Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · FEBRUARY 1996

Impact Factor: 2.91 · DOI: 10.1021/jf9503467

4 AUTHORS, INCLUDING:

Liisa T Vahteristo
European Chemicals Agency
24 PUBLICATIONS **984** CITATIONS
SEE PROFILE

Velimatti Ollilainen
University of Helsinki
50 PUBLICATIONS **1,575** CITATIONS
SEE PROFILE

Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography

Liisa T. Vahteristo,* Velimatti Ollilainen, Pekka E. Koivistoinen, and Pertti Varo

Department of Applied Chemistry and Microbiology, Food Chemistry, P.O. Box 27, FIN-00014 University of Helsinki, Finland

A reversed-phase high-performance liquid chromatographic method with fluorescence and ultraviolet detection was used to evaluate the different steps in folate analysis to detect and quantify the most abundant folate forms naturally present in foods. Rapid heat extraction by microwave heating and an enzymatic deconjugation with hog kidney conjugase and chicken pancreas is presented. The extracts were purified with strong anion-exchange cartridges before injection. The combined use of ascorbic acid and mercaptoethanol in the extraction step noticeably improved the stability of tetrahydrofolate, making it also possible to analyze some of the most labile folate vitamers. Tetrahydrofolate was also shown to be easily degraded during the deconjugation step. The compounds 5-methyltetrahydrofolate and, to a lesser extent, 5-formyltetrahydrofolates were also found as important vitamers in foods. In addition, folic acid and 10-formylfolic acid could be determined with the described method.

Keywords: *Folates; folic acid; food; HPLC*

INTRODUCTION

The folate content in food and tissues is usually determined by a microbiological assay (Finglas et al., 1993). When the distribution of folates and their levels are also of interest, separation of various folate forms must be accomplished. These water-soluble compounds are well suited for liquid chromatographic separation because of small differences in ionic character and hydrophobicity. A number of chromatographic methods have been reported in which the folates are separated as their corresponding monoglutamates by conventional reversed-phase separation (e.g., Day and Gregory, 1981; Gounelle et al., 1989; Hahn et al., 1991) or ion-pairing techniques (e.g., Holt et al., 1988; White et al., 1991), and, in some instances, as intact polyglutamates of the folates present (Selhub, 1989; Varela-Moreiras et al., 1991).

The low concentrations of folates present in most foods or other tissues limit the applicability of any separation technique and emphasize the need for sensitive detection techniques. Fluorescence, ultraviolet, and electrochemical detection are widely used for this detection purpose. Detection selectivity reduces the number of interfering compounds in a chromatogram and is therefore of critical importance in identification and quantification of the folate forms present. The relative instability of folates to heat, strong acids, and oxygen is prone to cause underestimation of the folate content in foods because of analytical losses, at least if the most labile folate forms constitute a large proportion of the actual forms present.

The purpose of the present study was to develop procedures applicable to determination of folate forms present in food and other biological samples. A method for extracting folates from the matrix followed by deconjugation of folate polyglutamate derivatives to their respective monoglutamate forms with a combination of hog kidney (HK) and chicken pancreas (CP) conjugases is presented. The digest was further purified and concentrated with strong anion-exchange (SAX) solid-phase extraction (SPE) cartridges and subsequently separated and quantified by reversed-phase high-performance liquid chromatography (HPLC) with a combination of fluorescence and ultraviolet detectors. The method was validated with 5,6,7,8-tetrahydrofolate (H_4folate), 5-methyl-5,6,7,8-tetrahydrofolate (5-CH_3-H_4-folate), 5-formyl-5,6,7,8-tetrahydrofolate (5-HCO-H_4-folate), and folic acid; 10-formylfolic acid (10-HCO-folic acid) can also be determined by the same purification and separation procedure at different detection parameters.

MATERIALS AND METHODS

Folate Compounds. The H_4 folic acid, 5-CH_3-H_4 folic acid (barium salt), and 5-HCO-H_4 folic acid (calcium salt) were obtained from Sigma (St. Louis, MO). Tetrahydrofolic acid trihydrochloride and folic acid were obtained from Merck (Darmstadt, Germany). Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-HCO-folic acid were obtained from Dr. Schirck's Laboratories (Switzerland). The folate decomposition products pterin-6-carboxylic acid (Pt-6-COOH) and *p*-aminobentsoyl-glutamic acid (*p*ABG) were obtained from Sigma. Stock standard solutions of folate compounds were prepared by dissolving in 0.1 M phosphate buffer (pH 7.0); aliquots were taken for checking the concentration spectrophotometrically (Blakley, 1969). This dissolving was immediately followed by addition of 1.0% (w/v) sodium ascorbate. The H_4folate was dissolved in 0.1 M phosphate buffer (pH 7.0) containing 0.1% (v/v) mercaptoethanol (MCE) for determination of the concentration because this compound was very unstable without added antioxidant. The rapid degradation would otherwise cause shifts in absorbance maxima and give false low results for the purity determined. Standard solutions were flushed with nitrogen and stored at −20 °C for no more than 4 months. Aliquots of these solutions were taken for preparing a standard mixture (H_4-, 5-CH_3-H_4-, 5-HCO-H_4folates and folic acid) in 0.01 M acetate containing sodium ascorbate 1.0% (w/v) at pH 4.9. These mixtures were stored as just indicated and renewed every 4 weeks.

* Author to whom correspondence should be addressed (e-mail Liisa.Vahteristo@Helsinki.Fi).

0021-8561/96/1444-0477$12.00/0

Samples. Food samples were cut into 1-cm cubes, and a subsample was further cut into cubes several millimeter in size and weighing 2–5 g. The samples were placed into several plastic containers into which two volumes of extraction buffer (see below) were added. The contents were then flushed with nitrogen, and the samples were stored at −20 °C until analysis. The samples and standards were handled and prepared under subdued light and with opaque containers.

Enzymes. The HK conjugase (γ-carboxypeptidase) was prepared according to the method of Gregory et al. (1984). The yield from a 500-g portion of fresh kidneys was ~100 mL of conjugase preparation, and successive extractions yielded comparable preparations. Each enzyme preparation was assayed for its activity using $PteGlu_3$ as substrate at pH 4.9 in 0.1 M acetate buffer, 1% ascorbate at 37 °C. Conversion of the substrate to folic acid was assayed after 30 and 40 min of incubation. The pH activity profile of HK conjugase was determined in 50 mM phosphate and acetate buffers in the presence of 0.5% sodium ascorbate with 0.5 pH intervals over the range of pH 4.0–7.0. Fifty microliters of enzyme preparation were mixed with 30 μg of $PteGlu_3$ in 1.3 mL of the aforementioned buffers at each pH tested. Activity was determined by analyzing the amount of folic acid produced from $PteGlu_3$ in 45 min at 37 °C.

The CP was obtained from Difco (Detroit, MI). It was dissolved in deionized water (1 mg/mL), centrifuged at 2000 rpm after 30 min of stirring, and stored at −20 °C. The activity of CP was similarly tested, and the amount of $PteGlu_2$ produced was analyzed under the chromatographic conditions reported by Day and Gregory (1985). Human plasma purchased from Sigma and plasma from outdated laboratory stores was tested. The lyophilized plasma was prepared according to Finglas et al. (1993).

Extraction and Incubation. Samples (2–5 g, stored in extraction buffer) were homogenized in 5–6 volumes of extraction buffer [75 mM K_2HPO_4 containing 52 mM ascorbic acid/ascorbate mixture and 0.1% (v/v) 2-mercaptoethanol, pH 6.0, adjusted with phosphoric acid] under nitrogen flow with an Ultra-Turrax T25 homogenizer (IKA, Germany) for 20 s at 13 500 rpm; 2-octanol was used to reduce foaming. Flour and other powdered samples were dissolved in extraction buffer under nitrogen flow without further homogenization. The homogenate, divided into two 50-mL polycarbonate copolymer tubes (Nalgene, Rochester, NY) was flushed with nitrogen (15 s), heated for 1 min in a microwave oven (MDS-81, CEM Corp., Matthews, NC) at 75% power and shaken once. After capping the tubes tightly, the tubes were kept in a boiling water bath for an additional 10 min. The tubes were shaken another two times during heat extraction. The extracts were rapidly cooled in ice and centrifuged (Sorvall RC2-B) at 11000*g* for 30 min at 2–4 °C. The residue was redissolved with 5–10 mL of extraction buffer and recentrifuged for 10 min, and the supernatants were combined and filled to 50 mL with extraction buffer in a volumetric flask. In a regular extraction, the tubes were immediately capped after flushing with nitrogen and placed in a boiling water bath, omitting only the microwave heating step.

The pH of the extract (3–5 mL) was adjusted to 4.9 with acetic acid and 0.8–1.2 mL of HK conjugase and 0.2 mL of CP were added. The mixture was flushed with nitrogen, capped, and incubated in a water bath at 37 °C for 2 h unless otherwise stated. The extracts were then kept in a boiling water bath for 5 min to inactivate the enzymes and cooled in ice. Deconjugation efficiency in each sample type was tested by adding 46 nmol of $PteGlu_3$ to the aforementioned reaction mixture by the method of Engelhardt and Gregory (1990). The substrate $PteGlu_3$ was added only after heat extraction of the sample to avoid effects of endogenous deconjugases.

Purification. SPE was used to purify and concentrate the sample extracts with a Baker spe-21 vacuum manifold (J. T. Baker Chemical). Several products were tested, and 3-mL, SAX cartridges [quaternary amine (N^+), Baker 7091-3] were chosen. Elution and stabilities of calibrants after elution were tested with chloride at 0.9 M (5%) and 1.7 M (10%) sodium chloride levels, and citrate (0.5 M sodium citrate) and iodide (0.3 M potassium iodine) at pH 7.0 with 1% sodium ascorbate. Sodium chloride (10%) was further tested as a counterion in three buffers: pH 4 acetate, pH 4 citrate–phosphate, and pH 6.5 citrate–phosphate. Sodium chloride (10%) in 0.1 M acetate was then chosen for elution of folates from 3-mL SAX cartridges. A slightly modified purification method of Gounelle et al. (1989) was used, in which 10 mL of 0.01 M phosphate buffer containing 0.1% 2-mercaptoethanol (pH 7.0, conditioning buffer) was applied on the column after its activation. The sample extract (3–5 mL) was diluted with water (6–10 mL), and 15 μL of mercaptoethanol was added before applying it on a SAX column. The mixture was passed through slowly (<1 mL/min). The column was washed with 2 × 1.5 mL of conditioning buffer, and the folate compounds were eluted with 2.5 mL of 0.1 M sodium acetate containing 10% (w/v) sodium chloride and 1% (w/v) ascorbic acid. The eluent was passed through at a rate of <0.3 mL/min. Inactivated samples containing a lot of denatured proteins were filtered with syringe filters (Acrodisc, 0.8-μm pore size, 25 mm diameter) before chromatographic purification. The filter was washed with water.

HPLC Analysis. A Varian Vista 5500 liquid chromatograph equipped with a cooled Waters 700 Satellite WISP autosampler and Waters 470 scanning fluorescence detector together with a Varian UV-200 detector were used. A data acquisition system (Millennium 2010 Chromatography Manager) was used to collect chromatographic data and to evaluate peak height. The folate vitamers were separated with a Shandon Hypersil ODS column (3 μm, 150 × 4.6 mm i.d.) protected with a guard column (Novapak C_{18} Guard-Pak; Waters, Milford, MA). The column temperature was maintained at 30 °C. The excitation and emission wavelengths were set at 290 and 356 nm, respectively, for H_4-, 5-CH_3-H4- and 5-HCO-H_4folates, and at 360 and 460 nm, respectively, for 10-HCO-folic acid. Folic acid and $PteGlu_3$ were detected with the UV detector at a wavelength of 290 nm.

Gradient elution with acetonitrile and 30 mM potassium phosphate buffer (pH 2.2) was used to separate the vitamers. The run time was 15 min and the time between injections was 32 min. The gradient was started at 10% acetonitrile with a lag of 4 min, after which the acetonitrile proportion was raised to 24% within 8 min and again back to 10% after 3 min; the flow rate was 0.8 mL/min. In the event that separation was not efficient for some sample matrixes or when further verification of the peak identity was required, two columns were used. A Spherisorb ODS column (5 μm, 250 × 4.6 mm) was placed in front of the usual separation column, the gradient was modified to obtain separation of the vitamers within 30 min; the flow rate was reduced to 0.5 mL/min.

Quantification. Quantification was based on an external standard method in which the peak height was plotted against the concentration. Calibration plots using least-squares regression analysis for each compound were prepared every day. The calibrant mixture was diluted with the buffer, which was used to elute the sample from the SPE cartridge. The amounts were expressed in micrograms (μg) of pure calibrant in its free acid form. Recovery studies were performed by adding the calibrant mixture to the sample before purification (SPE recovery) or homogenization.

RESULTS AND DISCUSSION

Extraction. Stability of the folate calibrants during sample treatment was affected by the level of antioxidant or combination of antioxidants present (Table 1). The results indicate better stability with a combination of the antioxidants ascorbic acid and mercaptoethanol than if only ascorbic acid was added. The necessity of flushing nitrogen through the sample was also observed, at least if only ascorbic acid was used as an antioxidant.

The combined use of ascorbic acid and mercaptoethanol in extraction of folates has also been reported by Wilson and Horne (1984) and Duch et al. (1983). The concentrations used by Wilson and Horne (1984) were high (2% ascorbic acid, 0.2 M MCE), but Duch et al. (1983) used concentrations (1% ascorbate, 10 mM MCE) similar to the ones used in this study. Use of two

Table 1. Relative Stability (Percent) of Folate Vitamers in Different Combinations of Antioxidants during Heat Extraction and Incubation Experiment at pH 4.9[a]

conditions	H_4folate	5-CH3-H_4folate	5-HCO-H_4folate	folic acid	av
A (0.5% AA)[b]	44[c]	86	86	112	82 ± 28.1
B (1.0% AA)	56	91	89	97	83 ± 18.5
C (0.5% AA, 20 mM MCE)	97	97	95	114	101 ± 8.9
D (1% AA, flushed with N_2 on surface only)	51	86	85	91	78 ± 18.4
E (1% AA, incubated with HK)	74	90	86	108	90 ± 14.1

[a] Each sample, except sample D, was flushed with nitrogen (15 s through the sample); heat extraction was for 50 min in capped glass tubes; incubation was for 18 h at 37 °C; heat denaturation was for 10 min; and other conditions as described in text. [b] AA, ascorbic acid. [c] Retention was calculated as percentage of peak area left when compared with the chromatogram of untreated calibrant mixture; all figures are means of duplicate determinations, and the amount of each compound was 1.2–2.5 μg.

Table 2. Comparison of Folate Extraction at pH 6 by Microwave Heating and Heating in a Boiling Water Bath

extraction method	standard recovery ($n = 4$), %[c]	folates in milk powder ($n = 3$), μg/100 g	temp after 10 min, °C
microwave[a]	103 ± 13	40 ± 3	100
waterbath[b]	99 ± 7	40 ± 5	85

[a] One minute in microwave, 10 min in a boiling waterbath. [b] Ten minutes in a boiling waterbath. [c] Calibrant mixture was 3, 3, 4, and 9 ng mL^{-1} of H_4-, 5-CH_3-H_4-, and 5-HCO-H_4folate and folic acid, respectively.

antioxidants at pHs above neutral is based on the results of Wilson and Horne (1983) and on the relative instability of ascorbic acid at high pH. However, the necessity of using mercaptoethanol (combined with the use of ascorbic acid) in protecting folates also at pH 4.9 was observed in this study. Neither of the reducing agents used interfered with the chromatographic separation of the studied folate forms. Breakdown products of mercaptoethanol produced during heating of samples also had to be separated from H_4folate when peak identity was verified by UV detection. Mercaptoethanol did not interfere with fluorescence detection.

Heating of the sample by a microwave procedure (see Materials and Methods) produced similar results to the regular extraction method (Table 2). Heating with the microwave procedure did not affect the amount of folates in milk powder nor the recovery of standards when compared with regular 10-min heat extraction in a water bath at pH 6. On the other hand, after a 10-min extraction at pH 4.9, the amount of 5-CH_3-H_4folate found in milk powder was 30 μg/100 g (duplicate determination) compared with 40 μg/100 g after both types of extraction at pH 6. When the regular heat extraction method was used, the temperature of the extract attained 80 °C only after 15 min under the conditions used here (25 mL in 50-mL plastic tubes, surface of the liquid under water). Microwave heating was therefore chosen due to the rapid heating of the sample extract. The rapid rise in temperature minimizes possible interconversions and deconjugation due to inactivation of endogenous enzyme activities (Tamura, 1990; Keagy, 1985; Krumdieck et al., 1983).

The possible presence of 10-formyl-H_4folate (10-HCO-H_4folate) in foods was not monitored. The method of Gregory (1984), in which the 10-HCO-H_4folate is quantitatively converted to 5-HCO-H_4folate, was tested and found to produce a higher amount of HCO-H_4folate (as sum of those two forms). However, the sum of all detected vitamers in pig liver, for example, was lower than that produced with the extraction method presented here. Most probably, the conditions used in this study cause only partial conversion of 10-HCO-H_4folate to 5-HCO-H_4folate, but this phenomenon was not tested. Other interconversions might have also taken place. Wagner et al. (1995) recently demonstrated interconversion of 5- and 10-HCO-H_4folates to H_4folate in liver cytosol. More evidence of the presence of 5-HCO-H_4folate in cells has also been gained (Kruschwitz et al., 1994). In the literature, the existing data on 10-HCO-H_4folate in foods is very scarce (Seyoum and Selhub 1993).

Conjugation. The pH–activity profile of the HK conjugase preparation attained its maximum at pH 4.8–5.0 in both acetate and phosphate buffers. The long incubation time may have shifted the apparent pH optimum to 0.5 pH unit higher than previously reported by Bird et al (1946) and Engelhardt and Gregory (1990). This higher optimum could be due to higher pH stability at pH 5.0 than at pH 4.5, as shown by Engelhardt and Gregory (1990). The amount of folic acid produced in phosphate buffer was ~20% higher than the amount produced in acetate buffer. Acetate was shown to inhibit deconjugation of added $PteGlu_3$ to the same extent as in wheat extract after a 2-h incubation at pH 4.9. Goli and Vanderslice (1992) earlier reported weak inhibition effects in plasma conjugase and CP conjugase caused by acetate and other salts.

Added $PteGlu_3$ in spinach extract was completely deconjugated to pteroylglutamic acid (folic acid) in 2 h with HK conjugase. In buffer, the deconjugation was complete within only 1 h. In white cabbage extract, the amount of folic acid produced from $PteGlu_3$ in 1 h with HK conjugase was ~20% less than in buffer, indicating lower inhibition action than in spinach. This inhibition effect due to sample matrix requires careful attention and optimization with each sample and enzyme type, as discussed by Engelhardt and Gregory (1990). In the case of inadequate deconjugation, the amount of enzyme and/or time of incubation was increased for determining the total folates present.

Long incubation times (>3 h) were avoided because of the lability of H_4folate during incubation (Figure 1). Diminishing amounts of H_4folate, even in the presence of ascorbic acid, were determined after 1 h of incubation of spinach and white cabbage extracts. The stability of H_4folate improved when mercaptoethanol was added to the reaction mixture, and its use in the extraction buffer was taken as a basis for further experiments.

Use of human plasma as a deconjugase source was discontinued because of the very low activity found in both commercial (Sigma) and our own preparations, both fresh and outdated; however, Lakshmaiah and Ramasastri (1975, 1980) reported the successful use of human plasma with pH optima of 4.5–6.0. In our study, the plasma deconjugase activity using $PteGlu_3$ as substrate in phosphate buffer at pH 6.0 or in white cabbage extract at pH 6 and 4.9 in the presence of 0.2% mercaptoethanol was very low. In our later trials, HK conjugase and lyophilized human plasma (Sigma) in the presence of mercaptoethanol produced similar, repeatable results with lyophilized pig liver, milk powder, and whole wheat flour as matrixes. Carboxypeptidase G (*Pseudomonas* species) EC 3.4.22.12 (Sigma C-4053)

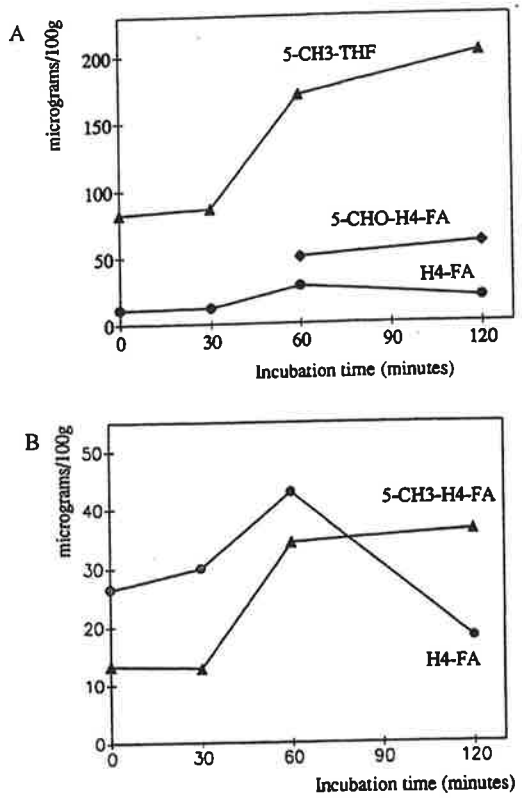

Figure 1. Effect of incubation time on detected natural folate monoglutamates in (A) spinach extract and in (B) white cabbage extract. Samples were extracted and incubated with HK and CP conjugases in acetate buffer (pH 4.9) containing 1% ascorbate. H4-FA, tetrahydrofolate; 5-CH3-H4-FA, 5-methyltetrahydrofolate; 5-HCO-H4-FA, 5-formyltetrahydrofolate.

showed no quantitative activity toward $PteGlu_3$ substrate in the presence of 0.5% ascorbate at pH 4–7 in phosphate or acetate buffers in the concentrations previously reported by Laukkanen et al. (1988).

The activity of CP-producing pteroyldiglutamic acid during 40 min of incubation at pH 5 in phosphate and acetate buffers was low, but rapidly increased at higher pH, as expected from the literature (Leichter et al., 1977). However, CP was added to the sample extract (at pH 4.9) together with HK conjugase on a routine basis to minimize inhibition effects by producing diglutamates with an enzyme not so susceptible to inhibition effects, at least to some inhibitors from plant sources (Keagy, 1985). CP also contains residual proteolytic and amylase activities, as pointed out by De Souza and Eitenmiller (1990) and by Pedersen (1988), which can be advantageous in samples high in proteins or carbohydrates.

Purification. Most matrixes tested contained large amounts of substances that interfered with quantitation and identification of folate forms unless extracts were properly purified. SPE could be used for concentrating sample extracts 1.5–2-fold. The adsorbent capacity was tested in case larger amounts had to be applied. Dilution of the incubated extract with two volumes of water before application on the SPE cartridge adequately lowered the salt concentration, which can be critical to isolate retention on the adsorbent.

SAX and weak anion exchanger DEA (diethylaminopropyl, Varian Bond Elut, 100 mg) quantitatively retained the folate forms studied. The standard mixture and added standards from wheat germ extract were similarly eluted from both SAX and DEA cartridges (Table 3). Folic acid was most strongly retained and not eluted effectively enough with 5% sodium chloride

Table 3.[1] Distribution and Recovery of Added Folate Vitamers from Wheat Germ Extract in Solid-Phase Extraction Elution Fractions[a]

	recovery, %[b]							
	H_4folate		5-CH_3-H_4folate		5-HCO-H_4folate		folic acid	
fraction	SAX[b]	DEA[c]	SAX	DEA	SAX	DEA	SAX	DEA
elution 1	87[c]	67	92	99	89	90	75	98
elution 2	4	3	7	6	9	6	21	nd
sum	91	70	99	105	98	96	96	98

[a] 100-mg cartridges; elution fractions, 0.8 mL of 10% NaCl, 1% ascorbate, 0.1 M acetate (pH 3.6); all the fractions during SPE were collected; all vitamers were retained, but loss of 5-CH_3-H_4 folate during washing was 0.1–0.2%; others eluted only after the application of elution buffer. [b] Recovery as percent calculated from standard mixture injected before purification; vitamer amounts from sample subtracted; applied amount of sample was 0.34 g fresh weight. [c] SAX = strong anion-exchange cartridge; DEA = diethylaminopropyl weak anion-exchange cartridge.

(data not shown). These findings support the results of Rebello (1987), who reported inefficient elution of folates with 0.1 M phosphate buffer (pH 7.5) without high counter ion concentration. When HK conjugase treatment in the absence of mercaptoethanol was also carried out, recovery of H_4folate was only ~40–50%, the others remaining the same as without incubation (5-CH_3-H_4folate, 107%; 5-HCO-H_4folate, 114%; folic acid, 112%). SPE recoveries of added folate vitamers from white cabbage and spinach extracts were similar to that from wheat germ extract.

Purification of the extract was of critical importance to the success of the final analysis. The method to be used must be validated under exactly those conditions used with the real samples. The use of SAX cartridges instead of a weak anion exchanger makes the method more robust because small changes in the matrix do not affect the performance of the sorbent to a great extent. With dilute samples and standard mixtures, both types of anion-exchange purifications could be applied. Anion-exchange purification as a chromatographic technique based on separation principles different from those used in the analytical column is also expected to improve the specificity of the analysis because, primarily, only anionic compounds are retained.

Chromatography and Method Performance. The fluorescence intensity of 5-HCO-H_4folate was slightly higher at the pH of the gradient buffer (2.2) than at pH 2.3, as also reported by Gounelle et al. (1989). The detection limits [signal-to-noise (S/N) ratio ≥2] in this liquid chromatographic system were estimated to be <0.03, 0.02, 0.1, and 0.1 ng for H_4-, 5-CH_3-H_4- and 5-HCO-H_4folates, and folic acid, respectively. The responses of fluorescence and UV detectors were linear in the normal working concentration range. The retention times were repeatable (CV< 2%) when the temperature of the column was kept constant. Repeatability of the analysis method was tested with milk powder and lyophilized pig liver. The variations within a day were 4 and 14% for 5-CH_3-H_4folate, and 19 and 17% for H_4folate in milk powder and pig liver, respectively. The variation within a day for folic acid in milk powder was 2.8% and for 5-HCO-H_4folate in pig liver 8%. The variations within a day (CV% of three samples) for total folate content were 2.4 and 11.6% for milk powder and lyophilized pig liver, respectively. The high relative coefficient of variation in pig liver was mainly due to the lability of H_4folate, which also caused its high variation within a day. Recoveries of added folate

Table 4. Percentage Recovery of Folate Vitamers Added to Pig Liver, Milk Powder, and Pollack Fillet[a]

	recovery, %			
food item	H_4folate	5-CH_3-H_4folate	5-HCO-H_4folate	folic acid
pig liver, lyophilized	50–70	70–90	70–80	—[b]
milk powder	60–70	90	—	75
baked fish (pollack)	56	72–77	65	59

[a] The vitamers were added before extraction and quantitated after incubation and purification steps with external calibration plots; the added folate amounts were in the same range as found in the samples. [b] —, Not determined.

monoglutamates taken through the entire sample treatment for pig liver, milk powder, and pollack fillet are presented in Table 4.

The chromatographic system was a modification of the method used by Gregory et al. (1984) and further modified by Case and Steele (1989). Application of the gradient elution in this study improved peak symmetry and enabled more rapid elution of strongly retained impurities. The gradient elution used allowed good separation of the four vitamers studied as well as the breakdown products Pt-6-COOH and pABG within 14 min. Retention of the compounds studied was very sensitive to even small changes in acetonitrile concentrations. The capacity factors (k') for H_4-, 5-CH_3-H_4-, and 5-HCO-H_4folates, and folic acid were 4.0, 4.6, 5.2, and 5.5, respectively (Figure 2). A typical chromatogram of the main natural folate monoglutamates present in foods is shown in Figure 2. Pt-6-COOH and pABG were separated from folate vitamers; both eluted earlier ($k' = 1$ for Pt-6-COOH and $k' = 1.9$ for pABG). Native fluorescence of reduced folates at low pH was used for detection (Figure 2A), and folic acid was detected with its absorbance maximum at 290 nm (Figure 2B). In the event of high vitamer quantities, UV detection could also be used for quantitation because it also was used for verification of peak identity and purity by comparing ratios of fluorescence and UV absorbance responses of the standard compound with those of the sample. Other folate vitamers (5,10-methenyl-H_4folate, etc.) with emission maxima at 460 nm (Blakley, 1969) can be detected at an excitation wavelength of 360 nm.

Folate Monoglutamates in Some Foods. The results obtained for various food matrixes with the reported method (Table 5) were in agreement with previously published data, although the existing data were very variable. The total amounts of folates detected as sums of the studied vitamers in beef liver (single organ, 535 μg/100 g) and in chantarelles (*Cantarellus cibarius*; pooled sample, 40 μg/100 g) were similar to the amounts found in Finnish food tables (330 μg/100 g and 44 μg/100 g, respectively; Rastas et al., 1994), but the amount found in freeze-dried white cabbage (pooled sample, 20 μg/100 g calculated for fresh product) was only about half (55 μg/100 g). The amount of 5-CH_3-H_4folate detected in white cabbage was consistent with the findings of Müller (1993), who also reported this vitamer to be the main folate form present in white cabbage. As apparent from Figure 1B, the presence of H_4folate in fresh white cabbage was also detected in the present study. The amount of 5-HCO-H_4folate in liver and chantarelles is close to the detection limits, resulting in the high standard deviation in these samples. In an intercomparison study organized by the European Union Measurement and Testing Programme, the results for lyophilized pig liver and milk powder obtained with the method described here

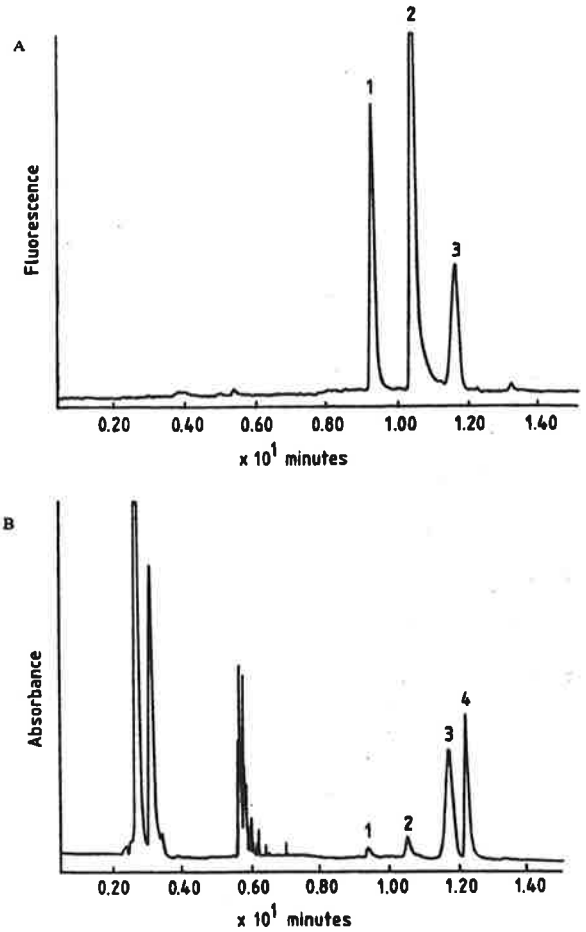

Figure 2. Reversed-phase chromatogram of the main folate forms present in foods. A Shandon Hypersil ODS column (3 μm, 200 × 4.6 mm i.d.) and a mobile phase of acetonitrile:30 mM phosphate buffer (pH 2.2) were used. Fluorescence detection was at ex 290 nm and em 356 nm (A) and UV detection was at 290 nm (B). (1) H_4folate (40 ng); (2) 5-CH_3-H_4folate (30 ng); (3) 5-HCO-H_4folate (100 ng); (4) folic acid (60 ng).

Table 5. Distribution of Folate Monoglutamates in Some Example Foodstuffs

	concentration, μg/100 of product[a]		
food	H_4folate	5-CH_3-H_4folate	5-HCO-H_4folate
white cabbage (lyophilized)	—[b]	210 ± 24	—
beef liver	360 ± 53	170 ± 27	5 ± 1.9
chantarelles	17 ± 8.3	20 ± 3.0	4 ± 2.0

[a] The values are means ± SD of three determinations. [b] —, Not detected.

were in good agreement with those obtained by a microbiological method (Seale et al., 1995). Application of the method to the analysis of cereal products requires further purification of the incubated extract to be adequate for quantitation. Affinity chromatography could possibly be the choice for improving the purification efficiency and specificity (Selhub et al., 1988; Selhub, 1989).

In conclusion, a rapid method for the heat extraction and analysis of folates in foods and other tissues is presented. Adequate prevention of folate degradation during the analysis is stressed, together with an effective deconjugation step. The chromatographic system, because of its excellent separation efficiency, provided data on distribution of folate vitamers in various foods and could be applied to other biological materials, including plasma. The liquid chromatographic apparatus combined with a cooled autosampler can easily

be automated, allowing high sample throughput in the laboratory.

ACKNOWLEDGMENT

We thank K. Passila and M. Akkanen for technical assistance.

LITERATURE CITED

Bird, O. D.; Robbins, M.; Vandenbelt, J. M.; Pfiffner, J. J. Observations on vitamin B_c conjugase from hog kidney. *J. Biol. Chem.* **1946**, *163*, 649–659.

Blakley, R. L. The biochemistry of folic acid and related pteridines. In *Frontiers of Biology, 1969*; North-Holland Publishing: Amsterdam, 1969.

Case, G. L.; Steele, R. D. Determination of reduced folate derivatives in tissue samples by high-performance liquid chromatography with fluorometric detection. *J. Chromatogr.* **1989**, *487*, 456–462.

Day, B. P.; Gregory, J. F. Determination of folacin derivatives in selected foods by high-performance liquid chromatography. *J. Agric. Food Chem.* **1981**, *29*, 374–377.

Day, B. P.; Gregory, J. F. Determination of folypoly-γ-glutamate carboxypeptidase (folacin conjugase) activity using reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1985**, *318*, 387–392.

De Souza, S.; Eitenmiller, R. Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay. *J. Micronutrient Anal.* **1990**, *7(1)*, 37–57.

Duch, D. S.; Bowers, S. W.; Nichol, C. A. Analysis of folate cofactor levels in tissues using high-performance liquid chromatography. *Anal. Biochem.* **1983**, *130*, 385–392.

Eigen, E.; Shockman, G. D. The folic acid group. In *Analytical Microbiology*; Kavanaugh, F., Ed.; Academic: New York, 1963; pp 431–448.

Engelhardt, R.; Gregory, J. F. Adequacy of enzymatic deconjugation in quantification of folate in foods. *J. Agric. Food Chem.* **1990**, *38*,15–158.

Finglas, P. M.; Faure, U.; Southgate, D. A. T. First BCR-intercomparison on the determination of folates in food. *Food Chem.* **1993**, *46*,199–213.

Goli, D. M.; Vanderslice, J. T. Investigation of the conjugase treatment procedure in the microbiological assay of folate. *Food Chem.* **1992**, *43*, 57–64.

Gounelle, J.-C.; Ladjimi, H.; Prognon, P. A rapid and specific extraction procedure for folates determination in rat liver and analysis by high-performance liquid chromatography with fluorometric detection. *Anal. Biochem.* **1989**, *176*, 406–411.

Gregory, J. F.; Sartain, D. B.; Day, B. P. F. Fluorometric determination of folacin in biological materials using high performance liquid chromatography. *J. Nutr.* **1984**, *114*, 341–353.

Hahn, A.; Stein, J.; Rump, U.; Rehner, G. Optimized high-performance liquid chromatographic procedure for the separation and quantification of the main folacins and some derivatives. I. Chromatographic system. *J. Chromatogr.* **1991**, *540*, 207–215.

Holt, D. L.; Wehling, R. L.; Zeece, M. G. Determination of native folates in milk and other dairy products by high-performance liquid chromatography. *J. Chromatogr.* **1988**, *449*, 271–279.

Keagy, P. M. Folacin. Microbiological and animal assays. In *Methods of Vitamin Assay*; Augustin, J., Klein, B. P., Becker, D. A., Venugopal, P. B., Eds.; Wiley: New York, 1985; pp 445–471.

Krumdieck, C.; Tamura, T.; Eto, I. Synthesis and analysis of the pteroylpolyglutamates. In *Vitamins and Hormones. Advances in Research and Applications*; Aurbach, G. D., McCormick, D. B., Eds.; Academic: New York, 1983; pp 45–104.

Kruschwitz, H. L.; McDonald, D.; Cossins, E. A.; Schirch, V. 5-Formyltetrahydropteroylpolyglutamates are the major folate derivatives in *Neurospora crassa* conidiospores. *J. Biol. Chem.* **1994**, *269*, 28757–28763.

Lakshmaiah, N.; Ramasastri, B. V. Folic acid conjugase from plasma. III. Use of the enzyme in the estimations of folate activity in foods. *Int. J. Vit. Nutr. Res.* **1975**, *45*, 262–272.

Lakshmaiah, N.; Ramasastri, B. V. Plasma folic acid conjugase. *Methods Enzymol.* **1980**, *66*, 670–678.

Laukkanen, M.; Antila, P.; Antila, V.; Salminen, K. The water-soluble vitamin contents of Finnish liquid milk products. *Meijeritiet. Aikakauskirja* **1988**, *46* (1), 7–24.

Leichter, J.; Butterworth, C. E., Jr.; Krumdieck, C. L. Partial purification and some properties of pteroylpolyglutamate hydrolase (conjugase) from chicken pancreas. *Proc. Soc. Exp. Biol. Med.* **1977**, *154*, 98–101.

Müller, H. Bestimmung der Folsäure-Gehalt von Gemuese und Obst mit Hilfe der Hochleistungsfluessigchromatographie (HPLC). *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 137–141.

Pedersen, J. C. Comparison of γ-glytamyl hydrolase (conjugase; EC 3.4.22.12) and amylase treatment procedures in the microbiological assay for food folates. *Br. J. Nutr.* **1988**, *59*, 261–271.

Rastas, M.; Seppänen, R.; Knuts, L.-R.; Karvetti, R.-L.; Varo, P., Eds. *Ruoka-aineiden ravintoainesisältö (Nutrient composition of foods)*; Kansaneläkelaitos: Helsinki, 1993.

Rebello, T. Trace enrichment of biological folates on solid-phase adsorption cartridges and analysis by high-pressure liquid chromatography. *Anal. Biochem.* **1987**, *166*, 55–64.

Seale, R.; Finglas, P. M.; Vahteristo, L. *Final report of 3rd EU MAT Intercomparison Study: HPLC Methods*; Institute of Food Research: Norwich, 1995; 35 pp.

Selhub, J.; Darcy-Vrillon, B.; Fell, D. Affinity chromatography of naturally occurring folate derivatives. *Anal. Biochem.* **1988**, *168*, 247–251.

Selhub, J. Determination of tissue folate composition by affinity chromatography followed by high-pressure ion pair liquid chromatography. *Anal. Biochem.* **1989**, *182*, 84–93.

Seyoum, E.; Selhub, J. Combined affinity and ion pair coulumn chromatographies for the analysis of food folate. *J. Nutr. Biochem.* **1993**, *4*, 488–494.

Tamura, T. Microbiological assay of folates. In *Folic Acid in Health and Disease*; Picciano, M. F., Stokstad, E. L. R., Gregory, J. F., Eds.; Wiley-Liss: New York, 1990; pp 121–138.

Varela-Moreiras, G.; Seyoum, E.; Selhub, J. Combined affinity and ion pair liquid chromatographies for the analysis of folate distribution in tissues. *J. Nutr. Biochem.* **1991**, *2*, 44–53.

Wagner, C.; Briggs, W. T.; Horne, D. W.; Cook, R. J. 10-Formyltetrahydrofolate dehydrogenase: Identification of the natural folate ligand, covalent labeling, and partial tryptic digestion. *Arch. Biochem. Biophys.* **1995**, *316*, 141–147.

White, D. R.; Lee, H. S.; Krüger, R. E. Reversed-phase HPLC/EC determination of folate in citrus juice by direct injection with column switching. *J. Agric. Food Chem.* **1991**, *39*, 714–717.

Wilson, S. D.; Horne, D. W. Evaluation of ascorbic acid in protecting labile folic acid derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6500–6504.

Wilson, S. D.; Horne, D. W. High-performance liguid chromatographic determination of the distribution of naturally occurring folic acid derivatives in rat liver. *Anal. Biochem.* **1984**, *142*, 529–535.

Received for review June 6, 1995. Revised manuscript received October 19, 1995. Accepted October 31, 1995.[⊗] This work was financially supported by Finnish Academy, University of Helsinki and Ministry of Agriculture and Forestry, Finland.

JF9503467

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.