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Comparative Study on Pressure and Temperature Stability of 5-Methyltetrahydrofolic Acid in Model Systems and in Food Products

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A comparative study on the pressure and temperature stability of 5-methyltetrahydrofolic acid (5-CH_3-H_4folate) was performed in model/buffer systems and food products (i.e., orange juice, kiwi puree, carrot juice, and asparagus). Effects of pH and ascorbic acid (0.5 mg/g) on 5-CH_3-H_4folate stability in buffer systems were studied on a kinetic basis at different temperatures (from 65 to 160 °C) and different pressure/temperature combinations (from 100 to 700 MPa/from 20 to 65 °C). These studies showed that (i) the degradation of 5-CH_3-H_4folate in all model systems could be described by first-order reaction kinetics, (ii) the thermostability of 5-CH_3-H_4folate was enhanced by increasing pH up to 7, (iii) 5-CH_3-H_4folate was relatively pressure stable at temperatures lower than 40 °C, and (iv) ascorbic acid enhanced both the thermo- and barostabilities of 5-CH_3-H_4folate. In food products, temperature and pressure stabilities of 5-CH_3-H_4folate were studied at different temperatures (70−120 °C) and different pressure/temperature combinations (from 50 to 200 MPa/25 °C and 500 MPa/60 °C). 5-CH_3-H_4folate in orange juice and kiwi puree was relatively temperature (up to 120 °C) and pressure (up to 500 MPa/60 °C) stable in contrast to carrot juice and asparagus. Addition of ascorbic acid (0.5 mg/g) in carrot juice resulted in a remarkable protective effect on pressure (500 MPa/60 °C/40 min) and temperature degradation (120 °C/40 min) of 5-CH_3-H_4folate.

KEYWORDS: Folate; temperature; pressure; stability; model systems; food products

INTRODUCTION

Folates have come into focus due to their protective role against birth defects (e.g., neural tube defects), cardiovascular diseases, and cancer (e.g., colorectal). In some countries, folic acid fortification programs have been initiated to increase folic acid intake by women in the reproductive age category. In the United States, for instance, fortification of cereals and grains with folic acid began in 1996, and since January 1998, all cereal grain products are fortified with 140 μg of folic acid/100 g. In the United Kingdom, fortification of foods with folic acid is still voluntary (*1*). There are some concerns with regard to the danger of such a policy to public segments with unrecognized vitamin B12 deficiency, because folic acid can mask the hematological abnormalities and allow the neurological complications to progress when intakes of folic acid exceed 1 mg per day (*2*−*5*). Up to now, in other European countries, folic acid fortification is not permitted and still being discussed. Hereto, strategies for increasing the intake of food folates endogenously present in foods are being explored.

Folates exist in a large variety of foods including vegetables, fruits, meat products (especially liver), beans, fermented dairy products, and cereals (*6*). Plant foods, for example, fruits and vegetables, are by far the most important contributor to the folate intake of adults (*7*). Folates in fruits and vegetables mostly exist as 5-methyltetrahydrofolic acid (5-CH_3-H_4folate). Before consumption, many of these raw materials are often processed, thereby potentially affecting the folates present.

Several authors (*8*−*10*) have reported degradation kinetics of folate such as folic acid, 5-CH_3-H_4folate, tetrahydrofolic acid (H_4folate), or 5-formyltetrahydrofolic acid (5-CHO-H_4folate) during thermal and high hydrostatic pressure treatments. Most kinetic studies have been carried out in buffer systems or in fortified food products. Thermostability of folates (i.e., 5-CH_3-H_4folate and other folate derivates such as folic acid, H_4folate,

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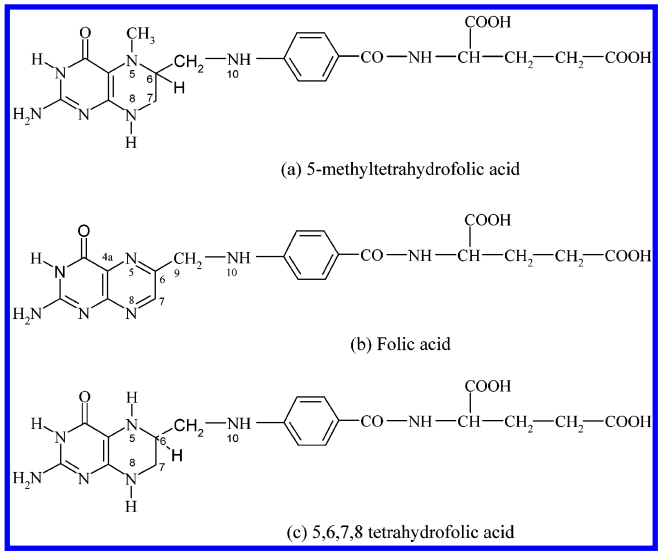

Figure 1. Chemical structures of folates used in this study.

and 5-CHO-H_4folate) in different buffer systems and in fruit juices has been reported in the past three decades. Most thermal treatments were carried out at temperatures between room temperature and 100 °C to study the degradation kinetics of folic acid, 5-CH_3-H_4folate, H_4folate, and 5-CHO-H_4folate (*8*, *10*−*14*), whereas some researchers provided data at higher temperatures (>100 °C) for folic acid and 5-CH_3-H_4folate (*9*, *10*, *15*, *16*). In the literature, it was generally observed that the thermal degradation kinetics of 5-CH_3-H_4folate and other folate derivates followed first-order reaction kinetics. The presence of oxygen influences the degradation kinetics of folates; for example, Ruddick and co-workers (*14*) have reported that the degradation kinetics of 5-CH_3-H_4folate follow pseudo-first-order reaction kinetics under unlimited oxygen concentration and second-order reaction kinetics when the oxygen concentration was limited. With respect to the thermostability of folates, folic acid and 5-CHO−H_4folate have a similar "high" stability at neutral pH, whereas at low pH, 5-CHO-H_4folate is less stable than folic acid. In contrast, 5-CH_3-H_4folate and H_4folate are very thermolabile.

Unfortunately, stability of endogenous folates, especially 5-CH_3-H_4folate, in fruits and vegetables during thermal or high-pressure treatments has been investigated to only a limited extent. Hereto, the purpose of this study was to compare temperature and pressure stability of 5-CH_3-H_4folate in buffer/model systems with that of endogenous 5-CH_3-H_4folate in food products/in situ, for example, in orange juice, kiwi puree, carrot juice, and asparagus. At the same time, the effect of an antioxidant such as ascorbic acid on pressure and temperature stability of 5-CH_3-H_4folate in both systems was also considered.

MATERIALS AND METHODS

Folate Stability in Model Systems. *Sample Preparation.* In this study, 5-methyltetrahydrofolic acid (5-CH_3-H_4folate, **Figure 1a**) was obtained from Schircks Laboratory (Jona, Switzerland). The 5-CH_3-H_4folate stock solution (1 mg/mL) was prepared under subdued light by dissolving 5-CH_3-H_4folate in sodium borate solution (0.05 M, pH 9.22) with 0.4% (v/v) mercaptoethanol (i.e., in order to stabilize the folate during storage). The stock solution was flushed with nitrogen, divided in 0.5 mL portions, and immediately stored at −80 °C until use. The stock solution was stable for 12 weeks at −80 °C.

The working solution (0.4 μg/mL) was prepared on the day of use by diluting the stock solution with different types of buffer solutions, that is, acetic acid buffer (0.1 M, pH 3 and 5), phosphate buffer (0.2 M, pH 7), sodium borate solution (0.05 M, pH 9), and citric acid−phosphate (McIlvaine) buffer (0.2 M; pH 3, 4, and 7). The concentration of 5-CH_3-H_4folate and the ratio of 5-CH_3-H_4folate/dihydro derivates in the stock solution were spectrophotometrically determined according to procedure of Konings (*17*). To study the effect of ascorbic acid on 5-CH_3-H_4folate stability, sodium−ascorbic acid (Sigma, Darmstadt, Germany) (0.5 mg/g) was used.

All organic solvents (methanol and acetonitrile) were obtained from Merck (Darmstadt, Germany). For HPLC analysis, Milli-Q water was used. Samples were covered with aluminum foil, and direct contact with air was avoided at all times.

Thermal Treatment. The working solution was filled under vacuum in capillary tubes (Hirschmann, d = 1.5 mm and l = 150 mm). The samples were immersed in a silicone oil (M1028/50, Roger Coulon pvba, Brussels, Belgium) bath (60−160 °C) (Grant Instruments Ltd., Cambridge, U.K.) during different preset time intervals under isothermal condition. To stop the heating effect, the samples were immediately withdrawn from the oil bath and cooled in an ice bath. The residual concentration of folates was measured using HPLC. The blank (A_0) was defined as the folate concentration of non-heat-treated samples.

High-Pressure Treatment. The working solution was filled in polyethylene flexible microtubes (Elkay, 500 μL). The samples were treated under isobaric−isothermal conditions in a laboratory pilot scale, multivessel, high-pressure apparatus (Resato, Roden, The Netherlands) consisting of eight thermostated 8 mL pressure vessels, which allowed high pressures (up to 800 MPa) to be combined with temperatures from 10 to 65 °C. An oil/glycol mixture (TR15, Resato) was used as pressure transmitting fluid. The pressure was built at a constant rate of 100−125 MPa/min, and an equilibration period for 2 min, allowing the temperature inside the vessels to evolve to its desired value, was taken into account. At that moment, the first pressure vessel was decompressed and the residual folate concentration of the corresponding sample was considered to be a blank (A_0). The other vessels were then decompressed as a function of time. After withdrawal, the samples were stored in an ice bath until the residual folate concentration was measured.

Folate Stability in Food Products (in Situ). *Sample Preparation.* Orange (*Citrus sinensis*, Navelina, category I, Spain), carrot (*Daucus carota*, Delhaize, category I, Belgium), kiwi (*Actinidia deliciosa*, Zespri Green 4030, New Zealand), and asparagus (*Asparagus officinalis* L., Flandria, Belgium, category I, diameter = 16−22 mm) were purchased at a local market. Descriptions of the samples used are summarized in **Table 1**. Orange and carrot juices were prepared by squeezing the oranges and carrots in a fruit mixer (Le Duo, Magimix, Montceau-en Bourgogne, France) and centrifuged at 20300*g* and 4 °C (Beckman J2-HS centrifuge, Palo Alto, CA) for 10 min. Kiwi puree was prepared by mixing the peeled kiwi in a mixer (Büchi mixer B-400, Flawil, Switzerland). The samples were divided in 40 mL portions and packed in polyethylene plastic bottles. The samples were frozen in liquid nitrogen and stored at −80 °C until use. Fresh asparagus was bought on the same day of the treatments, and only the white part of the asparagus was used. Before treatments, the asparagus was peeled using a cylindrical knife (diameter = 1.2 cm), and afterward the peeled samples were sliced in small pieces (~1 cm length).

To study the effect of ascorbic acid on 5-CH_3-H_4folate stability, sodium−ascorbic acid was directly added to the carrot juice (0.5 mg/g), whereas for asparagus, sodium−ascorbic acid diluted in distilled water (0.5 mg/g) was used as brine liquid during thermal and pressure treatments (see further on).

In this study, folic acid, 5-CH_3-H_4folate, and tetrahydrofolic acid (H_4folate) (Schircks Laboratory, Jona, Switzerland) were used as standard solutions (**Figure 1**). The stock solutions were prepared by diluting the purified folates (1 mg/mL) in sodium borate solution (0.05 M, pH 9.22) in the presence of 0.4% mercaptoethanol and ascorbic acid (0.5 mg/mL). The stock solutions were flushed with nitrogen and stored at −80 °C (maximum of 12 weeks) until use. The concentrations of those folate derivates in the stock solutions were spectrophotometrically determined according to the procedure of Konings (*17*).

Thermal Treatment. The samples of orange juice, kiwi puree, and carrot juice (~5 g per tubes) were filled in glass tubes (Pyrex, 20 mL volume, diameter = 1 cm; VWR International, Leuven, Belgium), whereas the cylindrical asparagus pieces (~9.65 ± 0.4 g per tube) were

Table 1. Description of Sample and Extraction Conditions Used for Study in Food Products

	orange juice	kiwi puree	carrot juice	asparagus
pH	3.76	3.41	6.52	5.60
concn of ascorbic acid[a] (mg/g)	0.51	0.53	0.11	0.13
concn of 5-CH_3-H_4folate (as free folate) (μg/g)	0.13 ± 0.03	0.07 ± 0.04	0.08 ± 0.03	0.08 ± 0.03
total concn of 5-CH_3-H_4folate (μg/g)	0.16 ± 0.04	0.23 ± 0.06	0.12 ± 0.03	0.20 ± 0.05
total folate (μg/g)	0.16 ± 0.04	0.23 ± 0.06	0.12 ± 0.03	0.34 ± 0.04
matrix bound and polyglutamates (%)	20	68	34	77
folate derivates found	5-CH_3-H_4folate	5-CH_3-H_4folate	5-CH_3-H_4folate, H_4folate[b]	5-CH_3-H_4folate, H_4folate
total concn of 5-CH_3-H_4folate (μg/g) (*6*)	0.18 ± 0.01	0.23 ± 0.04	0.11 ± 0.03	0.58 ± 0.01
total folate (μg/g) (*6*)	0.20 ± 0.02	0.23 ± 0.04	0.13 ± 0.03	0.56 ± 0.01
polyglutamates (%) (*6*)	73	83	66	93
folate derivates found (*6*)	5-CH_3-H_4folate, H_4folate[b]	5-CH_3-H_4folate, H_4folate[b]	5-CH_3-H_4folate, H_4folate,[b] 10-CHO-H_4folate[b]	5-CH_3-H_4folate
ratio sample and extraction buffer (w/w)	1:1	1:1 up to 1:8	1:1 up to 1:3	1:5 up to 1:10

[a] Measured by titration method. [b] Concentration was very low (~ <0.01 μg/g).

packed in stainless steel tubes (diameter = 1.2 cm; l = 10 cm) containing distilled water (~3.5 ± 0.25 g per tube). The tubes were tightly closed and heated in an oil bath (70−120 °C) (Grant Instruments, Cambridge, U.K.) for 30 min and at 120 °C for different preset time intervals. To stop the heat treatment, the samples were cooled in an ice bath. Afterward, the treated samples (~10 g) were mixed with extraction buffer (Ches/Hepes buffer, 50 mM, pH 7.85, with 114 mM ascorbic acid and 200 mM β-mercaptoethanol), using a sample/extraction buffer ratio as mentioned in **Table 1**, and subsequently homogenized using a mixer (Ultraturrax T25 Basic IKA Labortechnik, Janke & Kunkel GmbH & Co. KG, Staufen, Germany). The sample crude extract was stored at 4 °C for <24 h.

High-Pressure Treatment. The samples of orange juice, carrot juice, and kiwi puree were filled in plastic tubes (Falcon, 15 mL, VWR International), avoiding air bubbles in the samples. The tubes were covered with Parafilm and tightly closed. To avoid contamination of pressure medium in the samples, the tubes were double-packed with polyethylene plastic bags (Medisch Labo Service, Menen, Belgium; 22 cm length, 6 cm length) using a vacuum-sealing machine (Multivac A300/16, Wolfertschwenden, Germany) up to 11 mbar. For asparagus, the cylindrical sample pieces were double-vacuum-packed in the polyethylene plastic bags.

High-pressure treatments were carried out in a single-vessel high-pressure apparatus (warm isostatic press, SO. 5-7422-0, 590 mL, Epsi, Temse, Belgium), which allowed high pressures up to 550 MPa to be combined withtemperatures between −35 and 60 °C. The vessel was thermostated by a cryostat (N8-KT 50W, ThermoHaake, Karlsruhe, Germany) using 56% ethylene glycol as heating/cooling medium. A propylene/glycol mixture (60% DowcalN, The Dow Chemical Co., Horgen, Switzerland) was used as pressure-transferring medium.

In this study, samples were treated at 60 °C/500 MPa for different time intervals (5−100 min) and at 25 °C combined with pressures of 50−200 MPa for 10 and 30 min. The pressure was automatically built and manually released. After treatments, the samples were stored in an ice bath. Afterward, the treated samples (~10 g) were mixed with the extraction buffer using a sample/extraction buffer ratio as shown in **Table 1** and homogenized as previously described. The sample crude extract was stored at 4 °C for <24 h before extraction.

Extraction Procedure. The extraction was performed as described by Konings (*17*). The mixture of sample and extraction buffer was contained in centrifuge tubes (50 mL, Oak Ridge centrifuge tubes, 3119, polypropylene copolymer, Nalgene Labware, New York). The extraction was carried out by immersing the mixture into boiling water for 10 min and subsequently cooling in an ice bath. The pH of the mixture was adjusted to 7 by adding KOH (0.6 g/mL) and, afterward, it was divided in two portions. To the first portion were added no enzymes to determine free folates in the samples, whereas on the second portion was carried out a trienzyme extraction by adding 500 μL of rat plasma conjugase (rat plasma Wistar male S/Hep. Lithium, Iffa-Credo, Charles River Laboratories), 50 μL of protease (peptidase, 1 mg/mL in distilled water, P-7500, Sigma-Aldrich, Steinheim, Germany), and 50 μL of amylase (Thermamyl 120L type L, Novo Nordisk, Bagsværd, Denmark) in order to determine the concentration of total folates. The difference between the total and free folate concentrations (estimated as monoglutamates) gives the concentration of bound folates and polyglutamates. The rat plasma was first dialyzed as described by Konings (*17*) and, afterward, centrifuged at 2500*g* and 4 °C for 10 min. The dialyzed plasma was divided in small portions (500 μL) and stored at −80 °C until use. The activity of rat plasma conjugase was evaluated using yeast (*Saccharomyces cerevisiae* type I, Sigma-Aldrich).

The samples (with or without enzymes) were incubated for 4 h at 37 °C in a shaking water bath (Certomat WR, B. Braun, Melsungen, Germany) at a speed of 200 shakings per minute. The deconjugation process was terminated by heating the mixtures in boiling water for 5 min in order to inactivate the enzymes. Afterward, the mixture was cooled in an ice bath. The samples were centrifuged at 4360*g* and 4 °C for 20 min. The supernatant was collected in a centrifuge tube. One milliliter of extraction buffer was added to the sediment and mixed. The latter mixture was centrifuged at 4360*g* and 4 °C for 20 min, and the supernatant was gathered with the first obtained supernatant. The entire supernatant was stored at −80 °C prior to the folate isolation and purification using affinity chromatography.

Isolation and Purification Procedure. The affinity columns were prepared as described by Konings (*17*). Folate-binding protein (FBP; 1 mg/mL, Scripps Laboratories, San Diego, CA) and Affi-gel 10 (Bio-Rad Laboratories, Hercules, CA) were used, respectively, as ligand and binding matrix.

The isolation and purification procedure was performed as described by Konings (*17*). Before application to the FBP columns, the sample extract was thawed and centrifuged at 14600*g* and 4 °C for 15 min. The columns were equilibrated with 5 mL of phosphate buffer (0.1 M, pH 7), and the sample extract was transferred into the FBP column. The column was rinsed with 5 mL of phosphate buffer (25 mM, pH 7, containing 0.1 M NaCl) and subsequently with 5 mL of phosphate buffer (25 mM, pH 7). The folates were eluted with 4.6 mL of elution buffer (0.02 M trifluoroacetic acid/0.02 M 1,4-dithioerythritol, pH 6.35) and collected in a 5 mL volumetric flask containing 200 μL of ascorbic acid solution (0.25 g/mL), 40 μL of KOH solution (0.60 g/mL), and 5 μL of β-mercaptoethanol. The volume was adjusted to 5 mL using elution buffer. The concentration of folates was measured by a reverse phase liquid chromatography (RP-HPLC) analysis.

RP-HPLC Assay. A reverse phase HPLC analysis (AKTA purifier, Amersham Biosciences, Uppsala, Sweden) at 25 °C using a Prevail C18 column (250 mm × 4.6 mm, 5 μm particle size, Alltech, Deerfield, IL) and Unicorn 4.0 data analysis software (Amersham Biosciences) was applied to quantify and identify folate derivates in the samples. The column was isocratically equilibrated using a mixture of acetonitrile (5%) and phosphate buffer (330 mM; pH 2.15) with a flow rate of 1.5 mL/min for 4 min before the sample was applied to the column. A linear gradient from 5 to 17% acetonitrile was carried out within 10 min and, afterward, the column was washed with a mixture of acetonitrile (17%) and phosphate buffer (330 mM; pH 2.15) for 4 min before the assay was terminated. The retention time of 5-CH_3-H_4folate was situated between 12 and 13 min. In this study, UV (290 nm, AKTA

Table 2. Effect of pH and Ascorbic Acid (AA; 0.5 mg/g) on Thermostability of 5-CH_3-H_4folate in Different Buffer Solutions

	k ($\times 10^{-3}$ min^{-1})							
T (°C)	citric acid–phosphate (pH 3)	acetic acid (pH 3)	citric acid–phosphate (pH 4)	citric acid–phosphate with AA (pH 4)	acetic acid (pH 5)	phosphate (pH 7) (*10*)	citric acid–phosphate (pH 7)	borate (pH 9)
65	nd[a]	10.18 ± 1.21[b]	nd	nd	14.41 ± 2.25	9.73 ± 0.83	nd	9.61 ± 0.81
70	5.07 ± 1.13	22.45 ± 2.55	20.45 ± 0.94	nd	31.27 ± 4.70	13.06 ± 1.12	2.32 ± 0.15	15.22 ± 1.03
80	nd	79.98 ± 14.57	73.06 ± 16.84	nd	80.78 ± 8.72	28.14 ± 0.09	nd	55.29 ± 3.28
90	125.07 ± 12.28	165.62 ± 23.66	115.08 ± 20.85	nd	105.86 ± 11.06	68.31 ± 5.86	14.21 ± 0.94	112.36 ± 11.81
100	257.38 ± 27.33	nd	331.27 ± 28.71	nd	nd	nd	35.14 ± 1.48	nd
120	nd	nd	nd	2.24 ± 0.33	nd	nd	nd	nd
140	nd	nd	nd	3.94 ± 0.24	nd	nd	nd	nd
160	nd	nd	nd	4.71 ± 0.58	nd	nd	nd	nd
E_a (kJ/mol)	144 ± 20	114 ± 11	89 ± 10	26 ± 7	81 ± 16	80 ± 5	96 ± 2	104 ± 8
r^2	0.98	0.98	0.97	0.93	0.93	0.99	0.99	0.99

[a] Not determined. [b] Standard error of regression.

purifier, Amersham Biosciences) and fluorescence (λ_{em} = 359 nm and λ_{ex} = 280 nm, RF-10Axl, Shimadzu, Kyoto, Japan) detectors were used to measure 5-CH_3-H_4folate for a concentration >0.2 μg/mL and <0.2 μg/mL, respectively. The folate concentration was calculated on the basis of the peak area and peak height in comparison to the standard solutions. The regression correlation (r^2) of the standard curves in this study were at least 0.98.

Data Analysis. In the literature, it has been reported that the degradation kinetics of 5-CH_3-H_4folate during thermal and high-pressure treatments follow first-order reaction kinetics (*8*−*10*, *15*). Hence, in this study, eq 1 was used to estimate the degradation rate constants of 5-CH_3-H_4folate for both thermal and high-pressure treatments.

$$\ln(C) = \ln(C_0) - kt \quad (1)$$

C is the concentration of 5-CH_3-H_4folate at treatment time t, C_0 is the concentration of 5-CH_3-H_4folate at time = 0, and k is the degradation rate constant. The degradation rate constant can be estimated on the basis of a linear regression analysis of the natural logarithm of the residual folate concentration as a function of treatment time.

Linearized Arrhenius (eq 2) and Eyring equations (eq 3) were used to estimate, respectively, the temperature and pressure dependences of the k values. Activation energy (E_a) and activation volume (V_a) values were calculated by linear regression analysis, respectively, by plotting the natural logarithm of k values as a function of the reciprocal of the absolute temperature or by plotting the natural logarithm of k values as a function of pressure.

$$\ln(k) = \ln(k_{refT}) + \left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (2)$$

$$\ln(k) = \ln(k_{refP}) - \left[\frac{V_a}{RT}(P - P_{ref})\right] \quad (3)$$

k_{refT} is the degradation rate constant at reference temperature T_{ref} and k_{refP} is the degradation rate constant at reference pressure P_{ref}.

RESULTS AND DISCUSSION

Temperature and Pressure Stability of 5-CH_3-H_4folate in Model Systems. *Effect of pH and Ascorbic Acid on Thermostability of 5-CH_3-H_4folate in Model Systems.* In this study, the stability of 5-CH_3-H_4folate in model systems at different pH values using different types of buffer solutions and in the presence/absence of ascorbic acid was investigated at temperatures >65 °C. In all model systems, 5-CH_3-H_4folate was degraded during thermal treatment (>65 °C), and the kinetics of 5-CH_3-H_4folate degradation was adequately described by a first-order reaction model (r^2 > 0.96). The estimated k values are summarized in **Table 2**.

On the basis of the estimated kinetic parameter (k and E_a) values, the thermostability of 5-CH_3-H_4folate was maximal at pH 7 (in phosphate buffer). At both lower and higher pH values, a decrease in stability was observed. The thermostability of 5-CH_3-H_4folate was largely increased by adding ascorbic acid (decrease of rate constant) and, in addition, the E_a value was decreased by ~70% in the presence of ascorbic acid (indicating less pronounced temperature sensitivity). It is very clear that ascorbic acid has a strong protective effect and strongly retards folate degradation during thermal treatments.

In this investigation, a mixture of citric acid and phosphate buffer (McIlvaine buffer) was used because this buffer had a broad pH range from acid to neutral pH. At pH 3, the thermostability of 5-CH_3-H_4folate in McIlvaine buffer was higher than in acetic acid, but the estimated E_a values for both buffers were not significantly different (95% confidence interval). In contrast, the thermostability of 5-CH_3-H_4folate in McIlvaine buffer at pH 7 was lower than that in phosphate buffer. The E_a value of 5-CH_3-H_4folate in phosphate buffer (pH 7) was situated in the same order of magnitude as reported by Barret and Lund (*13*) but higher than that found by Ruddick and co-workers (*16*). In this study, the temperature dependence of k values in McIlvaine buffer at pH 7 was slightly more sensitive than that in phosphate buffer (**Table 2**).

Effect of pH and Ascorbic Acid on Pressure Stability of 5-CH-H_4folate in Model Systems. The pressure stability of 5-CH_3-H_4-folate in McIlvaine buffer (pH at atmospheric pressure was 4) was studied in a pressure/temperature range of 100−700 MPa and 20−65 °C. The pressure degradation kinetics of 5-CH_3-H_4folate could be described by a first-order reaction model (eq 1) as previously reported by Nguyen and co-workers (*10*). The estimated k values are summarized in **Table 3**. At constant pressure, the degradation rate of 5-CH_3-H_4folate in McIlvaine buffer was enhanced by increasing temperature as previously observed by Nguyen and co-workers (*10*). The barostability of 5-CH_3-H_4folate in phosphate buffer (pH at atmospheric pressure was 7) (*10*) was slightly higher than that in McIlvaine buffer; however, the effect of initial buffer pH on the pressure stability of 5-CH_3-H_4folate was less pronounced than that on its thermostability. It could be that citric acid−phosphate buffer was relatively not pH stable under pressure. The pH was decreased by ~0.3 unit for a pressure increase of 100 MPa (*18*−*20*).

The effect of ascorbic acid on the pressure degradation of 5-CH_3-H_4folate was studied up to 800 MPa combined with temperatures up to 65 °C. No degradation of 5-CH_3-H_4folate

Table 3. Estimated k Values of 5-CH_3-H_4folate Degradation in Citric Acid–Phosphate Buffer (0.2 M, pH 4 at 0.1 MPa) during Combined Pressure and Temperature Treatments

P/*T* combinations		estimated values	
P (MPa)	*T* (°C)	k ($\times 10^{-3}$ min^{-1})	r^2
100	40	5.35 ± 0.62[a]	0.95
	45	7.35 ± 1.62	0.87
200	35	3.27 ± 0.26	0.98
	40	11.16 ± 0.77	0.99
	45	33.96 ± 5.17	0.93
	50	54.28 ± 8.04	0.94
300	35	3.31 ± 0.16	0.99
	40	8.41 ± 1.03	0.94
	45	10.51 ± 2.69	0.88
400	30	3.42 ± 0.46	0.90
	35	4.48 ± 0.70	0.93
	50	21.87 ± 3.58	0.93
500	40	0.92 ± 0.40	0.73
	50	31.35 ± 5.51	0.92
700	40	4.84 ± 0.78	0.93
	50	41.70 ± 6.24	0.92
	60	59.53 ± 10.82	0.94

[a] Standard error of regression.

Table 4. Estimated Activation Energy Values of 5-CH_3-H_4folate Degradation in Citric Acid–Phosphate Buffer (0.2 M, pH 4 at 0.1 MPa) at Different Constant Pressure Levels

P (MPa)	E_a (kJ·mol^{-1})
100	nd[d]
200	158 ± 19[b] ($r^2 = 0.97$)
300	94 ± 32 ($r^2 = 0.89$)
400	78 ± 9 ($r^2 = 0.99$)
600	nd
700	110 ± 43 ($r^2 = 0.87$)

[a] Not determined. [b] Standard error of regression.

in McIlvaine buffer was noticed even at 800 MPa and 65 °C for 6 h in the presence of ascorbic acid.

The temperature dependence of k values under pressure could be described by a linearized Arrhenius relation. The estimated E_a values at different constant pressure levels are given in **Table 4**. The highest temperature dependence of k values occurred at 200 MPa. However, the estimated E_a values of 5-CH_3-H_4folate in McIlvaine buffer (pH at atmospheric pressure was 4) at pressures >200 MPa are situated in the same order of magnitude (i.e., no significant difference at a confidence level of 95%).

From the data we can conclude that (i) pressurization at elevated temperatures (≥40 °C) enhanced the 5-CH_3-H_4folate degradation and (ii) the presence of ascorbic acid has a stabilizing effect on the pressure degradation of 5-CH_3-H_4folate. As a consequence, 5-CH_3-H_4folate concentrations will not be largely affected at combined high pressure and room temperature conditions (i.e., mostly applied for high-pressure pasteurization). However, a much pronounced effect is to be expected at elevated/high temperatures (>60 °C) (i.e., mostly applied for high-pressure sterilization). In the latter treatment, 5-CH_3-H_4-folate was easily degraded as also noticed by Nguyen and co-workers (*10*). Again, the presence of ascorbic acid strongly enhances folate stability during high-pressure/thermal processing.

Temperature and Pressure Stability of 5-CH_3-H_4folate in Food Products (in Situ). In this study, orange juice, kiwi puree, carrot juice, and asparagus were selected in order to study the effect of (i) pH and (ii) ascorbic acid content (**Table 1**) on the thermo- and barostability of endogenous 5-CH_3-H_4folate. As it has been noticed that folate loss is mainly caused by leaching, a closed system was used in this investigation.

As indicated in **Table 1**, the concentration of 5-CH_3-H_4folate in orange juice, carrot juice, and kiwi puree obtained in this study was situated in the same order of magnitude as reported by Konings and co-workers (*6*); however, values for asparagus are somewhat lower. In this investigation, the total percentage of matrix bound and polyglutamates was lower than that found by Konings and co-workers (*6*).

The thermostability of 5-CH_3-H_4folate was studied at different temperatures (70−120 °C) for a fixed treatment time of 30 min (**Figure 2**) and as a function of treatment time at 120 °C (**Figure 3**). For orange juice and kiwi puree, 5-CH_3-H_4folate concentration was not or only slightly decreased during thermal treatment. In contrast, 5-CH_3-H_4folate in carrot juice and asparagus was significantly degraded (40−70%) during thermal treatments. This trend is observed in both **Figures 2** and **3**. It could be correlated with a difference in ascorbic acid content (**Table 1**). With respect to the stability of other folate derivates in carrot juice, the H_4folate in carrot juice was found to be below the detection limit (no peak found in the chromatogram) after thermal treatment at 120 °C for 2 h. The H_4folate derivate was relatively more heat sensitive than 5-CH_3-H_4folate.

Folate stability in situ during high-pressure treatment at 500 MPa and 60 °C is shown in **Figure 4**. The same finding as previously observed during thermal treatment was noticed at 500 MPa and 60 °C. 5-CH_3-H_4folate in orange juice and kiwi puree was relatively stable during high-pressure treatment (e.g., **Figure 4A**), in contrast to carrot juice and asparagus (e.g., **Figure 4B**). With respect to pressure stability of other folate derivates in carrot juice, the peak of H_4folate disappeared after pressurization at 500 MPa and 60 °C for 1 h. Again, H_4folate in carrot juice was more pressure sensitive than 5-CH_3-H_4folate.

A previous study (*21*) showed that high pressure increased the free folate concentration (and consequently increased the folate bioavailability) in leek at 25 °C combined with pressures between 50 and 200 MPa. In this study, the same pressure/temperature combinations have been applied to orange juice, kiwi puree, carrot juice, and asparagus. 5-CH_3-H_4folate in all fruits and vegetables studied was relatively stable (i.e., no degradation) at those pressure/temperature combinations (**Figure 5**). No increase in the free 5-CH_3-H_4folate concentration of asparagus, kiwi puree, and carrot juice was noticed after pressure treatments up to 200 MPa (e.g., **Figure 5A**). In this study, an increase in free 5-CH_3-H_4folate concentration of asparagus, kiwi puree, and carrot juice was not noticed, probably because polyglutamates had partly been converted to monoglutamates by endogenous conjugase due to mechanical texture breakdown during sample preparation. On the contrary, the free 5-CH_3-H_4-folate concentration of orange juice (**Figure 5B**) was increased after pressure treatments at 150 and 200 MPa as previously observed by Melse-Boonstra (*21*).

On the basis of the study in food products, it was concluded that 5-CH_3-H_4folate in situ was relatively stable to thermal and high-pressure treatments as compared to model systems. This might be caused by the presence of endogenous ascorbic acid. Different folate stabilities among orange juice, kiwi puree, carrot juice, and asparagus seem to coincide with different levels of ascorbic acid content (**Table 1**). In an additional experiment, the effect of ascorbic acid (0.5 mg/g) on the temperature and pressure stability of 5-CH_3-H_4folate in carrot juice and asparagus

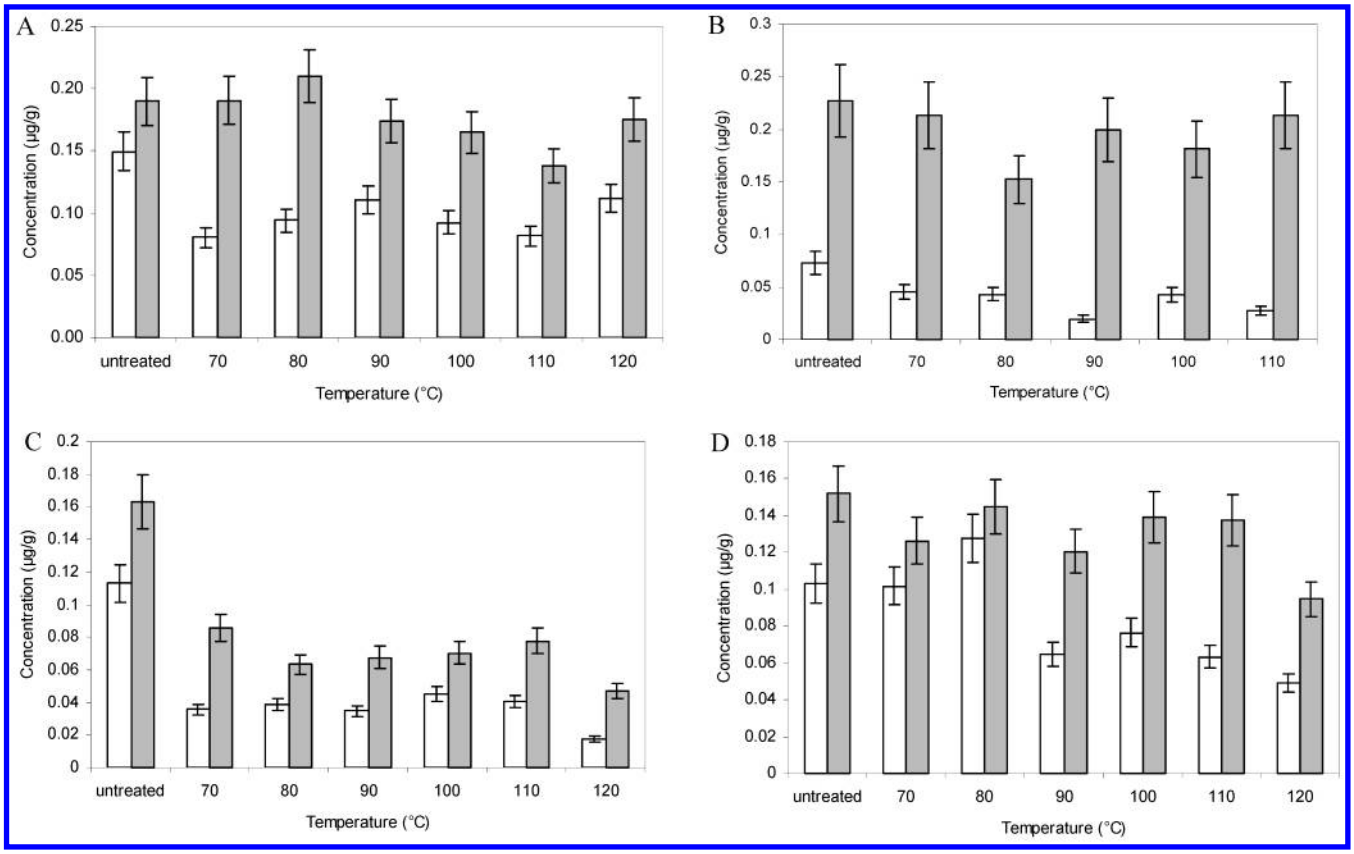

Figure 2. Influence of temperature on the stability of free (white column) and total (shaded column) 5-CH_3-H_4folate in orange juice (**A**), kiwi puree (**B**), carrot juice (**C**), and asparagus (**D**) for treatment time of 30 min. Error bars represent standard deviation of the measurements.

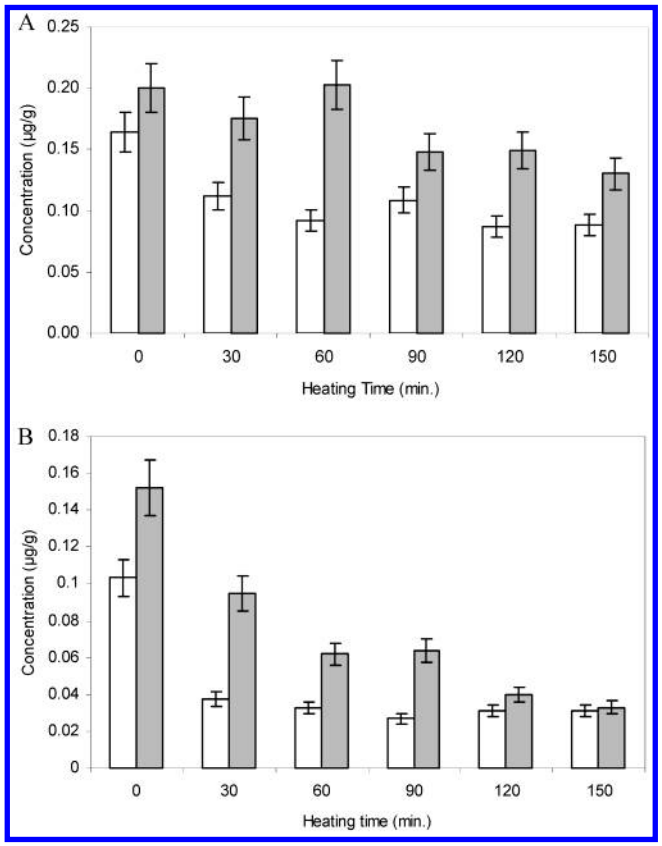

Figure 3. Influence of heating time on the stability of free (white column) and total (shaded column) 5-CH_3-H_4folate in orange juice (**A**) and asparagus (**B**) at 120 °C. Error bars represent standard deviation of the measurements.

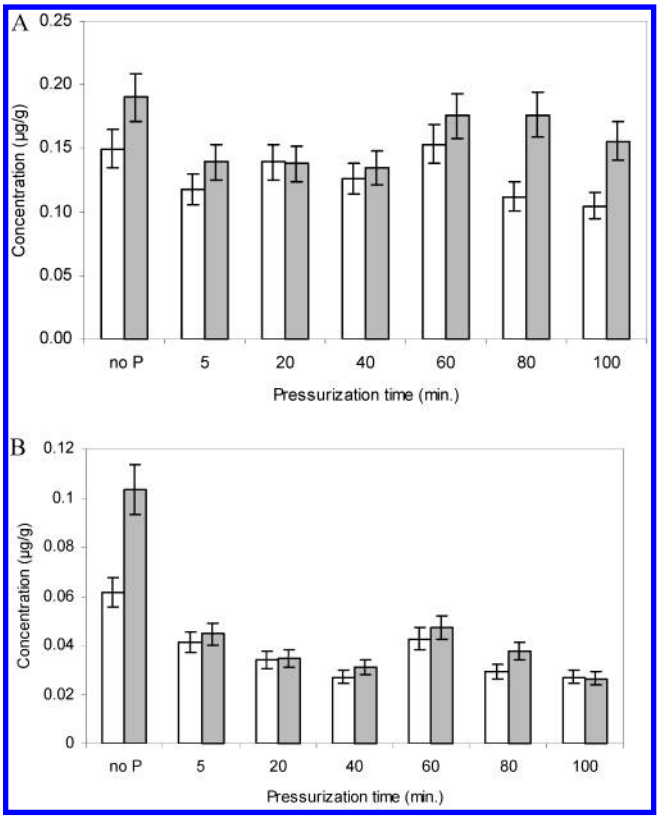

Figure 4. Influence of pressurization time on the stability of free (white column) and total (shaded column) 5-CH_3-H_4folate in orange juice (**A**) and carrot juice (**B**) at 500 MPa and 60 °C. Error bars represent standard deviation of the measurements.

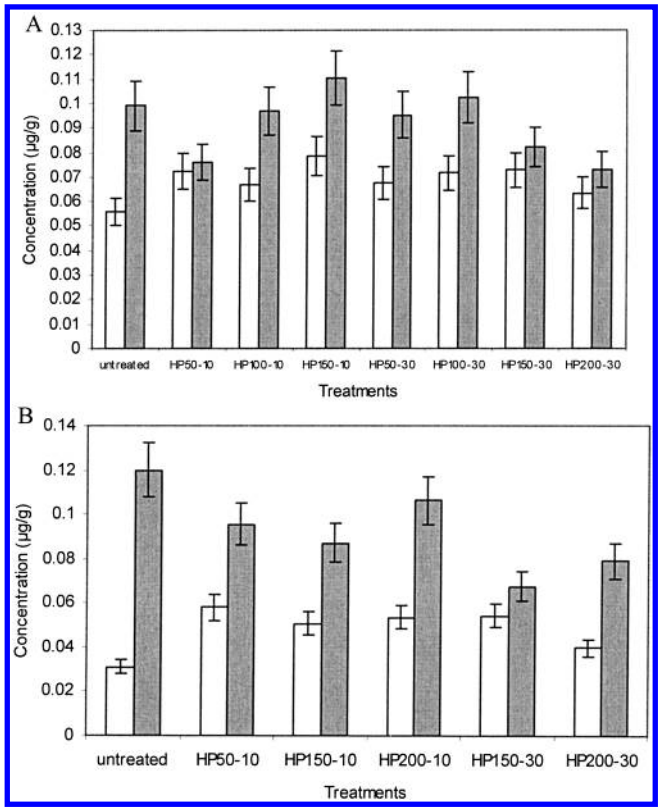

Figure 5. Influence of different pressure levels (50–200 MPa) at 25 °C on the stability of free (white column) and total (shaded column) 5-CH_3-H_4folate in carrot juice (**A**) and orange juice (**B**). Error bars represent standard deviation of the measurements. Treatments are symbolized as "HP (pressure level)–(treatment time)", e.g., HP150–10 (pressure treatment at 150 MPa for 10 min.).

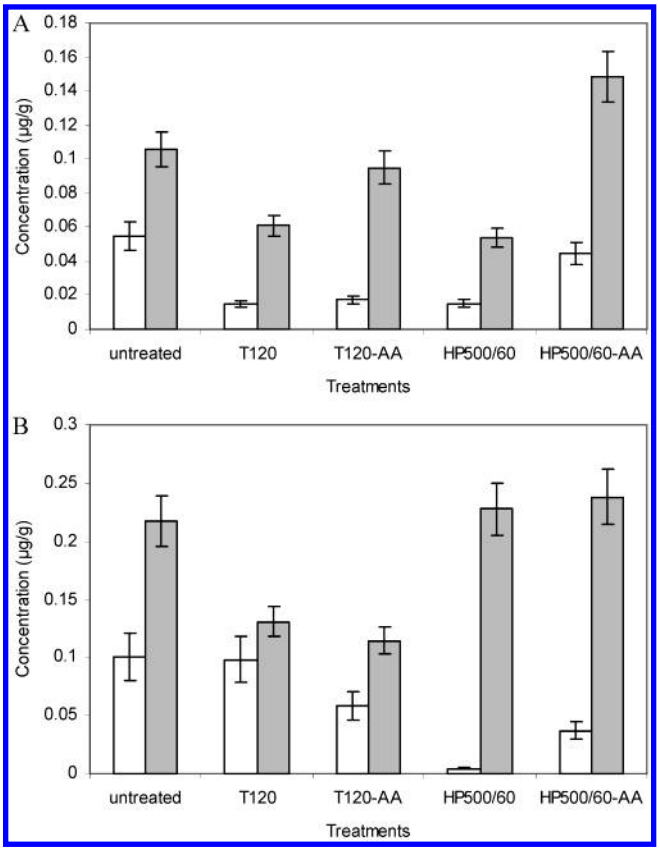

Figure 6. Effect of ascorbic acid (0.5 mg/g) on temperature and pressure stability of free (white column) and total (shaded column) 5-CH_3-H_4folate in carrot juice (**A**) and asparagus (**B**). T120 and T120-AA represent thermal treatments at 120 °C for 40 min in absence and presence, respectively, of ascorbic acid. HP500/60 and HP500/60-AA represent pressure treatments at 500 MPa and 60 °C for 40 min in absence and presence, respectively, of ascorbic acid. Error bars represent standard deviation of the measurements.

was further studied. The results showed that ascorbic acid significantly increased the thermo- and barostability of 5-CH_3-H_4folate in carrot juice; however, the effect of ascorbic acid on 5-CH_3-H_4folate stability in asparagus was less pronounced (**Figure 6**) because the exogenous ascorbic acid was present in only the brine for asparagus. These results confirm that the protective effect of ascorbic acid observed in model systems can be extrapolated to food systems

Conclusions. In this study, folate stability (especially 5-CH_3-H_4folate) in model systems during thermal and pressure treatments has been studied as a comparison to folate stability in food products. It was concluded that (i) 5-CH_3-H_4folate was less stable in model systems than in food products during temperature−pressure treatments and (ii) endogenous ascorbic acid in foods plays an important and positive role in increasing folate stability and seems to be more important than the pH of the food medium.

Ascorbic acid addition could be considered as a strategy in preventing folate degradation during processing; however, further investigations must be carried out to quantify the most efficient ascorbic acid fortification level needed. It could be also of interest to investigate whether other antioxidants can increase folate stability.

ABBREVIATIONS USED

5-CH_3-H_4folate, 5-methyltetrahydrofolic acid; 5-CHO-H_4folate, 5-formyltetrahydrofolic acid; 10-CHO-H_4folate, 10-formyltetrahydrofolic acid; C, concentration of 5-CH_3-H_4folate at treatment time t; C_0, concentration of 5-CH_3-H_4folate at time = 0; E_a, activation energy (kJ·mol^{-1}); H_4folate, 5,6,7,8-tetrahydrofolic acid; k, degradation rate constant (min^{-1}); V_a, activation volume (cm^3·mol^{-1}); RP-HPLC, reverse phase high-pressure liquid chromatography.

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LITERATURE CITED

(1) Hughes, J.; Buttriss, J. An update on folates and folic acid: contribution of MAFF-funded research. *Br. Nutr. Found. Nutr. Bull.* **2000**, *25*, 113−124.
(2) Kelly, P.; McPartlin, J.; Goggins, M.; Weir, D. G.; Scott, J. M. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. *Am. J. Clin. Nutr.* **1997**, *65*, 1790−1795.
(3) Rotherberg, S. P. Increasing the dietary intake of folate: pros and cons. *Semin. Hematol.* **1999**, *36*, 65−74.
(4) Scott, J. M. Folate and vitamin B12. *Proc. Nutr. Soc.* **1999**, *58*, 441−448.
(5) Flynn, C.; Enright, H. Fortification of foods with folic acid. Correspondence to the editor. *N. Engl. J. Med.* **2000**, *343*, 970−972.

(6) Konings, E. J. M.; Roomans, H. H. S.; Dorant, E.; Goldbohm, R. A.; Sari, W. H. M.; Van den Brandt, A. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *Am. J. Clin. Nutr*. **2001**, *73*, 766−776.
(7) Scott, J.; Rébeille, F.; Fletcher, J. Review: folic acid and folates: the feasibility for nutritional enhancement in plant foods. *J. Sci. Food Agric*. **2000**, *80*, 795−824.
(8) Paine-Wilson, B.; Chen, T.-S. Thermal destruction of folacin: effect of pH and buffer ions. *J. Food Sci.* **1979**, *44*, 717−722.
(9) Mnkeni, A. P.; Beveridge, T. Thermal destruction of pteroylglutamic acid in buffer and model food systems. *J. Food Sci*. **1982**, *47*, 2038−2041, 2063.
(10) Nguyen, M. T.; Indrawati; Hendrickx, M. Model studies on the stability of folic acid and 5-methyltetrahydrofolic acid degradation during thermal treatment in combination with high hydrostatic pressure. *J. Agric. Food Chem.* **2003**, *51*, 3352−3357.
(11) Cooper, R. G.; Chen, T.-S.; King, M. A. Thermal destruction of folacin in microwave and conventional heating. *J. Am. Diet. Assoc*. **1978**, *73*, 406−410.
(12) Chen, T.-S.; Cooper, R. G. Thermal destruction of folacin: effect of ascorbic acid, oxygen and temperature. *J. Food Sci.* **1979**, *44*, 713−716.
(13) Barrett, D. M.; Lund, D. B. Effect of oxygen on thermal degradation of 5-methyl-5,6,7,8-tetrahydrofolic acid. *J. Food Sci.* **1989**, *54*, 146−149.
(14) Ruddick, J. E.; Vanderstoep, J.; Richards, J. F. Kinetics of thermal degradation of methyltetrahydrofolic acid. *J. Food Sci.* **1980**, *45*, 1019−1022.
(15) Viberg, U.; Jägerstad, M.; Öste, R.; Sjöholm, I. Thermal processing of 5-methyltetrahydrofolic acid in the UHT region in the presence of oxygen. *Food Chem*. **1997**, *59*, 381−386.
(16) Mnkeni, A. P.; Beveridge, T. Thermal destruction of 5-methyltetrahydrofolic acid in buffer and model food systems. *J. Food Sci*. **1983**, *48*, 595−599.
(17) Konings, J. M. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. *J. AOAC Int*. **1999**, *82*, 119−127.
(18) Neuman, R. C.; Kauzmann, W.; Zipp, A. Pressure dependence of weak acid ionization in aqueous buffers. *J. Phys. Chem*. **1973**, *77*, 2687−2691.
(19) Kitamura, Y.; Itoh, T. Reaction volume of protonic ionization for buffering agents. Prediction of pressure dependence of pH and pOH. *J. Solution Chem*. **1987**, *16*, 715−725.
(20) Hayert, M.; Perrier-Cornet, J. M.; Gervais, P. A simple method for measuring the pH of acid solutions under high pressure. *J. Phys. Chem*. **1999**, *103*, 1785−1789.
(21) Melse-Boonstra, A.; Verhoef, P.; Konings, E. J. M.; Van Dusseldorp, M.; Matser, A.; Hollman, P. C. H.; Meyboom, S.; Kok, F. J.; West, C. E. Influence of processing on total, monoglutamate and polyglutamate folate contents of leeks, cauliflower, and green beans. *J. Agric. Food Chem.* **2002**, *50*, 3473−3478.

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