DO AVAILABLE FOOD COMPOSITION DATA FOR FOLATE MEET CURRENT RESEARCH NEEDS?

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Abstract

Reliable food composition data for folate are needed: a) for use in estimating and evaluating the adequacy of folate intakes of populations, b) for formulation of experimental diets in folate nutrition studies, and c) as a component in the development of dietary recommendations (e.g. RDA and RDI values). Most of the current values for folate in databanks are derived from assay procedures that are now known to underestimate folate content. Improvement and optimization of several key components of the folate assay are needed. The ineffectiveness of common extraction techniques causes folate values to be erroneously low in many assays of food folate using traditional methods. Application of "trienzyme" procedures to folate extraction, in addition to selection of proper extraction conditions, improves the accuracy of folate measurements. Data will be presented indicating substantial underestimation of folate content in representative foods and diets when assayed by traditional methods as used in major databanks.

A review of estimates of folate intake (derived from food intake estimates and food composition databank values) indicates apparent intakes in the range of ~200-300 µg/d for many adults. Such estimates of folate intake have been shown to be correlated with measures of nutritional status in several studies, although it is highly likely that actual intake is underestimated. Precisely controlled nutritional studies have shown that an intake of 200 µg/d in highly bioavailable form is inadequate for nonpregnant women, while observational studies have concluded that an estimated dietary folate intake of ~200 µg/d maintains adequate folate nutriture in spite of a typical bioavailability of most food folate of only ~50%. Such conflicting data can only be explained by the fact that estimated intakes are erroneously low because of the inadequacy of current food composition data.

There is a great need for further refinement and optimization of methods for measurement of folate in foods. An optimized method should be applied with the objective of replacing existing databank values for the folate content of foods. Emphasis should be placed initially on classes of foods whose consumption level and concentration of naturally occurring folate make them significant dietary sources of this vitamin. Additional emphasis should be placed on providing accurate values for all foods containing added folic acid.

INTRODUCTION

Our understanding of the connections between folate nutritional status and public health is becoming firmly established, but there is a great need to define more closely the relationships between folate intake and many aspects of health. Among the metabolic functions of this vitamin are: a) participation in recycling of methionine and related formation and transfer of methyl groups, b) regulation of the concentration of plasma homocysteine, and c) involvement in nucleic acid synthesis through its role in the formation of purines and thymidylate. Through these roles, folate is essential for cellular development and homeostasis. Epidemiological studies have shown that inadequate folate status is associated with increased incidence of neural tube defects and certain other birth defects, elevated risk of various forms of vascular disease including coronary heart disease, and increased risk of several types of cancer. Since January 1, 1998, folic acid enrichment has been required in many types of cereal-grain based foods (FDA 1996). This enrichment is estimated to provide approximately 100 µg of additional folate daily per capita.

Data regarding the folate content of foods are used in many ways. Such data allow estimation of dietary intakes, from which dietary adequacy can be evaluated. Food composition data are also essential in the formulation of diets for experimentally controlling folate intake and in the development of dietary advice by health professionals. Finally, food composition data provide one criterion in formulating dietary recommendations (e.g. Recommended Dietary Allowances). For example, a folate intake that provides adequate nutritional status for a population may be viewed as meeting the nutritional needs of that population and, thus, provides indirect evidence of the requirement for available dietary folate (see reviews: Herbert 1987, NAS 1989). The reliability of all such applications of dietary folate data depends on the quality and accuracy of the analytical values in the database(s) used. The purpose of this discussion is to illustrate how existing food composition data do not fulfill the needs of the nutrition and public health communities, with primary focus on: a) inadequacies of analytical methods upon which most databanks are based; b) the probable inaccuracy of estimates of folate intake of the U.S. population; and c) the impact of inaccurate analytical data on many currently held views of folate requirements.

ANALYTICAL LIMITATIONS OF FOOD COMPOSITION DATA

Nearly all databank values regarding the folate content of foods have been gathered using microbiological assay procedures. Although many advances have been made in the determination of the individual forms of folates in foods by liquid chromatographic techniques (e.g. Pfeiffer et al. 1997b, Seyoum and Selhub 1993), microbiological assays probably will remain the method of choice for routine use in determining total folate. The key elements of this assay include extraction of folates from the sample matrix, enzymatic deconjugation of polyglutamyl folates, and quantification using the folate-dependent

bacterium *Lactobacillus casei* that responds nearly equally to all forms of the vitamin (Tamura 1990, 1998). The array of possible folates in foods is summarized in Figure 1. Major naturally occurring forms of the vitamin are polyglutamates of 5-methyl-tetrahydrofolate, and to a lesser extent 5- and 10-formyl-tetrahydrofolate, and unsubstituted tetrahydrofolate. Synthetic folic acid added to enriched or fortified foods also comprises a significant fraction of total folate intake. Assays must be able to provide a reliable measure of all forms present in a particular food sample. Although unresolved issues concerning the conditions of the microbiological growth assay still remain (e.g. optimal pH; Phillips and Wright 1983), many more problems can arise from inadequate extraction and/or enzymatic deconjugation (Engelhardt and Gregory 1990, Gregory et al. 1990, Pfeiffer et al. 1997b).

It is important to recognize that there is no current official method for measurement of total folate in foods. Since 1960, the Association of Official Analytical Chemists has listed a microbiological assay method for folic acid in vitamin preparations (AOAC 1990). This method is <u>not suitable</u> for food analysis because it employs *Streptococcus faecalis* (also called *Enterococcus hirae* and *Streptococcus faecium*, ATCC 8043) as the assay organism. This organism does not respond to 5-methyl-tetrahydrofolate, the most common naturally occurring form of the vitamin. A microbiological assay using *Lactobacillus casei* has been collaboratively validated and accepted as an AOAC official method for measurement of total folate in infant formula (AOAC 1995). The key aspects of sample preparation (i.e. extraction and deconjugation) needed for reliable application of this method to other foods have not been optimized.

The critical importance of proper sample extraction is illustrated by the following example (Gregory et al. 1990). Representative foods (frozen peas and calf liver) were subjected to microbiological assay for total folate using *L. casei* following thermal extraction using a pH 4.5 acetate buffer (with 50 mM ascorbate), a pH 7.0 phosphate (with 50 mM ascorbate), or a pH 7.85 HEPES-CHES buffer (with 101 mM ascorbate + 200 mM 2-mercaptoethanol). As shown in Table 1, choice of extraction buffer has a large impact on total measured folate in these and undoubtedly other types of foods. It should be noted that the most effective extraction method yields a value for total folate that exceeds the USDA Nutrient Database value by ~144% for frozen peas and 319% for calf liver. Most traditional folate analyses involve homogenization and heating of the sample in a buffer of ~pH 7 containing ascorbate. Even though the pH 7.85 buffer used in this study does not yield fully optimal results (as will be discussed later), these results strongly suggest that the traditional extraction procedures would cause the assay to underestimate substantially the actual folate content. It should also be noted that this phenomenon is observed when analyzing by HPLC; thus, the increase in folate yield is not simply an artifact of the microbiological assay.

The need for optimization of enzymatic deconjugation also exists. In this regard, the time of incubation and/or quantity of enzyme must be determined for each type of food sample

because of the presence of conjugase inhibitors in many foods (Engelhardt and Gregory 1990, Pfeiffer et al. 1997b). Insufficient enzymatic deconjugation yields underestimation of folate in either microbiological or HPLC assays (Engelhardt and Gregory 1990, Pfeiffer et al. 1997b). This effect probably contributes to the underestimation of food folate in databanks.

As discussed in this symposium by Dr. Jeanne Rader of the FDA, a great deal of evidence indicates that the use of protease and amylase treatments, in addition to deconjugation with folate conjugase, enhances the yield of measurable folate in many foods (e.g. DeSouza and Eitenmiller 1990, Martin et al. 1990, Pfeiffer et al. 1997b, Tamura et al. 1997, Tamura 1998). It is apparent to many analysts that this "trienzyme" approach is the method of choice for measurement of folate in foods. Dr. Rader will describe FDA activities in optimizing and testing a trienzyme procedure which, in conjunction with the *L. casei* microbiological assay, will probably become the official method for analysis of cereal-grain foods for regulatory purposes. It is possible that a systematic evaluation of extraction conditions (time, temperature, sample/extractant ratio, etc.) might eventually eliminate the need for the lengthy enzymatic treatment. However, the trienzyme procedure provides a great advance over traditional approaches to folate analysis, and its use should be continued to improve our understanding of the actual content and dietary intake of folate. An example of data for total dietary folate analysis of eight different diet composites is provided in Table 2 which illustrates the value of trienzyme analysis. It is clear from these data that conventional assays not employing the trienzyme approach would underestimate total folate content or intake regardless of the extraction buffer employed.

One caveat is needed regarding trienzyme methods of folate assays at this time: only in the case of cereal-grain foods has it been established that the response to amylase and protease treatment is due solely to enhanced extraction of folate (Pfeiffer et al. 1997). The potential for enzymatic generation of growth factors for *L. casei* cannot be totally ruled out at this time when trienzyme-based extraction methods are applied to other types of foods. Recent studies regarding the analysis of cereal-grain products have shown that the results of properly calibrated HPLC and microbiological assays are equivalent for samples conventionally extracted and prepared (i.e. conjugase alone) and those prepared with a trienzyme treatment (Pfeiffer et al. 1997). These findings support the validity of trienzyme-based folate analysis, similar to that used in the FDA method, for measurement of folate in cereal-grain foods. Although it is anticipated by this author that trienzyme-based folate analysis will be shown to be accurate for other types of foods (e.g. Table 2), comparisons with validated HPLC results are needed to confirm the specificity of the trienzyme-based *L. casei* assay prior to its widespread application.

The details of folate assays used to generate the major nutrient databanks often have not been reported. In view of the many apparent limitations of traditional analytical methods used in generating the food composition data for folate, it appears highly likely that current databank values, the majority of which were generated prior to current applications of

trienzyme methods, underestimate actual folate content. It is likely most of the USDA databank values were generated using an extraction method employing a buffer such as 0.1 M phosphate buffer, pH 7.0, with 1% ascorbate followed by conjugase treatment (personal communication from J. Holden, Nutrient Data Laboratory, USDA/ARS). This is analogous to the phosphate buffer extraction that yielded a large underestimation of folate content in analysis of peas and liver shown in Table 1. As shown in Table 2 regarding the measurement of total folate in diet composites, trienzyme treatment provides further enhancement of extraction above that obtained with the pH 7.85 buffer and conjugase alone. As shown in Table 3, the use of the trienzyme method in analysis of these food composites has a great effect on estimated daily folate intake calculated from this analysis. Unfortunately, the difference between results of folate assays conducted with or without trienzyme methods between probably varies from food to food (Table 2). Trienzyme treatment gave a 35.4% mean increase, but the range was 10.1% to 94.2%. Thus, there is no way to predict actual folate content from the databank values by applying a "correction factor." There is a growing consensus (Pfeiffer et al. 1997b, Tamura et al. 1997, Tamura 1998) that trienzyme-based analysis should be used to generate reliable food composition data. However, as mentioned above, there is a need for further confirmation of the specificity of this assay prior to its general acceptance.

LIMITATIONS OF FOOD COMPOSITION DATA FOR ESTIMATING FOLATE INTAKE IN NUTRITION RESEARCH STUDIES

Many researchers have estimated the folate intake of various experimental groups and subsets of the population by using food composition databanks and measures of food intake (e.g. food records, food frequency techniques, etc.). A representative but not exhaustive survey of such estimates is shown in Table 4.

Mean estimated intake of dietary folate in women ranged from ~200-300 µg/d, while that of men ranged from ~280-325 µg/d. Most of these estimates were based largely or entirely on data from the USDA Nutrient Database. Variation in estimated folate intake among these studies could reflect differences in assessing food consumption, number of subjects evaluated, and real differences in food selection patterns (e.g. intake of fortified breakfast cereals). However, the accuracy of all estimates rests primarily on the accuracy of the values in the databank(s) used. For analytical reasons discussed previously, it is highly likely that all of the estimates of dietary folate intake underestimate actual intake. Aside from the analytical issues, it is also likely that the apparent lower intakes in some studies may be related in part to incomplete food tables, especially in early studies. Also, published values do not take into account seasonal variation in nutrient content or cooking/preparation methods that differed from those used in samples analyzed to generate databank values.

In spite of the underestimation of folate intake, another aspect of whether food composition data meet research needs is whether estimated intakes are associated with actual folate

nutritional status. Jacques et al. (1993) reported initial evidence that estimates of folate intake derived from food frequency analysis were significantly correlated with folate nutritional status. In a more recent study that evaluated incidence of coronary heart disease in over 80,000 nurses, relative risk of coronary heart disease was inversely related to estimated total folate intake from food or supplements (Rimm et al. 1998). These authors also stated that "The relative risk for a diet high in folate was virtually identical after excluding the 33.7% of women who reported use of vitamin supplements." Thus, the food composition data used (derived largely from the USDA Nutrient Database) was sufficiently reliable to allow classification of subjects by quintile of folate intake, which indicated that intake was inversely related to relative risk of coronary heart disease. Similar associations have been observed in the elderly population of the Framingham study (Selhub et al. 1993, Tucker et al. 1996b). In these studies, estimated folate intake (diet + supplements) was classified by decile and shown to be proportional to plasma folate concentration and inversely related to plasma homocysteine concentration. Thus, available food composition data are at least suitable for classifying intakes, such as by quintiles (Rimm et al. 1998) or deciles (Selhub et al. 1993, Tucker et al. 1996b). It is interesting to note that the intake of folate-rich foods is also predictive of folate nutritional status. In this regard, Tucker et al. (1996a) observed strong relationships between measures of folate status (plasma homocysteine and folate concentrations) and weekly frequency of intake for breakfast cereals as well as fruits and vegetables.

HOW ACCURATE ARE OUR CURRENT ESTIMATES OF FOLATE INTAKE?

The exact nutritional requirement for folate is unclear, although clear experimental evidence indicates that the 1989 Recommended Dietary Allowance (RDA) values are insufficient. The author and colleagues (O'Keefe et al. 1995) conducted a study in which young women were fed for 10 wk a low-folate diet (30 µg/d folate from food sources). The women were supplemented with synthetic folic acid dissolved in apple juice to provide total folate intakes of either 200, 300, or 400 µg/d. Under the conditions of this protocol, with careful dietary control and an accurate knowledge of intake, the 200 µg/d intake did not provide sufficient folate as reflected by changes in plasma and red cell folate and plasma homocysteine concentration. This is strong evidence that the requirement is higher than previously believed.

A "Recommended Dietary Intake" of 3 µg folate/kg body weight was reported by Herbert (1987), which was based in part on observations from several studies of apparent nutritional adequacy of populations with dietary folate intakes in the range of ~200 µg/d. This approach also was used in developing the 1989 RDA values (180 µg/d for women, 200 µg/d for men; NAS 1989).

How can one reconcile these observations? On one hand are studies and recommendations indicating that ~200 µg/d of folate from dietary sources (having incomplete folate bioavailability) would be adequate, while on the other hand is a

controlled study which showed that 200 µg/d primarily from highly absorbable folic acid did not meet nutritional needs (O'Keefe et al. 1995).   Differences in these views of folate requirements are amplified when one considers the bioavailability of the folate consumed. The bioavailability of naturally occurring dietary folate is incomplete, perhaps 50% on the average (Cuskelly et al. 1996, Sauberlich et al. 1987, Gregory 1995), while the bioavailability of folic acid consumed with food is considerably higher (Pfeiffer et al. 1997a). The answer to this dilemma is that the studies employing current food composition databanks substantially underestimate actual intake. Thus, conclusions regarding dietary allowances should not be based solely on estimated folate intake from current food composition databanks.

The Food and Nutrition Board, Institute of Medicine has released new dietary recommendations (IOM 1998). They defined an Estimated Average Requirement (EAR) for adults (men and women 19-50 yr) of 320 µg dietary folate equivalents per day and a Recommended Dietary Allowance (RDA) of 400 µg dietary folate equivalents per day (IOM 1998). These contrasted markedly with the 1989 RDA values for folate of 180 and 200 µg per day for women and men (NAS 1989), respectively. The rationale behind this increase was recent metabolic data indicating higher requirements and, as described in this paper, the mounting evidence that actual folate intakes are greater than previously believed.

SUMMARY and RECOMMENDATIONS

There are two take-home lessons from this discussion regarding the significant limitations of existing food composition data for folate. First, there is a great need for refinement of methods for measurement of folate in foods. Although recent advances have been made, standardization and optimization are still needed. Second, an optimized and validated method should be applied to foods with the objective of replacing existing databank values for the folate content of foods. Emphasis should be placed initially on classes of foods whose consumption level and concentration of naturally occurring folate make them significant dietary sources of this vitamin. Additional emphasis should be placed on accurate values for all foods containing added folic acid. Differences in bioavailability have been shown to exist between naturally occurring folate and added folic acid. For this reason, data for enriched/fortified foods would be most useful if separate listings were provided for total folate and added folic acid, as recommended recently (IOM 1998).

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Figure 1. Structures of common folates.

Folic (Pteroyl-L-Glutamic) Acid
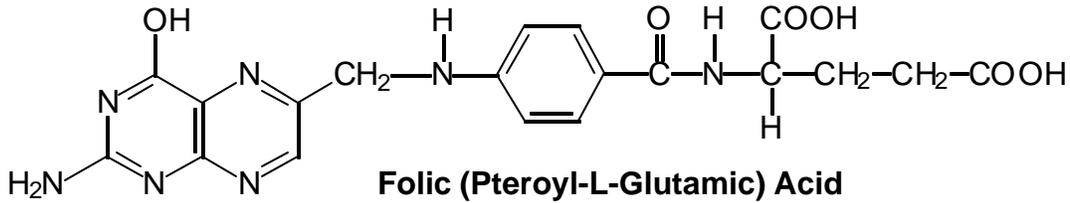
Polyglutamyl Tetrahydrofolates
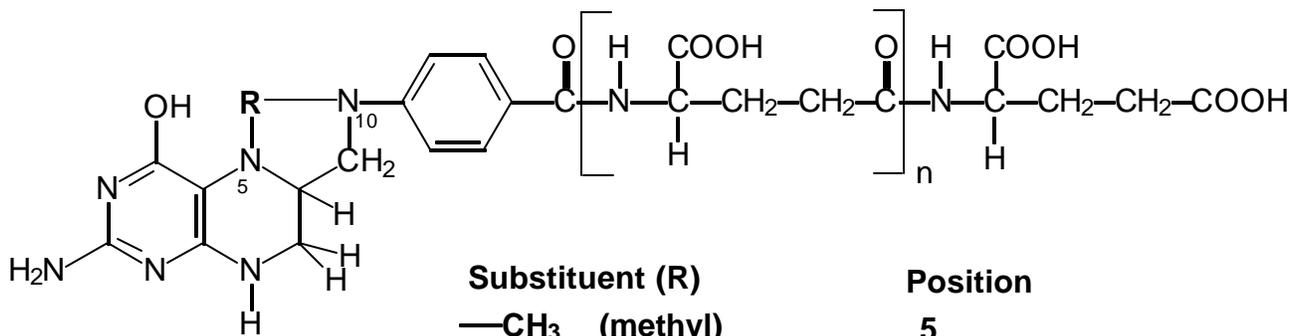
Substituent (R)		Position
—CH$_3$	(methyl)	5
—CHO	(formyl)	5 or 10
—CH=NH	(formimino)	5
—CH$_2$—	(methylene)	5 and 10
—CH=	(methenyl)	5 and 10

Table 1. Effect of extraction buffer pH and composition on total measured folate (Gregory et al. 1990). Current values from USDA Nutrient Database (USDA 1997) are provided for comparison.

Food Sample	Extraction Buffer			USDA Nutrient Database value[#]
	pH 4.9 acetate buffer (+ ascorbate)	pH 7.0 phosphate (+ ascorbate)	pH 7.85 HEPES-CHES buffer (+ ascorbate & mercaptoethanol)	
Frozen peas, not prepared (μg/g)	0.494 ± 0.044	0.538 ± 0.009	0.979 ± 0.053*	0.402
Raw calf liver (μg/g)	11.3 ± 5.6	13.0 ± 0.44	26.9 ± 1.3*	6.42

*Significantly greater than other extraction methods, $P<0.05$.
[#]Data from USDA Nutrient Database for Standard Reference, Release 11-1 (1997).

Table 2. Analysis of eight different diet composites for total folate by *Lactobacillus casei* assay with conjugase treatment alone or with trienzyme treatment to enhance extraction.

Experimental Diet	Total Folate Concentration (µg/100g homogenate)		Increase (%)
	Conjugase alone	**Trienzyme**	
a	4.20	5.99	42.6
b	5.84	7.29	24.8
c	4.57	5.81	27.1
d	6.27	7.42	18.3
e	5.72	7.05	23.3
f	7.07	10.1	42.9
g	19.8	21.8	10.1
h	7.62	14.8	94.2
Mean±SD			35.4 ± 26.3

Values are means of duplicate analyses (A. D. Mackey and J. F. Gregory, unpublished) by method of Pfeiffer et al. 1997. Samples extracted using pH 7.85 HEPES-CHES buffer (ascorbate + mercaptoethanol). Samples obtained from composites of eight different diets from a controlled folate nutrition study conducted by Drs. P. B. Duell and M. R. Malinow, unpublished.

Table 3. Estimated folate intakes derived from analysis of diet homogenates for total folate by *Lactobacillus casei* assay with conjugase treatment alone or with trienzyme treatment to enhance extraction.

Experimental Diet	Total Dietary Folate Intake (µg per day)		Increase (%)
	Conjugase alone	Trienzyme	
a	95	135	42.6
b	140	175	24.8
c	97	123	27.1
d	147	175	18.3
e	192	236	23.3
f	221	317	42.9
g	577	634	10.1
h	220	427	94.2
Mean±SD			35.4 ± 26.3

Values are means of duplicate analyses (A. D. Mackey and J. F. Gregory, unpublished) by method of Pfeiffer et al. 1997. Samples extracted using pH 7.85 HEPES-CHES buffer (ascorbate + mercaptoethanol). Samples obtained from composites of eight different diets from a controlled folate nutrition study conducted by Drs. P. B. Duell and M. R. Malinow, unpublished.

Table 4. Summary of representative estimates of folate intake from dietary sources. All values shown are for intakes prior to inclusion of folic acid in cereal-grain enrichment in ~1997.

Investigators or Study	Estimated Folate Intake ± SEM* (µg/d)
CSFII, 1985-86; women, 20-49 yr (LSRO 1989)	193 ± 3.1 (n=2056)
NHANES II / Subar et al. (1980)	
women, 19-74 yr	207 ± 2.9 (n=5835)
men, 19-74 yr	281 ± 3.6 (n=5331)
Brown et al. (1997)	
women, 22-35 yr	255 ± 9 (n=189)
Tucker et al. (1996a)	
men and women, 67-95 yr	276 (n=667)
Rimm et al. (1998); women, 30-55 yr, (values	366 (mean, n=80082)
include supplements at 26% of intake)	277 (median)
CSFII (1989-91), Guenther et al. (1997)	
women, not pregnant or lactating, $\geq$ 20 yr	221 (n=4621)
men, $\geq$ 20 yr	292 (n=3381)
CSFII (1994-96), cited by Koehler et al. (1997)	
women, $\geq$ 20 yr	226 (n=4816)
men, $\geq$ 20 yr	301 (n=5056)
Koehler et al. (1997)	
men and women, 65-94 yr	300 ± 6 (n=308)
NHANES III (Dodd & Carriquiry, 1997)	
women, 19-30 yr	254 ± 3.6 (n=1972)
women, 31-50 yr	255 ± 3.0 (n=2988)
women, 51-70 yr	269 ± 3.6 (n=2076)
women, $\geq$ 71 yr	275 ± 4.2 (n=1368)
men, 19-30 yr	313 ± 5.1 (n=1942)
men, 31-50 yr	317 ± 4.1 (n=2533)
men, 51-70 yr	322 ± 4.9 (n=1942)
men, 71$\geq$ yr	302 ± 5.4 (n=1255)

*RDA values (1989): 180 µg/d for women $\geq$ 19 yr, 200 µg/d for men $\geq$ 19 yr. Values shown are means±SEM, with supplement use excluded, unless otherwise indicated. CSFII, USDA Continuing Study of Food Intakes of Individuals; NHANES, National Health and Nutrition Examination Survey.