JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Protocol for the Production of Concentrated Extracts of Food Folate for Use in Human Bioavailability Studies

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To provide a tool to study folate bioavailability under controlled conditions, a methodology was developed to produce extracts representative of natural food folates but removed from their matrix and sufficiently concentrated so as to elicit a response in biomarkers of folate status without distorting usual dietary intake patterns. Egg, spinach, and yeast were selected to represent the wide range in extent of folate conjugation found in foods (0, 60, and 100% polyglutamyl folate, respectively). The protocol, which was based on extracting food folates using only reagents safe for human consumption, was optimized in the laboratory (thermal extraction for 10 min in a 2% ascorbate solution at pH 5) and then adapted for industrial scale production in a food-processing facility. Results showed that the extracts were 2.3−12 times more concentrated in folate compared with their corresponding food sources. Neither the mono- to polyglutamate ratio nor the distribution of the main folate derivatives was altered during processing, making these extracts suitable for use in human bioavailability studies.

KEYWORDS: Food folate; bioavailability; polyglutamate; monoglutamate

INTRODUCTION

Folate has attracted major interest in recent years as having an established role in the prevention of neural tube defects (NTD) (*1*, *2*) and possible preventive roles against cardiovascular disease (*3*) and certain cancers (*4*−*7*). For the prevention of NTD, various official bodies worldwide (*8*−*10*) recommend an additional 400 μg of folate/day for women prior to conception and in early pregnancy. Because of the public health difficulties in achieving this recommendation through supplementation of the target group, mandatory fortification of grain foods with folic acid has recently been introduced in the United States (*11*). Similar policies are under consideration elsewhere, but universal fortification is controversial for various reasons, in particular, the concern that it may mask the hematological changes associated with vitamin B_{12} deficiency, thereby allowing the concomitant irreversible nerve degeneration to go undetected (*12*). The third approach to increase folate status, which does not have the health concerns associated with food fortification, is to increase consumption of foods naturally rich in folate. The effectiveness of this strategy as a means of optimizing folate status has, however, been shown to be limited (*13*−*15*), primarily as a result of the poor bioavailability of natural food folates compared with the synthetic vitamin, folic acid.

Although food folate bioavailability is considered to be on average 50% that of folic acid (*16*), a great deal of uncertainty exists in our knowledge of folate bioavailability from natural food sources. The bioavailability of folates from various foods is considered to be dependent on the food matrix, the relative content of monoglutamyl and polyglutamyl folates, and the presence of components that could inhibit both intestinal folate deconjugation and specific transport processes of folate (*17*). However, the relative influence of each of these factors in determining the overall bioavailability of food folates is unknown. In particular, although disruption of the food matrix was shown to enhance the bioavailability of micronutrients such as β-carotene and lycopene (*18*), few studies have addressed the role of the food matrix as a determinant of folate bioavailability from natural food sources. In addition, previous bioavailability studies in free-living subjects involving the provision of folate-rich foods may be confounded by factors including the poor stability of food folates during cooking, resulting in variable folate losses prior to ingestion (*19*), the poor compliance of subjects with demanding intervention protocols in long-term studies, or dietary displacement of usual food folate sources with intervention foods, all of which may result in considerably less food folate being consumed than that targeted. In contrast, the provision of folic acid in tablet form (against which

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10.1021/jf0262312 CCC: $25.00
Published on Web 06/17/2003

responses to food folates are usually compared) not only provides the vitamin in a very stable form but is likely to be associated with much greater subject compliance (*13*). Such factors may compromise the interpretation of many published folate bioavailability studies.

The overall aim of this study was to provide a tool to examine food folate bioavailability under controlled conditions. Specifically, we developed methodology to produce extracts representative of natural food folates (in terms of the mono- to polyglutamate ratio and folate derivative content) but removed from their matrix and sufficiently concentrated so as to elicit a response in biomarkers of folate status without distorting usual dietary intake patterns and without subjecting the food folate to cooking. We addressed the optimization of a laboratory protocol for the extraction of natural folates from the food matrix with industrial up-scaling and concentration by freeze-drying in a food-processing facility. Spinach, yeast, and egg yolk were selected because they encompass a wide range of mono- to polyglutamate ratios considered to be of relevance to the efficiency of the intestinal absorption of food folates and, therefore, bioavailability.

MATERIALS AND METHODS

The initial protocol adopted was based on the well-documented procedures for folate extraction and stabilization prior to food folate determination as reviewed by Tamura (*20*). One important limitation on protocol development for this study was the intended use of the final product in human studies. As a result, only reagents certified as being safe for human consumption were considered for inclusion in the current protocol. Thus, reagents such as 2-mercaptoethanol (commonly used in food folate methodology) had to be eliminated. As an alternative, folate extraction was performed using a food grade replacement, that is, ascorbic acid (British Pharmacopoeia, BP), the pH of which was adjusted by the addition of a solution of sodium bicarbonate (BP) or sodium ascorbate (BP), both commonly used in the food industry.

Optimization of Extraction of Folates from Food (Laboratory Scale Protocol). The liberation of folate from the food matrix is reported to be dependent on the pH of the extraction buffer (*21*) and the duration of thermal extraction (*20*). The pH of the extraction buffer also influences the stability of food folates during extraction (*22*). Therefore, a series of experiments was conducted, as described below, to ascertain the optimum conditions of extraction buffer pH and duration of thermal extraction that maximized the folate yield in food extracts. To obtain a uniform, concentrated product with an increased shelf life for use in these preliminary experiments, baker's yeast (Andrews of Hillsborough, Hillsborough, U.K.) and fresh spinach (Tesco, Coleraine, U.K.) were freeze-dried on receipt. Dried egg powder was commercially sourced (Frampton Ltd., Shepton Mallet, U.K.).

Experiments To Determine Optimum Conditions of Extraction Buffer pH and Duration of Thermal Extraction. Solutions of pH 3, 5, and 7 were achieved by the addition of 0.4 g of ascorbic acid to 20 mL volumes of 0, 1, and 5% sodium bicarbonate (Favourite brand, Belfast, U.K.), respectively. Duplicate 20 mL volumes of each of the sodium bicarbonate solutions were added to 50 mL oak ridge centrifuge tubes (Nalgene Co., Rochester, NY) and taken to a temperature of 80−100 °C by immersion in a boiling water bath for 5 min. To each heated buffer were added 2 g of dried food (yeast, spinach, or egg yolk) and 0.4 g of ascorbic acid. The sample was purged of oxygen by flushing with nitrogen for 30 s prior to tube capping. Samples were held at 80−100 °C by immersion in a boiling water bath for 10 min.

Experiments to determine the optimal duration of thermal extraction were investigated by immersion of duplicate samples in a boiling water bath for periods of 0, 5, 10, or 15 min. Food samples (yeast, spinach, or egg yolk) (2 g) plus ascorbic acid (0.4 g) were added to 20 mL of 1% sodium bicarbonate extraction buffer in 50 mL oak ridge centrifuge tubes that had been heated to 80−100 °C by immersion in a boiling water bath for 5 min. To remove oxygen, samples were purged with nitrogen for 30 s prior to tube capping.

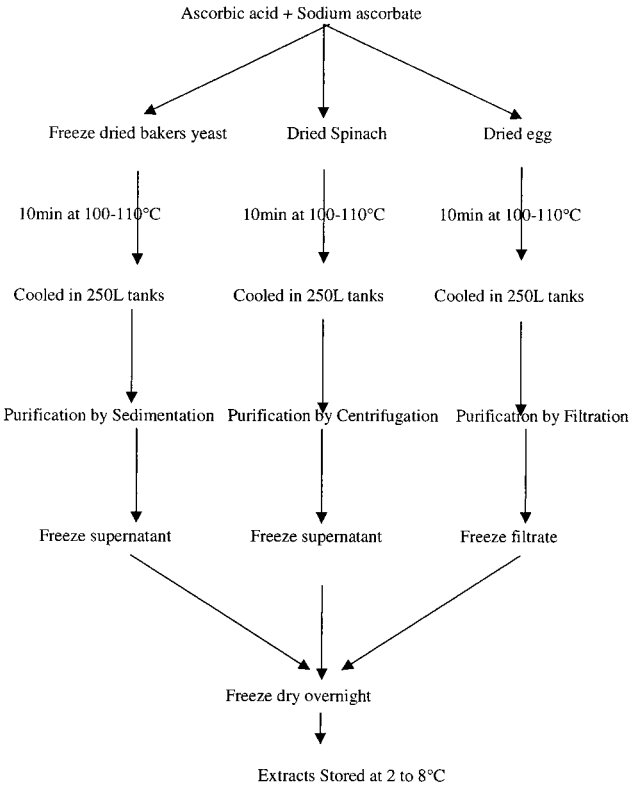

Figure 1. Industrial protocol for food folate extract production.

Thermal extraction (for both sets of experiments) was terminated by cooling on ice. Samples were homogenized using an Ultra Turrax homogenizer (IKA) and then centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was transferred to a disposable universal tube, rotated vigorously in liquid nitrogen to coat the sides with a thin layer of ice, and placed in a −70 °C freezer for 1 h prior to freeze-drying overnight (Hetosice, Birkerod, Denmark). Universal tubes containing the dried samples were sealed and stored at −70 °C until analysis. Experiments investigating the effect of buffer pH and duration of thermal extraction described above were repeated on three separate occasions.

Industrial Protocol for the Production of Food Folate Extracts. The production of sufficient quantities of extracts required for human bioavailability studies was carried out at Loughry College—The Food Centre, Cookstown, U.K. Dried spinach and egg powder were purchased directly from Kanegrade, Stevenage, U.K., and Frampton Ltd., Shepton Mallet, U.K., respectively. Baker's yeast was sourced from Andrews of Hillsborough, Hillsborough, U.K., and freeze-dried in an industrial food-grade freeze-dryer by Icon Foods, Sligo, Ireland. The industrial procedure adopted involved two steps: thermal extraction of folate from the initial product and freeze-drying of the resulting extract (**Figure 1**).

Thermal Extraction. For the thermal extraction of folate from spinach powder, egg yolk powder, or freeze-dried yeast, 4 kg of each of these food folate sources, 800 g of sodium ascorbate (BP) (Takeda Chemical Industries Ltd., Osaka, Japan), 75 g of ascorbic acid BP (Takeda Chemical Industries Ltd.), and 40 L of water (to achieve a final pH of 5) were added to the Scanima A/S 501 high-speed shear mixer (Aalborg, Denmark). With gentle mixing, the temperature of the mixture was raised to 100−110 °C and held for 10 min followed by rapid cooling by water circulation. Following heating, the solid matter was removed by a different procedure for each of the three extracts.

Spinach extracts were transferred to 250 L tanks, each lined with a sterile liner, and stored at 2−8 °C for a maximum of 3 days. The spinach extracts were then passed through a CA225 decanter (Westfalia Separator, Cork, Ireland). The liquid component was filled into 25 kg lined boxes and frozen rapidly in a blast freezer (Superfreeze, Magherafelt, U.K.). Egg yolk extracts were transferred to a 250 L tank lined with a sterile liner. The solid component was removed by cheesecloth filtration. The liquid component was transferred to 25 kg

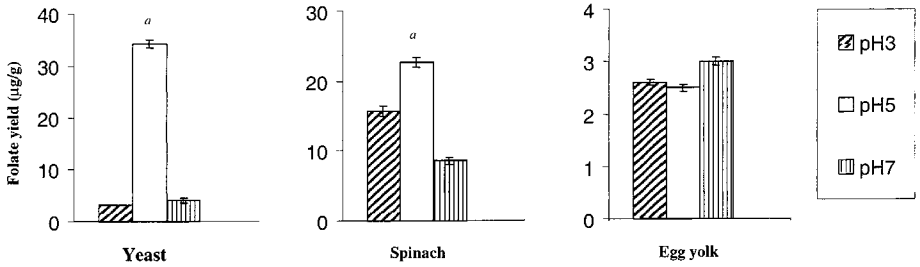

Figure 2. Effect of pH during thermal extraction (10 min) on folate yield in food folate extracts. Extraction solutions of pH 3, 5, and 7 were achieved by the addition of 0.4 g of ascorbic acid to 20 mL volumes of 0, 1, and 5% sodium bicarbonate, respectively. Experiments were repeated on three separate occasions, and values are mean ± SEM. [a]Significantly different from other pH values by ANOVA with Bonferroni post hoc test ($P < 0.05$).

lined boxes and frozen rapidly in a blast freezer. The fat component of the egg yolk extract, which had risen to the top of the liquid prior to freezing, was subsequently removed by cutting away the fat layer from the frozen block with a band saw (AEW Engineering Co. Ltd., Norwich, U.K.). Yeast extracts were transferred to a 250 L tank lined with a sterile liner and allowed to settle overnight at 2−8 °C. The following morning the supernatant was siphoned into 25 kg lined boxes, sealed, and frozen rapidly in a blast freezer. The frozen spinach, egg yolk, and yeast extracts were then stored at −20 °C prior to freeze-drying.

Freeze-Drying. The frozen blocks of extracts were crushed in a Palmia renderer (Sonndnarem, Sweden), the component parts of which were cooled prior to rendering of ice blocks to reduce the thawing of the crushed ice. Frozen crushed extracts were spread evenly over a metal tray and placed in a −20 °C freezer for 1 h prior to freeze-drying. Freeze-drying was conducted in a Radly Engineering (Waterford, Co. Cork, Ireland) freeze-dryer at 60 °C and 0.5 mbar for 8 h. The dried folate extracts were milled in a KitchenAid Professional Mixer fitted with a grain mill attachment (KitchenAid, St. Joseph, MI) prior to transfer to sterile liners and storage at 2−8 °C.

Food Folate Analysis. The folate content of samples of foods and industrially produced extracts was determined by microbiological assay.

Sample Preparation for Microbiological Assay. Folates in extract samples were deconjugated with rat serum conjugase prior to analysis. Rat serum obtained from Sigma Chemical Co. (S7648) (Poole, Dorset, U.K.) was purified according to the protocol of Pfeiffer et al. (*23*). The crude rat serum was filtered through a 0.2 μm syringe filter (Satorious, Gottingen, Germany), aliquoted into 1 mL portions, and stored at −80 °C. The folate conjugase activity of serum enzyme preparations was determined by the formation of folic acid from pteroyltriglutamate incubated with rat serum for 1 h in 0.1 M/L phosphate buffer, pH 7 at 37 °C. Folic acid and pteroyltriglutamate were determined using the HPLC method of Belz and Nau (*24*) with a UV detector set at 280 nm. The retention time was 20.8 min for folic acid and 53.4 min for pteroyltriglutamate. The quantity of rat serum used under the conditions of the assay was found to completely hydrolyze 0.1 μM pteroyltriglutamate (to folic acid), which is much in excess of the typical amount of polyglutamates present in food and extract samples. An identical procedure was followed for the determination of folate in food samples, with the modification that thermal extraction and trienzyme treatment (i.e., α-amylase and protease in addition to conjugase) were applied according to the procedure of Tamura et al. (*20*) prior to analysis by microbiological assay.

Microbiological Assay. The total folate content of foods and industrially produced extracts was determined after conjugase treatment by *Lactobacillus casei* (NCIMB10463) microassay as described by Molloy and Scott (*25*) using folic acid (FA) as a standard. Under the conditions of the assay in our laboratory (pH 6.7 of the assay medium) *L. casei* shows equivalent responses to the main folate derivatives: FA, 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), 10-formyltetrahydrofolate (10-CHO-H_4folate), and tetrahydrofolate (THF). The assay was carried out in 96-well microtiter plates, which were read by an automated plate reader (Rainbow, Austria). The buffer (0.5% ascorbate) and inoculum were prepared as per the method of Molloy and Scott (*25*). All dilutions were carried out in 0.5% sodium ascorbate solution using an automated Hamilton dilutor (Hamilton, Bonadur AG, Switzerland). For quality control, spinach samples were used, prepared by thermal extraction and trienzyme treatment with α-amylase, conjugase, and protease (*20*). Aliquots of these samples were stored at −80 °C for up to 3 months. The coefficient of interassay variation for folate content of quality control spinach samples was 5.5% ($n = 48$). Recovery studies were performed by spiking the quality control samples with FA, 5-CH_3-H_4 folate, and 5-CHO-H_4folate at three different levels (0.25, 0.5, and 1.0 μg). The spiked samples underwent the entire procedure of food folate analysis (thermal extraction, trienzyme treatment, and microbiological assay). The percentage recovery for different folate derivatives was between 87 and 101%. A linear response was demonstrated for each of the folate standards in the spiked reference samples.

HPLC Separation of Folate Derivatives. The folate derivative profile of foods and industrially produced extracts was determined by a combined HPLC/microbiological method, in which folate derivatives were separated by HPLC and eluted fractions quantified by microbiological assay, according to the procedure of Belz and Nau (*24*) with the following modifications. A Waters 600 (Watford, U.K.) system was equipped with a Waters 717 autosampler and a Waters 2487 dual-wave absorbance detector. A data acquisition system (Millennium, 2000) was used to control equipment and monitor separation. Folate monoglutamates were separated using a 0.2 μm filter in series with a Spherisorb (Waters) ODS2 guard cartridge (10 × 4.6 mm, 5 μm) and a Spherisorb ODS2 column (150 × 4.6 mm, 3 μm). Isocratic elution was performed with a mobile phase consisting of 81% 100 mM sodium acetate buffer with 5 mM TBAP (pH 6) and 19% methanol at a flow rate of 1 mL/min. Folate monoglutamate standards (5-CH_3-H_4folate, 5-CHO-H_4folate, 10-CHO-H_4folate, DHF, THF, 5,10-CH-H_4folate, and 5,10-CH_2-H_4folate, donated by Eprova, Schaffhausen, Switzerland) in 50 μM concentrations were chromatographed at 280 nm using a Waters 2487 dual-wavelength UV detector to establish their elution position on the chromatogram. Fractions were collected into 3 mL tubes (Starsted, Wexford, Ireland) every 0.5 min by an automated fraction collector (LKB FRAC-100, Pharmacia). The folate content of each fraction was then determined by microbiological assay as described above. The profile was generated on Excel (version 6.1) by plotting fraction concentration against fraction time points. The peak area was calculated by manual integration.

Statistical Analysis. Statistical analysis was performed by DataDesk, version 6 (Data Description Inc.), statistical package. Differences in folate yield for different pH values and duration of thermal extraction in laboratory scale experiments were evaluated by analysis of variance with post hoc Bonferroni test. The two-sample t test was used to compare the relative content of folate derivatives in food folate extracts with that in the corresponding food source. Differences were considered to be significant at $P < 0.05$.

RESULTS

Optimization of Extraction of Folates from Food (Laboratory Scale Protocol). Analysis of results from experiments to establish the optimal conditions for thermal extraction showed that the highest folate yield in all three extracts could be achieved by thermal extraction for 10 min in a 2% ascorbate solution at pH 5 (**Figures 2** and **3**).

Table 1. Total Folate and Percentage Polyglutamate Content of Yeast, Spinach, and Egg Yolk Extracts Compared with Corresponding Food Sources

	yeast			spinach			egg yolk		
product	mean[a] (μg/100 g)	SD	% polyglutamate[b]	mean (μg/100 g)	SD	% polyglutamate	mean (μg/100 g)	SD	% polyglutamate
food[c]	1625	187	98	197	21	60	178	16	0
extracts[d]	3721	560	99.6	2300	380	50	1000	120	0

[a] Values presented are the mean of three separate measurements. [b] Polyglutamate values were determined as mean differences in folate content with and without conjugase treatment, expressed as a percentage of total folate. [c] Dried yeast, fresh spinach, and raw egg yolk were purchased from a local retail outlet (Tescos). [d] Industrially produced food folate extracts (see **Figure 1**).

Table 2. Percentage Distribution of Folate Derivatives in Food Folate Extracts and Corresponding Food Sources[a]

	yeast		spinach		egg yolk	
	food[b]	extract[c]	food[b]	extract[c]	food[b]	extract[c]
5-CH_3-H_4folate	46.2 ± 3.9	47.7 ± 3.8	43.2 ± 2.1	46.5 ± 8.5	84.5 ± 5.7	80.6 ± 5.5
5-CHO-H_4folate	23.0 ± 3.2	35.1 ± 5.6*	21.2 ± 2.0	43.0 ± 9.5*	15.5 ± 5.7	14.1 ± 3.4
10-CHO-H_4folate	11.1 ± 2.3	5.4 ± 2.5*	3.0 ± 1.2			
DHF	11.6 ± 1.4	9.2 ± 1.6	20.0 ± 4.5	4.6 ± 0.1*		5.3 ± 2.0
THF	5.5 ± 0.6	1.0 ± 0.1**	9.2 ± 4.4	4.2 ± 1.2*		
5,10-CH-H_4folate	2.6 ± 0.5	1.6 ± 0.8	3.3 ± 0.6	1.7 ± 0.5*		

[a] Values are presented as means ± SD, based on three independent analyses. Significant difference from the relative folate derivative content of corresponding food, two-sample *t* test, is indicated (*, $P < 0.05$; **, $P < 0.01$;). [b] Dried yeast, fresh spinach, and raw egg yolk were purchased from a local retail outlet (Tescos). [c] Industrially produced food folate extracts (see **Figure 1**).

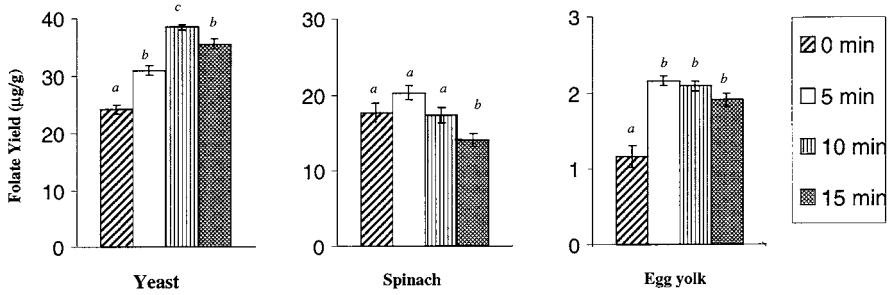

Figure 3. Effect of duration of thermal extraction on folate yield in food extracts. Extraction solution contained 0.4 g of ascorbic acid and 1% sodium bicarbonate adjusted to a pH of 5. Experiments were repeated on three separate occasions, and values are mean ± SEM. Values were compared using ANOVA with the Bonferroni post hoc test.

Protocol for the Production of Food Folate Extracts (Industrial Scale). The folate contents of the industrially produced yeast, spinach, and egg yolk folate extracts were found to be 2.3, 11.6, and 5.6 times more concentrated than the corresponding natural food folate source as typically purchased by the consumer (**Table 1**). When the folate contents of extracts and foods are expressed as dry matter (micrograms per 100 g) the comparisons were as follows: 3721 in extract versus 1625 in food for yeast folate, 2300 in extract versus 374 in food for spinach folate; and 1000 in extract versus 269 in food for egg. The industrial protocol did not alter the percentage polyglutamate content of the folate extracts compared with the corresponding food sources (**Table 1**).

The folate derivative profile of each food was compared with the corresponding folate extract (**Table 2**). In all cases, 5-CH_3-H_4folate and 5-CHO-H_4folate were found to be the main folate derivatives, accounting for over 95% of egg yolk folate, 64% of spinach folate, and 69% of yeast folate. In yeast and spinach, small percentages of other folate derivatives were also present—10-CHO-H_4folate, DHF, THF, and 5,10-CH-H_4folate. Whereas the relative amount of 5-CH_3-H_4folate remained unchanged as a result of processing, there was a significant increase in 5-CHO-H_4folate detected in both yeast and spinach extracts, but not in egg yolk extract, compared with their corresponding food sources. The relative amounts of some derivatives were found to be significantly decreased in extracts compared with their food sources: 10-CHO-H_4folate and THF in yeast extract; DHF, THF, and 5,10-CH-H_4folate in spinach extract.

Industrially produced folate extracts were tested in an independent laboratory (Beechwood, Ballyclare, U.K.) for total colony count and presence of *Coliforms*, *Staphylococcus aureus*, *Listeria*, and *Salmonella* species. In total, four batches of spinach, four batches of egg yolk, and three batches of yeast were produced and tested. In the cases of spinach and yeast, all batches tested were found to be within the limit set for dried heat processed foods, ready to eat after rehydration (*26*), and were therefore combined and stored at −20 °C for later use. One batch of egg yolk was found to be contaminated with *Listeria*, and another had a higher than acceptable total colony count, so both were rejected for use in subsequent human studies. A final two batches of egg yolk found to be acceptable were retained, combined, and stored at −20 °C for later use in human bioavailability studies.

DISCUSSION

The overall aim of this study was to develop methodology to produce extracts of natural food folates that would be suitable for use in controlled human bioavailability studies. The optimal liberation of folate from the food matrix at laboratory scale was

achieved by thermal extraction at 100 °C for 10 min in a 2% ascorbic acid buffer adjusted to pH 5. Gregory (*27*) has reported that a pH of 4.9 maximizes folate stability in ascorbate buffers during thermal challenge. This is in good agreement with our findings showing significantly greater yield for yeast and spinach folate at pH 5, indicating that folate stability is a key determinant of folate yield under these conditions. This is further supported by the observation of a significant reduction in folate yield from both spinach and yeast as a result of exposure to 100 °C for 15 min compared with 10 min. Folate yield from egg was unresponsive to pH changes of extraction buffer or increased duration of thermal extraction, which could be the result of the predominance in egg of the cofactor form 5-CH_3-H_4folate, found in the present study as elsewhere (*28*−*30*). This cofactor form is reported to be very stable in a low-O_2 environment (*28*), as achieved by purging with nitrogen in the current protocol.

Using the optimal conditions for folate extraction established in the laboratory, the production was scaled up to a food-processing facility. The folate contents of the industrially produced folate extracts were found to be considerably more concentrated than the food as typically consumed, that is, 2.3, 5.6, and 11.7 times higher than those of dried yeast, fresh egg yolk, and fresh spinach, respectively. The degree of concentration of folate as a result of the production of food folate extracts as described here was dependent to an extent on the water content of the starting material. For example, in the case of yeast, the concentration of folates during the production of extracts was low compared to the other extracts because the product as purchased is in dried form, whereas the concentration of folates in spinach extract was high compared with fresh spinach, which contains a very high amount (90%) of water. However, even when the water content is taken into account, the folate concentration in these extracts remains considerably higher than in the corresponding food sources. In addition, in the case of egg yolk, removal of the fat during the industrial process greatly enhanced the concentration of folates in the final egg yolk extract.

The folate polyglutamate contents reported here for yeast (100%), spinach (60%), and egg yolk (0%) are in close agreement with published data (*30*), indicating that in yeast virtually all of the folates are present as polyglutamates of five or greater glutamate residues, whereas eggs contain folates in the deconjugated form. The polyglutamate content of spinach found here (60%) is somewhat lower than the 80% reported elsewhere for vegetables (*31*, *32*). It is possible that our approach to the estimation of percentage of polyglutamate, which was based on the difference in *L. casei* response by assaying with and without folate conjugase, may have resulted in a small degree of underestimation of the polyglutamate content in spinach due to partial (20%) response of the microorganism to pentaglutamate (*33*), the predominant form of polyglutamates in spinach (*34*). However, we calculate the extent of this potential underestimation to be just over 10% at most. In addition, it has been suggested that the content of monoglutamates relative to polyglutamates increases with storage of spinach, which may also account for the difference between our findings and the polyglutamate content of spinach reported by Chen et al. (*32*). Importantly, the mono- to polyglutamate ratio was largely unaltered as a result of processing in the current study, with similar ratios for extracts compared to their corresponding food sources.

Folates in foods and food folate extracts were separated by HPLC, and fractions were collected for the determination of the folate derivative content by microbiological assay. 5-CH_3-H_4folate was found to be the main folate derivative in the three food sources, comprising 40−50% of folates in yeast and spinach and 85% of folates in egg yolk. The relative content of this main folate derivative was remarkably similar between extracts produced by the current protocol and their corresponding food sources. However, as expected, other folate derivatives, that is, THF, DHF, 10-CHO-H_4folate, and 5,10-CH-H_4folate, were found to be less stable. Thus, although interconversion between folate derivatives was for the most part prevented during the production of extracts, some degree of interconversion of the more labile cofactor forms appears to have occurred during production or analysis.

There is a large degree of variation in published data on the distribution of specific folate derivatives in various foods. Vegetables in general are reported (*31*, *35*) to contain 70−80% of their folate content in the form of 5-CH_3-H_4folate, whereas other reports indicate that 100% of spinach folate is in the 5-CH_3-H_4folate form (*36*), considerably higher than shown here for spinach. Seyoum and Selhub (*30*) reported that 86% of folates in baker's yeast exists in the form of 5-CH_3-H_4folate, again higher than that found in yeast in the current study. Egg yolk has been reported to contain 87−100% of folate in the form of 5-CH_3-H_4folate (*29*, *30*), which is in good agreement with the current study. Variability in the folate derivative profile of foods depends on enzymatic activity, which is itself influenced by the storage conditions, although the largest factor for the difference is likely to be methodological. The high sensitivity and specificity of the microbiological assay used in this study to quantify folates separated by HPLC permits the measurement of all relevant derivatives.

The development of food folate extracts described here provides a suitable tool for addressing the problematic area of folate bioavailability. Factors such as the food matrix and the ratio of monoglutamyl to polyglutamyl folates are reported to influence the bioavailability of folates from different foods, but the relative impact of these factors is unknown (*17*). By employing the use of the food folate extracts produced here in controlled intervention studies in humans, together with parallel studies in which folate is administered in its usual matrix (i.e., whole food), we can estimate the extent of the effect of the food matrix in determining the overall folate bioavailability. Results from such studies just completed at this Centre show very similar biological responses to equivalent doses of food folates, whether they are administered in the form of the extracts developed here (*37*) or as intact food folate (*38*). This suggests that in fact the food matrix does not exert a major impact on folate bioavailability under normal physiological conditions. One potential limitation of using these extracts for the determination of food folate bioavailability is that they could contain organic acids, nucleic acids, or other soluble components in concentrated form, which may have inhibitory effects on intestinal folate deconjugation and therefore could potentially diminish folate absorption. However, our recent intervention studies using extracts produced by the current protocol (*37*) show very comparable responses to equivalent doses of different food folate extracts irrespective of the extent of folate conjugation (0% polyglutamate in egg yolk extract versus 50% polyglutamate in spinach extract versus 100% polyglutamate in yeast extract). This indicates that the presence of any soluble components in the extracts does not appear to have an important influence on the extent of folate bioavailability in vivo, at least under the conditions of their use in our intervention studies. Such observations are in good agreement with previous findings (*39*)

showing that the extent of folate conjugation is not a limiting factor for folate bioavailability.

In summary, we have developed a protocol for the production of concentrated extracts, representing food folate sources with a wide range of mono- to polyglutamate ratios, that can be used in human bioavailability studies. We have demonstrated that both the mono- to polyglutamate ratio and the folate derivative profile of these extracts are comparable with the corresponding natural food source. In this way the folate extracts of spinach, egg yolk, and yeast produced by the current protocol can be considered to be representative of the folates in the foods from which they have been derived. Use of these concentrated food folate extracts in human bioavailability studies, comparing the effects of food folate with equivalent doses of folic acid, may overcome the many practical difficulties encountered in long-term feeding studies in humans.

ABBREVIATIONS USED

NTD, neural tube defect; FA, folic acid; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate; 10-CHO-H_4folate, 10-formyltetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-CH-H_4folate, 5,10-methenyltetrahydrofolate;

ACKNOWLEDGMENT

We are grateful to Eprova, Schaffhausen, Switzerland, for the generous gift of folate standards.

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Received for review December 18, 2002. Revised manuscript received March 13, 2003. Accepted March 18, 2003. This work was supported by the U.K. Food Standards Agency (Grant NO5013).

JF0262312