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Pressure and Temperature Stability of 5-Methyltetrahydrofolic Acid: A Kinetic Study

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Detailed kinetic studies of [6*S*] and [6*RS*] 5-methyltetrahydrofolic acid (5-CH_3-H_4folate) degradation during thermal (from 60 to 90 °C) and high pressure/thermal (from 30 to 45 °C; from 200 to 700 MPa) treatments were carried out. The results confirmed that the temperature and pressure induced degradation kinetics of [6*S*] 5-CH_3-H_4folate were identical (within 95% confidence interval) with those of [6*RS*] 5-CH_3-H_4folate. Under equal processing conditions, the estimated degradation rate constants (k), activation energy (E_a), and activation volume (V_a) values of [6*S*] and [6*RS*] 5-CH_3-H_4folate were the same (95% confidence interval). The modified thermodynamic model proposed by Nguyen and co-workers (*J. Agric. Food Chem.* **2003**, *51*, 3352−3357) to describe the pressure and temperature dependence of the rate constant for folate degradation was reevaluated.

KEYWORDS: 5-CH_3-H_4folate; kinetics; degradation; temperature; pressure

INTRODUCTION

Folates (i.e., folic acid and its derivates) play an important role in the metabolism of amino acids and nucleic acids (*1*−*3*). An inadequate folate intake can cause birth disorders such as neural tube defects (spina bifida), cancers, and several diseases, for example, megaloblastic anemia, cardiovascular disease, and Alzheimer's disease (*4*−*7*). Therefore, a sufficient daily folate intake is important, and it can be achieved either by fortification or by increasing the consumption of food products such as leafy green vegetables, fruits, liver, etc,, that contain endogenous folates.

In some countries, for example, in the United States, food fortification using synthetic folic acid is a common practice, and it has been proven that the fortification of grain products with folic acid was associated with a substantial improvement in folate status in a population of middle-aged and elderly adults. However, folic acid can mask the hematological abnormalities of vitamin B_{12} deficiency while the neurological complications remain in progress (when folic acid intake is >1 mg/day) (*8*−*12*).

Recently, the commercial bioactive and natural form of [6*S*] 5-methyltetrahydrofolic acid (i.e., [6*S*] 5-CH_3-H_4folate) has successfully been synthesized. In theory, this folate derivate does not encounter the masking problem of vitamin B_{12} deficiency. Hereto, the use of [6*S*] 5-CH_3-H_4folate for food fortification is being explored to replace folic acid (*12*). The bioavailabilities of [6*S*] 5-CH_3-H_4folate and folic acid are found to be equivalent in men (*13*); however, the stability of 5-CH_3-H_4folate is lower than that of folic acid. Up to now, detailed kinetic information regarding [6*S*] 5-CH_3-H_4folate stability is still limited, whereas the degradation kinetics of the racemic enantiomer mixtures of 5-CH_3-H_4folate, that is, [6*RS*] 5-CH_3-H_4folate have been studied (*14*−*20*). Because the information on the latter is abundant in the literature, it can be questioned whether the stability of [6*S*] could be scientifically claimed to be identical with [6*RS*] stability. Empirically, enantiomers have identical physical and chemical properties except their optical activity.

The purpose of this investigation was to study, on a kinetic basis, the pressure and temperature stability of 5-CH_3-H_4folate and to verify whether the degradation kinetics of [6*S*] 5-CH_3-H_4folate was identical with that of [6*RS*] 5-CH_3-H_4folate. In this study, both [6*S*] and [6*RS*] were treated under the same intrinsic (i.e., pH, buffer type, ionic strength, concentrations of oxygen and folate) and extrinsic (i.e., pressure, temperature) conditions to allow an adequate comparison. Second, the modified thermodynamic model of Hawley (*21*) proposed by Nguyen and co-workers (*18*) to describe the pressure and temperature dependence of the degradation rate constants of 5-CH_3-H_4folate was reevaluated for both [6*S*] and [6*RS*]. Such kinetic information is indispensable for the design, evaluation, assessment, and optimization of processes.

MATERIALS AND METHODS

Sample Preparation. In this investigation, [6*S*] and [6*RS*] 5-CH_3-H_4folates were obtained, respectively, from Merck Eprova (Schaffhausen, Switzerland) and 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 a sodium borate solution (0.05 M, pH 9.22) containing 0.4% (v/v) β-mercaptoethanol (i.e., to stabilize the folate during storage). After flushing with nitrogen, the stock

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solution was divided in 0.5 mL portions and immediately stored at −80 °C until further use. The stock solution was stable for 12 weeks at −80 °C. The working solution (0.2 μg/mL containing 0.8 ppm of β-mercaptoethanol and 8.11 ppm of dissolved oxygen) was prepared on the day of use by diluting the stock solution with phosphate buffer (0.1 M, pH 7). In this study, 0.8 ppm of β-mercaptoethanol was used because it stabilizes [6*S*] 5-CH_3-H_4folate during experiments for maximally 3 h at 4 °C to obtain highly reproducible kinetic data (data not shown). As a consequence, the presence of β-mercaptoethanol in the sample must be taken into account when the obtained kinetic data are interpreted because it slightly increases the stability of folate during processing (*22*). 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 the procedure of Konings (*23*). In this study, the ratio of 5-CH_3-H_4folate/dihydro derivates of the working solution had to be above 3.3 to eliminate the contamination of dihydro derivates. All organic solvents were obtained from Merck (Darmstadt, Germany), and bi-distilled water was used. During the experiments, samples were covered with aluminum foil to avoid direct contact with air at all times and stored at 4 °C prior to the treatments and the HPLC measurements.

Thermal Treatment. The working solution was filled in capillary tubes (Hirschmann, $d = 1.5$ mm and $L = 150$ mm) under vacuum. The samples were treated in a water bath (60−90 °C) (Y14 Grant Instruments Ltd., Cambridge, U.K.) during different preset time intervals under isothermal conditions and, subsequently, cooled in an ice bath after their withdrawal from the water bath to stop the heating effect. The residual concentration of 5-CH_3-H_4folate was measured using HPLC as soon as possible after the treatment (<3 h of storage in an ice bath).

Combined Pressure and Temperature Treatment. The working solution was filled in polyethylene flexible microtubes (Elkay, 500 μL) and, afterward, vacuum-packed (up to 0.11 mbar) using polyethylene bags. The samples were treated in laboratory pilot scale, multivessel high-pressure equipment (Resato, Roden, The Netherlands) consisting of eight thermostated 8 mL pressure vessels at pressures up to 700 MPa combined with temperatures between 30 and 45 °C. An oil/glycol mixture (TR15, Resato) was used as the pressure transmitting fluid. The pressure was built up manually at a constant rate of 100−125 MPa/min. In this investigation, an equilibration period of 2 min was used, allowing the temperature inside the vessels to evolve to the desired value. After the equilibration time, the first pressure vessel was decompressed and the residual folate concentration of the corresponding sample was considered as the folate concentration at $t = 0$ (i.e., C_0 under isobaric isothermal condition). The other vessels were then decompressed as a function of time. After withdrawal, the samples were stored in an ice bath (maximum 3 h) until the residual folate concentration was measured. Several authors (*24*, *25*) mentioned that the pH of the phosphate buffer decreases by ∼0.3 unit per 100 MPa of pressure increase. Therefore, the pH of phosphate buffer could be <7 during the pressure treatment.

RP-HPLC Assay To Identify and Quantify 5-CH_3-H_4folate. To identify and quantify 5-CH_3-H_4folate derivates in the samples, a reverse phase HPLC analysis (AKTA purifier, Amersham Biosciences, Uppsala, Sweden) using a PrevailC18 column (250 mm × 4.6 mm, 5 μm particle size, Alltech, Deerfield, IL) and a fluorescence detector ($\lambda_{em} = 359$ nm and $\lambda_{ex} = 280$ nm, RF-10Axl, Shimadzu, Kyoto, Japan) was used. The HPLC analysis was carried out following the modified procedure of Konings (*23*). The column was thermostated (Spark Mistral, Emmen, The Netherlands) at 25 °C and isocratically equilibrated with a mixture of acetonitrile (5%) and phosphate buffer (330 mM; pH 2.15) at a flow rate of 1 mL/min for 3 min before sample injection (100 μL) and for 4 min after the injection. A linear gradient from 5 to 17% acetonitrile was applied 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. The folate concentration was calculated on the basis of the peak area and peak height as compared to the standard solutions using Unicorn 4.0 data analysis software (Amersham Biosciences). The correlation coefficients (r^2) of the standard curves in this study were at least 0.98.

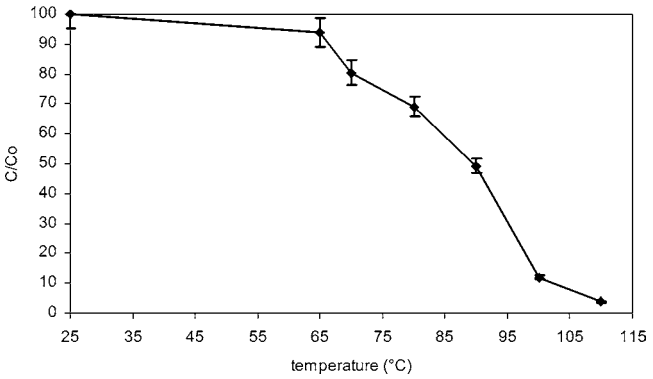

Figure 1. Stability of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) in phosphate buffer (0.1 M, pH 7, 0.8 ppm of β-mercaptoethanol) at various temperatures during thermal treatment for 15 min.

Data Analysis. Previous studies (*14*−*16*, *18*−*20*) have shown that 5-CH_3-H_4folate degradation during thermal and high-pressure treatments in an excess of oxygen can be described by "pseudo"-first-order reaction kinetics (the term "first-order kinetics" is used in this paper). Hereto, in this study, eq 1 was used to estimate the degradation rate constants of 5-CH_3-H_4folate

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

where 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 the predicted slope of the linear regression analysis when the natural logarithm of the residual folate concentration is plotted as a function of treatment time.

To estimate the temperature and pressure dependencies of the k values, the linearized Arrhenius (eq 2) and Eyring equations (eq 3) were respectively used. Activation energy (E_a) and activation volume (V_a) values were calculated using linear regression analysis 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, respectively

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

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

where k_{refT} is the degradation rate constant at reference temperature T_{ref}, k_{refP} is the degradation rate constant at reference pressure P_{ref}, and R is the universal gas constant (8.314 J mol^{-1} K^{-1}).

RESULTS AND DISCUSSION

Kinetics of 5-CH_3-H_4folate Degradation during Thermal Treatment. The [6*S*] 5-CH_3-H_4folate stability was initially screened at various temperatures to define the temperature range for detailed degradation kinetic studies. **Figure 1** illustrates the folate retention after thermal treatments for 15 min at different constant temperatures. It can be noted that folate degradation already occurred around 65 °C. Hence, a detailed kinetic study of [6*S*] 5-CH_3-H_4folate degradation was investigated in the temperature range between 60 and 90 °C.

In this study, the degradation kinetics of [6*RS*] 5-CH_3-H_4folate and [6*S*] 5-CH_3-H_4folate were studied simultaneously under the same intrinsic and extrinsic conditions. To identify a primary kinetic model describing the evolution of [6*S*] 5-CH_3-H_4folate concentration as a function of treatment time, the kinetic approach already described for [6*RS*] 5-CH_3-H_4folate degradation was initially used. As reported by several authors

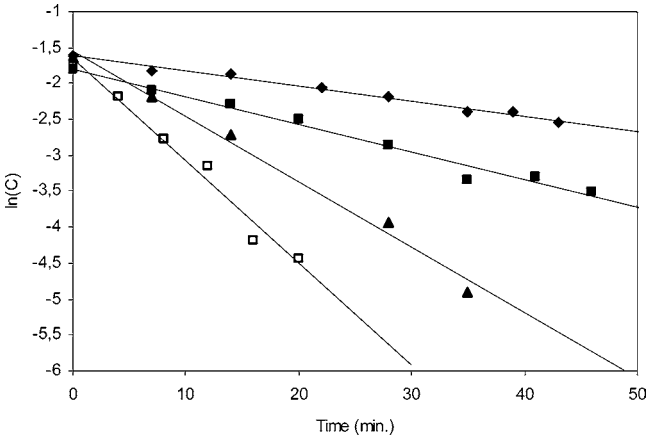

Figure 2. Degradation kinetics of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) in phosphate buffer (0.1 M, pH 7, 0.8 ppm of β-mercaptoethanol) under isothermal conditions: (◆) 60 °C; (■) 70 °C; (▲) 80 °C; (□) 90 °C.

Table 1. Estimated *k* Values and E_a Values of [6*S*] and [6*RS*] 5-CH_3-H_4folate (0.2 μG/mL) Degradation in Phosphate Buffer (0.1 M; pH 7[a]) under Isothermal Conditions

	k values ($\times 10^{-2}$ min^{-1})	
temp (°C)	[6*S*] 5-CH_3-H_4folate	[6*RS*] 5-CH_3-H_4folate
60	1.95 ± 0.17[b] ($r^2 = 0.96$)	2.15 ± 0.08 ($r^2 = 0.99$)
70	4.34 ± 0.42 ($r^2 = 0.96$)	4.48 ± 0.35 ($r^2 = 0.98$)
80	9.34 ± 0.74 ($r^2 = 0.99$)	8.04 ± 0.45 ($r^2 = 0.99$)
90	15.02 ± 18.0 ($r^2 = 0.94$)	18.03 ± 1.59 ($r^2 = 0.98$)
E_a (kJ mol^{-1})	69.34 ± 4.33 ($r^2 = 0.96$)	69.97 ± 3.57 ($r^2 = 0.99$)

[a] Containing 0.8 ppm of β-mercaptoethanol. [b] Standard error of regression.

(*14*−*16*, *18*, *20*, *28*, *29*), the [6*RS*] 5-CH_3-H_4folate degradation followed first-order kinetics. By plotting the natural logarithm of the residual folate concentration as a function of treatment time, it was confirmed that the [6*S*] 5-CH_3-H_4folate degradation also followed first-order kinetics as illustrated in **Figure 2**. On the basis of the estimated kinetic parameters (**Table 1**), it can be concluded that the estimated *k* and E_a values of both [6*S*] and [6*RS*] (**Table 1**) are of the same order of magnitude (95% confidence interval).

As compared to the results previously obtained by Viberg et al. (*16*) and Nguyen et al. (*18*), the estimated E_a value of [6*RS*] 5-CH_3-H_4folate in this study was lower. Viberg et al. (*16*) did not report the use of any antioxidant in the buffer system even though a higher folate concentration (2.6 μg/mL) and a lower oxygen concentration (6.8 ppm) were applied. As compared to the results of Nguyen et al. (*18*), this study used the same ratio of folate and β-mercaptoethanol (1:4) but a different ratio between folate and dissolved oxygen concentrations. This calls for further studies on the effect of the ratio of oxygen to folate and antioxidant on the folate degradation kinetics.

Kinetics of 5-CH_3-H_4folate Degradation during Pressure and Temperature Treatment. The pressure stability of [6*S*] 5-CH_3-H_4folate was initially screened by treating the samples for 15 min at constant temperature combined with different pressure levels. **Figure 3** shows that (i) folate retention is decreased by increasing both pressure and temperature, (ii) pressure has a synergistic effect on the thermal degradation of folate (i.e., a faster degradation was obtained when pressure was applied to the system at a given temperature), and (iii) a remarkable pressure degradation occurs at temperatures beyond 30 °C. Hence, the detailed kinetics of [6*S*] 5-CH_3-H_4folate degradation was studied in the temperature range between 30 and 45 °C combined with pressures from 200 to 700 MPa.

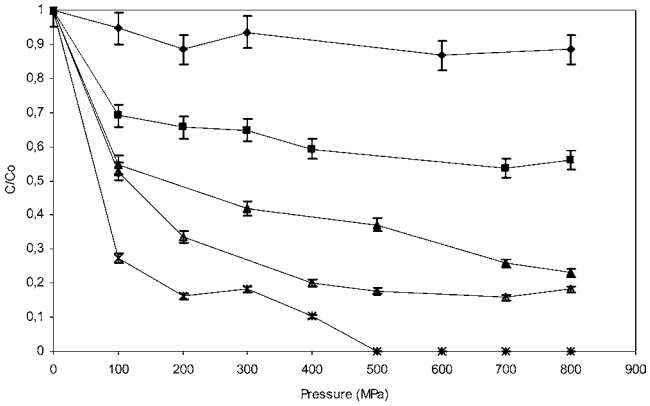

Figure 3. Pressure and temperature stability of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) in phosphate buffer (0.1 M, pH 7, 0.8 ppm of β-mercaptoethanol) during treatments of 15 min: (◆) 20 °C; (■) 30 °C; (▲) 40 °C; (△) 45 °C; (∗) 50 °C.

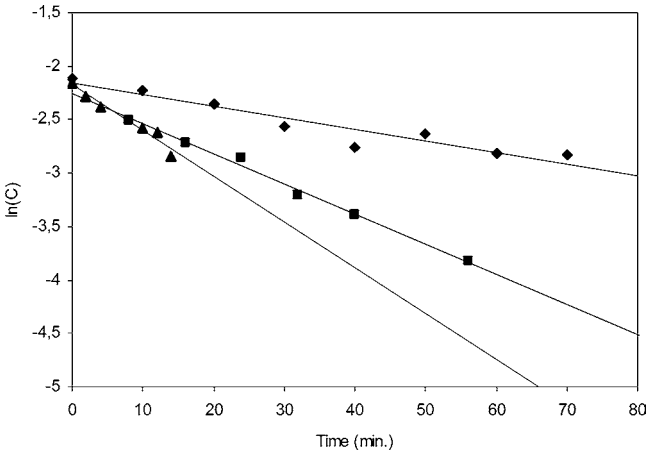

Figure 4. Degradation kinetics of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) in phosphate buffer (0.1 M, pH 7, 0.8 ppm of β-mercaptoethanol) at 400 MPa and various temperatures: (◆) 30 °C; (■) 35 °C; (▲) 40 °C.

As previously noted in the thermal experiments, the kinetics of [6*S*] 5-CH_3-H_4folate degradation were similar to that of [6*RS*] 5-CH_3-H_4folate. Therefore, the same modeling approaches as already described for [6*RS*] 5-CH_3-H_4folate (*18*, *20*) were applied to describe the degradation kinetics of [6*S*] 5-CH_3-H_4-folate during combined pressure and temperature treatments. First-order kinetics was used to describe the evolution of residual folate concentration as a function of treatment time at defined constant pressure and temperature combinations. Arrhenius (eq 2) and Eyring (eq 3) equations were used as secondary models to describe, respectively, the temperature and pressure dependencies of the *k* values. In this study, the kinetics of [6*RS*] 5-CH_3-H_4folate degradation during pressure treatment were investigated under the same conditions as [6*S*] 5-CH_3-H_4folate to allow mutual comparison.

In the whole pressure and temperature range studied, the [6*S*] 5-CH_3-H_4folate degradation could be described by first-order kinetics (**Figure 4**). In the pressure (from 300 to 500 MPa) and temperature (from 30 to 40 °C) areas studied, the estimated *k* values of [6*S*] and [6*RS*] 5-CH_3-H_4folate were situated in the same range (95% confidence interval) (**Table 2**). It can be noted from **Table 2** that the *k* values increase either with increasing pressure at constant temperature or with increasing temperature at constant pressure.

Table 2. Estimated *k* Values of [6*S*] and [6*RS*] 5-CH_3-H_4folate (0.2 μg/mL) Degradation in Phosphate Buffer (0.1 M; pH 7[a]) during Isobaric Isothermal Conditions

		k value ($\times 10^{-3}$ min^{-1}) at temperature of			
pressure (MPa)	5-CH_3-H_4folate	30 °C	35 °C	40 °C	45 °C
200	[6*S*]	5.88 ± 0.64[b] (r^2 = 0.97)	16.76 ± 1.64 (r^2 = 0.95)	35.65 ± 3.11 (r^2 = 0.98)	45.58 ± 8.99 (r^2 = 0.84)
	[6*RS*]	nd[c]	nd	17.42 ± 1.93 (r^2 = 0.95)	nd
300	[6*S*]	9.09 ± 1.36 (r^2 = 0.94)	22.32 ± 3.18 (r^2 = 0.92)	42.66 ± 5.21 (r^2 = 0.94)	49.30 ± 1.81 (r^2 = 0.99)
	[6*RS*]	9.82 ± 0.74 (r^2 = 0.98)	18.76 ± 0.85 (r^2 = 0.99)	39.36 ± 3.64 (r^2 = 0.97)	nd
400	[6*S*]	11.79 ± 1.85 (r^2 = 0.91)	27.64 ± 1.10 (r^2 = 0.99)	51.81 ± 7.30 (r^2 = 0.93)	nd
	[6*RS*]	11.58 ± 1.68 (r^2 = 0.95)	22.72 ± 1.19 (r^2 = 0.99)	46.19 ± 3.95 (r^2 = 0.97)	nd
500	[6*S*]	15.73 ± 3.47 (r^2 = 0.84)	32.34 ± 7.43 (r^2 = 0.86)	59.16 ± 9.27 (r^2 = 0.93)	nd
	[6*RS*]	15.45 ± 1.54 (r^2 = 0.95)	26.57 ± 2.35 (r^2 = 0.98)	59.92 ± 10.85 (r^2 = 0.94)	nd
600	[6*S*]	15.88 ± 1.70 (r^2 = 0.95)	nd	nd	nd
	[6*RS*]	nd	nd	nd	nd
700	[6*S*]	18.27 ± 1.34 (r^2 = 0.98)	nd	nd	nd
	[6*RS*]	nd	nd	nd	nd

[a] Containing 0.8 ppm of β mercaptoethanol. [b] Standard error of regression. [c] Not determined.

Table 3. Estimated E_a and V_a Values of [6*S*] and [6*RS*] 5-CH_3-H_4folate (0.2 μg/mL) Degradation in Phosphate Buffer (0.1 M; pH 7[a])

Estimated E_a Values (kJ mol^{-1})		
pressure (MPa)	[6*S*] 5-CH_3-H_4folate	[6*RS*] 5-CH_3-H_4folate
0.1	72.08 ± 10.54[b] (r^2 = 0.96)	59.19 ± 7.81 (r^2 = 0.97)
200	110.93 ± 19.33 (r^2 = 0.94)	nd[c]
300	92.02 ± 18.38 (r^2 = 0.93)	109.42 ± 5.29 (r^2 = 0.99)
400	116.81 ± 9.09 (r^2 = 0.99)	109.06 ± 2.64 (r^2 = 0.99)
500	104.49 ± 4.34 (r^2 = 0.99)	106.75 ± 13.34 (r^2 = 0.98)
Estimated V_a Values[d] (cm mol^{-1})		
temperature (°C)	[6*S*] 5-CH_3-H_4folate	[6*RS*] 5-CH_3-H_4folate
30	–5.49 ± 0.87[b] (r^2 = 0.91)	–5.71 ± 0.90 (r^2 = 0.98)
35	–5.60 ± 0.53 (r^2 = 0.98)	–4.46 ± 0.26 (r^2 = 0.99)
40	–4.46 ± 0.24 (r^2 = 0.99)	–5.47 ± 0.75 (r^2 = 0.98)

[a] Containing 0.8 ppm of β-mercaptoethanol. [b] Standard error of regression. [c] Not determined. [d] In the pressure range between 300 and 500 MPa.

To describe the temperature and pressure dependencies of the *k* values, the linearized Arrhenius and Eyring equations were, respectively, used. It can be noted from **Table 3** that (i) the estimated E_a values of both [6*S*] and [6*RS*] are situated in the same order of magnitude (95% confidence interval) but higher than the results obtained by Nguyen and co-workers (*18*); (ii) the estimated E_a value at ambient pressure (0.1 MPa) is lower than that at elevated pressure levels, indicating that the *k* values of folate degradation under pressure were more sensitive to temperature changes than were those at 0.1MPa; (iii) the estimated V_a values have a negative sign, indicating that folate degradation is enhanced by a pressure increase; and (iv) the estimated V_a values of both [6*S*] and [6*RS*] are situated in the same order of magnitude (95% confidence interval) and similar to the results obtained by Nguyen and co-workers (*18*).

The estimated *k* values were interpolated as a function of pressure and temperature to depict the iso rate pressure temperature contour diagram of [6*S*] 5-CH_3-H_4folate degradation (**Figure 5**). It is clearly seen that pressure has a synergistic effect on the thermal degradation of folate as also previously noted (*18*, *20*).

Pressure and Temperature Dependence of the Rate Constants for 5-CH_3-H_4folate Degradation. To describe the combined pressure and temperature dependence of the rate constants for [6*S*] 5-CH_3-H_4folate degradation, the same modeling approach as suggested for [6*RS*] 5-CH_3-H_4folate based on the thermodynamic model of Hawley (*22*) (eq 4) was used.

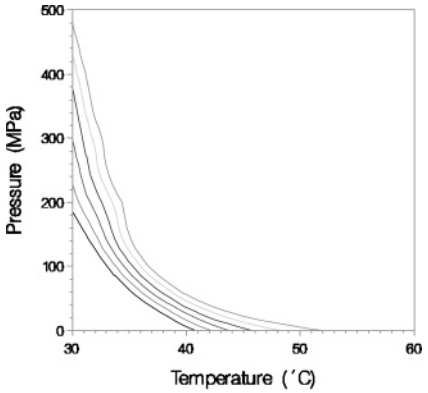

Figure 5. Pressure temperature iso rate contour diagram of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) degradation in phosphate buffer (0.1 M, pH 7, 0.8 ppm of β-mercaptoethanol). The inner and outer lines indicate *k* values equal to 0.005 and 0.015 min^{-1}, respectively.

$$\ln(k) = \ln(k_{ref}) - \frac{\Delta\kappa^{\ddagger}}{2R_gT}(P - P_{ref})^2 - \frac{\Delta V_0^{\ddagger}}{R_gT}(P - P_{ref}) + \frac{\Delta S_0^{\ddagger}}{R_gT}(T - T_{ref}) + \frac{\Delta C_p^{\ddagger}}{R_gT}\left[T\left[\ln\left(\frac{T}{T_{ref}}\right) - 1\right] + T_{ref}\right] + \frac{\Delta\zeta^{\ddagger}}{R_gT}(P - P_{ref})(T - T_{ref}) \quad (4)$$

In this equation, $\Delta\kappa^{\ddagger}$ is the compressibility factor (cm^6 J^{-1} mol^{-1}), $\Delta V_0^{\ddagger}$ is the volume change (cm^3 mol^{-1}), $\Delta S_0^{\ddagger}$ is the entropy change (J mol^{-1} K^{-1}), $\Delta\zeta^{\ddagger}$ is the thermal expansibility absolute (cm^3 mol^{-1} K^{-1}), $\Delta C_p^{\ddagger}$ is the heat capacity (J mol^{-1} K^{-1}), and R_g is the universal gas constant (8.31577 cm^3 MPa K^{-1} mol^{-1}). When the whole data set of [6*S*] 5-CH_3-H_4folate degradation was analyzed using eq 4, the term $\Delta\zeta^{\ddagger}$ became redundant, which was indicated by a large standard error (>100%). This was also previously observed by Nguyen and co-workers (*18*) for the [6*RS*] form. Hence, the term $\Delta\zeta^{\ddagger}$ was omitted and a reduced version of eq 4 (eq 5) was applied.

$$\ln(k) = \ln(k_{ref}) - \frac{\Delta\kappa^{\ddagger}}{2R_gT}(P - P_{ref})^2 - \frac{\Delta V_0^{\ddagger}}{R_gT}(P - P_{ref}) + \frac{\Delta S_0^{\ddagger}}{R_gT}(T - T_{ref}) + \frac{\Delta C_p^{\ddagger}}{R_gT}\left[T\left[\ln\left(\frac{T}{T_{ref}}\right) - 1\right] + T_{ref}\right] \quad (5)$$

Table 4. Estimated Values of Model Parameters for Combined Pressure and Temperature Degradation of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) in Phosphate Buffer (0.1 M; pH 7) at P_{ref} = 400MPa and T_{ref} = 40 °C

kinetic parameter	[6*S*] 5-CH_3-H_4folate[a] (eq 5)	[6*RS*] 5-CH_3-H_4folate[b] (eq 5)	[6*RS*] 5-CH_3-H_4folate[c] (eq 5)	[6*S*] 5-CH_3-H_4folate[a] (eq 7)
ΔV_0 (cm mol^{-1})	–5.91 ± 1.67[d]	–8.86 ± 0.51[d]	–8.86 ± 0.51[d]	
ΔS_0 (J mol^{-1} K^{-1})	246.80 ± 38.68	297.90 ± 9.50	249.80 ± 8.35	
$\Delta\kappa$ ($\times 10^{-2}$ cm^6 J^{-1} $mole^{-1}$)	3.76 ± 1.33	2.12 ± 0.37	2.12 ± 0.37	
ΔC_p (J mol^{-1} K^{-1})	–881.60 ± 572.30	–1258.60 ± 201.5	–1258.60 ± 201.5	
k_{ref} ($\times 10^{-3}$ min^{-1})	43.30 ± 6.47	9.62 ± 0.62	2.71 ± 0.17	43.20 ± 6.25[d]
a ($\times 10^{-3}$ MPa^{-1})				3.42 ± 0.71
b ($\times 10^{-2}$ K^{-1})				12.48 ± 2.03
c ($\times 10^{-4}$ MPa^{-1} K^{-1})				1.81 ± 0.48
corrected r^2 [e]	0.991	0.996	0.996	0.991
SD[f]	0.359	0.273	0.273	0.347

[a] Containing 0.8 ppm of β-mercaptoethanol. [b] Reanalyzed data of Nguyen et al. (*18*) using T_{ref} = 40 °C and the system containing 40 ppm of β-mercaptoethanol. [c] Cited from Nguyen et al. (*18*) using T_{ref} = 50 °C. [d] Asymptotic standard error. [e] Corrected $r^2 = [1 - ((m-1)(1 - (SSQ_{regression}/SSQ_{total}))/(m-j)]$, where SSQ is the sum of square. [f] Standard deviation of model.

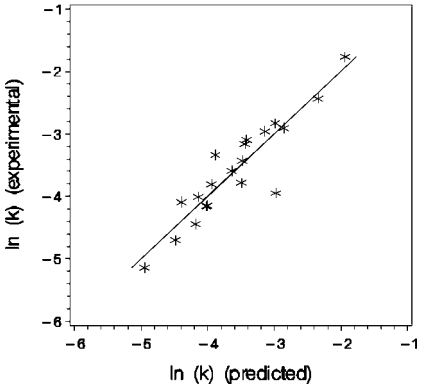

Figure 6. Relationship between the natural logarithm of the experimentally determined *k* values and the natural logarithm of the *k* values predicted using eq 5.

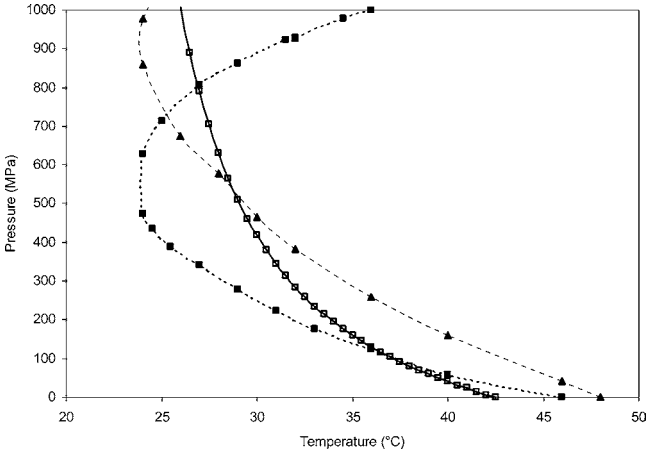

Figure 7. Predicted pressure and temperature iso rate (k = 0.01 min^{-1}) contour diagram of [6*S*] 5-CH_3-H_4folate (0.2 μg/mL) based on eq 6 (■) and eq 7 (□) compared to [6*RS*] 5-CH_3-H_4folate (▲, 10 μg/mL) based on eq 6 by reanalyzing data from Nguyen et al. (*18*) (T_{ref} = 40 °C and P_{ref} = 400 MPa).

The concomitant kinetic parameters of eq 5 were estimated using nonlinear regression analysis, and the estimated values are summarized in **Table 4**. In this study, the corrected regression coefficient (corrected r^2) was used to measure the accuracy of the model fitting to the data because the statistical parameters largely depend on the model structure and on the number of observations (m) and parameters (j). In **Figure 6**, a good agreement between the predicted ln(k) and the experimentally determined ln(k) can clearly be observed. In the experimental pressure temperature range, no trend in residuals (differences between predicted and experimental values) was noted. From **Table 4**, it is observed that the estimated kinetic parameters for [6*S*] 5-CH_3-H_4folate degradation were similar to those for [6*RS*] 5-CH_3-H_4folate degradation (*18*), except for the k_{ref} value. The estimated k_{ref} value of [6*S*] 5-CH_3-H_4folate degradation (T_{ref} = 40 °C and P_{ref} = 400 MPa) obtained in this study was higher (i.e., less stable) than that of [6*RS*] 5-CH_3-H_4folate [reanalyzed data from Nguyen et al. (*18*)]. It was presumably caused by lower concentrations in both folate and β-mercaptoethanol as compared to those in Nguyen et al. (*18*). The standard error of $\Delta C_p^{\ddagger}$ was almost >50% of its predicted parameter value. It could be that evaluation of the unknown kinetic parameters of eq 5, by means of computerized curve-fitting, would require additional data from large pressure and temperature intervals (500 MPa and 100 °C) (*31*), whereas the pressure and temperature range in this study was quite limited.

Omitting $\Delta\zeta^{\ddagger}$ implies that V_a is independent of temperature and pressure; however, this implication is not relevant for other parameters such as $\Delta\kappa^{\ddagger}$ and $\Delta V_0^{\ddagger}$. Because the parameters of eq 5 have no longer thermodynamic meanings, it is better to symbolize them as dummies and rewrite the equation as an empirical one (eq 6).

$$\ln(k) = \ln(k_{ref}) - \frac{A}{T}(P - P_{ref})^2 - \frac{B}{T}(P - P_{ref}) + \frac{C}{T}(T - T_{ref}) + \frac{D}{T}\left[T\left[\ln\left(\frac{T}{T_{ref}}\right) - 1\right] + T_{ref}\right] \quad (6)$$

Kinetic information is indispensable for the design, optimization, and assessment of high-pressure processing. Hence, it was verified whether eq 6 was suitable to predict the pressure and temperature dependence of the k values outside the pressure and temperature area studied. By inserting the estimated values into the concomitant kinetic parameters of eq 6, the pressure and temperature combinations resulting in the same k value (e.g., 0.010 min^{-1}) are illustrated in **Figure 7**. As depicted in **Figure 7**, eq 6 was adequate to predict the k value in the pressure and temperature range studied, but not outside the range because the contour diagram turned to an elliptical curve.

This elliptical curvature can be avoided by eliminating the quadratic terms from the equation. Referring to eq 4, this means that the terms $\Delta\kappa^{\ddagger}$ and $\Delta C_p^{\ddagger}$ must be omitted. The thermodynamic implication of this reduction is that E_a and V_a values are

not dependent, respectively, on temperature [i.e., $\Delta C_p^{\ddagger} = 0$ when $E_a \neq f(T)$] and on pressure [i.e., $\Delta\kappa^{\ddagger} = 0$ when $V_a \neq f(P)$]. This implication can be defended by the fact that the Arrhenius and Eyring equations are adequate to describe the temperature and pressure dependencies of k values in certain pressure and temperature areas studied (**Table 3**). By eliminating the quadratic terms, eq 4 was reduced and rewritten using dummies as described in eq 7.

$$\ln(k) = a(P - P_{ref}) + b(T - T_{ref}) + c(P - P_{ref})(T - T_{ref}) + \ln(k_{ref}) \quad (7)$$

The estimated values of the concomitant parameters (eq 7) are summarized in **Table 4**. Indeed, the elliptical form at higher pressure could be avoided by inserting the predicted values in eq 7 (**Figure 7**). The corrected r^2 and standard deviation of eq 7 were similar to those of eq 5; however, this model overestimated the predicted pressure and temperature combinations in the pressure and temperature area studied (compare the iso rate contour diagrams in **Figures 5** and **7**). On the basis of the whole simulation (**Figure 7**), eq 5 (or empirically described as eq 6) still gave the best fit for folate degradation, particularly in the pressure and temperature area studied.

Conclusions. On the basis of the kinetic data obtained in this study, we confirmed that the thermal and pressure degradation kinetics of [6*S*] and [6*RS*] 5-CH_3-H_4folate were comparable. Under identical experimental conditions and setup, the temperature and pressure stability of the single enantiomer [6*S*] was identical (within 95% confidence interval) with that of the racemic mixture [6*RS*].

Up to now, no general kinetic model is available to describe the combined pressure and temperature dependence of the k values for nutrient degradation. There is still a lack of information defining the detailed degradation mechanism of folate (e.g., 5-CH_3-H_4folate). This information can be used as a fundamental basis to evaluate which approaches have to be chosen in designing an appropriate kinetic model based on the chemical reaction scheme. Hereto, further investigations in this area are greatly needed.

Such a kinetic approach is valuable not only for high-pressure processing optimization and assessment in industrial applications but also for experimental design at laboratory scale. On the basis of the kinetic data obtained, the experiments can be more efficiently set up, especially for experiments in which extraction, isolation, and assay procedures are laborious and time-consuming such as folate.

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