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Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products

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January 1, 1998 was the effective date for FDA regulations that mandated the fortification in the USA of a wide range of enriched cereal-grain products with folic acid at levels specified in federal regulations. Because data on pre-fortification levels of folate in such products are limited, we measured folate in 56 enriched foods, including enriched breads and rolls, flours, corn grits and meals, rices, and macaroni and noodle products. Folate was measured by a modification of the Association of Official Analytical Chemists' microbiological method 992.05 using *Lactobacillus casei*. Foods were composited, suspended in 0.1 M phosphate buffer containing 1% ascorbic acid (pH 7.8), autoclaved and cooled. Chicken pancreas conjugase was added and the suspensions were incubated for 16 h at 37°C. Values for folate in enriched products were (μg/100 g): bread and rolls, 24–40; flours, 19–24; corn grits and meals, 22–32; macaroni and noodle products, 27–42; rice, 19–32. Because the single-enzyme method is usually insufficient to liberate food-bound folates, suspensions of foods were also incubated with α-amylase and conjugase followed by treatment with protease to determine the effects of the tri-enzyme digestion on release of folates. For many foods, total folate was 20–30% higher after the tri-enzyme digestion than after incubation with conjugase alone. The modifications of AOAC method 992.05 described here provide a microbiological assay method for the determination of folates in cereal-grain products that may be appropriate for collaborative testing.

INTRODUCTION

Regulations published on March 5, 1996 by the US Food and Drug Administration (FDA) require that folic acid be added to a wide range of enriched cereal-grain products including breads, rolls and buns, wheat flours, corn meals, rice, noodles and macaroni. The effective date for these regulations was January 1, 1998 (FDA, 1996*a*). The FDA took this action to assist women of childbearing age in meeting the Public Health Service recommendation that they consume 400 μg folate per day to reduce their risk of having a pregnancy affected with a neural tube birth defect (Department of Health and Human Services, Public Health Service, 1992). The FDA's rationale in developing its folic acid fortification program and in determining the levels of fortification have been described previously (FDA, 1993*a*,*b*; 1996*a*,*b*,*c*; Crane *et al.*, 1995; Yetley and Rader, 1995, 1996).

While folates in foods have traditionally been measured by microbiological assay procedures (Gregory, 1989; Tamura, 1990, for review), there is considerable interest in the development and validation of more rapid procedures (e.g. high-performance liquid chromatography (HPLC) methods). A number of such procedures were reported to be in developmental stages in 1993 (Finglas *et al.*, 1993). More recently, HPLC methods for determination of folates in meat and dairy products (Vahteristo *et al.*, 1997) and in cereal-grain food products (Pfeiffer *et al.*, 1997) have been described.

Despite progress in the development of HPLC methods, the Association of Official Analytical Chemists (AOAC) official methods that are currently used for compliance purposes are microbiological methods. For example, AOAC official method 944.12 (Folic Acid (Pteroyl-monoglutamic Acid), in Vitamin Preparations, Microbiological Methods) is applicable to preparations

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containing free folic acid (AOAC, 1995*a*). Method 944.12 specifies use of the microorganism *Enterococcus hirae* ATCC No. 8043 (*Streptococcus faecalis*). *E. hirae* does not respond to many food folate forms and thus is not useful for the assay of total folates. AOAC official method 992.05 (Folic Acid (Pteroyl-monoglutamic Acid) in Infant Formula-Microbiological Methods) uses *Lactobacillus casei* (*L. rhamnosus*) as the assay microorganism (AOAC, 1995*b*). AOAC method 992.05 also utilizes a single-enzyme digestion with chick pancreas conjugase (folate conjugase; γ-glutamyl-carboxypeptidase; pteroylpoly-gammaglutamyl hydrolase; EC 3.4.22.12) to liberate mono- or diglutamates from naturally occurring folylpolyglutamates. The treatment of foods with folate conjugase is necessary because most foods are known to contain polyglutamyl forms of the vitamin, which must be hydrolyzed to simpler forms that will promote growth of *L. casei*. *L. casei* has been the preferred test microorganism for many years because it responds to most metabolic forms of folate found in biological systems.

AOAC official method 992.05 is applicable only to the determination of folate in infant formula, since infant formula was the matrix in which the method was successfully collaboratively studied and for which the performance characteristics of the method have been determined (Tanner *et al.*, 1993). In 1990, the Institute of Medicine, US National Academy of Sciences (IOM/NAS) noted that the Official Methods Board of AOAC had taken a restrictive view of applications of official methods, recommending that extension of a specific method to commodities other than those commodities for which the method was approved be subjected to a mini-collaborative study. The IOM further noted that while the scientific rationale for this view is justified, it effectively removes AOAC official method status from many analytical methods used in determining compliance in nutrition labeling (IOM, 1990).

As noted above, AOAC official method 992.05 is not appropriate for analyzing total folates in foods. While method 992.05 was not intended for measurement of total folate, it can measure folate indigenous to infant formula ingredients other than added folic acid because it includes the deconjugation step with chick pancreas conjugase (Eitenmiller and Landen, 1995). Thus, among the AOAC folate methods available, method 992.05 is the method most appropriate for studies aimed at developing a microbiological assay that can be used for determination of this vitamin in foods, particularly in the cereal-grain products that are the subject of the new fortification regulations.

A number of authors have reported that treatment with folate conjugase alone is usually insufficient to liberate food-bound folates. For example, treatment with protease (EC 3.4.24.31) increased the assayed folate contents of breast milk and liver (Yamada, 1979; Tamura *et al.*, 1997), and folate contents of foods high in starch and glycogen were increased following treatment with α-amylase (EC 3.2.1.1) (Cerna and Kas, 1983). Based on reports that extraction of total folates from complex food matrices could be improved by treatment with protease and α-amylase in addition to the traditional heating at 100°C and conjugase digestion steps, DeSouza and Eitenmiller (1990) developed a tri-enzyme digestion procedure that increased measurable folate from a variety of food matrices and was particularly effective on cereal-based and milk and milk-based products. DeSouza and Eitenmiller (1990) showed that values for folate in high-protein foods and in dairy foods were higher after treatment with protease, and that values for folate in high-starch foods increased after treatment with α-amylase. The results of DeSouza and Eitenmiller (1990) and Martin *et al.* (1990) showed that digestion of food extracts with α-amylase and protease in combination with folate conjugase treatment was needed to accurately determine the total folate content of many foods. The tri-enzyme procedure of Martin *et al.* (1990) has been adapted into protocols at the FDA's Atlanta Center for Nutrient Analysis for the analysis of food folate in FDA Total Diet Survey samples.

Tamura *et al.* (1997) determined total folate contents in complete food composites and extended earlier observations regarding the need for a tri-enzyme digestion prior to analysis. The findings of Tamura *et al.* (1997) and others indicate that the total folate content of foods may be significantly underestimated unless it is determined after treatments with α-amylase and protease in addition to treatment with the traditional conjugase. Pfeiffer *et al.* (1997) also addressed the need for improved methods for the analysis of folate and recently described an optimized adaptation of components of several procedures, including a modified tri-enzyme extraction, to quantify folates in unfortified and fortified cereal-grain products. These recent results provide strong evidence for the essentiality of a tri-enzyme treatment in folate analysis.

The levels of naturally occurring folates in enriched cereal-grain products prior to fortification and those resulting from fortification are currently of considerable interest. The purpose of this study was three-fold: (1) to determine, by AOAC official method 992.05, the pre-fortification levels of folate in a variety of cereal-grain products, (2) to test modifications of method 992.05 that might lead to a method suitable for a collaborative study for the determination of folates in cereal-grain products and (3) to compare results obtained by AOAC official method 992.05 (i.e. the single-enzyme method) with results obtained using the tri-enzyme procedure developed in (2). The latter portion of the study was undertaken in order to estimate the extent to which current methodology may underestimate the folate content of foods and to present the results of studies designed to improve the extraction step of the analysis.

MATERIALS AND METHODS

Reagents

Preparation of reagents has been described previously by Angyal (1996). Distilled water was used throughout. Agar culture medium (Lactobacilli Agar AOAC, No. 0900-15), culture suspension medium (Lactobacilli Broth AOAC, No. 0901-15) and folic acid *Lactobacillus casei* assay medium (No. 0822-15) were obtained from Difco Laboratories (Detroit, MI, USA). The assay medium was prepared according to label instructions and the designated amount of ascorbic acid was added.

The test organism, *L. casei* subsp. *Rhamnosus* (ATCC No. 7469) (American Type Culture Collection, Rockville, MD, USA), was stored at refrigerator temperatures and maintained through weekly transfer on agar maintenance medium incubated for 16–21 h at 37°C. Inocula were prepared one day preceding the assay. Cells were transferred from the stock culture of *L. casei* to sterile tubes containing liquid culture medium and incubated in a constant temperature bath for 16 h at 37°C. The culture was centrifuged and the supernatant decanted. The cells were washed three times with 10 ml sterile saline. After the third wash, the cells were resuspended in sterile folate-free basal medium and incubated at 37°C until used. The final inoculum was prepared by adding five drops of the resuspended folate-depleted cell suspension to 10 ml of sterile folate-free basal medium.

Standard solutions

Folic acid

Folic acid was obtained from the United States Pharmacopeia (Rockville, MD, USA). Water content was found to be 7.67%. Folic acid was dissolved in deaerated 0.1 M phosphate buffer, pH 7.0. A portion was taken immediately for spectrophotometric determination of the concentration (Blakley, 1969; Pfeiffer *et al.*, 1997).

A folic acid standard solution of 100 $\mu g\,ml^{-1}$ was prepared according to AOAC official method 944.12 (AOAC, 1995*a*). 50 mg USP folic acid (purity 97.94%; dried to constant weight) was suspended in about 30 ml 0.01 N NaOH. About 300 ml distilled water was added and the pH adjusted to 7.5 with HCl. This stock solution (100 $\mu g\,ml^{-1}$) was stored refrigerated under toluene. An intermediate solution (1 $\mu g\,ml^{-1}$) was prepared by diluting 5 ml of the stock solution with water and adjusting the pH to 7.5 with HCl. The volume was brought to 500 ml with additional water and the solution stored refrigerated under toluene. Working solutions of concentrations from 0.12 $ng\,ml^{-1}$ to 10 $ng\,ml^{-1}$ were prepared fresh on the day of the assay by diluting the intermediate solution with water.

The working standard solution was usually 0.20 $ng\,ml^{-1}$. Suitable volumes of the stock solution were diluted with water to obtain desired concentrations. Final volumes contained 2 ml 1% ascorbic acid/1.42% Na_2HPO_4 buffer (pH 6.7 ± 0.1) per 100 ml. The working standard solution was used to construct a five-point standard curve using the following volumes (ml): 1, 2, 3, 4, 5 (i.e. 0.02–0.10 $ng\,ml^{-1}$).

5-Methyltetrahydrofolic acid

5-Methyltetrahydrofolic acid, disodium salt, was purchased from Fluka (Milwaukee, WI, USA). The compound was dissolved in deaerated 0.1 M phosphate buffer, pH 7.0. A portion was taken immediately for spectrophotometric determination of the concentration using the molar extinction coefficient of 30 800 at 290 nm (Gupta and Huennekens, 1967), followed by rapid addition of sodium ascorbate to a final concentration of 0.5%. Working solutions were corrected for the spectrophotometrically-determined concentrations and the DL racemic mixture and were prepared fresh on the day of use.

5-Formyltetrahydrofolic acid

5-Formyltetrahydrofolic acid, calcium salt (folinic acid, calcium salt) was purchased from Sigma Chemical Company (St Louis, MO, USA). The compound was dissolved in deaerated 0.1 M phosphate buffer, pH 7.0. A portion was taken immediately for spectrophotometric determination of the concentration (Blakley, 1969). Working solutions were handled as described above for 5-methyltetrahydrofolate.

Enzyme preparations

HPLC-grade water was used for all enzyme preparations.

Conjugase (5 $mg\,ml^{-1}$)

Chicken pancreas conjugase (Difco Laboratories, Detroit, MI, USA; No. 0459-12) was suspended in 1.42% Na_2HPO_4 buffer, pH 7.8, containing 1% ascorbic acid and stirred vigorously for 10 min. The suspension was transferred to test tubes and centrifuged for 10 min at about 2000 rpm. The supernatant was decanted through a glass wool pledget into a beaker, covered with parafilm and stored refrigerated.

α-Amylase (20 $mg\,ml^{-1}$)

0.5 g α-amylase (Sigma Chemical Co., St Louis, MO, USA; No. A-0273) was suspended in 25 ml of glass-distilled water and stored refrigerated.

Protease (Pronase E; 2 $mg\,ml^{-1}$)

0.05 g protease (Sigma Chemical Co., St Louis, MO, USA; No. P-5147) was suspended in 25 ml glass-distilled water, filtered through a glass wool pledget if necessary and stored refrigerated.

Foods

Foods representative of the classes of enriched cereal-grain products that were required to be fortified with folic acid by January 1, 1998 were purchased from local supermarkets. Additional products included rice flour, ready-to-eat cereals, other breakfast foods (e.g. pancakes, toaster pastries and cereal bars) and products such as cake, cookie and pizza crust mixes. The majority of the additional products identified an enriched cereal-grain component (e.g. enriched flour) in their ingredient lists. Refrigerated or frozen products were stored according to label directions until prepared for analysis.

Several of the foods were also used in testing the modifications described below. These included an unfortified shredded whole-wheat cereal, an enriched stone-ground yellow corn meal and an enriched medium-grain rice.

Sample preparation

Laboratory samples were ground in a Waring blender or small coffee grinder. Preparation was carried out under subdued light and care was taken to minimize contact with air. The ground samples were stored at room temperature or frozen in tightly sealed glass bottles.

Digestion procedures

Test portions of the composites equal to about 0.25 to 1.0 g dry solids and containing about 1 μg folic acid were placed in 125 ml Erlenmeyer flasks containing 10 ml buffer (1.42% Na_2HPO_4 and 1% ascorbic acid, pH to 7.8 with 4 N NaOH) and mixed thoroughly. A standard containing 1 μg folic acid/ml was analyzed with each set of samples. An additional 10 ml buffer was added and 0.1 ml octanol was added as an antifoaming agent. The flask was covered with a small beaker, autoclaved for 15 min at 121–123°C, cooled and an additional 10 ml buffer was added.

Conjugase treatment
The same lot of Difco chicken pancreas conjugase preparation was used for all studies reported here (Difco Lot 97043JA; expiration date, July 2000). Use of the same lot throughout the studies eliminated concern regarding lot-to-lot variation. In addition, the relative potency of Lot 97043JA was determined as described below. The unfortified shredded whole-wheat cereal product was used in these studies.

Concentration of conjugase preparation Varying amounts of chicken pancreas conjugase preparation (0–50 mg) were added to 1 g samples of the unfortified shredded whole-wheat cereal and the assay performed according to usual procedures. Prior to the study, the product was assayed with and without conjugase to confirm the need for the conjugase step in measuring folate in unfortified products. Results without conjugase treatment and with conjugase treatment (20 mg) for the product were 10.4 ± 5.13 and 43.9 ± 0.64 μg folate/100 g, respectively.

Time of incubation with conjugase preparation Values for folate in the unfortified shredded whole-wheat cereal were not significantly different whether determined by the single- or tri-enzyme procedure (Table 11). We therefore performed the assay varying only the length of time the samples were exposed to the conjugase preparation (i.e. α-amylase and protease were omitted). 20 mg of the chicken pancreas conjugase preparation was added to 1 g samples of the unfortified shredded whole-wheat cereal product. Incubation times with conjugase were 0, 1, 2 and 4 h at 37°C. Samples were autoclaved to terminate the deconjugase reaction and the assay was performed as usual.

The data obtained in these studies showed that digestion conditions using 20 mg of the conjugase preparation for 4 h at 37°C were sufficient for the cereal-grain products we examined (see Tables 1 and 2). Unless otherwise noted, we used these conditions throughout the remainder of the studies.

Single-enzyme procedure
4 ml of conjugase preparation was added to each flask and the flasks were incubated for 16 h or overnight at 37°C. The enzyme was inactivated by autoclaving the samples and standard for 3 min at 100°C, followed by cooling. The samples and standard were adjusted to pH 4.5 with HCl, diluted to a final volume of 100 ml with water and filtered. An aliquot of the clear filtrate was diluted to a final volume such that the folate concentration was about 0.2 ng ml^{-1}. The final volume of each solution to be assayed contained ≥10 ml of pH 6.7 ± 0.1 buffer (1.42% Na_2HPO_4; pH adjusted to 6.7 ± 0.1 with 4 N NaOH).

Table 1. Liberation of folate from an unfortified shredded whole-wheat cereal by varying amounts of chicken pancreas conjugase preparation

Conjugase (mg)	Folate liberated (μg/100 g)
0	0
0.05	7.3 ± 0.9
0.25	17.5 ± 0.6
1.0	23.2 ± 0.6
5.0	28.1 ± 0.5
20.0	30.0 ± 1.6
50.0	31.8 ± 0.1

Treatment: α-amylase + chicken pancreas conjugase, 4 h; pronase, 16 h. Chicken pancreas conjugase preparation (0–50 mg) was added to 1 g samples of the unfortified shredded whole-wheat cereal product and the usual assay at pH 6.7 ± 0.1 was performed. Values are means ± SD of two to four determinations. Results are expressed as μg folate liberated per 100 g product. The amount of folate measured in the absence of conjugase treatment was subtracted from the amount measured with varying amounts of conjugase to obtain the value for 'folate liberated'.

Table 2. Effect of length of incubation with 20 mg chicken pancrease conjugase on the liberation of folate from an unfortified shredded whole-wheat cereal

Time (h)	μg folate/100 g
0	12.1
1	41.2
2	40.7
4	40.8

Chicken pancreas conjugase preparation (20 mg) was added to 1 g samples of an unfortified shredded whole-wheat cereal and incubated for 0–4 h. Values are means of two determinations. Results are expressed as μg folate/100 g product. The amount of folate liberated can be calculated by subtracting the amount of folate measured at 0 time from the amounts measured after 1, 2 or 4 h of incubation with conjugase.

Tri-enzyme procedure after Martin et al. *(1990)*
4 ml of conjugase preparation and 1 ml of α-amylase preparation were added to each flask. Flasks were covered and incubated for 4 h at 37°C. After 4 h, 1 ml of the protease enzyme was added and the flasks were incubated overnight at 37°C. The enzymes were inactivated by autoclaving the sample and standard for 3 min at 100°C, followed by cooling. The samples and standard were adjusted to pH 4.5 with HCl, diluted to a final volume of 100 ml with water and filtered. An aliquot of the clear filtrate was diluted to a final volume such that the folate concentration was about 0.2 ng ml^{-1}. The final volume of each solution to be assayed contained ≥10 ml of pH 6.7 ± 0.1 buffer (1.42% Na_2HPO_4; pH adjusted to 6.7 ± 0.1 with 4 N NaOH).

Modifications of the tri-enzyme procedure The effects of modifications in the tri-enzyme procedure on the yield of total folates were studied with four cereal-grain products: enriched all-purpose flour, rotelle (a pasta product), a long-grain and wild-rice product and a ready-to-eat bran cereal. For the enriched all-purpose flour and rotelle products, total folate values measured by the tri-enzyme procedure were significantly higher than values obtained following conjugase treatment alone (Tables 9 and 10). For the rice product and the ready-to-eat bran cereal, values obtained following the conjugase or tri-enzyme digestion procedures were not significantly different (Tables 10 and 11).

Order of addition of enzymes The effect of order of addition of the enzymes on the yield of total folates was studied by varying the order in which conjugase, α-amylase, and protease were added. In the procedure of Martin *et al.* (1990), α-amylase is added prior to treatment with protease. Two sequences were tested following the initial heating step:

1. α-amylase followed by protease: α-amylase was added and the digestate incubated for 2 h at 37°C. Protease was then added and the digestate incubated for an additional 3 h at 37°C. The digestion mix was heated to 100°C, cooled and conjugase was added for the overnight incubation
2. protease followed by α-amylase: Protease was added for 3 h at 37°C. The reaction mixture was heated to 100°C, cooled and brought to pH 7.8 with buffer. α-amylase was added and the incubation continued for an additional 2 h at 37° C. Finally, conjugase was added for the overnight incubation.

The concentrations of the enzymes were the same as those described previously. The final steps (inactivation of enzymes, cooling, pH to 4.5, dilution and assay at pH 6.7 ± 0.1) were the same for all modifications.

Initial results showed that when α-amylase was added first, there was little change in the values compared to those obtained with the tri-enzyme method (data not shown). However, when protease was added first, there were significant increases in total folates measurable by the microbiological assay for the enriched flour and rotelle products. For this reason, the effects of variations in pH during the extraction were determined in samples to which protease was added first.

Effects of pH during extraction For these studies, the samples were adjusted to pHs of 4.3, 6.0, 6.8 or 7.8 and the digestions carried out as described above. Protease was added for 3 h at 37°C. The digestion mixture was heated to 100°C, cooled and restored to the initial pH with buffer. α-amylase was added and the incubation continued for an additional 2 h at 37°C. Finally, conjugase was added for the overnight incubation. The final steps (inactivation of enzymes, cooling, pH to 4.5, dilution and assay at pH 6.7 ± 0.1) were the same for all modifications.

Statistical analysis

Differences between values obtained by the conjugase and tri-enzyme methods were determined by 1-tailed *t*-tests. Differences with $p < 0.05$ were considered to be statistically significant. An analysis of variance (ANOVA) was used to determine whether values obtained when extractions were performed at various pHs were significantly different from values obtained by the usual tri-enzyme procedure.

Assay

Total folates were determined by microbiological assay using a modification of AOAC official method 992.05 (AOAC, 1995*b*; Angyal, 1996). Background information is provided in AOAC (1995*c*), Ford *et al.* (1969), Flynn (1965) and Toepfer *et al.* (1951). *L. casei* (ATCC 7469) was the assay microorganism. 12 tubes containing four different amounts of extract (i.e. 1–4 ml) in triplicate were used to assay each test sample. The assay buffer consisted of 1 g ascorbic acid (reagent grade)

dissolved in 100 ml 1.42% Na_2HPO_4. The pH was adjusted to 6.7 ± 0.1 with 4 N NaOH. Growth was read after 22 h of incubation at 37°C in a Bausch and Lomb Spectronic 20 spectrophotomer equipped with a flow cell. The assay tube reading apparatus consisted of a Gilson escargot fractionator Model 222 sample changer modified with an air-agitator system and connected to the spectrophotometer and a printer or computer. Enzyme blank preparations were assayed for folic acid contribution at different dilution levels similar to those used to dilute test preparations containing different levels of folate. Negligible responses were observed for dilutions corresponding to those used for products containing the lowest levels of folic acid. For this reason, blank corrections were deemed unnecessary.

An eight-point fourth degree polynomial regression plot and a computer program designed according to the official AOAC protocol were used to calculate ng folic acid/ml extract and μg total folate/100 g sample. Assay results were considered acceptable if the assay met the AOAC criteria for microbiological assays (i.e. assays with less than two-thirds of the tubes (67%) within the acceptable range were not used to determine the folate content of product sample). All 56 samples were analyzed by the AOAC single enzyme procedure. 37 of the samples were also analyzed by the tri-enzyme procedure, and four samples were analyzed following the modifications described above.

Assay at pH 6.2 and use of reduced folates as calibrants

The microbiological assay is typically run at pH 6.7 ± 0.1, as specified for AOAC method 992.05 and in instructions for use of the Difco media. We also carried out the assay at pH 6.2 because of reports that 5-methyltetrahydrofolate showed a reduced response relative to folic acid under assay conditions with an initial pH of 6.8 (Phillips and Wright, 1982, 1983).

We also performed the assay with calibrants other than folic acid. For these studies, we constructed standard curves with 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, and assayed the unfortified shredded-wheat cereal-grain product against these calibrants.

Recovery studies

Recovery of folic acid added at levels of 0.4 to 1.0 μg to samples of three types of cereal-grain products was also determined. The samples were spiked with known amounts of folic acid, carried through the entire procedure and assayed. The amount of folic acid added for recovery purposes was equivalent to the amount present in the sample. Recoveries were performed at pH 6.2 and at pH 6.7 ± 0.1. Recoveries of folate were calculated as follows: (Concentration of folate measured in spiked sample−concentration of folate measured in unspiked sample)/(concentration of folate added in spiked sample) × 100) = % recovery.

Analysis of reference material and check samples

Standard Reference Material SRM 1846 is a spray-dried milk-based infant formula that is intended primarily for use in validating methods for determining proximates, minerals and certain vitamins in infant formula and similar matrices. SRM 1846 was used as an in-house quality control material. The mass fraction value for folic acid in SRM 1846 of 1.29 ± 0.28 mg kg^{-1} was determined by microbiological assay. Portions of this SRM were analyzed with each set of folate analyses and with all modifications.

Cereal-grain samples from the Vitamin, Mineral and Proximate Check (VMP) series, available from the American Association of Cereal Chemists International Check Sample Service (AACC, St Paul, MN, USA), were also analyzed by the tri-enzyme procedure. Preparation of the check samples was handled as described above for foods.

RESULTS AND DISCUSSION

Need for a microbiological assay for folates in cereal-grain products

There is a need to develop more broadly applicable microbiological methods for analysis of folates in foods. Among the available AOAC official microbiological methods for folate analysis, method 992.05 for infant formula is most amenable to extension to other matrices because it utilizes *L. casei* as the assay microorganism and because it includes treatment of samples with a conjugase preparation. In this paper, we report on a microbiological method that includes a tri-enzyme digestion and describe its use in the analysis of folates in cereal-grain products. The method described here may serve as the basis for a mini-collaborative study that, if successful, may provide an official method applicable to cereal-grain products.

Optimizing conditions for the assay

Conjugase treatment

The results of studies to determine the adequacy of the concentration and time of exposure to the chicken pancreas conjugase preparation are shown in Tables 1 and 2. 20 mg of conjugase preparation was sufficient for the cereal-grain products we examined (Table 1), as was the 4 h incubation with the preparation (Table 2).

While these conditions of concentration and time of incubation with chicken pancreas conjugase were adequate for these studies, we do not know whether the conditions established above for cereal-grain products will be adequate for all types of food. For this reason, it is essential to optimize the concentration and time for deconjugation in folate assays before extensive use of a specific set of conditions. In the present study, we also

sought to reduce the likelihood that conditions were not optimum by using samples containing 0.25 to 1.0 g dry solids and about 1 μg folic acid.

Modification of the tri-enzyme procedure

We used four cereal-grain products for these studies. For two of the four products examined (i.e. enriched all-purpose flour and rotelle), total folate values measured by the tri-enzyme procedure were significantly higher than values obtained following conjugase treatment alone, while for the rice product and the ready-to-eat bran cereal, values obtained following single-enzyme and tri-enzyme digestion procedures were not significantly different (Table 3).

In determining the effects of order of addition of α-amylase, protease and conjugase on the yield of total folate, we observed that when α-amylase was added prior to protease and conjugase there was little change in values compared with those obtained with the tri-enzyme assay. However, when protease was added first, there were significant increases in total folates measurable by the microbiological assay for two of the four products (see below and Table 3). For this reason, studies in which the pH of the extraction was varied were carried out in suspensions in which digestion with protease preceded addition of α-amylase and conjugase (Table 3).

For the enriched flour product, results obtained by the usual tri-enzyme procedure were significantly lower than results obtained when protease was added first and the extraction was performed at pH 4.3, 6.0, 6.8 or 7.8 ($p < 0.0199$). There were no significant differences among values obtained at the four pHs tested, however. This suggests that, for this product, the more significant aspect of the modification was the order of addition of the enzymes, rather than the pH at which the extraction was performed.

There were also similar significant differences among the results for the rotelle product ($p < 0.0449$). Folate values obtained following digestion at pHs 4.3 and 6.0 were higher than values obtained using the usual tri-enzyme procedure. However, results obtained following digestion at pHs 6.8 and 7.8 were not significantly different from values obtained by the unmodified tri-enzyme procedure. In addition, there were no significant differences among results obtained at the four pHs tested. For the rice product and the ready-to-eat bran cereal product, there were no significant differences among groups (1) to (5) (Table 3).

Tamura *et al.* (1997) observed that the optimal pH for enzyme treatment may differ for each type of food and that further studies may be needed to establish the best method for obtaining the maximum folate content of individual food items. Tamura *et al.* (1997) found, for example, that for complex food composites (e.g. composites of fruits, vegetables, cereal-grain products, meats, etc.), tri-enzyme treatment gave the highest values at pH 4.1 (vs pHs of 6.3 and 7.85). Tamura *et al.* (1997) expressed uncertainty that pH 4.1 was the best pH for other food items, however, noting that the folate contents of other foods were often higher at pH 7.85 after tri-enzyme digestion. Our data also indicate that additional studies will be needed to further optimize extraction conditions, and that examination of the order of addition of the enzymes should also be considered.

Analysis of reference material 1846 and AACC check samples

We used SRM 1846 as an in-house quality control material and analyzed portions of this SRM with each set of folate analyses and with all modifications. Values found for folate in SRM 1846 were $1.37 \pm 0.12\ mg\ kg^{-1}$ ($n = 35$; conjugase assay (single-enzyme)) and $1.40 \pm 0.10\ mg\ kg^{-1}$ ($n = 18$; tri-enzyme assay) vs the mass fraction value of $1.29 \pm 0.28\ mg\ kg^{-1}$ determined by microbiological assay for this material. All values fell within those specified for SRM 1846. As expected, there were no differences in values in SRM 1846 determined by the single-enzyme or tri-enzyme procedures, since the greatest part of folic acid in the infant formula material is added folic acid. We noted, however, that use of the tri-enzyme assay with this material resulted in extracts that appeared to be more homogeneous and clearer

Table 3. Effects of modifications in the tri-enzyme procedure on extraction of folates from cereal-grain products

Product	Conjugase	Tri-enzyme		pH 4.3	pH 6.0	pH 6.8	pH 7.8
	(conj).[a] (μg folate/100 g)	(1) (μg folate/100 g)	$p <$	(2) (μg folate/100 g)	(3) (μg folate/100 g)	(4) (μg folate/100 g)	(5) (μg folate/100 g)
All-purpose flour	26.1 ± 2.5	33.1 ± 0.6	0.05	42.2 ± 2.1	43.7 ± 3.9	39.4 ± 2.4	40.4 ± 3.3
Rotelle	31.4 ± 3.3	39.6 ± 2.6	0.05	53.8 ± 4.2	52.9 ± 4.5	46.6 ± 1.4	46.7 ± 6.9
Long-grain and wild rice	50.7 ± 16.6	59.4 ± 2.6	NS	69.7 ± 0.4	74.8 ± 10.1	67.2 ± 4.1	72.1 ± 10.3
Bran cereal	629.3 ± 96.1	677.8 ± 104.4	NS	721.3 ± 39.4	653.4 ± 69.2	655.8 ± 33.9	643.8 ± 91.0

Values are means ± standard deviations for four, four, four, and six determinations at pHs 4.3, 6.0, 6.8 and 7.8, respectively. Effects of variations in pH during the extraction were determined in samples to which protease was added, and subsequently inactivated, prior to addition of α-amylase and conjugase.

[a]Results obtained by the single enzyme (conj.) and tri-enzyme procedures (1) were compared by one-tailed *t*-tests. Values obtained by the tri-enzyme procedure (1) and modifications (2), (3), (4) and (5) were compared by ANOVA.

than the original digestion mixtures and required fewer filtrations prior to analysis.

The behavior of a particular food in an analytical method is primarily determined by the relative proportions of protein, fat and carbohydrate in the food. A recent report of the AOAC Task Force on Methods for Nutrition Labeling depicted a scheme in which all foods could be organized by their relative proportions of protein, fat and carbohydrate. The composition of SRM 1846 (i.e. 27.1% fat, 11.1% protein and 57.2% carbohydrate) places it in a sector that includes foods of 0–33% fat content, 0–33% protein content and 33–67% carbohydrate content (Wolf, 1993; Sharpless *et al.*, 1997). Thus, SRM 1846 may be considered to be a matrix representative of a variety of other foods that fall in the same sector (e.g. cocoa, spaghetti with meatballs, whole milk yogurt, wheat germ and canned asparagus) (Sharpless *et al.*, 1997). A number of the cereal-grain products assayed in this study (e.g. breads, pastries, rolls, some pastas and some cereals) fall in the same fat–protein–carbohydrate composition sector as SRM 1846, while other cereal-grain foods, such as cornflakes, noodles and biscuit dough, have higher carbohydrate contents (67–100%) but similar protein and fat contents (i.e. $\leq$33%) as SRM 1846. Thus, while SRM 1846 is not as applicable for method validation as, for example, cereal-grain samples, we have found it useful as a quality control material.

In addition to analysis of SRM 1846, we analyzed five cereal-grain check samples from the AACC (Table 4). In all cases, results obtained by the tri-enzyme method fell within the range of values reported for the cereal-grain check samples.

pH of assay and response to reduced folates

The microbiological assay is typically run at pH 6.7 ± 0.1 as specified for AOAC method 992.05 and in instructions for use of the Difco media. We also carried out the assay at pH 6.2 because of reports that *L. casei* showed a showed a reduced response to 5-methyltetrahydrofolate relative to folic acid under conventional assay conditions (i.e. initial pH 6.8; Phillips and Wright, 1982, 1983). Approximately equivalent responses were reported when the initial pH of the assay medium was adjusted to pH 6.2 (Phillips and Wright, 1982; Wright and Phillips, 1985). Concern has been expressed that assays performed with media at an initial pH of 6.8 with folic acid as the calibrant may underestimate the folate content of foods containing predominantly 5-methyltetrahydrofolate (Wright and Phillips, 1985). Wilson and Horne (1984), however, reported that the *L. casei* response to folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate is equivalent using pH 6.7 medium from Difco laboratories. Under conditions of their assay, Wilson and Horne (1984) reported that folate and (6*R*,*S*)-5-formyl-FAH_4, (6*R*,*S*)-5-CH3-FAH_4, and (6*S*)-5 CH_3-FAH_4 (as the active (*S*) isomers) gave equal growth responses for *L. casei*. Tamura *et al.* (1972) made similar observations using pH 6.7 medium prepared in their own laboratories. Chen *et al.* (1978) assayed both folic acid and 5-methyltetrahydrofolate on a molar-equivalent basis by a radiometric method. *L. casei* showed essentially identical metabolic activity with each of these folates. Shane *et al.* (1980) also reported that *L. casei* responded equivalently to 5-methyltetrahydrofolate and folic acid. O'Broin *et al.* (1975) reported that the monoglutamates of 5-methyl- and 5-formyl-tetrahydrofolate gave essentially the same response for *L. casei*, while Newman and Tsai (1986) reported slight differences in the molar response of *L. casei* to folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate.

Because of these apparently contradictory findings and because inequalities in the growth of *L. casei* on different reduced folates could significantly affect the validity of the assay, we conducted several studies of the response of *L. casei* to different calibrants at pHs of 6.2 and 6.7 ± 0.1.

We constructed standard curves of folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate and carried out the microbiological assays at pHs of 6.2 and 6.7 ± 0.1. Our results showed that the relative sensitivities of the assay microorganism, expressed as picomoles of standard required for 50% transmittance, were slightly greater at pH 6.2 than at pH 6.7 ± 0.1 for folic acid and 5-methyltetrahydrofolate (Fig. 1 and Table 5). Good growth was obtained with all three calibrants at both pHs. Assays performed at pH 6.2 appear to be somewhat more sensitive than those performed at pH 6.7 ± 0.1.

We assayed an enriched corn meal, an enriched rice product and unfortified shredded whole-wheat cereal at pH 6.2 and pH 6.7 ± 0.1 using folic acid as the calibrant. Growth was slightly better at pH 6.2 than at pH 6.7 ± 0.1 in both the standard and sample tubes. Similar results for the folate content of the three products, expressed as μg folate/100 g product, were obtained at both pHs (Table 6).

Analysis of the unfortified shredded whole-wheat cereal at pH 6.2 and pH 6.7 ± 0.1 against folic acid and 5-methyltetrahydrofolate as the calibrants gave comparable results (Table 7). A single assay at both pHs with

Table 4. Values for folate in Vitamin, Mineral and Proximate (VMP) check samples from the AACC determined by the tri-enzyme procedure

Sample	Tri-enzyme procedure	AACC values	
	(folate mg/100 g)	(folate mg/100 g)	(*N*)
VMP-3	0.444	0.385 ± 0.062	(13)
VMP-4	1.401	1.433 ± 0.708	(13)
VMP-5	1.533	1.508 ± 0.168	(12)
VMP-6	0.488	0.443 ± 0.036	(13)
VMP-1	0.482	0.443 ± 0.037	(10)

AACC values are means ± SD of *N* values determined by laboratories subscribing to the AACC check sample program. Tri-enzyme values are the result of a single analysis.

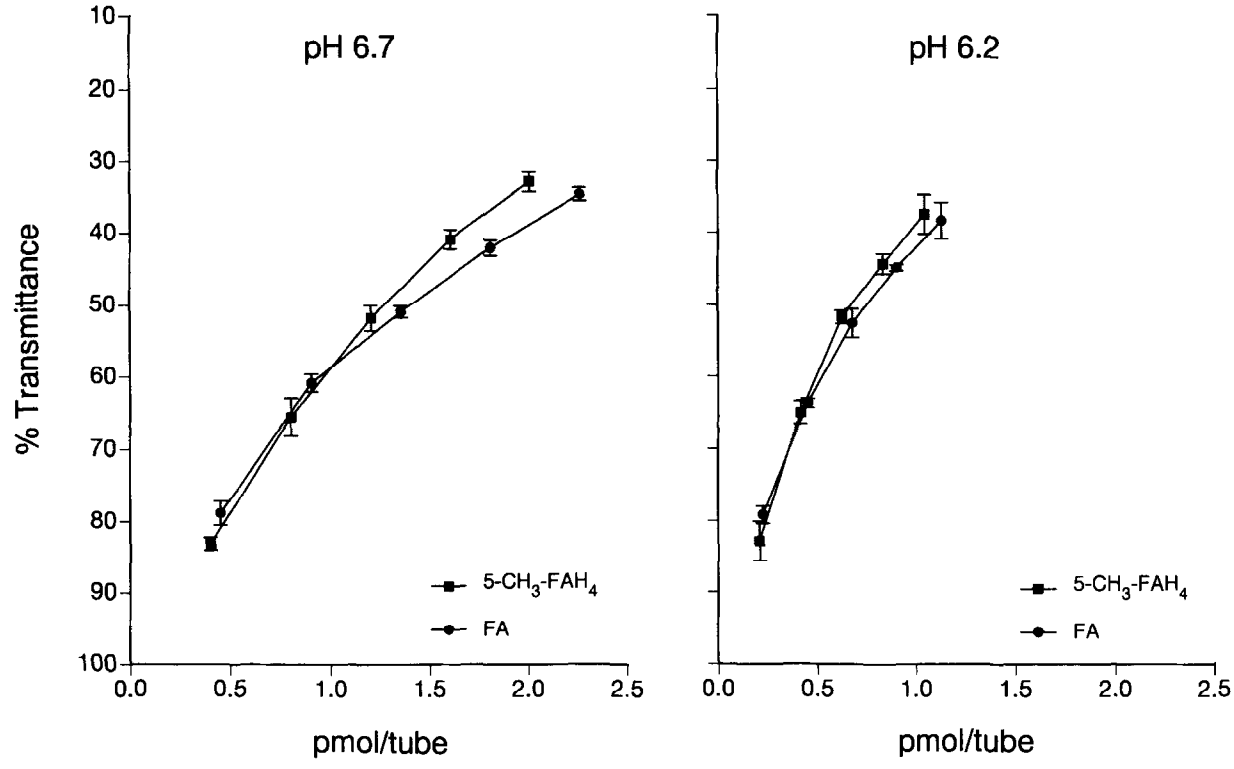

Fig. 1. Comparative responses of *L. casei* to folic acid and 5-methyltetrahydrofolate. pHs of the assay were 6.2 and 6.7 ± 0.1. Concentrations of the calibrants (picomol/tube) were determined spectrophotometrically. For 5-methyltetrahydrofolate, calculations were based on the amount of the (6*S*)-5-methyltetrahydrofolate in the (6*R,S*) mixtures (i.e. one half of the amounts determined spectrophotometrically).

5-formyltetrahydrofolate as the calibrant gave a slightly lower mean value at pH 6.2 than at pH 6.7 ± 0.1.

Recovery of folic acid added to samples

Three cereal-grain samples and a standard were spiked with known amounts of folic acid, carried through the entire procedure and assayed at pH 6.2 and pH 6.7 ± 0.1. The amount of folic acid added for recovery purposes was equivalent to the amount present in the sample. For two of the three spiked cereal-grain products and the spiked standard, mean recoveries were 91–101% (Table 8). Recoveries of folic acid added to the enriched rice product were consistently lower (82% at pH 6.7 ± 0.1 and 75% at pH 6.2).

Table 5. Relative sensitivity of microbiological assay with *L. casei* to folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate

Standard	Picomoles/tube for 50% transmittance	
	pH 6.2	pH 6.7 ± 0.1
Folic acid	0.7 ± 0.1	1.1 ± 0.2
5-methyltetrahydrofolate	0.7 ± 0.0	1.0 ± 0.3
5-formyltetrahydrofolate	0.5	0.7

Values for folic acid and 5-methyltetrahydrofolate are means ± SD of three independent determinations. Values for 5-formyltetrahydrofolate are results of a single assay that met AOAC criteria for microbiological assays. The pH of the media was determined before and immediately after autoclaving, with the following results: pH 6.2 media, 6.32 ± 0.01 (before), 6.17 ± 0.01 (after); pH 6.7 media, 6.81 ± 0.01 (before), 6.64 ± 0.01 (after). Concentrations of the folate compounds were determined spectrophotometrically. For 5-methyltetrahydrofolate and 5-formyl-tetrahydrofolate, calculations were based on the amounts of the (6*S*)-5-methyltetrahydrofolate or (6*S*)-5-formyltetrahydrofolate in the (6*R,S*) mixtures (i.e. one half of the amounts determined spectrophotometrically).

Folate in enriched cereal-grain products

The need to establish a credible database for folates in foods containing naturally occurring folates and for foods that are fortified with folate has focused attention

Table 6. Effect of pH of microbiological assay with *L. casei* on determination of folate in three cereal-grain products

Product	Total folate (μg/100 g)	
	pH 6.2	pH 6.7 ± 0.1
Enriched stone-ground yellow corn meal	282.0 ± 17.7	277.2 ± 4.0
Enriched rice, medium grain	192.4 ± 14.6	200.2 ± 5.6
Shredded whole-wheat cereal	45.1 ± 3.0	42.7 ± 2.4

Assays were carried out at pH 6.2 or 6.7 ± 0.1. Values are means ± SD of two or three determinations. The calibrant was folic acid.

on the importance of reliable folate analyses. One of the objectives of this study was to determine the extent to which the single-enzyme microbiological assay, which is among the methods most commonly used, underestimates total food folates, and to improve the methodology for the analysis of total folates by the microbiological method.

Current folate levels in enriched bakery products, wheat flours, corn grits and corn meals are listed in Table 9. Values for folate determined by the conjugase (single-enzyme) method were (μg/100 g): bread and rolls, 24–40; flours, 19–24; corn grits and meals, 22–32. Under the current regulations, whole-wheat bread and whole-wheat flour will not be fortified; values are included for comparative purposes only. Folate values in 8 of the 16 products were significantly higher when assayed following the tri-enzyme treatment.

Folate levels in enriched rice and enriched macaroni and noodle products are listed in Table 10. Of the 13 products assayed by both the single-enzyme and tri-enzyme procedures, four showed increased levels of folate when assayed by the tri-enzyme procedure. Two of the enriched macaroni products (large shells and trio shells) were found to be fortified with folic acid at levels consistent with the March 5, 1996 regulations.

Table 7. Microbiological assay with *L. casei* of an unfortified shredded whole-wheat product using standard curves constructed with folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate

Standard curve	Folate acid (μg/100 g)	
	pH 6.2	pH 6.7 ± 0.1
Folic acid	44.8 ± 1.9	44.0 ± 0.2
5-methyltetrahydrofolate	41.5 ± 4.3	40.9 ± 0.1
5-formyltetrahydrofolate	35.9	39.5

Values obtained with standard curves constructed with folic acid and 5-methyltetrahydrofolate are means ± SD of three independent determinations. Values obtained with a standard curve constructed with 5-formyltetrahydrofolate represent the results of a single assay. Concentrations of the folate compounds were determined spectrophotometrically. For 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, calculations were based on the amounts of the (6*S*)-5-methyltetrahydrofolate or (6*S*)-5-formyltetrahydrofolate in the (6*R*,*S*) mixtures (i.e. one half of the amounts determined spectrophotometrically).

Table 8. Recovery of folic acid added to the standard and to three cereal-grain products

Sample or product	Total folate recovered (%)	
	pH 6.2	pH 6.7 ± 0.1
Spiked standard	98.3	100.7 ± 2.7
Enriched stone-ground yellow corn meal	100.6	94.9 ± 5.7
Enriched rice, medium grain	74.8	82.1 ± 4.4
Shredded whole-wheat cereal	90.9	99.2 ± 10.6

Percentage recoveries are means ± SD of two determinations at pH 6.7 ± 0.1 and of one determination at pH 6.2. The standard curve for this study was constructed with folic acid.

Table 9. Folate levels in enriched bakery products, wheat flours, corn grits and corn meals measured after conjugase and tri-enzyme digestions

Product	Conjugase (μg/100 g)	Tri-enzyme (μg/100 g)	Tri-/conj. ×100 (%)	*p*
Enriched bread, rolls and buns				
White pita bread	23.8 ± 0.8	30.3 ± 1.6	127	≤0.019
White bread	43.4 ± 5.7	ND	—	—
Seeded rolls	88.4 ± 24.6	92.3 ± 8.3	104	NS
Whole-wheat bread	48.9 ± 6.4	46.9 ± 1.7	96	NS
Enriched flour				
All-purpose baking mix	19.8 ± 1.4	24.8 ± 2.0	125	≤0.050
Wheat flour	24.8 ± 1.5	32.2 ± 2.1	130	≤0.029
All-purpose flour	26.1 ± 2.5	33.1 ± 0.6	127	≤0.031
Hot-roll mix	33.5 ± 3.6	38.4 ± 5.0	115	NS
Bread mix (white)	39.2 ± 0.7	39.6 ± 0.5	101	NS
Bread mix (whole-wheat and white)	64.4 ± 3.9	64.7 ± 11.0	100	NS
Whole-wheat flour	51.5 ± 8.1	58.5 ± 3.9	113	NS
Enriched corn grits				
Instant grits with butter	25.2 ± 1.4	28.7 ± 0.7	114	≤0.042
Quick grits	27.5 ± 6.2	31.5 ± 1.6	115	NS
Enriched corn meals				
White corn meal	19.7 ± 3.1	26.1 ± 1.3	133	≤0.057
Yellow corn meal	25.3 ± 1.2	29.2 ± 0.7	115	≤0.029
Yellow corn meal	32.6 ± 1.1	37.5 ± 0.3	115	≤0.012
Corn muffin mix	24.1 ± 3.3	28.3 ± 0.9	118	NS

Values are means ± SD of three to four determinations per product. Percentage differences in results obtained by the two digestion procedures were calculated as follows: (Tri-/conj.) × 100.
ND, not determined; NS, not significant.

Table 10. Folate levels in enriched rice and enriched macaroni and noodle products measured after conjugase and tri-enzyme digestions

Product	Conjugase (μg/100 g)	Tri-enzyme (μg/100 g)	Tri-/conj. ×100 (%)	*p*
Enriched rice				
Medium-grain rice	20.1 ± 2.0	25.6 ± 2.7	127	≤0.076
Brown rice, natural	23.7 ± 2.3	31.3 ± 1.4	132	≤0.029
Instant rice	28.0 ± 4.6	28.3 ± 0.8	101	NS
Long-grain and wild rice	50.7 ± 16.6	59.4 ± 2.6	117	NS
Enriched macaroni products				
Penne rigate	27.6 ± 2.9	32.5 ± 3.6	118	NS
Pasta salad	28.4 ± 2.3	ND	—	—
Pasta	28.9 ± 4.8	ND	—	—
Rotelle	31.4 ± 3.3	39.6 ± 2.6	126	≤0.055
Pasta	32.1 ± 1.3	ND	—	—
Pasta	33.0 ± 3.8	ND	—	—
Thin spaghetti	35.1 ± 5.7	39.0 ± 4.7	111	NS
Thin spaghetti	35.6 ± 4.4	37.8 ± 3.9	106	NS
Large shells	179.9 ± 9.2	203.5 ± 20.1	113	NS
Trio shells	181.5 ± 21.6	211.7 ± 26.8	117	NS
Enriched noodle products				
Noodles without yolk	28.3 ± 1.4	32.9 ± 2.9	116	≤0.0921
Egg noodles	42.9 ± 1.3	45.2 ± 3.8	105	NS
Noodles and sauce	83.2 ± 0.7	80.4 ± 5.7	97	NS

Values are means ± SD of two to four determinations per product. Percentage differences in results obtained by the two digestion procedures were calculated as follows: (Tri-/conj.)×100.
ND = not determined; NS = not significant.

Table 11. Current levels of folate in selected foods measured after conjugase and tri-enzyme digestions

Product[a]	Conjugase (μg/100 g)	Tri-enzyme (μg/100 g)	Tri-/conj. ×100 (%)	*p*
Flours				
Rice flour	22.7 ± 1.1	25.2 ± 1.3	111	≤0.089M
Ready-to-eat cereals				
Shredded whole-wheat cereal	44.2 ± 3.2	47.3 ± 4.5	107	NS
Toasted rice cereal*	397.6 ± 48.6	450.1 ± 70.0	113	NS
Whole-grain oat cereal*	416.6 ± 36.7	419.5 ± 33.2	101	NS
Wheat-bran/corn-bran cereal*	629.3 ± 96.1	677.8 ± 104.4	108	NS
Toasted corn, oats, wheat and rice cereal*	1,326.1 ± 51.8	ND	—	—
Other breakfast foods				
Buttermilk pancakes	22.5 ± 2.4	ND	—	—
Toaster pastry	24.0 ± 2.0	ND	—	—
Whole-grain wheat waffles*	67.8 ± 19.6	72.2 ± 13.6	106	NS
Toaster tart*	111.0 ± 7.9	ND	—	—
Toaster pastry*	158.1 ± 5.0	ND	—	—
Waffles*	158.1 ± 18.5	143.9 ± 7.9	91	NS
Cereal bar*	255.5 ± 22.8	ND	—	—
Multi-grain cereal bar (1)*	278.7 ± 108.3	292.5 ± 77.9	105	NS
Multi-grain cereal bar (2)*	311.4 ± 28.6	ND	—	—
Miscellaneous				
Cake mix	13.6 ± 1.5	ND	—	—
Cake and cookie mix	21.9 ± 2.1	ND	—	—
Pizza crust mix	48.2 ± 4.6	ND	—	—
Stuffing mix	53.2 ± 12.1	ND	—	—
Rice cereal-based snack*	182.2 ± 1.6	ND	—	—
Active dry yeast	2,632.5 ± 425.8	ND	—	—
Fruit-flavored drink mix*	3,214.2 ± 261.5	ND	—	—

Values are means ± SD of three to four determinations per product. Percentage differences in results obtained by the two digestion procedures were calculated as follows: (Tri-/conj.)×100.
ND = not determined; NS = not significant.
[a]Products marked * are fortified with folic acid at 40 to 400 μg/serving.

Current levels of folate were also determined in foods such as rice flour, ready-to-eat cereals, breakfast foods (e.g. pancakes, toaster pastries and cereal bars) and products such as cake and cookie and pizza crust mixes (Table 11). The majority of these products identified an enriched cereal-grain component (e.g. enriched flour or

Table 12. Fortification requirements for enriched cereal-grain products in the USA

Standardized product	Folic acid	
	mg/lb	μg/100 g
Part 136-Bakery Products		
§136.115 Enriched bread, rolls, buns	0.43	95
Part 137 Cereal Flours and Related Products		
§137.165 Enriched flour	0.7	154
§137.185 Enriched self-rising flour	0.7	154
§137.235 Enriched corn grits	0.7–1.0	154–220
§137.260 Enriched corn meals	0.7–1.0	154–220
§137.305 Enriched farina	0.7–0.87	154–192
§137.350 Enriched rice	0.7–1.4	154–308
Part 139 Macaroni and Noodle Products		
§139.115 Enriched macaroni products	0.9–1.2	198–264
§139.122 Enriched non-fat milk macaroni products	0.9–1.2	198–264
§139.155 Enriched noodle products	0.9–1.2	198-264

Specifications for standardized products are found in 21 CFR Parts 136, 137 and 139. Values are expressed as amounts of folic acid in mg/lb and μg/100 g that are required to be added to enriched cereal-grain products. The values also apply to cross-referenced standards of identity for enriched vegetable macaroni products (§139.135) and enriched vegetable noodle products (§139.165). The standard of identity for enriched corn grits has been revoked. However, the food additive regulation for folic acid (CFR §172.345) was amended to include fortified grits among the non-standardized foods to which folic acid may be added. The level of addition will be the same as that permitted in the original standard for enriched corn grits.

enriched rice) in their ingredient lists. Many of these foods are currently fortified with folic acid (e.g. four of the five ready-to-eat cereals, seven of the nine other breakfast foods, and the cereal-based snack and fruit-flavored drink mix in the miscellaneous category). A subset of these products was assayed by the tri-enzyme procedure. No significant differences were found in values determined by the two methods. Rice flour will not be fortified; values for folate in this product were determined for informational purposes only.

In most cases, values found for folate in this study are in good agreement with previously reported data on the folate content of cereal-grain products. For example, Ranhotra *et al.* (1985*a*,*b*) reported an average folate content of 20–54 μg/100 g for breads (Ranhotra *et al.*, 1985*a*) and 19–26 μg folate/100 g for selected dry pasta products (Ranhotra *et al.*, 1985*b*). However, average folate contents of white long-grain rice and dry spaghetti are listed as 8 and 18 μg/100 g, respectively, in USDA Handbook 8-20 (USDA, 1989). These values are lower than those reported here.

Following fortification, folate levels in enriched cereal-grain products will increase from current levels to (μg/100 g): bread and rolls, 95; flours, 154; corn grits and meals, 154–220; rices, 154–308; macaroni and noodle products, 198–264 (Table 12). The fortification thus represents significant increases above current levels. Levels of folate in products containing an enriched cereal-grain ingredient (e.g. cake and cookie mixes, pizza crust mix, stuffing mixes, etc.) will increase in proportion to the amount of the enriched folate-fortified cereal-grain ingredient they contain.

As noted above, at the time of sample collection for this study some cereal-grain foods were already fortified with 10 to 100% of the daily value for folic

Table 13. Consistency between label values and analyzed values obtained by microbiological assay for selected folic acid-fortified foods

Product	Label information			Analyzed values, conjugase assay		% of declared folic acid (analyzed/label×100)
		Folate				
	Serving size (g)	% DV/serving	μg/serving	μg/100 g	μg/serving	
Ready-to-eat cereals						
Toasted rice cereal	30	25	100	398	119	119
Whole-grain oat cereal	30	25	100	417	125	125
Wheat and corn bran cereal	30	25	100	629	189	189
Toasted corn, oats, wheat and rice cereal	30	100	400	1,326	398	100
Other breakfast foods						
Whole-grain wheat waffles	78	10	40	68	53	133
Toaster tart	52	10	40	111	58	145
Toaster pastry	52	20	80	158	82	103
Waffles	68	20	80	158	107	134
Cereal bar	37	25	100	256	95	95
Multi-grain cereal bar (1)	37	25	100	311	115	115
Multi-grain cereal bar (2)	37	30	120	279	103	86
Miscellaneous						
Rice cereal-based snack	22	10	40	182	40	100
Fruit-flavored drink mix	3	20	80	3,214	96	120

Information on folic acid content from product labels was compared with results obtained following analysis of the products. Serving sizes are based on amounts referenced in 21 Code of Federal Regulations Section § 101.12(b). Values for folic acid on food labels are declared as the percentage of the daily value (DV) that is present in one serving of the food. The daily value for folate is 400 μg. We compared label declarations for folic acid with values found on analysis for 13 products fortified with folic acid and determined the consistency between label values and analyzed values.

acid (40 to 400 μg/serving). We compared label declarations for folic acid with values found on analysis for 13 such products. For 12 of 13 products, analyzed values for folate were 95 to 189% of the label declaration (Table 13). We do not know whether the highest values found represent manufacturers' excess addition of folic acid or whether the assay is measuring endogenous folate as well as folic acid added as a fortificant.

CONCLUSIONS

In 1993, Finglas *et al.* (1993) reported the results of the Commission of the European Communities' Community Bureau of Reference (BCR) project to improve the quality of analyses of vitamins in food. The project, undertaken to meet the requirements of the proposed Community legislation on nutrition labeling of foods, included research into improvements in vitamin methodology, comparisons of methods between laboratories and preparation of suitable reference materials for use during analysis of nutrients in foods. With respect to folate, the results of the first BCR intercomparison study showed that the use of HPLC and radioprotein binding assays was still in the developmental stage, but that reasonable agreement between laboratories for the determination of folates could be obtained by use of the microbiological assay. Finglas *et al.* (1993) noted that further improvements should be possible by optimization of the extraction and deconjugation steps of the assay. Several recent reports have addressed these issues.

Tri-enzyme methods utilizing α-amylase, protease and conjugase are currently under investigation for use in measuring the total folate content of foods and for determining the endogenous folate levels in specific enriched cereal-grain products. Tamura *et al.* (1997) evaluated the effect of α-amylase and protease digestions in addition to folate conjugase treatments on folate content of complete food composites and concluded that the tri-enzyme treatment is essential for the accurate determination of the folate content of food. Pfeiffer *et al.* (1997) evaluated a tri-enzyme procedure followed by HPLC for quantifying folates in unfortified and fortified cereal-grain products, and concluded that tri-enzyme extraction is applicable both for HPLC and microbiological assays. They also concluded that the traditional extraction and folate conjugase treatments are not appropriate for analysis of cereal-grain products (Pfeiffer *et al.*, 1997).

This paper provides data on the folate contents of a wide range of cereal-grain products and confirms the need for tri-enzyme treatment during the preparation of cereal-grain foods for folate analyses. For about 30% of the foods examined, results obtained by the tri-enzyme method were significantly higher than those obtained by the single-enzyme (conjugase) assay. Our data also suggest that modifying the order of addition of enzymes during the tri-enzyme extractions may increase the yield of folates for some products. Specifically, addition of protease prior to addition of α-amylase and conjugase increased extraction of folates in some foods. At the present time, however, it is not possible to identify those foods for which a specific modification will provide better results. More investigations are needed to determine the best extraction procedure to use with specific foods.

Folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate are suitable calibrants. The stability of folic acid and the availability of preparations of >98% purity provide significant advantages for the use of folic acid. An assay pH of 6.7 ± 0.1 is satisfactory. Increased sensitivity can be achieved when the assay is performed at pH 6.2 and this modification may be useful for samples of low folate content. Recoveries of 91–101% are achievable at both pHs. Rice products may pose some difficulties and additional work with these products appears necessary.

While there are still uncertainties as to the optimum conditions (e.g. order of addition of the enzymes and pH) for the pre-treatment of foods prior to analysis, all investigations to date point to the importance of using a tri-enzyme procedure in determining the folate contents of foods. There is also a need to establish rigorous quality control/quality assurance procedures for use during routine folate analyses. Our results with the AACC check samples indicate that these materials are applicable for such purposes. Our results with the infant formula reference material SRM 1846 suggest that this may also be a useful in-house quality control material. Other potentially useful reference materials include lyophilized Brussels sprouts (Finglas *et al.*, 1993) and four recently developed CRMs (wholemeal flour, milk powder, lyophilized mixed vegetables and pig's liver) with certified values for total folate by microbiological assay and indicative values for 5-methyltetrahydrofolate by HPLC (Finglas, pers. commun.).

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