

Determining bioavailability of food folates in a controlled intervention study¹⁻³

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ABSTRACT

Background: The concept of dietary folate equivalents (DFEs) in the United States recognizes the differences in bioavailability between natural food folates and the synthetic vitamin, folic acid. However, many published reports on folate bioavailability are problematic because of several confounding factors.

Objective: We compared the bioavailability of food folates with that of folic acid under controlled conditions. To broadly represent the extent to which natural folates are conjugated in foods, we used 2 natural sources of folate, spinach (50% polyglutamyl folate) and yeast (100% polyglutamyl folate).

Design: Ninety-six men were randomly assigned according to their screening plasma homocysteine (tHcy) concentration to 1 of 4 treatment groups for an intervention period of 30 d. Each subject received (daily under supervision) either a folate-depleted "carrier" meal or a drink plus 1) placebo tablet, 2) 200 μ g folic acid in a tablet, 3) 200 μ g natural folate provided as spinach, or 4) 200 μ g natural folate provided as yeast.

Results: Among the subjects who completed the intervention, responses (increase in serum folate, lowering of tHcy) relative to those in the placebo group ($n = 18$) were significant in the folic acid group ($n = 18$) but not in the yeast folate ($n = 19$) or the spinach folate ($n = 18$) groups. Both natural sources of folate were significantly less bioavailable than was folic acid. Overall estimations of folate bioavailability relative to that of folic acid were found to be between 30% (spinach) and 59% (yeast).

Conclusion: Relative bioavailability estimates were consistent with the estimates from the metabolic study that were used as a basis to derive the US DFE value. *Am J Clin Nutr* 2004;80:911-8.

KEY WORDS Food folate, bioavailability, homocysteine, folic acid

INTRODUCTION

Folate is attracting considerable interest as having an established role in the prevention of neural tube defects (NTDs; 1, 2) and possible preventive roles against cardiovascular disease (3), certain cancers (4), and neuropsychiatric conditions (5). For the prevention of NTDs, official bodies worldwide recommend women to take an additional 400 μ g folate/d before conception and in early pregnancy. However, the achievement of such recommendations is problematic. Although folic acid supplements are effective in optimizing folate status in women who receive them (6), they do not offer an effective strategy for the primary

prevention of NTDs because of poor compliance (7). Therefore, mandatory fortification of grain products with folic acid was introduced in the United States (8) and elsewhere (9). Despite impressive decreases in the incidence of NTDs since the introduction of these policies (10, 11), fortification remains controversial. It is untargeted; therefore, delivering the required nutrient levels to the at-risk group inevitably results in a proportion of the general population being exposed to high levels. Of greatest concern is the potential for high intakes of folic acid to mask the anemia of vitamin B-12 deficiency in elderly people, thereby allowing the concomitant irreversible nerve degeneration to go undetected (12). The third approach to optimize folate status, which does not have such health concerns, is by increased consumption of foods naturally rich in folate. However, the effectiveness of this approach was found to be somewhat limited (13-15), a finding generally attributed to the poor bioavailability of natural food folates compared with the synthetic vitamin, folic acid.

Previous human studies estimated the bioavailability of food folates relative to folic acid, depending primarily on the methodologic approach used, to range anywhere between 10% and 98% (16-21). The uncertainty about folate bioavailability (15) is of particular concern for countries without folic acid fortification (including some which do not even permit it on a voluntary basis) (22), and, therefore, a high dependency on natural food folates as a means to optimize status. In addition, although mandatory folic acid fortification in the United States means that relatively less reliance is placed on natural folate sources, US dietary recommendations are now based on the greater bioavailability of folic acid added to food compared with natural food folates, with the introduction of dietary folate equivalents (DFEs) (23). The estimated DFE conversion factor of 1.7 is largely based on one metabolic study in nonpregnant women that estimated the bioavailability of food folates to be no more than 50% that of folic

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acid (19) and other evidence showing that folic acid added to food had $\approx 85\%$ the bioavailability of free folic acid (24).

The aim of this study was to compare the bioavailability of food folates with folic acid under controlled conditions. The approach was to administer natural sources of folate under supervision at a dose (of predetermined folate content) within the physiologic range, but sufficiently concentrated to elicit serum folate and plasma homocysteine (tHcy) responses, for comparison with an equivalent dose of folic acid. To broadly represent the extent to which natural folates are conjugated in foods, we used 2 folate-rich sources, spinach and yeast, in which folates are present as 50% and 100% polyglutamyl folate, respectively.

SUBJECTS AND METHODS

Subject recruitment and screening

The Research Ethical Committee of the University of Ulster granted ethical approval, and subjects gave written informed consent at the time of recruitment to the study. Healthy men, aged 18–45 y, were recruited between December 2000 and September 2001 from the staff and student population at the University of Ulster, the Causeway Health and Social Services Trust, Coleraine, and the FG Wilson engineering firm, Belfast. All potential subjects were interviewed, using a short medical questionnaire about their general health, medication, and supplement use, to identify those subjects meeting the following inclusion criteria: no history of gastrointestinal, vascular, hepatic, renal disease, or hematologic disorders; not taking B vitamin supplements or consuming folic acid–fortified foods; and not taking drugs known to interfere with folate metabolism. In addition, a blood sample was collected to screen volunteers for identification of the 677C→T (thermolabile) variant of the methylenetetrahydrofolate reductase (*MTHFR*) gene and to determine tHcy concentrations. Individuals who were found to be homozygous for the 677C→T mutation (ie, *TT* genotype) were excluded from the study.

Intervention

Suitable subjects were stratified assigned on the basis of their screening tHcy concentrations to 1 of 4 treatment groups. The subjects received either a folate-depleted “carrier” meal or a drink plus 1) placebo tablet, 2) 200 μg folic acid in a tablet, 3) 200 μg folate provided as spinach, or 4) 200 μg folate provided as yeast.

Preintervention treatment

For a 4-wk run-in period before commencement of the folate intervention, all subjects (irrespective of the treatment group) were administered oral supplements of vitamin B-6 (1.6 mg/d) and vitamin B-12 (1.5 μg /d), dosages equivalent to UK Reference Nutrient Intake values (25). To monitor compliance, subjects were provided with these supplements on a weekly basis in a 7-d pill organizer box (Carepac, Farringdon, United Kingdom) and asked to return the box at each visit; any missed doses were recorded. This treatment was continued for the duration of the entire study, ie, until completion of the folate intervention.

Folate intervention

The folate intervention was conducted as a placebo-controlled, blind study, which was carried out over a 6-wk period, during which treatments were administered 5 d/wk (ie, in total, a

30-d folate intervention). To ensure compliance, subjects were supervised while taking the treatments on a daily basis. Each of the 4 treatments was administered in 1 of 2 ways, either as a meal (with other food present) or as a drink (with no other food present).

Administration of treatments as a meal. Each morning a carrier meal was freshly prepared by the catering staff at the School of Hotel, Leisure, and Tourism, University of Ulster, Portrush, Northern Ireland, under the supervision of 2 colleagues (MHF and MAK) from the Northern Ireland Center for Food and Health, University of Ulster, Coleraine. A total of 4 carrier meals were devised and rotated on a weekly menu cycle. Ingredients selected for use in the carrier meals were of low-folate content, according to the British Food Composition Tables (26). The ingredients (for full list, see **Appendix A**) were thrice boiled to reduce the water-soluble micronutrient content (ie, the ingredients were placed in cold water and taken to boiling temperature for a minimum of 1 min, and the water was removed and replaced with fresh cold water; this was carried out 3 times before the food was finally cooked). For each of the carrier meals, a duplicate meal was retained and stored for later analysis of total folate content; this was repeated on 2 separate occasions during the folate intervention.

Volunteers attended our catering center daily between 1200 and 1400 to receive their intervention treatments under supervision. The carrier meal was provided as a lunch to all volunteers irrespective of their treatment group allocation. Each subject received either the carrier meal alone (placebo treatment) or the carrier meal enriched to provide 200 μg natural folate provided from 1 of 2 natural folate sources, either lyophilized spinach (7.8 g; Kanegrade, Stevenage, United Kingdom) or lyophilized yeast (4.1 g; Allinson, Castleford, United Kingdom), with polyglutamate:monoglutamate of $\approx 50:50$ and $100:0$, respectively. The natural folate source was added to the carrier meal after the meal was fully cooked, just before serving. The meal was maintained under heat lights while being served. In addition, after consuming half of the meal, all subjects received a pill, either placebo (all groups except folic acid group) or 200 μg synthetic folic acid (folic acid group only). Subjects drank only water and were not permitted to use additional sauces or seasoning with the meal. Volunteers were instructed to follow their usual dietary pattern for all other meals and snacks consumed during the intervention period.

Administration of treatments as a drink. Volunteers received either a placebo drink or a drink that provided 200 μg natural folate in a disposable plastic cup at their place of work midmorning (1000–1100) under the supervision of 2 colleagues (MHF and NCA). The drinks were prepared freshly before each administration as follows: 7.8 g lyophilized spinach or 4.1 g lyophilized yeast (the equivalent of 200 μg total folate) was added to 20 mL water. The drinks were mixed vigorously; 50 mL sugar-free lemonade was added and again mixed. The placebo drink consisted of 20 mL water and 50 mL sugar-free lemonade, with no other ingredient. All drinks were prepared at the same time each morning and consumed within 2 h. Volunteers also received a pill, either placebo or 200 μg synthetic folic acid, which was taken after consuming the first half of the drink. Any residue remaining in the cup was rinsed with a small volume of lemonade, which the subject was required to drink to ensure the ingestion of the entire treatment dose. Apart from this drink, subjects

were instructed to follow their usual dietary pattern for the duration of the study.

Laboratory methods

Blood sampling and analysis

Blood samples were collected after an overnight fast (minimum of 12 h) at screening, before intervention with folate, and after intervention with folate. For each time point (other than screening), 2 blood samples were collected 2–4 d apart (shown to be the optimal time interval between repeated blood sampling for measurement of tHcy) (27). A total of 22 mL blood was collected from each subject into EDTA-coated preevacuated blood tubes for full blood count, whole blood folate, plasma pyridoxal-5'-phosphate (PLP), and tHcy analysis, or into a Vacuette Serum Separator tube (Greiner Labortechnik, Kremsmünster, Germany) for analysis of serum folate and serum vitamin B-12. Samples for PLP and tHcy analyses were wrapped in foil and placed on ice immediately after collection. Sample preparation and fractionation were performed within 0.5–2.5 h of the time of sampling as described in detail elsewhere (28), and fractions were stored at -70°C for batch analysis at the end of the study and at -20°C for extraction of DNA. Full blood counts were carried out on whole blood with an automated Coulter Counter (Causeway Health and Social Services Trust Laboratories, Coleraine, Northern Ireland).

Plasma tHcy was measured by immunoassay (29). Red blood cell folate (30), serum folate (30), and serum vitamin B-12 (31) were measured by microbiological assay. Plasma PLP was measured by reversed-phase HPLC with fluorescence detection (32). For all assays, samples were analyzed blind, in duplicate, and within 6 mo of sampling. Quality control was provided by repeated analysis of stored batches of pooled plasma (for tHcy and PLP), serum (for folate and vitamin B-12), and red blood cell lysates (for folate), covering a wide range of values in each case.

From screening samples, DNA was extracted from frozen whole blood by incubating with proteinase K (Gibco Life Technologies, Paisley, United Kingdom) as described in detail by Kawasaki and Erlich (33) or with the use of the QIAamp DNA Blood Mini Kit (QIAGEN Ltd, Crawley, West Sussex, United Kingdom). The *MTHFR* 677C→T mutation (ie, *TT* genotype) was identified by polymerase chain reaction amplification followed by *Hinf*I restriction digestion (Gibco Life Technologies), as previously described (34).

Natural folate analysis

The total folate content of the yeast, spinach, and carrier meals was measured by microbiological assay with *Lactobacillus casei* NCIB 10463 (30) after thermal extraction and trienzyme (α -amylase, protease, and conjugase) treatment according to the procedure of Tamura (35). The calibration of the assay was performed with the use of folic acid (Sigma Chemical Co, Poole, Dorset, United Kingdom) 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 found in foods. Folate assays were performed both at the start and at the end of the intervention period (in each case, triplicate measurements on 2 separate occasions 2 d apart). The coefficient of interassay variation in folate content of quality control samples was 5.5% ($n = 48$). The folate polyglutamate content in yeast and

spinach was determined as the mean difference in total folate content of samples treated with and without folate conjugase.

Dietary assessment

Dietary intake was recorded during the intervention period by a self-administered 4-d food diary (2 weekdays and 2 weekend days). Food intake data were analyzed for energy and nutrient intakes with the use of the dietary analysis program WISP (WISP for WINDOWS version 1.28; Tinuviel Software, Warrington, United Kingdom).

Statistical analysis

SPSS version 11 (SPSS Inc, Chicago) was used to compare the effects of intervention among the treatment groups with the use of analysis of covariance. The pretreatment value was used as a covariate; pretreatment and posttreatment values were log transformed. Treatment comparisons were made with the use of Tukey's test multiple comparisons procedure, and $P < 0.05$ was considered significant.

To represent the response to intervention of food folates relative to that of folic acid, estimations of relative bioavailability (%) were calculated as follows:

$$\text{RB} = (\bar{x}_t - \bar{x}_p) / (\bar{x}_f - \bar{x}_p) \times 100 \quad (1)$$

where RB is the relative bioavailability, $\bar{x}_t$ is the mean response in the treatment group (yeast or spinach), $\bar{x}_p$ is the mean response in the placebo group, and $\bar{x}_f$ is the mean response in the folic acid group. The 95% CIs were calculated by bootstrapping and were truncated at zero (36).

RESULTS

Baseline data

Of 127 subjects initially recruited and screened, 96 satisfied the inclusion criteria and proceeded to intervention (24 to each of 4 treatment groups), of which 74 subjects completed the study **Figure 1**. Subjects either withdrew from the study voluntarily or were withdrawn if their attendance (compliance) was $<95\%$ (in practice this meant failure to attend on more than one occasion over the 30-d intervention). Subjects who successfully completed the intervention had an attendance rate of 100%. The baseline characteristics of this cohort expressed as median and 25th–75th quartiles are presented in **Table 1**. No subject was found to have deficient status of folate (serum or red cell folate) or related B vitamins (plasma PLP or serum vitamin B-12) at baseline.

After subjects were stratified according to tHcy concentrations at screening, no significant differences were seen between groups in tHcy, serum folate, or red cell folate, either before or after the 4-wk run-in period with physiologic doses of vitamins B-6 and B-12 (independent *t* test; data not shown). However, as expected, significant increases (before and after 4-wk run-in period, paired *t* test) in both PLP (73.4 nmol/L compared with 108 nmol/L; $P < 0.001$) and serum vitamin B-12 (297 pmol/L compared with 325 pmol/L; $P < 0.05$) were observed. Although treatment with vitamins B-6 and B-12 continued for a further 6 wk (ie, throughout folate intervention), no further increase in either marker was observed (ie, week 4 compared with week 10).

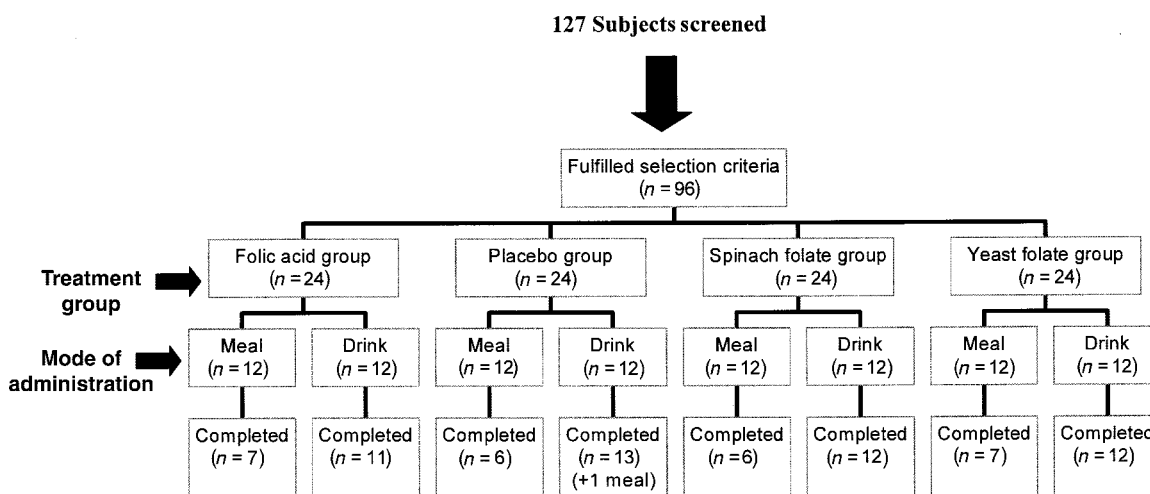


FIGURE 1. Subject group allocation and rates of completion. One subject, who was assigned to receive the placebo treatment as a meal, reported on day 1 that he would be unable to attend the catering center at the specified time each day. This subject agreed to be reassigned to receive the treatment as a drink to be administered daily (midmorning) at his place of work.

Food folate analysis

Analysis of the carrier meals (2 separate measurements for each of 4 meals, each assayed in triplicate) for total folate content showed a mean ($\pm$ SD) folate value of $44.89 \pm 16.5 \mu\text{g}/\text{meal}$ (Appendix A).

The total folate content of yeast and spinach, analyzed both at the start and at the end of intervention for each folate source (in each case, triplicate measurements on 2 separate occasions 2 d apart), showed that the quantity of natural folate sources required to provide $200 \mu\text{g}$ folate corresponded to mean weights of 4.1 g ($4.16 \pm 0.44 \text{ g}$ at the start; $3.78 \pm 0.46 \text{ g}$ at the end of intervention) lyophilized yeast and 7.8 g ($7.77 \pm 0.94 \text{ g}$ at start; $7.54 \pm$

0.86 g at end of intervention) lyophilized spinach. The polyglutamate:monoglutamate folate in spinach and yeast was found to be 50:50 and 100:0, respectively, whether this was measured at the start or at the end of intervention.

Intervention

To determine the relative effects of intervention with the various treatments, the response (postintervention value minus the preintervention value) of each treatment was compared among the 4 treatment groups (**Table 2**). The folic acid response (both tHcy and serum folate) was significantly different from placebo, spinach, and yeast; no other significant differences were observed. The overall bioavailability of these representative natural folate sources relative to that of folic acid (and adjusted for placebo effect) was estimated to be 30% (tHcy response: 23%; serum folate response: 36%) for spinach and 59% (tHcy response: 56%; serum folate response: 62%) for yeast. Thus, the average bioavailability of these representative food folate sources was estimated to be 45%. Analysis of food intake data for total energy and total folate are presented in **Table 3** and showed no significant differences between the 4 treatment groups.

Percentage responses to intervention (the postintervention value minus the preintervention value expressed as a percentage of the preintervention value) for both serum folate and tHcy are shown in **Figure 2**. The percentage response of both markers to folic acid was significantly different from the response to placebo, spinach, and yeast; no other significant differences were observed.

DISCUSSION

In devising the DFE conversion factor in the United States, experts drew heavily on one metabolic study in women conducted several years ago that estimated folate bioavailability from a mixed diet to be no more than 50% relative to that of folic acid (19). Reported estimates of food folate bioavailability since then are highly variable. Representative natural folate sources in the current study were found to be significantly less bioavailable than folic acid, with estimated relative bioavailability consistent

TABLE 1

General characteristics, dietary status, and laboratory nutrient status of subjects at screening¹

Variable	Reference range	Value ²
Age (y)	18–45	31.0 (23.3–38.5)
BMI (kg/m^2)	18.5–24.9 ³	26.3 (23.5–28.8)
Energy intake (MJ/d)	—	8.0 (7.0–11.0)
Folate intake ($\mu\text{g}/\text{d}$)	200 ⁴	183 (140–235)
Vitamin B-12 intake ($\mu\text{g}/\text{d}$)	1.5 ⁴	2.9 (2.0–4.4)
Vitamin B-6 intake (mg/d)	1.4 ⁴	1.9 (1.8–2.4)
Riboflavin intake (mg/d)	1.3 ⁴	1.3 (1.0–1.5)
Plasma homocysteine ($\mu\text{mol}/\text{L}$)	5–15 ⁵	10.5 (8.9–12.3)
Serum folate (nmol/L)	6.1–45 ⁶	16.67 (12.3–22.1)
Red blood cell folate (ng/mL)	150–1000 ⁶	385 (303–485)
Serum vitamin B-12 (pg/mL)	150–1000 ⁶	415 (291–554)
Plasma PLP ⁵ (nmol/L)	>30 ⁷	67.7 (48–89)

¹ $n = 74$. PLP, pyridoxal 5'-phosphate (vitamin B-6).

² Median; 25th–75th quartiles in parentheses.

³ Normal range for healthy males.

⁴ Reference nutrient intake in the United Kingdom for men between the ages of 19 and 50 y (26).

⁵ Reference range for normal homocysteine concentration (37).

⁶ Laboratory reference ranges for normal serum vitamin B-12 concentrations and normal serum and red blood cell folate concentrations from the Vitamin Research Laboratory, Trinity College, Dublin.

⁷ From reference 38.

TABLE 2Plasma total homocysteine (tHcy) and serum folate responses to a 30-d intervention with 200 μg natural folate or folic acid/d¹

	Preintervention ²	Postintervention ²	Response ^{2,3}	Relative bioavailability ⁴
				%
Plasma tHcy				
Placebo (<i>n</i> = 18)	11.6 $\pm$ 3.7 ⁵	11.8 $\pm$ 3.3	0.2 $\pm$ 1.2 ^b	—
Folic acid (<i>n</i> = 18)	11.5 $\pm$ 3.0	10.1 $\pm$ 1.9	-1.4 $\pm$ 2.1 ^a	—
Spinach folate (<i>n</i> = 18)	12.1 $\pm$ 2.9	11.7 $\pm$ 2.5	-0.4 $\pm$ 1.1 ^b	23
Yeast folate (<i>n</i> = 19)	11.9 $\pm$ 3.2	11.2 $\pm$ 2.7	-0.7 $\pm$ 0.9 ^b	56
Serum folate				
Placebo (<i>n</i> = 18)	15.9 $\pm$ 9.4	15.4 $\pm$ 8.4	-0.4 $\pm$ 4.2 ^b	—
Folic acid (<i>n</i> = 18)	17.2 $\pm$ 11.9	21.6 $\pm$ 13.1	4.4 $\pm$ 4.8 ^a	—
Spinach folate (<i>n</i> = 18)	13.5 $\pm$ 5.1	15.2 $\pm$ 6.5	1.8 $\pm$ 4.0 ^b	36
Yeast folate (<i>n</i> = 19)	15.0 $\pm$ 7.2	17.6 $\pm$ 5.69	2.6 $\pm$ 3.5 ^b	62

¹ Values in the same column with different superscript letters are significantly different, *P* < 0.05 (Tukey's test for multiple comparisons).² The units are $\mu\text{mol/L}$ for plasma tHcy and nmol/L for serum folate.³ Postintervention value minus preintervention value. Responses were compared by using analysis of covariance on log-transformed data (response = $\log^{\text{post}}_{\text{pre}}$).⁴ The response to yeast or spinach folate relative to the response to folic acid and corrected for the placebo response; for calculation, see text. [95% CIs were calculated by bootstrapping and were truncated at 0%: spinach (tHcy: 0%, 80%; serum folate: 0%, 90%); yeast (tHcy: 20%, 170%; serum folate: 20%, 170%).]⁵ $\bar{x} \pm \text{SD}$ (all such values). Values represent 2 fasting samples collected 2–4 d apart.

with the report of Sauberlich et al (19) that was based on a mixed diet.

The bioavailability of folates from various foods is considered to depend on the content of monoglutamyl and polyglutamyl folates and on the presence of components that can inhibit both intestinal folate deconjugation and specific transport processes of folate (17). Dietary folates (excluding fortified foods) comprise about one-third monoglutamate (derived mainly from bread and meat) and two-thirds polyglutamate (derived mainly from vegetables) (39). In the current study, we used 2 folate-rich sources, spinach and yeast, not because we wished to specifically study these foods as sources of folate per se, but rather to broadly represent the extent to which natural folates are conjugated in foods. Thus, although yeast is not an important dietary source of folate, its inclusion in our study enabled us to compare a folate source that was entirely in the polyglutamate form with another which had a much lower content (50%) of polyglutamyl folate. Although we showed no significant difference in the responses between these 2 food folate sources, the trend seen was consistently toward higher bioavailability (whether based on serum folate or tHcy responses) of folate from yeast than from spinach. Given that folate in yeast is all in the polyglutamate form and is even reported to contain potent inhibitors of certain conjugases (40), our results provide no support for the view that the extent of glutamation is a limiting factor in the bioavailability of folates from natural sources. Such observations are in good agreement with previous findings (41) from studies that used exogenous deuterium-labeled monoglutamyl and polyglutamyl folates

added to various foods, which showed equivalent bioavailability for the 2 folate forms. Results from the current study and the aforementioned study (41) are consistent with earlier observations (42), indicating that the activity of human jejunal brush border conjugase exceeds that needed for hydrolysis of polyglutamyl folates within the range of dietary intake and, therefore, was not rate limiting in the absorption process.

Apart from the activity of the conjugase enzyme, factors considered to influence the bioavailability of ingested folates include the presence or absence of other components in the diet or in the intestinal milieu that could inhibit or enhance absorption (43). Pfeiffer et al (24), for example, reported a small reduction in absorption of [¹³C₅] folic acid when administered after a light breakfast meal compared with its administration without food. Although in the current study all 4 treatments were administered in 1 of 2 ways, either in a drink with no other food present or as part of a meal, a subanalysis of the overall results comparing the responses according to the route of administration was not possible because of insufficient subject numbers completing the meal arm (across the 4 treatment groups). Further studies are clearly required to address this issue.

Reported estimates of relative bioavailability of food folates show great variation, ranging from 10% to 98% (16–21), depending on the methodologic approach and response index used. The interpretation of bioavailability studies in free-living subjects involving the provision of folate-rich foods could be particularly problematic as a result of several confounding effects. The current study, in which all treatments were administered

TABLE 3Total dietary folate and total energy intakes in the 4 treatment groups¹

	Folic acid	Placebo	Spinach folate	Yeast folate
Total dietary folate ($\mu\text{g/d}$)	202 $\pm$ 84	186 $\pm$ 70	184 $\pm$ 70	211 $\pm$ 86
Total energy (MJ/d)	9.11 $\pm$ 2.19	8.29 $\pm$ 2.17	8.70 $\pm$ 2.47	9.72 $\pm$ 2.37

¹ All values are $\bar{x} \pm \text{SD}$. Dietary intakes were measured in the middle of the intervention. Folate intake values do not include the contribution from the folate treatments administered daily (200 μg folic acid or folate/d). No significant differences between the treatment groups in total folate (*P* = 0.31) or total energy (*P* = 0.49) intake were observed (one-way ANOVA).

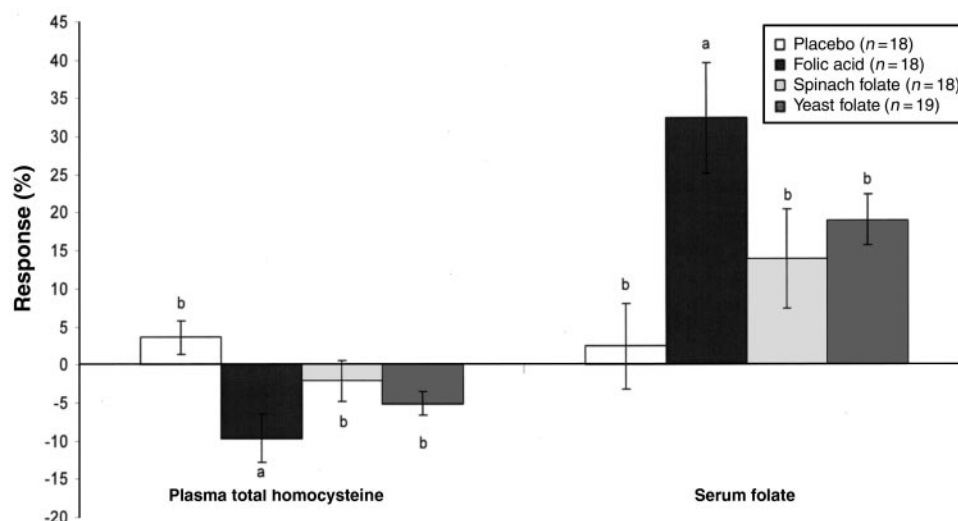


FIGURE 2. Mean ($\pm$ SEM) percentage response of plasma homocysteine and serum folate concentrations to a 30-d intervention with 200 μ g folate as either synthetic folic acid or natural folate source (spinach folate, 50% polyglutamyl folate; yeast folate, 100% polyglutamyl folate). Values represent 2 fasting samples collected 2–4 d apart. Percentage responses (homocysteine and serum folate) between the 4 groups were compared with the use of log-transformed data for normalization purposes. Bars with different letter are significantly different, $P < 0.05$ (Tukey's test for multiple comparisons).

daily under supervision and were of predetermined folate content, allowed several potential confounding effects to be overcome, including poor subject compliance and displacement of the usual dietary folate intake with intervention foods (13). In addition, all of the administered natural folate (provided as spinach or yeast) came from the same batch and was not subjected to cooking or further processing before ingestion, thereby eliminating the confounding effect of folate losses during cooking, which can be considerable in the case of green vegetables (44). Although stable-isotopic studies overcome these potential confounders, their applicability is limited somewhat in that, to improve the precision of short-term studies, presaturation of tissues with folate is recommended, thereby creating a nonphysiologic condition (24). The bioavailability of natural folate sources estimated in the current study is much lower than that from a previous long-term intervention study (21) that estimated the bioavailability of food folates relative to folic acid to be between 60% and 98% (depending on the endpoint used). The strength of the latter study (21) lies in its attempt to assess folate bioavailability from a mixed diet rather than from individual foods. The unexpected findings, however, could be the result of one or more of the following confounding factors. First, the response of folic acid was possibly underestimated as a result of administering 500 μ g folic acid every other day, on the assumption that it would be equivalent to 250 μ g daily. Higher intakes of folic acid (ie, doses in excess of 266 μ g) were shown to exceed the metabolic capacity of the intestinal mucosa, resulting in the appearance of un-reduced folic acid in the circulation (45), the uptake of which might not be equivalent to the reduced vitamin. A second limitation of the study is that the natural food folate and folic acid groups did not receive comparable doses of the vitamin (350 μ g natural folate daily compared with 500 μ g synthetic folic acid every other day), although this clearly was not intended in the study design. Some attempt was made to correct for the different doses at the analysis stage. Corrected values, however, may not necessarily represent relative food folate bioavailability to the same degree as a study, such as the current one, in which equivalent doses were administered daily throughout the intervention period.

We used 2 response indexes to assess folate bioavailability, serum folate and a functional biomarker of folate status, plasma tHcy, previously shown by us to be a reliable index that responds to low-dose folic acid in a dose-dependent manner (37). Our estimations of bioavailability of natural folate (relative to folic acid) are similar whether they are based on tHcy or serum folate responses (yeast folate 56% compared with 62%; spinach folate 23% compared with 36%, respectively). Thus, our results show good internal robustness in the estimation of folate bioavailability from natural sources. However, the use of tHcy responses in the determination of relative folate bioavailability required the inclusion in our study design of a 4-wk pretreatment period with physiologic doses of vitamins B-12 and B-6. This inclusion was necessary to ensure that any homocysteine lowering as a result of the presence of these vitamins in foods was corrected before the folate intervention. Red cell folate responses were not used in the estimation of folate bioavailability because we considered that the duration of the intervention (30 d) was insufficient to observe a complete turnover of the red cell folate population (ie, 120 d) and, therefore, fully reflect the effect of the red cell folate response.

In conclusion, the bioavailability of natural folate is now important, given that the alternatives only offer partial solutions for addressing suboptimal folate status in the general population, either because of limited compliance (in the case of folic acid supplementation) or safety concerns (in the case of fortification). By comparing the bioavailability of representative natural folate sources with folic acid, we estimate the relative bioavailability of natural folate to be $\approx 45\%$. In addition to losses of natural folates because of their incomplete bioavailability shown here, in practice losses before ingestion could also decrease the amount of available folate from natural sources, particularly in the case of green vegetables (44). Finally, the estimations of relative bioavailability in the current study are consistent with those estimated in the metabolic study by Sauberlich et al (19) that was the cornerstone of the derived US DFE value.

MPH-F had the lead role in writing the manuscript, data collection, and analysis. NCA assisted in manuscript preparation and had a lead role in data

collection and analysis. JMS was responsible for study design and assisted in manuscript preparation. KP was responsible for laboratory analysis and manuscript preparation. IB was responsible for statistical analysis and statistical aspects of manuscript preparation. MW was responsible for data analysis and assisted in manuscript preparation. JJS was responsible for study design and assisted in manuscript preparation. AAD supervised food delivery and assisted in dietary aspects of manuscript writing. AMM was responsible for laboratory analysis and assisted in manuscript preparation. MAS assisted in food delivery, laboratory aspects, and manuscript preparation. HM was study coordinator and was responsible for study design and writing of manuscript. None of the authors had any financial or personal interests, including advisory board affiliations, in any company or organization sponsoring the research.

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APPENDIX AIngredients used to prepare 4 "carrier" meals¹

Meal	Ingredients
Chicken duxelle	Onions, 100 g Mushrooms, 400 g Chicken, 600 g Margarine, 25 g Eggs, 2 Milk, 90 mL Bread crumbs, 100 g Nutmeg, 10 g Dry sherry, 150 mL Water, 360 mL Demiglaze, 70.35 g Turnips, 740 g Carrots, 438 g Salt, 3 g Pepper, 1.5 g
Pasta bake	Pasta, 200 g Mushrooms, 132 g Smoked bacon (without fat), 147 g Peppers (red), 124 g Margarine, 50 g Flour (plain white), 50 g Milk, 720 mL Salt, 1.5 g Cheese, 371 g Cloves, 2 Bay leaf, 1
Thai green curry	Chicken, 655 g Olive oil, 35 g Chicken stock, 250 mL (or one-half chicken stock cube) Garlic, 6 g Ginger root, 4 g Coconut milk, 115 mL Green curry paste, 5 g Coriander leaves, 10.5 g Lime juice, 9.5 g Salt, 3 g Rice, 200 g Black pepper, 1 g
Chicken and gammon pie	Chicken breast, 375 g Smoked gammon, 350 g Mushrooms, 100 g Margarine, 50 g Flour, 50 g Cream, 200 mL 1 chicken stock cube Water, 750 mL Carrots, 480 g Parsnips, 300 g Pastry (frozen)

¹ Each meal consisted of 4 servings. The mean ($\pm$ SD) folate contents of the chicken duxelle, pasta bake, Thai green curry, and chicken and gammon pie meals were 63.2 ± 22.6 , 38.1 ± 15.4 , 38.6 ± 5.3 , and 39.5 ± 15.2 μ g/meal, respectively. The general instructions for preparation of all the meals were as follows. All vegetables, meat, pasta, and rice were boiled 3 times. Dry ingredients, such as herbs, spices, stock cubes (chicken and beef), salt, and pepper, and other ingredients used for flavorings, such as Tabasco sauce (McIlhenny Co, Avery Island, LA), Worcestershire sauce (HP Foods Limited, Middlesex, United Kingdom), gravy browning (black jack), honey, brown sugar, mustard (dry), and fresh garlic, were not required to be preboiled. Bisto (Addlestone, Surrey, United Kingdom), soy sauce, and canned tomatoes were not used. After being boiled 3 times, the ingredients used in the sauce were pan fried in oil (peanut or olive) and garlic. Spices, herbs, and flavorings were added to the pan and cooked together with the meat and vegetables for a few minutes, and the stock (wine, sherry, etc) was finally added.

