FOOD COMPOSITION AND ADDITIVES

A Validated Liquid Chromatographic Method for Determining Folates in Vegetables, Milk Powder, Liver, and Flour

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A liquid chromatographic (LC) method was elaborated for determining folates in foods. Folates were extracted by homogenizing in buffer and heat treatment. A portion was incubated with an enzyme preparation containing conjugase, amylase, and protease. After purification by affinity chromatography, folate monoglutamates were determined by reversed-phase LC with fluorescence and diode array detection. Gradient elution with phosphate buffer and acetonitrile was used to separate vitamers. The most abundant folate forms naturally present in foods were detected, including tetrahydrofolic acid, 5-methyltetrahydrofolic acid, and 5-formyltetrahydrofolic acid. 10-Formylfolic acid could be detected by applying a second fluorescence detector. Folic acid, used for fortification, might also be quantitated with this system. The difference between folate concentrations in sample extracts, with and without treatment of conjugase, is a measure of the quantity of polyglutamates in the food matrixes. An additional treatment with conjugase, amylase, and protease reflects the amount of matrix-bound folates. The LC system gave a linear response over the range 0–100 ng/mL. Detection limit for these compounds were 7 pg/mL for tetrahydrofolic acid and 5-methyltetrahydrofolic acid and 59 pg/mL for 10-formylfolic acid (signal-to-noise ratio ≥ 3) when 100 µL was injected. Detection limits for 5-formyltetrahydrofolic acid and folic acid were 1 ng/mL. Repeatability relative standard deviation values for separate folates in 3 candidate Certified Reference Materials (CRMs)—mixed vegetables (CRM 485), pig liver (CRM 487), and wholemeal flour (CRM 121)—and a Certified Reference Material milk powder (CRM 421) varied from 3.3 to 21.0% for the concentration range 1.8–1440 µg/100 g. Recoveries ranged from 73 to 109%. Use of amylase and protease was advantageous. Use of a commercially available folate-binding protein for cleanup saved time and money and was effective. Results for 5-methyltetrahydrofolic acid were in good agreement with results obtained with other LC methods. Results for total folates were lower than results obtained with microbiological methods.

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Folates are polyglutamates (usually 5–7 glutamyl residues) of pteroic acid and related analogues exhibiting qualitative biological activity of the vitamin folic acid. Folic acid is the monoglutamate of pteroic acid. It is not a natural physiological form of the vitamin but is applied mainly for enrichment of foods.

Folates play a role in neural tube defects (1, 2). Epidemiological evidence suggests that elevated homocysteine in plasma is an independent risk factor for cardiovascular disease (3). Higher intakes of folic acid reduce homocysteine levels. A U.S. prospective cohort study found that high dietary intake of folates is negatively associated with colorectal adenomas (4). Dietary intake of folates could be an important issue in these diseases.

The average folate intake of adult men and women in various European countries ranges from 150 to 400 µg/day (5, 6). A comparison of folate intakes is not always possible because not all countries, including The Netherlands, own or have reliable databases for folates in food products. Most of the time, data from other (foreign) food tables are used.

The purpose of this study was to elaborate and validate a method for determining folates in foods that will be used to produce data for The Netherlands' food table. The total folate content of foods is usually determined by microbiological assay (7). This method cannot differentiate among various folates. The organism commonly used, *Lactobacillus rhamnosus* var. *casei*, responds to monoglutamate as well to di- and triglutamylfolates (8) but to unequal extents. Certain compounds can either stimulate or inhibit bacterial growth, resulting in unreliable data. As shown in recent papers (9–13), previously used methods might not have been applying optimal extraction conditions. Use of better methods to analyze folates in foods might result in more reliable data.

Bioavailability of folates from food has been estimated at about 50% (13). Bioavailability of monoglutamyl folates might vary between 70 and 120% with respect to folic acid (100%; 14). Monoglutamyl folates are almost fully absorbed in the je-

junum. Under certain circumstances, polyglutamates are not absorbed. For good evaluation of bioavailability studies, more detailed information about the folate content of foods is needed. Liquid chromatography (LC) may ensure separation and determination of different folate forms (10, 15, 16). It may also differentiate between amounts of monoglutamates and polyglutamates. Recent papers (17, 18) dealing with food analyses describe problems in identifying peaks when ion exchange is used at the purification and concentration stages of folate analysis before LC. Therefore, a more specific cleanup, like affinity chromatography, is used in this study to isolate folates in food matrixes.

Folates are extracted by homogenizing samples in buffer and heat treatment, followed by deconjugation of polyglutamates and destruction of matrix by protease and amylase. After cleanup of extracts by affinity chromatography, portions are injected into the LC system. Folates are determined by reversed-phase LC with fluorescence and diode array detection. Gradient elution with phosphate buffer and acetonitrile are used to separate vitamers. With this procedure, the most abundant folate forms naturally present in foods were determined, including tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), and 5-formyltetrahydrofolate (5-HCO-H_4folate). 10-Formylfolate (10-HCO-folate), 10-formyldihydrofolate (10-HCO-H_2folate), and folic acid could be detected as well. Analytical results for a mixed vegetable sample, milk powder, pig liver, and wholemeal flour are presented.

METHOD

Apparatus and Materials

Trade names and sources are for user information only.

(**a**) *Homogenizer.*—Ultra Turrax or equivalent.

(**b**) *Water bath.*—Boiling.

(**c**) *LC system.*—Equipped with secondary gradient pump, 100 µL injection loop, autosampler adjustable to 2°–4°C, column oven adjustable to 20°C, and 2 fluorescence detectors capable of excitation at 280 and 359 nm and emission at 360 and 460 nm, diode array detector (DAD), and data-handling system (Waters Chromatography, Milford, MA). Chromatogram at DAD was monitored at 280 nm.

(**d**) *Spectrometer.*—Adjustable between 200 and 400 nm (Perkin-Elmer Corp., Norwalk, CT, or equivalent).

(**e**) *LC column.*—(*1*) *Guard column.*—15 × 4.6 mm, stainless steel, packed with Vydac 201TP C_{18} (10 µm particle size). Ready-to-use columns from Alltech Associates, Inc., Deerfield, IL, are suitable. (*2*) *Separator column.*—250 × 4.6 mm id, stainless steel, packed with Vydac 201 TP 54 (5 µm particle size). Vydac (Hesperia, CA) is suitable.

(**f**) *Dialysis tubing.*—Membrane with molecular weight cutoff (MWCO) of 12 000–14 000. Maximum volume of 300 mL Spectra Por 4 from Spectrum (Houston, TX) is suitable.

(**g**) *Affinity chromatography columns.*—15 mL; Econo chromatography columns from Bio-Rad Laboratories (Hercules, CA) are suitable.

(**h**) *Vacuum manifold.*—For affinity chromatography columns (Waters).

(**i**) *Centrifuge.*—Adjustable to 5000 × *g* and 5°C.

(**j**) *Shaking waterbath.*—Adjustable to 37°C.

Reagents

All reagents should be of analytical purity unless otherwise stated. Water used should be Milli-Q grade or equivalent. *Caution:* Consult safety data sheets or labels for additional information on safe handling, toxicity, flammability, and explosiveness of chemicals. Trade names and sources are for user information only.

(**a**) *Solvents and reagents.*—Acetonitrile (LC grade), L(+)-ascorbic acid, dipotassium hydrogen phosphate, 2-mercaptoethanol, concentrated phosphoric acid, potassium hydroxide, sodium acetate, sodium ascorbate, sodium azide, sodium dihydrogen phosphate, sodium hydrogen carbonate, sodium tetrahydroboric acid decahydrate (E. Merck, Darmstadt, Germany); 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), dithioerytritol, ethanolamine hydrochloride, N-[2-hyroxyethyl]piperazine-N′-2-ethanesulfonic acid] (HEPES), and trifluoroacetic acid (Sigma Chemical Co., St. Louis, MO); acid-washed carbon powder (Fluka Chemie AG, Buchs, Switzerland); Affi-Gel 10 Gel (Bio-Rad); liquid enzyme preparations (Novo Nordisk, Bagsvaerd, Denmark) Flavourzyme 1000-L (aminopeptidase, EC 3.4.11.1) and Fungamyl 800-L (α-amylase, EC 3.2.1.1); folate-binding protein (FBP) bovine milk, Cat. No. F0524 (Scripps Laboratories, San Diego, CA).

(**b**) *Folate standards.*—Folic acid, 10-formylfolic acid (10-HCO-folate), (6*R,S*)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-HCO-H_4folate), (6*R,S*)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CH_3-H_4folate), (6*R,S*)5,10-methenyl-5,6,7,8-tetrahydrofolic acid hydrochloride (5,10-CH^+-H_4folate), pteroyltri-γ-L-glutamic acid ($PteGlu_3$), (6*R,S*)-5,6,7,8-tetrahydrofolic acid trihydrochloride (H_4folate) from B. Schircks Laboratories, Jona, Switzerland.

(**c**) *Sodium dihydrogen phosphate solution (0.1M).*—Dissolve 13.8 g $NaH_2PO_4 \cdot H_2O$ in 500 mL water, transfer to 1 L volumetric flask, dilute to volume with water, and mix.

(**d**) *Dipotassium hydrogen phosphate solution (0.1M).*—Dissolve 17.4 g K_2HPO_4 in 500 mL water, transfer to 1 L volumetric flask, dilute to volume with water, and mix.

(**e**) *Phosphate buffer (0.1M, pH 7).*—Mix 50 mL sodium dihydrogen phosphate solution, (**c**), with 80 mL dipotassium hydrogen phosphate solution, (**d**). Check pH and adjust when necessary to pH 7.0 with KOH (4M) or HCl (4M).

(**f**) *Potassium hydroxide solution (600 g/L).*—Dissolve 600 g KOH in 500 mL water, cool to room temperature, and dilute to 1 L with water, and mix.

(**g**) *Sodium borate solution (0.05M, pH 9.3).*—Degas ca 1.5 L water under reduced pressure to remove dissolved oxygen. Dissolve 19.07 g $Na_2B_4O_7 \cdot 10H_2O$ in 500 mL boiled water. Transfer to 1 L volumetric flask. Dilute to volume with water and mix. Check pH and adjust when necessary to pH 9.3 with KOH (4M) or HCl (4M). Prepare on day of use.

(**h**) *Sodium borate solution (0.05M) containing mercaptoethanol (0.4%, v/v).*—Bring 0.4 mL 2-mercaptoethanol into

a 100 mL volumetric flask. Dilute to volume with sodium borate solution, (**g**), and mix. Prepare on day of use.

(**i**) *Blank solution.*—Bring 1.0 mL sodium borate solution (0.05M) containing 2-mercaptoethanol (0.4%, v/v; **h**) in a 100 mL volumetric flask. Dilute to mark with phosphate buffer 0.1M, pH 7.0, (**e**). Prepare on day of use.

(**j**) *Ascorbic acid solution (1%, m/v).*—Dissolve 5 g ascorbic acid in 500 mL sodium borate solution, (**g**). Prepare fresh on day of use.

(**k**) *50 mM CHES–50 mM HEPES buffer.*—Dissolve in ca 80 mL water 1.192 g HEPES, 1.037 g CHES, and 2 g ascorbic acid. Add 1.39 mL 2-mercaptoethanol. Bring to pH 7.85 with KOH, (**f**). Transfer to 100 mL volumetric flask and dilute to volume with water. Prepare on day of use.

(**l**) *Wash solution I: Phosphate buffer (0.025M, pH 7) containing 1M NaCl.*—Dilute 25 mL phosphate buffer, (**e**), to 100 mL with water. Check pH and adjust when necessary to 7 with HCl (4M) or KOH (4M). Dissolve 5.85 g NaCl in 100 mL phosphate buffer pH 7.

(**m**) *Wash solution II: Phosphate buffer (0.025M, pH 7).*—Dilute 25 mL phosphate buffer, (**e**), to 100 mL with water. Check pH and adjust when necessary to pH 7 with HCl (4M) or KOH (4M).

(**n**) *Elution solution: 0.02M Trifluoroacetic acid–0.02M dithioerytritol.*—Dissolve 308 mg dithioerytritol in ca 40 mL water. Add 153 µL trifluoroacetic acid. Dilute to 100 mL with water and mix.

(**o**) *Ascorbic acid solution (25%, w/v).*—Dissolve 2.5 g ascorbic acid in 10 mL water. Prepare on day of use.

(**p**) *Sodium hydrogen carbonate solution (0.1M, pH 6.5).*—Dissolve 0.84 g $NaHCO_3$ in 80 mL water. Adjust to pH 6.5 with HCl and dilute to 100 mL with water. Store in refrigerator until use.

(**q**) *Sodium acetate solution (0.01M, pH 4.5).*—Dissolve 82 mg sodium acetate in 80 mL water. Adjust to pH 4.5 with HCl. Transfer to a 100 mL volumetric flask and dilute to volume with water. Store in refrigerator.

(**r**) *Ethanolamine hydrochloride (1M, pH 8).*—Dissolve 6.1 g $C_2H_5ONH_2 \cdot HCl$ in 80 mL water. Bring to pH 8 with KOH (4M). Dilute to 100 mL with water. Store in refrigerator.

(**s**) *Affinity chromatography columns.*—For preparation of 6 columns, bring 3 mL FBP (1 mg FBP/mL) with 9 mL cold $NaHCO_3$, (**p**), in a 25 mL capped bottle. Store in refrigerator until combination with Affi-Gel 10. Shake vial with Affi-Gel 10. Transfer 12 mL slurry into a glass fritted funnel. Drain supernatant solvent and wash gel with 3 bed volumes of cold sodium acetate, (**q**). Use vacuum but do not dry the gel bed. Transfer moist gel cake to the cold FBP solution within 20 min. Agitate sufficiently to make a uniform suspension. Continue gentle agitation overnight at 4°C and then add 1.2 mL ethanolamine solution, (**r**). Continue agitating at 4°C for 1 h. Distribute the gel over 6 columns, (**g**). Wash 2 times with 5 mL $NaHCO_3$ solution, (**p**), and 2 times with 5 mL phosphate buffer, (**e**). When not in use, store columns at 4°C in phosphate buffer, (**e**), containing 0.2% (w/v) sodium azide.

(**t**) *Dialyzing buffer: 50 mM CHES–50 mM HEPES, pH 7.85.*—Dissolve 23.8 g HEPES, 20.7 g CHES, 40 g sodium ascorbate, and 1.4 mL 2-mercaptoethanol in ca 1900 mL water. Adjust to pH 7.85 with KOH, (**f**), and dilute to 2 L with water. Add 4 g acid-washed carbon powder. Store in refrigerator until use.

(**u**) *Rat plasma conjugase.*—Fresh rat plasma was obtained from the local university animal laboratory. Collect rat blood in lithium heparin-coated tubes and centrifuge for 15 min at 3000 × *g*. A possible commercial source for rat plasma is Pel-Freez Biologicals, Cat. No. 36142 (Rogers, AR). From the collected plasma, transfer 100 mL into dialyzing tubing and dialyze in 2 L dialyzing buffer, (**t**), for 24 h at 4°C. Store dialyzed rat plasma in 0.5 mL portions for a maximum of 3 months at –80°C. Check activity of this plasma with $PteGlu_3$. About 40 nmol $PteGlu_3$ should be deconjugated within 20 min at 37°C.

(**v**) *Mobile phase.*—*(1) A: Phosphate buffer (0.033M, pH 2.1).*—Pipet 4.45 mL phosphoric acid, (**a**), in a 2 L conical flask. Add ca 1900 mL water. Adjust to pH 2.1 with KOH, (**f**). Transfer to a 2 L volumetric flask and dilute to the mark with water. *(2) B: Acetonitrile.*—Degas solutions.

(**w**) *Folate standard solutions.*—*(1) Stock solutions (1 mg/mL).*—Bring folate standards, (**b**), to room temperature. Dissolve by ultrasonic agitation ca 10 mg folic acid in 8 mL sodium borate solution, (**h**). Transfer into 10 mL volumetric flask, dilute to volume with borate solution, (**h**), and mix. Similarly, prepare stock solutions for 10-HCO-folate, 5-HCO-H_4folate, 5-CH_3-H_4folate, 5,10-CH^+-H_4folate, and H_4folate. *(2) Standard working solutions (10 µg/mL).*—Bring immediately 1.0 mL folate stock solution, (**w**)(*1*), into 100 mL volumetric flask containing 60 mL phosphate buffer, (**e**). Dilute to volume with phosphate buffer, (**e**), and mix. 5,10-CH^+-H_4folate is diluted in 0.01M HCl. Determine absorbance difference (*A* –

Table 1. Molar absorption coefficients (ε)[a], molar mass (M), and maximum wavelengths (λ_{max}) for folates

Folate vitamer	pH	M	λ_{max}, nm	ε, µmol/mL · cm
Folic acid	7	441.4	283	27.6
5-CH_3-H_4folate	7	457.4	290	31.7
H_4folate	7	445.4	297	29.1
5-HCO-H_4folate	7	473.5	285	37.2
10-HCO-folate	7	469.4	269	20.9
5,10-CH^+-H_4folate	2	456.4	352	25.0

[a] Data from reference 19.

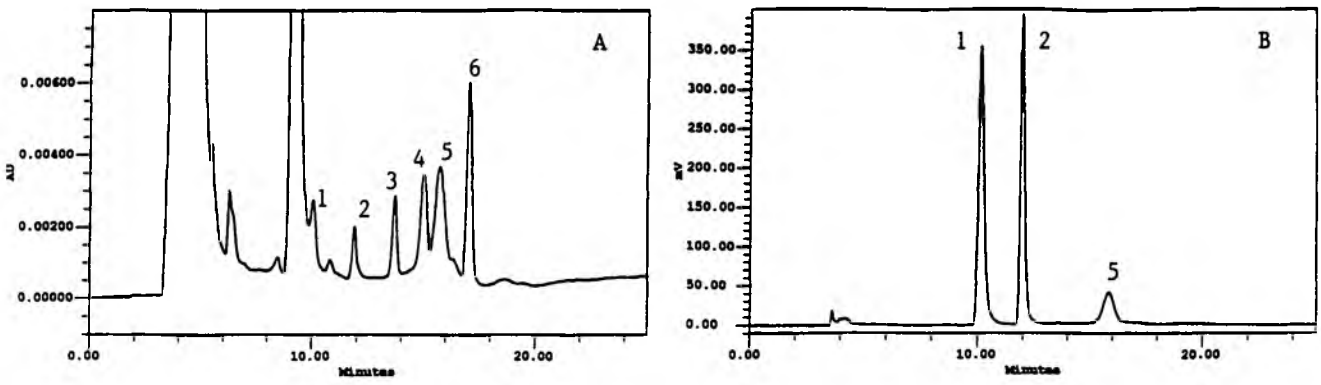

Figure 1. Chromatograms of folates in a standard mixture detected by UV (280 nm) for A and by fluorescence (λ_{ex} = 280 nm, λ_{em} = 359 nm) for B. Concentrations injected: H_4folate and 5-CH_3-H_4folate, ± 6 ng; folic acid, 5-HCO-H_4folate, 10-HCO-folate, and 10-HCO-H_2folate, ± 20 ng. Peaks: 1 = H_4folate, 2 = 5-CH_3-H_4folate, 3 = 10-HCO-H_2folate, 4 = 10-HCO-folate, 5 = 5-HCO-H_4folate, 6 = folic acid.

A_0) for each standard working solution within 5 min, with spectrometer at suitable wavelengths. (Use settings given in Table 1.) A is absorbance of standard solution, and A_0 is absorbance of blank, (i). Record absorbance spectra between 200 and 400 nm. Calculate concentration of each standard working solution. The blank for 5,10-CH^+-H_4folate is 0.01M HCl. Determine the absorbance for 5-CH_3-H_4folate also at 245 nm. Beware that the calculated absorbance ratio (290 nm/245 nm) does not exceed 3.3. Larger ratios indicate the presence of a dihydroderivative of 5-CH_3-H_4folate. (*3*) *Standard working solutions for storage (10 μg/mL).*—Immediately after preparation of each stock solution, (**w**)(*1*), pipet 2.0 mL into a 200 mL volumetric flask. Dilute to the mark with ascorbic acid solution, (**j**). Divide these solutions in 4 mL portions and store at –80°C. These solutions are stable for 3 months. (*4*) *Calibration solutions.*—Construct calibration curves with diluted standard working solutions, (**w**)(*3*). Pipet respectively 20, 40, 60, 80, and 100 μL H_4folate and 5-CH_3-H_4folate and 50, 100, 150, 200, and 250 μL 5-HCO-H_4folate, folic acid, and 10-HCO-folate in five 25 mL volumetric flasks. Accordingly, add to each flask 1 mL ascorbic acid solution, (**o**), 25 μL 2-mercaptoethanol, (**a**), and 200 μL KOH, (**f**). Dilute to volume with elution solution, (**n**). Concentration range of folate standards is 8–100 ng/mL. Prepare solutions fresh on day of use.

Materials

Materials analyzed in this study consisted of a lyophilized mixed vegetable sample (sweet corn–thinned tomatoes–carrot, 10 + 1 + 1, m/m/m; Certified Reference Material [CRM] 485), lyophilized pig liver (CRM 487), milk powder enriched with folic acid (CRM 421), and wholemeal flour (CRM 121). Milk powder was a spray-dried powder from cow's milk. Three materials are candidate CRMs: CRM 485, CRM 487, and CRM 121. CRM 421 is already a CRM.

The Institute for Reference Materials and Measurements, Geel, Belgium, provided these samples.

Extraction

Perform all samples and standard preparations under subdued light. Avoid contact with air and operate under nitrogen atmosphere when possible. Suspend milk powder by mixing 50.00 g sample with 50.00 g water at 37°C prior to analysis. Accurately weigh into separate 100 mL beaker mixed vegetables (1.50 g), pig liver (0.50 g), milk powder slurry (4.00 g), and wholemeal flour (5.00 g). Add ca 45 mL CHES–HEPES buffer, (**k**), and homogenize with the Ultra Turrax apparatus. Cover beaker and place in boiling water bath for 10 min. Swirl extracts occasionally during heat treatment. Homogenize again (Ultra Turrax) and cool immediately in water bath at 0°C.

Adjust to pH 7 with HCl (4M) and weigh each extract. Transfer for each material the following mass portions into three 10 mL centrifuge tubes for deconjugation: mixed vegetables, 4.00 g; pig liver, 2.00 g; milk powder, 5.00 g; and wholemeal flour, 8.00 g. The folate content in each tube should not exceed 500 ng. For each material, add nothing to the first tube (treatment 1), add 0.5 mL thawed rat plasma conjugase, (**u**), to the second tube (treatment 2), and add 0.5 mL thawed rat plasma conjugase, (**u**), along with 50 μL Fungamyl, (**a**), and 50 μL Flavourzyme, (**a**), to the third tube (treatment 3). For the wholemeal flour extract, perform 2 treatments. Add to the first tube 50 μL Fungamyl, (**a**), and 50 μL Flavourzyme, (**a**), (treatment 4), and add to the second tube thawed rat plasma conjugase, Fungamyl, and Flavourzyme as mentioned for treatment 3 above. Mix and incubate all tubes for 4 h at 37°C in shaking water bath. Stop deconjugation by keeping tubes for 5 min in boiling water bath. Cool immediately in ice bath and centrifuge for 20 min at 5000 × *g* and 2°–4°C. Transfer supernatant into a clean tube, resuspend residue in ca 2 mL CHES–HEPES buffer, (**k**), and centrifuge for 20 min at 5000 × *g* and 2°–4°C. Combine supernatants and store at –80°C until cleanup (usually within a few days).

Affinity Chromatography

Equilibrate FBP column, (**s**), by rinsing with 5 mL phosphate buffer, (**e**). Don't allow column to dry. Transfer thawed sample extracts into FBP columns and elute at flow rate of ca

Table 2. Results (dry-mass basis) and statistical findings for determinations (N = 10) of folates in mixed vegetables (CRM 485), pig liver (CRM 487), wholemeal flour (CRM 121), and milk powder (CRM 421)[a]

Vitamer	Treatment	Mean, µg/100 g	s_r, µg/100 g	RSD_r, %	r, µg/100 g	Total folates by LC as folic acid, µg/100 g	Microbiological value, µg/100 g[b]
				Mixed vegetables			
5-CH_3-H_4folate	1	189	19	9.9	53		
	2	205	14	6.7	38		
	3	202	15	7.3	41		
Total	1					182 ± 18	
	2					198 ± 13	315 ± 28
	3					195 ± 14	
				Pig liver			
5-CH_3-H_4folate	1	56	16	28.9	46		
	2	270	26	9.6	72		
	3	330	27	8.3	74		
H_4folate	1	60	11	19.0	32		
	2	800	256	32.4	716		
	3	1440	78	5.4	219		
Total	1					114 ± 14	
	2					1040 ± 180	1340 ± 140
	3					1750 ± 58	
				Milk powder			
5-CH_3-H_4folate	1	15.7	1.9	12.1	5.3		
	2	25	2	8.5	6.1		
	3	29.8	1.1	3.6	3.0		
H_4folate	1	1.20	0.15	12.7	0.43		
	2	1.5	0.3	23.8	1.0		
	3	1.8	0.4	21.0	1.1		
Folic acid	1	70	3	4.5	9		
	2	66	7	10.2	19		
	3	77	3	4.2	9		
Total	1					86 ± 2	
	2					92 ± 4	142 ± 14
	3					107 ± 2	
				Wholemeal flour			
5-CH_3-H_4folate	4	2.59	0.13	5.1	0.37		
	3	4.2	0.3	7.4	0.9		
H_4folate	4	—[c]	—	—	—		
	3	6.0	0.7	12.2	2.0		
5-HCO-H_4folate	4	3.6	1.2	32	3.3		
	3	18	6	32	16		
10-HCO-folate	4	6.1	0.4	6.6	1.1		
	3	11.6	0.4	3.3	1.1		
Total	4					12 ± 1	
	3					37 ± 3	50 ± 8

[a] s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation; r = repeatability ($2.8 \times s_r$). Treatment 1 = extraction without deconjugation or addition of enzymes. Treatment 2 = extraction of folates followed by deconjugation after addition of rat plasma conjugase. Treatment 3 = extraction of folates followed by deconjugation with rat plasma conjugase and addition of Fungamyl and Flavourzyme. Treatment 4 = extraction of folates followed by addition of Fungamyl and Flavourzyme.

[b] Microbiological results originate from EC Measurement and Testing Program.

[c] — = not detected, smaller than detection limit.

0.3 mL/min. Apply light vacuum if necessary. Rinse with 5 mL wash solution I, (**l**), followed by 5 mL wash solution II, (**m**). Elute folates with 4.6 mL elution solution, (**n**), in tube containing 0.2 mL ascorbic acid solution, (**o**), 40 µL KOH, (**f**), and 5 µL 2-mercaptoethanol, (**a**). Transfer to 5 mL volumetric flask and fill to the mark with elution solution, (**n**). Proceed with LC analysis.

LC Analysis

Prior to analysis, equilibrate LC system with a mixture of 95% phosphate buffer, (**v**)(*1*), and 5% acetonitrile, (**v**)(*2*). Adjust flow rate to 0.8 mL/min, inject 100 µL sample extract or working standard solutions, (**w**)(*4*), and subsequently run gradient program. Start gradient with a mixture of 95% phosphate buffer and 5% acetonitrile. After 3 min, change acetonitrile proportion to 10% within 10 min. Between 10 and 12 min, adapt the acetonitrile proportion to 5% and to 10% after 12.5 min. Between 13 and 14 min, change the mobile phase composition to 95% phosphate buffer and 5% acetonitrile. Regenerate column after 26 min by modifying eluant composition to contain phosphate buffer and acetontrile at respective volume fractions of 0.7 and 0.3 within 1 min. Maintain composition until 30 min and change again to 95% phosphate buffer and 5% acetonitrile within 0.5 min. Use a flow rate of 0.8 mL/min. Time between 2 consecutive injections is 40 min.

Construct linear regression plot of standard curve for each vitamer and calculate concentration in sample. Correct for blanks from rat plasma conjugase, Flavourzyme, and Fungamyl.

Results and Discussion

The method is suitable for determining folates in various food matrixes. The LC separation of individual folates is satisfactory for standards and folates in various food matrixes. Antioxidants and degradation products like *para*-aminobenzoylglutamate do not interfere with several folate vitamers. Figure 1 illustrates separation of the most important folates determined with the method using UV and fluorescence detection. With this procedure 5,10-CH^+-H_4folate and H_2folate are also separated. However 10-methylene-H_4folate is not separated from H_4folate.

Stabilities of folate stock solutions were tested regularly by injecting standard working solutions into the LC system. Concentrations were calculated and compared with those of freshly prepared stock solutions. It was found that folate stock solutions should not be used after 12 weeks of storage at –80°C.

The response of the LC system was linear for the concentration range 0–100 ng/mL for all folates (correlation coefficients >0.999). Detection limits (signal-to-noise ratio ≥3) were 7 pg/mL for H_4folate and 5-CH_3-H_4folate, 59 pg/mL for 10-HCO-folate, and 1 ng/mL for 5-HCO-H_4folate and folic acid.

Folates are extracted from food matrixes by several procedures. Gregory et al. (20) mentioned the superiority of the extraction buffer used by Wilson and Horne (21, 22). Vahteristo et al. (16) and Pfeiffer et al. (10) described the excellent stability of folates when extracted with a combination of ascorbic acid and 2-mercaptoethanol. Vahteristo et al. (16) investigated the procedure described by Gregory et al. (15) by comparing a 1 h extraction at pH 4.9 with a 10 min extraction at pH 6.0. The latter resulted in higher folate contents. Higher folate levels, especially H_4folate in pig liver, were obtained in this study when compared with the Vahteristo et al. (16) method, probably because of the more neutral pH conditions. De Souza and Eitenmiller (9) and Martin (8) used protease and α-amylase to obtain a more complete extraction of folates from food matrixes. Tamura et al. (12) stated that all food folate values in tables should be revised by using enzyme treatments to accurately establish food folate content. Pfeiffer et al. (10) applied protease and α-amylase to folate assays of cereal and grain products.

Table 3. Recovery of folates ($N = 10$)[a]

Vitamer	Amount in sample, µg/100 g	Spiked, µg/100 g	Found, µg/100 g	Recovery, %
		Mixed vegetables		
5-CH_3-H_4folate	202	172	356	90 ± 9
		Pig liver		
5-CH_3-H_4folate	330	283	641	109 ± 12
H_4folate	1440	820	2161	88 ± 16
		Milk powder		
5-CH_3-H_4folate	29.8	25.5	51.7	85 ± 10
Folic acid	77	70	140	90 ± 12
		Wholemeal flour		
5-HCO-H_4folate	18	60	63	76 ± 12
10-HCO-folate	11.6	6.7	16.4	73 ± 14

[a] Results are on a dry-mass basis.

In this study, 2 liquid enzyme preparations were used because of their coherent enzyme activities at different pHs. A combination of Fungamyl, Flavourzyme, and rat plasma conjugase used for 4 h at 37°C had no effect on the deconjugation of polyglutamates for pig liver, yeast powder, and $PteGlu_3$. Comparable results were obtained for the same samples treated with rat plasma conjugase and the combination of rat plasma conjugase, Fungamyl, and Flavourzyme. No starch was demonstrable after incubation of a potato sample with the 3 enzymes. For milk, the procedure delivered a clear sample after incubation with the combined enzymes.

For deconjugation of polyglutamates, hog kidney conjugase and rat plasma conjugase are commonly used in folate analysis. Application of hog kidney conjugase at pH 4.9 resulted in substantial losses of folates (23). Deconjugation at pH 6–7 resulted in improvements of analytical findings. Rat plasma conjugase has an optimum pH of 6.2–7.5 (24) and, therefore, is appropriate for deconjugation. A study was performed to determine kinetic parameters for both hog kidney conjugase and rat plasma

conjugase. Hog kidney conjugase was purified according to a method described by Gregory et al. (15). Fresh rat plasma was purified according to a method described by Pfeiffer et al. (10). The reaction mixture for hog kidney conjugase consisted of 75 mM K_2HPO_4, 1% ascorbic acid (w/v), and 0.1% (v/v) 2-mercaptoethanol, pH 4.5. The reaction mixture for rat plasma conjugase consisted of 50 mM HEPES, 50 mM CHES, 2% ascorbic acid (w/v), and 0.2M 2-mercaptoethanol, pH 7.5. $PteGlu_3$ was used as substrate. The Lineweaver–Burk method for calculating kinetic parameters yielded K_m values of 2.0 and 2.3 μM $PteGlu_3$ for hog kidney conjugase and rat plasma conjugase, respectively. The K_m for hog kidney conjugase was in good agreement with results of Engelhardt and Gregory (25), who calculated a K_m value of 2.6. The findings show that hog kidney conjugase and rat plasma conjugase have comparable affinities to polyglutamyl folates. Along with the higher folate results determined during neutral pH conditions, they support use of rat plasma conjugase for deconjugation. V_{max} for hog kidney conjugase was 0.3 nmol/(60 μL conjugase preparation × min). V_{max} for rat plasma conjugase was 0.3 nmol/(100 μL plasma × min). Every batch of conjugase preparation should be checked for activity with $PteGlu_3$ as substrate. No loss of activity was shown after 3 months storage at –80°C.

Sample extracts have to be purified and/or concentrated because many foods have low folate contents. Extracts may contain many interfering compounds with identical chemical and/or chromatographical properties. Several papers describe use of anion-exchange purification alone or, in some cases, combined with solid-phase extraction (SPE) to concentrate folates (15, 16, 26–28). We initially tested a strong-anion-exchange SPE procedure in this study.

Müller (18) stated that LC is not the right technique for determining folates in cereal grain food products because the presence of many interfering compounds from samples results in misinterpretations. Pfeiffer et al. (10) used affinity chromatography for cleanup and concentration of cereal grain products. However, the described preparation of FBP is labor-intensive. Commercial FBP proved to be a success and made the procedure more applicable under routine conditions.

Affi-Gel 10 with a slight negative charge was a suitable binder for FBP. To maximize coupling, FBP was dissolved in a solution with a pH lower than the pI of FBP. Iwai et al. (29) and Svendsen et al. (30) reported isoelectric points ranging from 6.8 to 8.5 for FBP from bovine milk. A pH of 6.5 was selected for the overnight coupling reaction at 4°C. Remaining coupling groups were blocked with ethanolamine. The actual binding capacity was estimated by overloading the column with folic acid stock solution. Retained folic acid was eluted with 5 mL elution solution, and the mean binding capacity of 18.5 nmol/column was determined. Sample folate loading should not exceed 25% of the binding capacity because of low 5-HCO-H_4folate recoveries (10, 31). After 6 samples (18 extracts) had been run, the binding capacity of a column decreased to 7.8 nmol/column (58% reduction).

The method was validated by application to analysis of folates in 4 CRMs (N = 10). Results are presented in Table 2. Samples represented the most important folate sources in human food and foodstuffs: pig liver, milk powder (enriched with folic acid), mixed vegetables, and wholemeal flour. To estimate monoglutamates, samples were analyzed without addition of enzymes (treatment 1). Folate concentrations were quantitated after addition of rat plasma conjugase to establish the sum of mono- and polyglutamates (treatment 2). Total folate concentrations were determined after treatment with rat plasma conjugase and with Fungamyl and Flavourzyme (treatment 3).

For wholemeal flour extracts, 2 treatments were performed. One was similar to treatment 3, and the other involved treatment with Flavourzyme and Fungamyl only (treatment 4) to quantitate folate monoglutamates. Recoveries were determined by spiking samples before extraction with different standards at various folate levels (Table 3). Quantitation of recoveries was performed after application of rat plasma conjugase, Flavourzyme, and Fungamyl.

Optimal conditions for pH, deconjugation time, and conjugase concentration were investigated. To check the deconjugation, an amount of $PteGlu_3$ was added prior to deconjugation of all 4 validated matrixes. Deconjugation was complete in 4 h at 37°C, revealing the absence of an inhibitor impact on the deconjugation process.

Folates were quantitated according to external calibration standards. Recoveries of standards were near 100%, except for H_4folate. Some H_4folate losses occurred during affinity chromatography. All H_4folate values reported here were corrected for a recovery value of 69% [N = 5, s (standard deviation) = 6.7%] from standard H_4folate.

Typical fluorescence and UV chromatograms are shown in Figure 2. H_4folate, 5-CH_3-H_4folate, and 10-HCO-folate were quantitated by fluorescence detection, whereas folic acid and 5-HCO-H_4folate were quantitated from UV chromatograms and confirmed by DAD.

Results were examined for outliers by the Grubb's test at the p = 0.05 level of significance. One outlier each was found for H_4folate in milk powder (treatment 1), 5-CH_3-H_4folate in milk powder (teatment 3), 10-HCO-folate in wholemeal flour (treatment 3), and 5-CH_3-H_4folate in wholemeal flour (treatment 4). Repeatability relative standard deviations (RSD_r) were lowest for treatment 3, varying from 3.3 to 21.0% (concentration range, 1.8–1440 μg/100 g). These values are acceptable for the levels studied according to the International Union of Pure and Applied Chemistry (1989) Harmonized Protocol (32) and AOAC's Peer-Verified Methods Program (33), which recommend acceptable within-laboratory method performance (RSD_r) ranges from 1/2 to 2/3 of the predicted reproducibility relative standard deviation (RSD_R) for the levels of interest. One exception is the RSD_r value of 32% for 5-HCO-H_4folate in wholemeal flour (treatment 3). The value is high, probably because of the relatively low UV absorbance of 5-HCO-H_4folate. The RSD_r value is acceptable when standard deviations of each vitamer were pooled, yielding 1 standard deviation for total amount of folates in wholemeal flour. For treatments 1, 2, and 4, all RSD_r values were acceptable except those for 5-CH_3-H_4folate and H_4folate in pig liver (treatment 1) and for H_4folate in pig liver (treatment 2). These high variations may have been caused by high concentrations of matrix-bound folates.

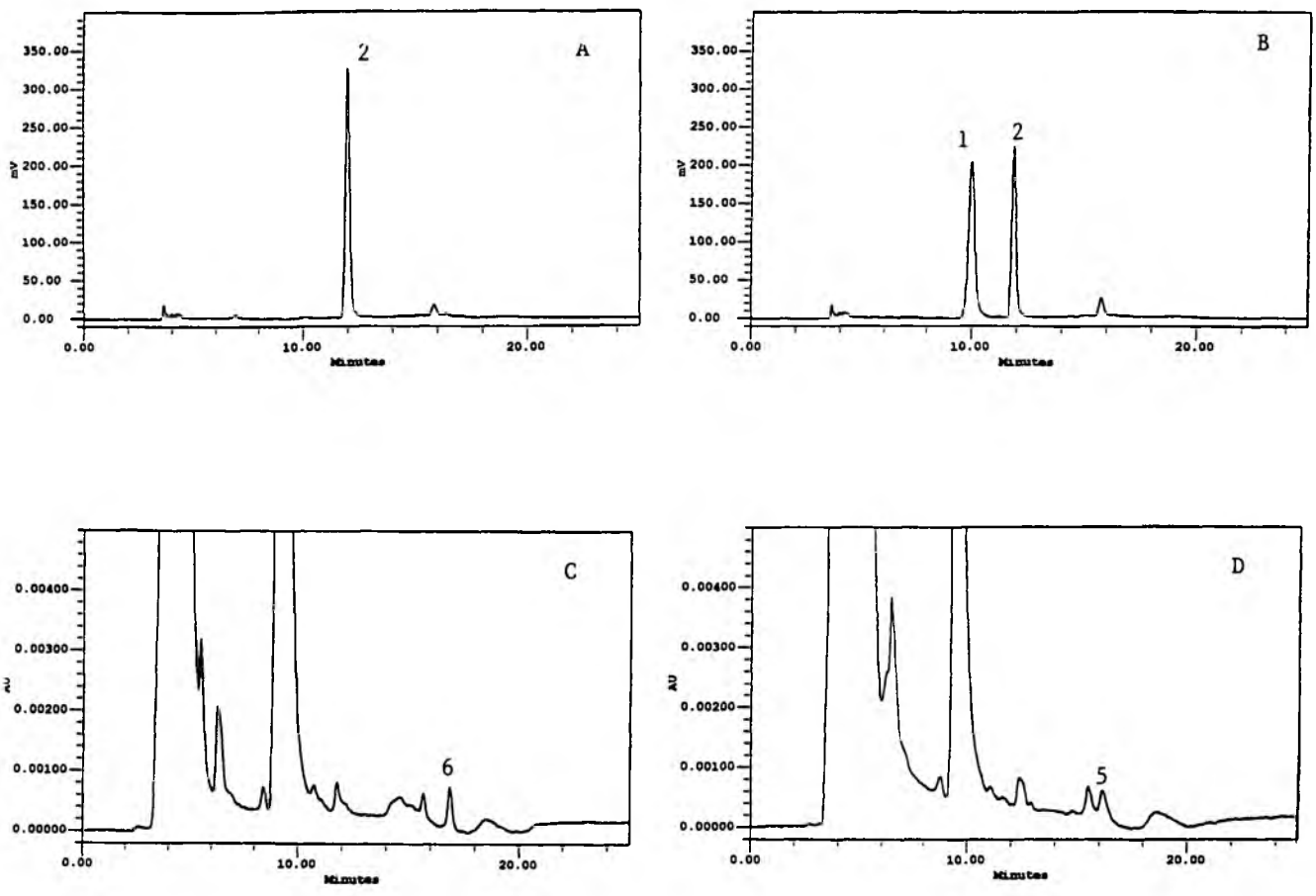

Figure 2. Chromatograms of folates detected by fluorescence (λ_{ex} = 280 nm, λ_{em} = 359 nm; samples A and B) and UV (280 nm; samples C and D). Peaks: 1 = H_4folate, 2 = 5-CH_3-H_4folate, 5 = 5-HCO-H_4folate, 6 = folic acid. Samples: A = mixed vegetables, B = pig liver, C = milk powder, D = wholemeal flour.

Mean standard recoveries for folates added to mixed vegetables, pig liver, milk powder, and wholemeal flour ranged from 73 to 109%. The acceptable recovery range for the levels determined is 80–110%, according to the AOAC Peer-Verified Methods Program (33).

Samples also were analyzed in intercomparison studies organized by the Institute of Food Research (Norwich, UK) under the European Commission Measurement and Testing Program. Fifteen participants assayed samples using their individual microbiological method with *L. rhamnosus* var. *casei*. The pH of the media for microbiological growth was 6.2. Microbiological results of this collaborative study are also summarized in Table 2 (34). A few participants used protease or amylase in sample pretreatment. Participants in the EC study were encouraged to include an α-amylase treatment only for the wholemeal flour sample (35). Treatment with Flavourzyme and Fungamyl in the present study resulted in 50% higher folates in pig liver and 17% higher folates in milk powder (treatment 3). The vitamer that caused the increase in pig liver was mainly H_4folate.

It is difficult to compare microbiological results from the EC study and the present LC values for the 4 materials because sample pretreatments and quantitations were different. Microbiological results were therefore used as guide values for comparison of LC results of treatment 2. Total folates in the 4 materials analyzed in the present study were 20–35% lower than microbiological results. It was not evident that the Flavourzyme and Fungamyl treatment resulted in higher values of folates in wholemeal flour analyzed by LC. Untreated wholemeal flour extracts could not be applied onto affinity chromatography columns because of their viscosity.

Three participants assayed CRMs by LC for 5-CH_3-H_4folate (34, 35) and obtained the following results: mixed vegetables, 214 ± 42 μg/100 g; pig liver, 260 (190–380) μg/100 g; milk powder, 25.0 ± 1.4 μg/100 g; wholemeal flour, 4 (3–8) μg/100 g. Samples were not treated with protease or amylase. These results are in good agreement with data from the present study (treatment 2).

With the described LC method (treatment 3), the exact amount of folic acid added to the milk powder sample (75 μg/100 g wet mass) was established.

No 10-HCO-H_2folate or 5,10-CH^+-H_4folate were found in the samples analyzed. Pfeiffer et al. (10) determined 10-HCO-H_2folate in bread, which is probably produced during processing. With the described procedure, it is not possible to determine 10-formyltetrahydrofolic acid (10-HCO-H_4folate) because this vitamer is converted to 5-HCO-H_4folate acid during heat treatment (36) or modified to 5,10-CH^+-H_4folate because of the low pH of the mobile phase (10). Therefore, 10-HCO-H_4folate might be quantitated indirectly when present.

The procedure determines folates in vegetables, milk powder, liver, and flour with satisfactory, reliable, and reproducible results. Good estimates of monoglutamates, polyglutamates, and matrix-bound folates in these matrixes can be made. To determine total folate contents, a combined treatment of the food extract with rat plasma conjugase, amylase, and protease is recommended. The method is suitable for surveying vegetables, milk powder, liver, and flour for folates.

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