were similar for all experiments. From these observations, we concluded that the photochemical reaction runs to completion under our conditions, and that the photolytic product is not further degraded. For routine analysis of foods, we selected a tubing length (50 ft) greater than the minimum length we tested, to ensure complete reaction, and less than the maximum length, to minimize peak broadening.

Limits of Detection. Using UV detection at 280 nm, the limit of detection (LOD) for MTHF (three times the signal-to-noise ratio of standard solutions) corresponded to a concentration level

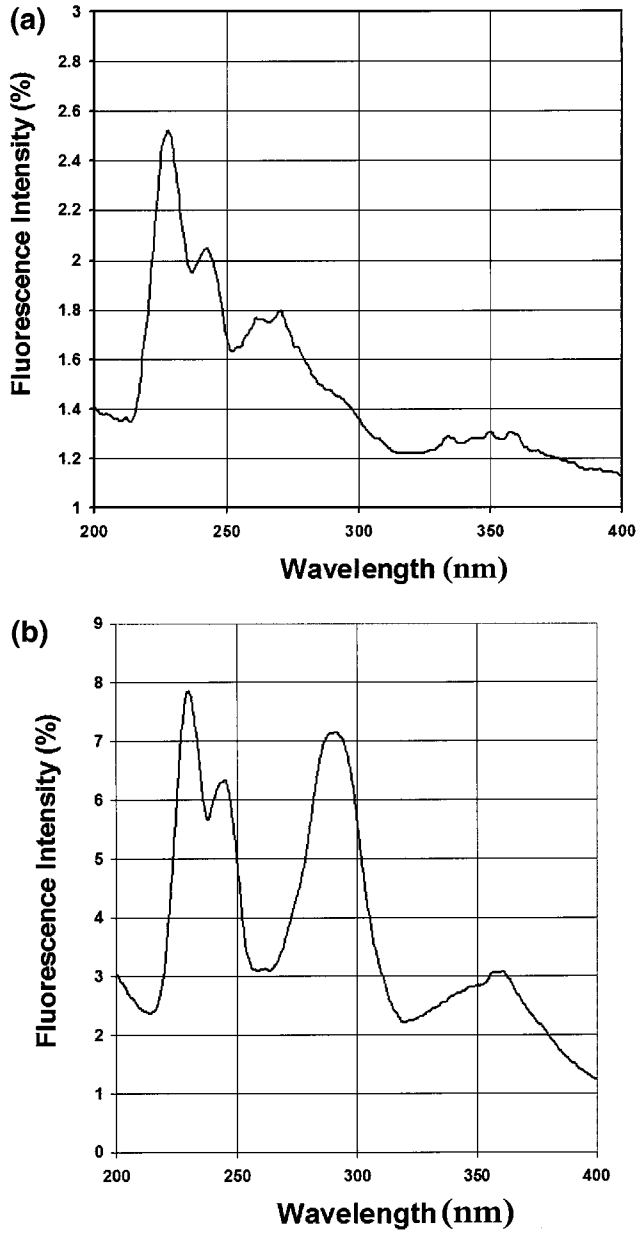

Figure 3. Fluorescence excitation spectra of (a) folic acid photolyzate and (b) pterin-6-carboxylic acid.

in foods of about 1 μg/100 g. For FA, again with UV detection at 280 nm and the parameters used for MTHF, the limit corresponded to a concentration of about 0.5 μg/100 g. For LV with UV detection at 280 nm, the LOD is 2 μg/100 g. Using fluorescence detection, the corresponding values were less than 0.1 μg/100 g for MTHF and FA, but the LOD was 5 μg/100 g for LV because of the weak fluorescence of the compound involved.

In food matrices, the LOD determined with standards is misleading, and standard addition to a matrix depleted in analyte gives a more realistic estimate. Food matrices depleted in MTHF and FA are not readily available, so we have formulated our results in terms of the level of positive identification (LOPI). This is the lowest concentration at which the UV spectrum of the analytical peak is indistinguishable from that of the synthetic standard. In our system, this level depends on the intensity of the UV spectrum of the analyte and on the contribution of the background components to the UV absorption. For both FA and MTHF, this level is somewhere between 10 and 20 μg/100 g

Table 2. Folate Values of (a) Certified Reference Materials and (b) Selected Foods

(a) Certified Reference Materials

	MTHF		FA	
CRM	HPLC value[a]	ref value[b]	HPLC value[a]	MB value[c]
BCR 121 Wholemeal Flour	(7 ± 2)[d]	4 (range = 3–8)	nd	50 ± 7
BCR 421 Dried Milk	(14.9 ± 1.1)	25 ± 2	69.4 ± 3.6	142 ± 14
BCR 485 Mixed Vegetables	196 ± 7	214 ± 42	nd	307 ± 28
SRM 1846 Infant Formula	(3.1 ± 0.2)	na	88 ± 7	129 ± 28

(b) Selected Foods

food	MTHF[e]	FA[e]	MB value[f]
orange juice[g]	88 ± 11	nd	104
refined flour	(9.5 ± 0.9)[d]	175 ± 18	180
frozen spinach	126 ± 15	nd	122
nonfat dried milk (NFDM)	35 ± 4	nd	

[a] Measurements made with the current HPLC system. Values (μg/100 g, dry weight basis) are averages of quadruplicate determinations ± standard deviation. nd, folic acid could not be quantified in food with the current system. [b] Reference value for MTHF provided on certificate for SRM or BCR material. na, reference value is not is not available for this SRM. [c] These values are the total folate content given in the certificates of analyses of the CRMs. [d] Values in parentheses represent values below the LOPI. [e] Measurements made with the current HPLC system. Values (μg/100 g) are averages of quadruplicate determinations ± standard deviation. nd, folic acid could not be quantified in food with the current system. [f] Values generated by microbiological assay for total folate content. These values were determined by Covance Laboratories (Madison, WI) on a wet weight basis. [g] Frozen concentrate.

in foods, depending strongly on the matrix. For LV, this value is at the upper end of this range, because of the characteristic broad chromatographic peak for this material. Although levels below the LOPI can easily be detected, there is no assurance that the measurement corresponds to the level of the putative analyte. In this report, levels below the LOPI are indicated by enclosure in parentheses. Since fluorescence is a more selective technique than UV, the LOPI based on the UV spectrum is probably an overestimate of the actual LOPI; however, in the absence of positive identification, we still treat these values as tentative. FLD is advantageous because chromatograms show a more stable baseline and less closely eluting peaks in the vicinity of both MTHF and FA as compared to chromatograms generated with absorbance (**Figure 2**), thereby enhancing precision and accuracy.

Analysis of Foods. The results of the analysis of several certified reference materials (CRMs) are tabulated in **Table 2a**. Several foods were also analyzed, and the results are tabulated in **Table 2b**. CRMs were analyzed in an attempt to indicate the accuracy of the proposed method. For two of the CRMs (BCR 121 and 485), values generated with this method were within acceptable ranges or uncertainties for each of the materials. However, for BCR 421, the value we determined for MTHF was below the value and uncertainty reported on its certificate. The reason for this discrepancy is unclear, although the UV spectrum of this peak is not unambiguously assignable to MTHF and thus is below the LOPI. For this material, the MTHF was not clearly separated from an impurity peak, and this also probably contributes to the discrepancy. SRM 1846 does not have a certified or information value for either MTHF or FA. The values generated by our procedure indicate substantial addition of FA to this formula and small amounts of MTHF which probably were contributed by one or more

ingredients of the mixture. The values determined by microbiological assay and reported on the CRM certificates were all substantially higher than the values determined by the new HPLC procedure. As evidenced in other comparisons of folate values determined chromatographically and microbiologically, microbiological values are frequently higher than concentrations determined by HPLC, especially at low levels (*49*, *50*). Although it is certainly possible that the reason for these discrepancies is the presence of other folate vitamers, LV (which has previously been reported in foods) was looked for and not found. Preliminary data from experiments employing recently developed liquid chromatographic/mass spectrometric techniques (*51*, *52*) and several of the same CRMs support the accuracy of this HPLC method (R. F. Doherty, unpublished data).

We also tested the new HPLC procedure on several foods and ingredients of processed foods (**Table 2**). In addition to the measurement of MTHF and FA in extracts of these foods by the new HPLC method, we also had total folate values determined by microbiological assay at a commercial analytical laboratory. The two sets of data agree quite well (**Table 2**). Only microbiological values for orange juice were slightly higher than the instrumental value for MTHF of this food. Values from other sources for orange juice show values substantially lower than ours, but no attempt was made to obtain representative samples of this food, and the microbiological values determined were also substantially higher than table values. These comparisons indicate the validity of the proposed method for the measurement of folates of foods.

We have looked for the existence of LV in all of the foods and CRMs we analyzed and could not positively identify it in any of them. In some cases, e.g., spinach and enriched flour, there was a peak which corresponded to the retention time of LV, but the UV spectrum did not correspond to that of LV.

Conclusions. The method described is a robust analytical method for the determination of folates in foods. It is applicable without modification to a wide variety of foods and produces results in essential agreement with CRM values. In addition, for foods, values determined by the proposed HPLC procedure are in close agreement with data from microbiological assay of total folates. It efficiently measures both synthetic and natural folates in one procedure and provides for identification of analytes on the basis of absorption spectra.

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