

# HPLC Determination of Stability and Distribution of Added Folic Acid and Some Endogenous Folates During Breadmaking<sup>1</sup>

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## ABSTRACT

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Bread flour was spiked with folic acid (1.40 mg/lb or 3.08 µg/g of flour) and processed into bread by the sponge and dough method. Changes that occurred to added folic acid and endogenous folate contents through different processing stages, including sponge formation, proofing, and baking, were assessed by reversed-phase ion-pair HPLC combined with UV and fluorometric detection. Sample extraction required α-amylase and rat plasma deconjugase digestion, and sample preparation required purification by solid-phase extraction. Added folic acid was measured by

monitoring UV absorption at 280 nm. Four selected forms of endogenous folates including tetrahydrofolate (THF), 5-formyl-THF, 10-formylfolate, and 5-methyl-THF were identified and quantified throughout the bread processing using a fluorescence excitation wavelength of 290 nm and emission wavelength of 350 or 450 nm. Data indicate a relatively good stability of added folic acid and native folates to the baking process, and increased endogenous folate contents in dough and bread as compared with the flour from which they were made.

Mandatory folic acid fortification in the United States was implemented on January 1, 1998, and as a result, U.S. food manufacturers are required to add the nutrient to certain specified cereal grain products (FDA 1996). One of the foods in which fortification is required is enriched bread. Required fortification levels range from 0.43 mg/lb to 1.4 mg/lb. Fortified bread is normally made with fortified flour, which is produced at the mill by means of a vitamin and mineral premix that is continuously fed into the final milled product. The proper amount of folic acid is simply included in the premix. Some bakeries may fortify flour by adding folic acid in the form of tablets or soluble packets but this probably constitutes <20% of the enriched bread produced (Ranum 1996).

The amount of folate available from processed foods varies depending on the preparation methods, and it is crucial to have appropriate information on the effects of processing variables such as fermentation and baking on folate retention. Calhoun et al (1958) noted that the folic acid level, on an equal moisture basis, was higher in bread than in its flour because of the contribution of other ingredients incorporated in the dough. Butterfield and Calloway (1972) reported folate contents of various bread products, as well as of different types of flours and bread dough. Most of the total folate in bread was contributed by the yeast and flour portions, with significantly higher levels present in whole wheat when compared with conventional bleached flour. Keagy et al (1975) determined stability of natural and added folates at various stages during bread processing. Total folate content increased during fermentation, and thus, time of proofing affected final folate values. Incorporation of additives such as benzoyl peroxide, potassium bromate, and ascorbic acid did not appreciably influence the folate content. These studies were conducted using microbiological methods that measure only total folate. Recent investigations using HPLC (Müller 1993; Pfeiffer et al 1997) succeeded in determining folic acid and some derivatives in cereal products including different types of bread. These studies, however, did not determine the stability and distribution of individual folates throughout bread processing.

The current study was designed to use HPLC measurement for monitoring different stages of breadmaking for added folic acid and some native folates, and determining the retention and distribution of these folates among flour, dough, and bread.

## MATERIALS AND METHODS

### Flour Fortification

The flour was commercial bread flour from Pillsbury (Minneapolis, MN) made from hard red spring wheat. It contained malted barley flour and was enriched with thiamin, riboflavin, niacin, and iron. Folic acid fortification was performed by adding folic acid to the commercial flour, and then blending in a rotating V-shaped blender (Patterson-Kelly Co., East Stroudsburg, PA) to reach a final concentration of 1.40 mg/lb or ≈3.08 µg/g, on an as-is basis.

### Baking Test

The bread dough formula was 700 g of flour (14% moisture), 420 g of water, 14 g of yeast (active dry), 3.5 g of yeast food, 35 g of sugar, 14 g of sodium chloride, 21 g of shortening. Sponge and dough bread loaves were baked using Approved Method 10-11 (AACC 2000). The sponge was made by mixing 420 g of the total flour with 252 g of water and all the yeast and yeast food. This mixture was allowed to ferment at 30 ± 1°C and 85% rh for 4 hr, then it was incorporated with the rest of the flour, water, and other ingredients to make dough. The dough was mixed to the point of minimum mobility, then proofed at 35.5 ± 1°C and 92% rh for an average of 75 min, and baked at 218 ± 5°C for 25 min.

### Sampling

Samples of sponge and proofed dough were pinched from the respective loaves immediately after fermentation and frozen before drying. The loaves were sliced after baking, and center slices were removed and used as samples. Sponge, proofed dough, and bread samples were freeze-dried overnight, then ground using a Janke and Kunkel grinder (model A-10, Tekmar Co., Cincinnati, OH) to pass through a 40-mesh sieve, and stored frozen until analysis. Portions of the ground samples were air-oven-dried at 130°C for 1 hr to determine moisture and dry weight of samples.

### Standard Solutions

Individual folate standards including tetrahydrofolic acid (THF), 5-methyl-tetrahydrofolic acid (5-CH<sub>3</sub>-THF), 5-formyl-tetrahydrofolic acid (5-CHO-THF), 10-formyl-folic acid (10-CHO-FA), and folic acid (FA) were purchased from Dr. Schirks Laboratory (Jona, Switzerland) and Sigma Chemical Co. (St. Louis, MO). Stock folate standards were made by dissolving each folate form in 0.1M dibasic potassium phosphate (pH 8–8.5) buffer containing 0.1% ascorbic acid and 0.1% 2-mercaptoethanol (MCE) to give a concentration of 200 µg/mL. Working solutions of folic acid and derivatives were made freshly just before use. Concentrations prepared were 0.1–2 µg/mL for FA, 10–120 ng/mL for 5-CHO-THF, and 5–40 ng/mL for THF, 5-CH<sub>3</sub>-THF, and 10-CHO-FA.

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## Enzymes

$\alpha$ -Amylase [EC 3.2.1.1] derived from *Aspergillus oryzae* (Type X-A, Sigma) was used. The enzyme was dissolved in distilled and filtered water to give a solution concentration of 25 mg/mL, 1 mL was used per gram of sample. Rat plasma [EC 3.4.12.10] suspension from Pel-Freez Biologicals (Rogers, AR) was used to convert native polyglutamylfolates into monoglutamate derivatives.

## Extraction

Samples of flour and freeze-dried dough and bread (5 g) were homogenized by stirring for 1 hr in 50 mL of 0.1M  $K_2HPO_4$  (pH 8–9) containing 0.1% ascorbate (w/v) and 0.1% MCE. The slurry was then adjusted to pH 6.6 corresponding to an intermediate pH value for optimal activity of both  $\alpha$ -amylase and rat plasma deconjugase which, when used individually, have optima of pH 6.9 and 6.5, respectively. Phosphoric acid was used to adjust the slurry pH, followed by  $\alpha$ -amylase treatment (1 hr) at 65°C for hydrolysis of starch.

After  $\alpha$ -amylase digestion, the extract was cooled and treated with rat plasma suspension at a concentration of 0.5 mL/10 mL of extract. The mixture was incubated for 3 hr at 37°C for deconjugation of polyglutamylfolates into monoglutamylfolates. After incubation, the extract was heated in boiling water for 5 min, then centrifuged for 20 min at 5,000  $\times g$ . The volume of the resulting supernatant was adjusted to 50 mL when necessary, and immediately stored at low temperature ( $-20^\circ C$ ), or an aliquot was immediately filtered and injected for the determination of added folic acid, or submitted to the purification procedure for the determination of native folic acid derivatives.

## Purification

Disposable strong anion-exchange (SAX) cartridges (Quaternary amine  $[N^+]$ , Baker 7091-3) were used for sample clean-up. The cartridges (3-mL SPE column, 500 mg) were conditioned by wetting with 3 mL of hexane, followed by 3 mL of methanol, and then equilibrated with 5 mL of 0.1M  $K_2HPO_4$  buffer (pH 8–9) containing 0.1% (w/v) ascorbic acid and 0.1% MCE. A portion of the extract (3 mL) was diluted with phosphate buffer (1–3 mL) before loading onto a cartridge, with an elution rate of  $\approx 0.6$  mL/min. It should be noted that the volume of the portion and the extent of dilution may be varied depending on the expected value of folates in the sample. The column was then rinsed with 2 mL of diluted (1:5) phosphate buffer. Analytes were eluted with at least 3 mL of 0.1M sodium acetate (pH 4.5) containing 5% (w/v)  $Na_2HPO_4$  (HPLC grade), 0.1% (w/v)

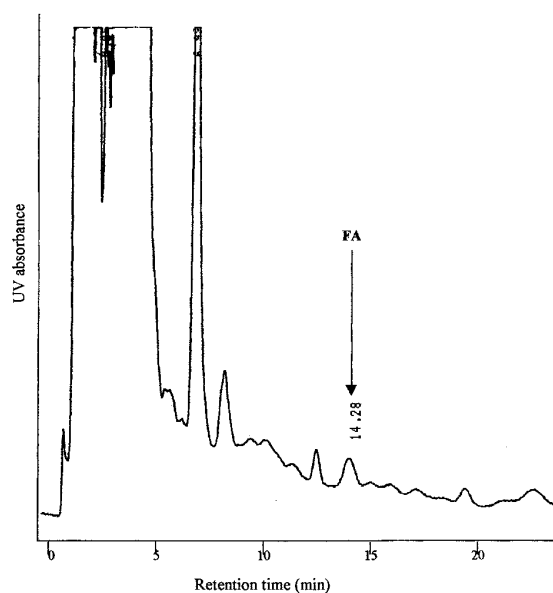


Fig. 1. Chromatographic separation of added folic acid in bread.

ascorbic acid, and 0.1% MCE. The eluent was filtered through a 0.45- $\mu m$  microporous filter before injection.

## Chromatography

Reversed-phased ion-pair HPLC was performed using a system consisting of two pumps: one low-pressure mixing pump (Hitachi model L-6200) and one model L-6000 solvent metering pump. Added folic acid was determined by a Hitachi L-4000 UV detector operating at 280 nm, and a Waters model 474 scanning fluorescence detector set at an excitation wavelength of 290 nm and emission wavelength of 350 or 450 nm was used in monitoring endogenous folates. The injection was made with a Rheodyne (Cotati, CA) loop-type injection valve (20  $\mu L$ ). A Brownlee (30 mm  $\times$  2.1 mm i.d.) guard column with 5  $\mu m$  ODS packing (Varian Chromatography Systems, Walnut Creek, CA) was installed before a Microsorb-MV  $C_{18}$  analytical column (150  $\times$  4.6 mm i.d., 3  $\mu m$  particle diameter). The mobile phase was composed of 24% methanol (v/v) in aqueous potassium phosphate buffer (3.5 mM  $KH_2PO_4$  and 3.2 mM  $K_2HPO_4$ ), pH 6.8, containing 5 mM tetrabutylammonium dihydrogen phosphate (Sigma) as an ion-pairing agent. Isocratic elution at ambient temperature was used, and the measurement of added folic acid was made at a flow rate of 1 mL/min with the UV detector sensitivity set at 0.01 absorbance units full scale. The determination of native folates required a flow rate of 1.3 mL/min, with the fluorescence detector gain set at 100. Postcolumn pH adjustment of the eluent stream was achieved upstream of the detector by pumping 0.2 mL/min of 0.5% (v/v) aqueous phosphoric acid through a reaction tee. Chromatograms were recorded, and peak areas and heights quantitated using a Hitachi model D-2500 chromatogram-integrator. The sensitivity-to-noise ratio of the recorder was  $\geq 4$ .

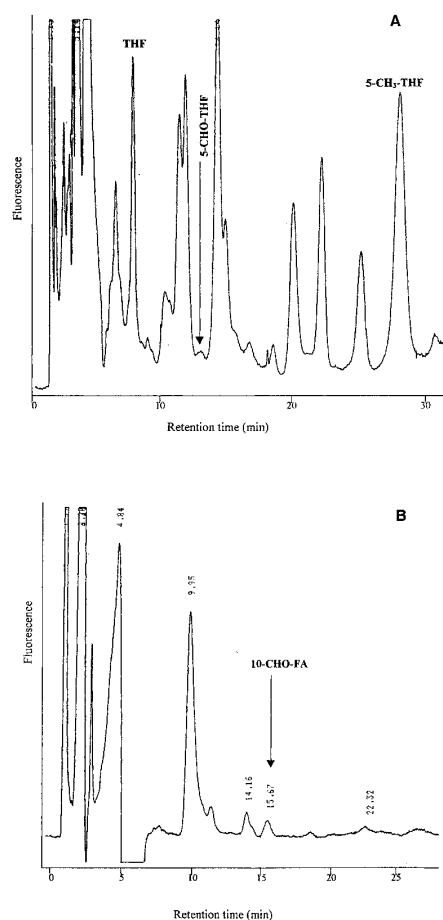


Fig. 2. Chromatographic separation of endogenous folates in bread. A, excitation/emission wavelengths 290/350 nm (upper panel); B, excitation/emission wavelengths 290/450 nm.

## Calculations and Statistical Analysis

The calibration curve for each folate measured by HPLC was obtained by the external standard method in which the peak area was plotted against the concentration of standard folate vitamer injected. However, peak height was used in the quantitation of 5-CHO-THF because of its weak fluorescence, which was near the detection limit. Each sample was analyzed in triplicate and results were expressed as mean with standard deviation. Folate contents in samples were quantified by using linear regression procedures (SAS Institute, Cary, NC) and expressed on a moisture-free basis. Differences among folate values of different bread processing stages were evaluated by one-way analysis of variance using Fisher's least significant difference (LSD). Differences were considered to be significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Added Folic Acid

The reversed-phase ion-pair procedure coupled with UV detection provided enough sensitivity to separate and detect the low level of added folic acid throughout the breadmaking process. Chromatograms of bread (Fig. 1) showed a near baseline resolution, making it unnecessary to conduct any sample purification.

Changes that occurred in the added folic acid level during bread processing are summarized in Table I. The results of the analysis of variance indicate a significant ( $P < 0.05$ ) decrease of added folic acid from flour to bread stage. A slight decrease was recorded at the sponge and proofed dough stages. This is probably due to the dilution effect resulting from the addition of other ingredients to flour. A greater loss attributable to the baking process occurred between proofed dough and bread. The total loss between spiked flour and bread was estimated to be  $\approx 20\%$  on a dry weight basis, suggesting a relatively good stability of folic acid to severe heat treatments such as baking. These results are in general agreement with those reported by Keagy et al (1975), who found a 14% loss of added folic acid due to baking.

### Endogenous Folates

The monitoring of native fluorescence permitted quantitation of four selected endogenous folates throughout the different stages of breadmaking from flour to bread (Fig. 2). Folate forms including

tetrahydrofolic acid (THF) and 5-formyltetrahydrofolic acid (5-CHO-THF) fluoresced naturally at a 350 nm emission wavelength. Optimum fluorescence of 5-CH<sub>3</sub>-THF at 350 nm was obtained only within mobile phase values of pH 2.7–3.0 achieved by a post-column mobile phase pH adjustment. Natural fluorescence of 10-formylfolic acid (10-CHO-FA) was obtained at 450 nm. Recovery of different folic acid derivatives during extraction was 89–91% for bread flour spiked at 1.40 mg/lb (3.08  $\mu\text{g/g}$ ). The recovery values from the SPE cartridge were  $91 \pm 2\%$  for THF,  $86 \pm 2\%$  for 5-CHO-THF,  $89 \pm 1\%$  for 10-CHO-FA, and  $88 \pm 1\%$  for 5-CH<sub>3</sub>-THF.

Significant differences ( $P < 0.05$ ) were observed among native folate contents in bread processing products. It appears that after 4 hr of fermentation, the resulting sponge gained about 72% extra folates as compared with the flour (Table II). This significant increase was achieved despite the dilution effect of other ingredients. The increase in folate concentration, as pointed out by Butterfield and Calloway (1972) and Keagy et al (1975), who obtained similar results, might be a result of a contribution or synthesis of folates from the yeast source. The 5-CH<sub>3</sub>-THF and to a lesser extent THF (Table III) are likely the main vitamer forms resulting from this contribution or synthesis. This result supports Seyoum and Selhub (1993), who, by studying polyglutamyl derivatives of folates in yeast extract, found that polyglutamyl derivatives of 5-CH<sub>3</sub>-THF were the most abundant, followed by those of THF and 10-CHO-THF (10-formyl-tetrahydrofolate). It was also observed that native folate contents dropped 15% from sponge to proofed dough state and  $\approx 20\%$  from proofed dough to bread, that is a total loss of 32% from sponge stage to bread. The apparent loss recorded between sponge and proofed dough is probably due in part to the addition of the rest of the ingredients that diluted the folate content. However, this loss is greater than can be accounted for by dilution effect alone. The incorporation of air during mixing of the other ingredients might have affected the distribution of folates. As a result, the THF content increased, whereas other folates were reduced at the proofed dough stage (Table III). The loss between proofed dough and bread results from the baking process (Calhoun et al 1958; Butterfield and Calloway 1972; Keagy et al 1975). The magnitude of native folate losses (20% on a dry weight basis) due to baking was equal to that recorded for folic acid, suggesting that the added and native

TABLE I  
Distribution of Added Folic Acid During Bread Processing as Determined by HPLC

Products	Folic Acid ( $\mu\text{g}/100\text{ g}$ ) <sup>a</sup>		
	First Batch	Second Batch	Mean
Flour (bread)	nd <sup>b</sup>	nd	...
Spiked flour (bread) <sup>c</sup>	334	340	337 $\pm$ 04
Sponge	327	304	316 $\pm$ 16
Proofed dough	307	299	303 $\pm$ 07
Bread	272	263	268 $\pm$ 06

<sup>a</sup> Values on a dry basis.

<sup>b</sup> Not determined.

<sup>c</sup> 1.40 mg/lb or 308  $\mu\text{g}/100\text{ g}$  of flour.

TABLE II  
Distribution of Endogenous Folates During Bread Processing by HPLC<sup>a</sup>

Products	Endogenous Folates <sup>b</sup> ( $\mu\text{g}/100\text{ g}$ )				Total Native Folates ( $\mu\text{g}/100\text{ g}$ )
	THF	5-CHO-THF	10-CHO-FA	5-CH <sub>3</sub> -THF	
Flour	8 $\pm$ 0.7	29 $\pm$ 0.7	8 $\pm$ 0.2	3 $\pm$ 0.3	48 $\pm$ 1.1
Sponge	18 $\pm$ 1.6	37 $\pm$ 0.1	10 $\pm$ 0.4	18 $\pm$ 0.7	83 $\pm$ 1.8
Proofed dough	23 $\pm$ 2.4	29 $\pm$ 0.7	8 $\pm$ 0.4	10 $\pm$ 1.5	70 $\pm$ 2.9
Bread	9 $\pm$ 0.3	16 $\pm$ 0.4	6 $\pm$ 0.1	25 $\pm$ 0.9	56 $\pm$ 1.0

<sup>a</sup> Mean  $\pm$  SD of triplicate bakes. Values on a dry basis.

<sup>b</sup> THF = tetrahydrofolic acid, FA = folic acid, 5-CHO- = 5-formyl-, 10-CHO- = 10-formyl-, 5-CH<sub>3</sub> = 5-methyl-.

TABLE III  
Percentage Distribution of Native Folates During Bread Processing

Products	Total <sup>a</sup> ( $\mu\text{g}/100\text{ g}$ )	Percent of Individual Native Folate <sup>b</sup> Relative to the Total			
		THF	5-CHO-THF	10-CHO-FA	5-CH <sub>3</sub> -THF
Flour	48 $\pm$ 1.1	16	61	16	7
Sponge	83 $\pm$ 1.8	22	44	12	22
Proofed dough	70 $\pm$ 1.8	33	42	11	14
Bread	56 $\pm$ 1.0	15	29	12	44

<sup>a</sup> Mean  $\pm$  SD of triplicate bakes. Values on a dry basis.

<sup>b</sup> THF = tetrahydrofolic acid, FA = folic acid, 5-CHO- = 5-formyl-, 10-CHO- = 10-formyl-, 5-CH<sub>3</sub> = 5-methyl-.

TABLE IV  
Total Folate Contents in Spiked Flour and Resulting Bakery Products<sup>a</sup>

Products	Added Folic Acid ( $\mu\text{g}/100\text{ g}$ ) <sup>b</sup>	Native Folates ( $\mu\text{g}/100\text{ g}$ ) <sup>c</sup>	Total Folates ( $\mu\text{g}/100\text{ g}$ )
Spiked flour <sup>d</sup> (bread)	337 $\pm$ 4.0	48 $\pm$ 1.1	385 $\pm$ 4.1
Sponge	316 $\pm$ 16.0	83 $\pm$ 1.8	399 $\pm$ 16.1
Proofed dough	303 $\pm$ 7.0	70 $\pm$ 2.9	373 $\pm$ 7.6
Bread	268 $\pm$ 6.0	56 $\pm$ 1.0	324 $\pm$ 6.1

<sup>a</sup> Values on a dry basis.

<sup>b</sup> Mean  $\pm$  SD of two replicates.

<sup>c</sup> Mean  $\pm$  SD of triplicate bakes.

<sup>d</sup> 1.40 mg/lb or 308  $\mu\text{g}$  of folic acid/100 g of flour.

folates would have similar sensitivity to baking heat treatment. Data obtained by Keagy et al (1975) using the sponge and dough method also showed a 20% loss of native folates. Baking reduced the level of all folate forms, except 5-CH<sub>3</sub>-THF, which for an unknown reason exhibited an apparent increase (Tables II and III). In general, the entire breadmaking process resulted in bread with 56 µg/100 g (dry basis) endogenous folate content, which is 16% higher than that of the flour (48 µg/100 g) from which it was made. This result is in accord with the findings of earlier investigations (Calhoun et al 1958; Butterfield and Calloway, 1972; Keagy et al 1975). A number of studies have reported various levels of total native folates in white bread: 56 µg/100 g (Butterfield and Calloway 1972), 46 µg/100 g (Keagy et al 1975), 40 µg/100 g (Calhoun et al 1958), 34 µg/100 g (USDA 1991), and 21 µg/100 g (Pfeiffer et al 1997). Folate values (56 µg/100 g) in bread prepared in the current study fall in the range of folate values of 40–56 µg/100 g obtained for white bread by earlier investigations that mostly used only hog kidney conjugase for folate extraction and *L. casei* for the detection. However, it is noticeably higher than folate values measured by later studies using HPLC methods and di- (Müller 1993) or tri-enzyme (Pfeiffer et al 1997) extraction procedures. Referring to the different folate contents in bread, it appears paradoxical that folate values resulting from earlier works that used microbiological methods, and supposedly less effective extraction procedures, are higher than those of later studies that used HPLC methods with improved extraction procedures. Microbiological assays may be subject to positive interference, while derivatization techniques as well as intricate purification procedures such as affinity chromatography may result in higher losses of compounds of interest. Differences in the results may also be accounted for by diversity in the processing methods and bread formulas, such as inclusion of milk products, along with other factors of experimental conditions including the type of antioxidant and conjugase. Pfeiffer et al (1997) did not detect any THF in cereal products, whereas the current study, as well as that of Müller (1993), did. Another important observation is that this study has confirmed the fact that folic acid molecules do not fluoresce without derivatization. According to Gregory (1984), folic acid is probably not a significant naturally occurring form of the vitamin, but it is often found in small quantities as an oxidation product of THF. Müller (1993) detected some native folic acid and stated that this might be an oxidation product of THF. Pfeiffer et al (1997) using UV detection, also measured native folic acid in unfortified bread samples, but they claimed that no significant interconversions or oxidation occurred during their analysis. Therefore, it is not clear whether folic acid occurs naturally in cereal products. More studies are needed to clarify this aspect.

Added folic acid contents were combined with those of native folates to determine total folate in bread. The resulting values (Table IV) indicate that the contribution of endogenous folates partly compensated for the loss of added folic acid. Although the total folate level in bread was numerically lower than that of flour, the analysis of variance found differences among total folate levels in the various bread processing stages to be nonsignificant.

## CONCLUSIONS

Reversed-phase ion-pair HPLC combined with UV and fluorometric detection provided an alternative approach for separating, identifying, and quantitating added folic acid and native folates in flour and bread. This research has confirmed some conclusions obtained by earlier studies that measured total folate by microbiological methods and has provided some insights into the retention and distribution of individual folate forms throughout bread processing.

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