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Implications of β-Mercaptoethanol in Relation to Folate Stability and to Determination of Folate Degradation Kinetics during Processing: A Case Study on [6*S*]-5-Methyltetrahydrofolic Acid

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The effect of β-mercaptoethanol (0−2%) addition to thermally and/or pressure-treated samples on [6*S*]-5-methyltetrahydrofolate was studied. Degradation of [6*S*]-5-methyltetrahydrofolate during both thermal and pressure treatments was mainly caused by oxidation, and the oxidized folates could be partly/completely reduced by β-mercaptoethanol. The addition of β-mercaptoethanol (2%) to the thermally and pressure-treated samples overestimated the "actual" stability of [6*S*]-5-methyltetrahydrofolate and misled the obtained kinetic information.

KEYWORDS: [6*S*]-5-Methyltetrahydrofolate; β-mercaptoethanol; stability; temperature; pressure

INTRODUCTION

Folates are important for human health because of their coenzyme role in carbon metabolism (*1*, *2*). Folate deficiency results in megaloblastic anaemia (*3*, *4*) and neural tube defects during pregnancy (*5*). Furthermore, it may cause neurocognitive dysfunction (*6*, *7*) and certain types of cancer (*8*). Some studies (*9*, *10*) have shown that low folate status can also be associated with a mild elevation of plasma homocysteine concentration, which may cause cardiovascular disease. Hereto, a sufficient daily folate intake is recommended. The folate intake can be increased by different strategies such as (i) supplementation, (ii) food fortification, or (iii) an adequate consumption of natural food products such as green leafy vegetables, fruit, liver, etc. In current situations, synthetic folic acid (the oxidized form of the vitamin) has been used in supplements and food fortification because it has a high bioavailability in humans and also a high stability during processing. However, folic acid fortification is not permitted, and it is still being discussed in some countries since a high folic acid intake (>1 mg per day) can mask the vitamin B_{12} deficiency. Hereto, the use of natural folates (e.g., [6*S*]-5-methyltetrahydrofolate) is being explored.

[6*S*]-5-Methyltetrahydrofolate ([6*S*]-5-CH_3-H_4folate) is the bioactive folate form, which naturally exists mostly in fruit and vegetables. This derivate is sensitive to oxygen, light, temperature, pressure, and others. Therefore, its stability can be affected during processing since many food products have to be processed before consumption. Previous studies (*11*−*15*) have shown that in the presence of oxygen, folate degradation is mostly caused by oxidation.

With regard to studies on folate stability in food products, extraction and purification steps are to be carried out prior to the identification and quantification assays such as high-performance liquid chromatography (HPLC), microbiological assays, etc. In literature, antioxidants such as β-mercaptoethanol or ascorbic acid are added to keep folate stable during extraction and purification. In the past decade, many studies have been conducted to optimize folate extraction from food products, often suggesting the use of high antioxidant concentrations (*16*−*18*). Unfortunately, the latter did not take into account that antioxidants can also act as a reductant to the oxidized folates (*13*, *19*, *20*). Hereto, the aim of this investigation was to study the effect of reductant addition such as β-mercaptoethanol to the thermally and high pressure-treated samples on evaluating the folate stability and its degradation kinetics. Moreover, the consequences of using antioxidants during folate extraction were also discussed.

MATERIALS AND METHODS

Sample Preparation. [6*S*]-5-CH_3-H_4folate (Metafoline, Merck Eprova, Schaffhausen, Switzerland) was stored at −80 °C in small ampules (±5−10 mg per ampule) under argon. Before sample preparation, the temperature of the phosphate buffer (0.1 M; pH 7) was kept constant at 25 °C to avoid variations in the initial oxygen concentration. For each experiment, the working solutions (0.2 μg/mL) were freshly prepared, and afterward, prior to the thermal and pressure treatments, the working solution was flushed with humidified air (debit = 350 cm^3 m) for 20 min at 25 °C to achieve 8.11 ppm oxygen content in the sample. After the solution was flushed, the oxygen concentration was measured with an oxygen meter (Strathkelvin Instruments oxymeter model 781, Glasgow, Schotland) and the sample was immediately filled in glass tubes with rubber septa (800 μL, 30 mm length, 8.2 mm diameter, Cleanpack, Belgium) and in flexible

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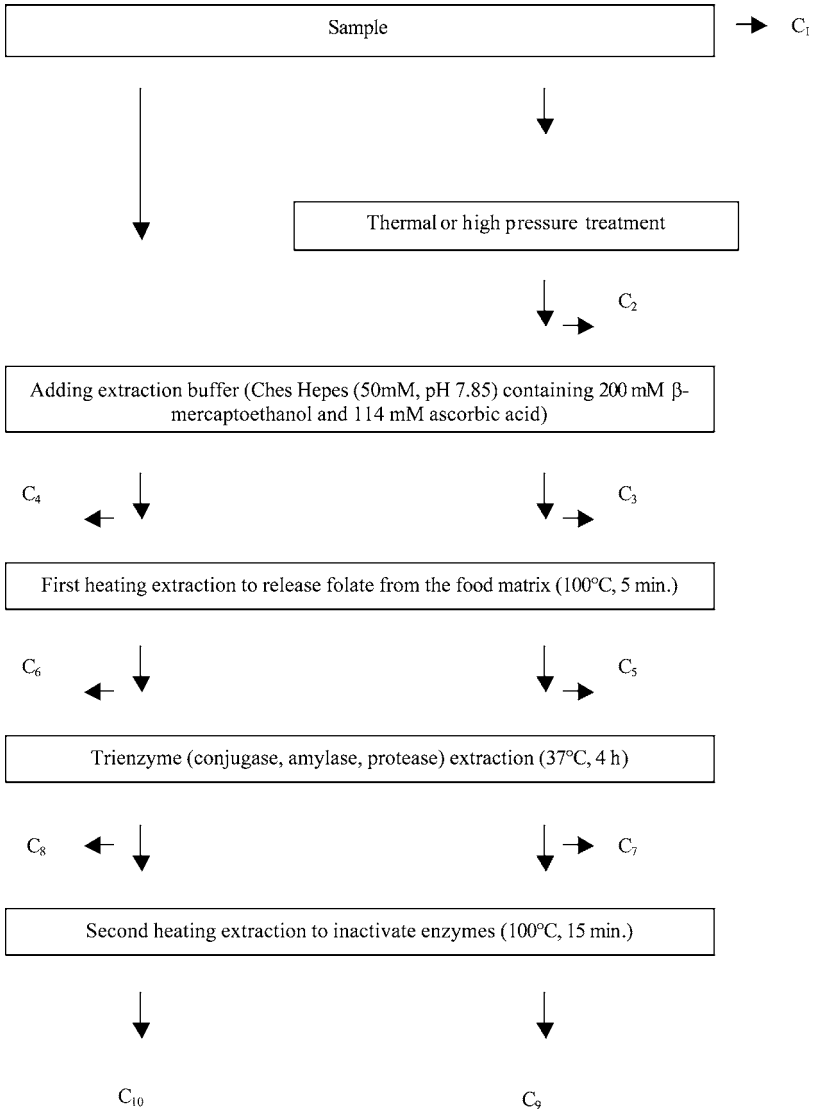

Figure 1. Folate extraction procedure described by Konings (*18*).

polyethylene plastic tubes (400 μL, Bioplastic, Landgraaf, Netherlands), respectively, for thermal and pressure treatments. After sample preparation, the working solution was immediately kept in an ice bath (0−4 °C) for less than 15 min to avoid further oxidation before the treatments. To avoid photodegradation, the working solution was covered with aluminum foil during experiments. Because phosphate buffer was used, a pH decrease of approximately 0.3 units for every 100 MPa pressure increase could occur (*21*−*23*). The folate concentration and the ratio of 5-CH_3-H_4folate/dihydro derivates were spectrophotometrically determined (*18*). The ratio of 5-CH_3-H_4folate/dihydro derivates of the working solution was above 3.3. The ratio lower than 3.3 indicated that the sample was contaminated with dihydro derivates of 5-CH_3-H_4folate.

Effect of β-Mercaptoethanol Concentrations on [6*S*]-5-CH_3-H_4folate. Different concentrations (0−2%) of β-mercaptoethanol were added to the samples before and after thermal and pressure treatments (described further). The latter mixture was incubated for 2 h at 25 °C to allow a reaction between the β-mercaptoethanol and the oxidized folates and afterward injected into the HPLC (described further). The peak evolution of [6S]-5-CH_3-H_4folate in the chromatogram was followed using Unicorn 4.0 software (Amersham Biosciences, Uppsala, Sweden).

Thermal Treatment. The samples were treated in a water bath at constant temperature (25 up to 90 °C) for different time intervals. After their withdrawal, the samples were immediately kept in an ice bath (<15 min). The treated sample was divided into two parts. To one part of the sample, β-mercaptoethanol (2%) was added and incubated for 2 h at 25 °C before the HPLC assay and another part (without 2% β-mercaptoethanol) was immediately used for the HPLC assay. The experiments were carried out in triplicate.

Combined Pressure and Temperature Treatment. The samples were treated in multivessel high pressure equipment (Resato, Roden, Netherlands) consisting of six thermostated vessels (40 mL volume) at 40 °C and different pressure levels up to 600 MPa for 15 min. The pressure was manually built up with a constant rate of 100 MPa/min. After decompression, the samples were kept in the vessel for 50 s. After their withdrawal, the samples were cooled in an ice bath (0−4 °C for 2 min) to stop the degradation. Afterward, one part of the treated sample was immediately used for the HPLC assay, and to the other part, 2% β-mercaptoethanol was added. The latter sample was incubated for 2 h at 25 °C before HPLC measurement. The experiments were carried out in triplicate.

Effect of Antioxidant on Folate Extraction. In this study, the extraction procedure of Konings (*18*) as depicted in **Figure 1** was chosen as an example in which β-mercaptoethanol and ascorbic acid were used as the antioxidant. First, the antioxidant free folate sample, i.e., [6*S*]-5-CH_3-H_4folate (0.2 μg/mL) dissolved in phosphate buffer (0.1 M, pH 7) was used and treated as described in **Figure 1**. The residual folate concentration for each extraction step (**Figure 1**, C_1...C_n) was measured using the HPLC assay. Second, a case study on folate stability in food products such as carrot juice was chosen as an example

to illustrate the problem using antioxidant in the extraction buffer. The experiments were carried out in triplicate.

Carrot Juice Preparation. Carrots (*Daucus carota*, category 1, Delhaize, Belgium) were purchased in a local market. The carrots were rinsed with distilled water and squeezed in a juice centrifuge (Magimix type Le Duo, Surrey, United Kingdom). The supernatant, obtained by centrifuging the crude extract for 10 min at 20400*g* and 4 °C (Beckman J2-HS, Palo Alto, CA), was divided into small portions (40 mL), instantaneously frozen in liquid nitrogen, and stored at −80 °C until use. Thawing of the frozen extract was standardized at 4 °C overnight. In this study, for each pressure or temperature treatment, carrot juice with and without addition of [6*S*]-5-CH_3-H_4folate (0.2 μg/mL) was freshly prepared. Before the treatments, the samples were kept in an ice bath (<30 min).

Thermal and Combined Pressure and Temperature Treatments of Carrot Juice. Thermal (70 and 120 °C; 15 min) and combined pressure temperature (500 MPa, 25 and 60 °C, 15 min) experiments were performed as described by Indrawati and co-workers (*24*). In the case of pressure treatment, the spiked and unspiked carrot juices were treated simultaneously in single vessel (590 mL) high pressure equipment (Epsi, Temse, Belgium). After the treatments, the samples were kept in an ice bath (<15 min) and immediately mixed with the extraction buffer (Ches/Hepes (50 mM, pH 7.85) containing 200 mM β-mercaptoethanol and 114 mM ascorbic acid). The ratio between carrot juice and extraction buffer was 1:3. If extraction and purification could not be conducted on the same day as the treatments, the sample mixture was frozen in liquid nitrogen and stored at −80 °C. The experiments were carried out in triplicate.

Extraction and Purification of 5-CH_3-H_4folate in Carrot Juice. The extraction procedure depicted in **Figure 1** was used in this investigation. Because the folate concentration used in this study was relatively high, the isolation and purification procedure of Konings (*18*) was modified. The folate binding capacity of the affinity column was doubled resulting in a doubled column volume. Hereto, the column was equilibrated with 10 mL of phosphate buffer (0.1 M, pH 7). Before the column was applied, the extract was filtered (regenerated cellulose filter, 0.45 μm). The amount of folates in the extract applied to the column was 10−20% of the binding capacity of the column (the binding capacity of the affinity column was regularly tested by applying 5 μg of [6*S*]-5-CH_3-H_4folate to the column). Afterward, the column was eluted with 10 mL of phosphate buffer (25 mM; pH 7; 0.1 M NaCl) and sequentially with 10 mL of NaCl free phosphate buffer (25 mM; pH 7). The folates were eluted two times with 4.6 mL of elution buffer (20 mM TFA/20 mM DTT) and collected in a 10 mL volumetric flask containing 400 μL of ascorbic acid solution (0.25 g/mL), 80 μL of KOH solution (0.50 g/mL), and 10 μL of β-mercaptoethanol. The volume of the volumetric flask was adjusted to 10 mL with the elution buffer. Before sample injection, the samples were double-filtered (regenerated cellulose filter, 0.45 μm).

HPLC Assay for Identification and Quantification of [6*S*]-5-CH_3-H_4folate. The RP-HPLC assay using a C_{18} column (Prevail C18, 250 mm × 4.6 mm, 5 μm particle size, Alltech, Deerfield, IL) was applied to identify and quantify [6*S*]-5-CH_3-H_4folate. The column was thermostated at 25 °C. An HPLC system (Äkta purifier, Amersham Biosciences) combined with UV−vis (at λ = 290 nm, UV-900, Amersham Biosciences) and fluorescence (at λ_{ex} = 280 nm and λ_{em} = 359 nm, RF-10AXL, Shimadzu, Kyoto, Japan) detectors was used. Before sample injection (100 μL), the column was equilibrated with 5% acetonitrile in phosphate buffer (330 mM, pH 2.10) at a flow rate of 1 mL/min for 2.5 min and for 4 min after the injection. A linear gradient was built from 0 to 100% within 10 min using 60% acetonitrile in phosphate buffer (330 mM, pH 2.10). Afterward, the column was washed with 60% acetonitrile in phosphate buffer (330 mM, pH 2.10) for 3.5 min. The elution time of [6*S*]-5-CH_3-H_4folate was around 11−12 min after the injection. To quantitate the folate concentration, the peak area and height of the samples were compared to that of the standard solutions with known concentrations (0−0.2 μg/mL). For this purpose, Unicorn 4.0 software (Amersham Biosciences) was used for data analysis, and the correlation coefficients (r^2) of the standard curves in this study were at least 0.98. The analytical measurements were carried out in duplicate.

Data Analysis. Previous studies have shown that folate degradation during temperature or pressure processing followed first-order reaction kinetics (*12*, *14*, *24*, *25*) as described in eq 1.

$$C_t = C_0 \times \exp(-kt) \tag{1}$$

where C_t is the residual folate concentration at treatment time t (μg/mL), C_o is the initial folate concentration (μg/mL), k is the degradation rate constants (min^{-1}) estimated using nonlinear regression analysis, and t is the treatment time (min). Equation 1 can be linearized to eq 2, and the k value can be estimated from the slope of the linear regression analysis.

$$\ln(C_t) = \ln(C_0) - kt \tag{2}$$

To describe the temperature dependence of the k values, the Arrhenius equation can be used and mostly integrated as eq 3 and linearized as eq 4.

$$k = k_{T_{ref}} \exp\left[-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \tag{3}$$

$$\ln(k) = \ln(k_{T_{ref}}) - \frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \tag{4}$$

where E_a is the activation energy (kJ mol^{-1}), R is the universal gas constant, $k_{T_{ref}}$ is the degradation rate constant at reference temperature (min^{-1}), T is the degradation temperature (K), and T_{ref} is the reference temperature (K). The kinetic parameters of eq 4 were estimated by linear regression analysis.

For a global analysis, eq 3 can be inserted into eq 1, yielding eq 5. The kinetic parameters of eq 5 were estimated using nonlinear regression analysis.

$$C_t = C_0 \times \exp\left\{-k_{T_{ref}} \exp\left[-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \times t\right\} \tag{5}$$

RESULTS AND DISCUSSIONS

Effect of β-Mercaptoethanol Concentrations on [6*S*]-5-CH_3-H_4folate. As a preliminary test, different concentrations of β-mercaptoethanol (0 up to 2%) were added to [6*S*]-5-CH_3-H_4folate (0.2 μg/mL) in phosphate buffer (0.1 M, pH 7) before and after thermal (80 °C, 15 min) and high pressure (400 MPa, 40 °C, 15 min) treatments. The effect of different β-mercaptoethanol concentrations on the folate retention is given in **Figure 2**. As expected, increasing β-mercaptoethanol concentrations added before treatment enhanced the temperature and pressure stability of [6*S*]-5-CH_3-H_4folate. The addition of β-mercaptoethanol after treatment resulted in a reduction of the oxidated folates, as reported by several authors (*13*, *19*, *26*). Applying a higher reductant concentration increased the magnitude of the reduction. However, it was also noticed that a high reductant concentration (e.g., 2% β-mercaptoethanol) did not guarantee a complete reduction of the oxidized folates. For example, the initial concentration of untreated [6*S*]-5-CH_3-H_4-folate can be completely recovered after 2% β-mercaptoethanol addition to the pressure (400 MPa, 40 °C, 15 min)-treated sample but not to the thermally (80 °C, 15 min) treated one (**Figure 2**).

On the basis of the chromatogram, the effect of β-mercaptoethanol addition after the treatment on [6*S*]-5-CH_3-H_4folate and the oxidized folates was qualitatively studied by following the peak evolution. It was noticed in the chromatogram of thermally and pressure-treated samples that the other peaks (presumably the oxidized folates) were found excluding the peak of [6*S*]-5-CH_3-H_4folate and the amount of the former peaks increased when the samples endured more severe treatment

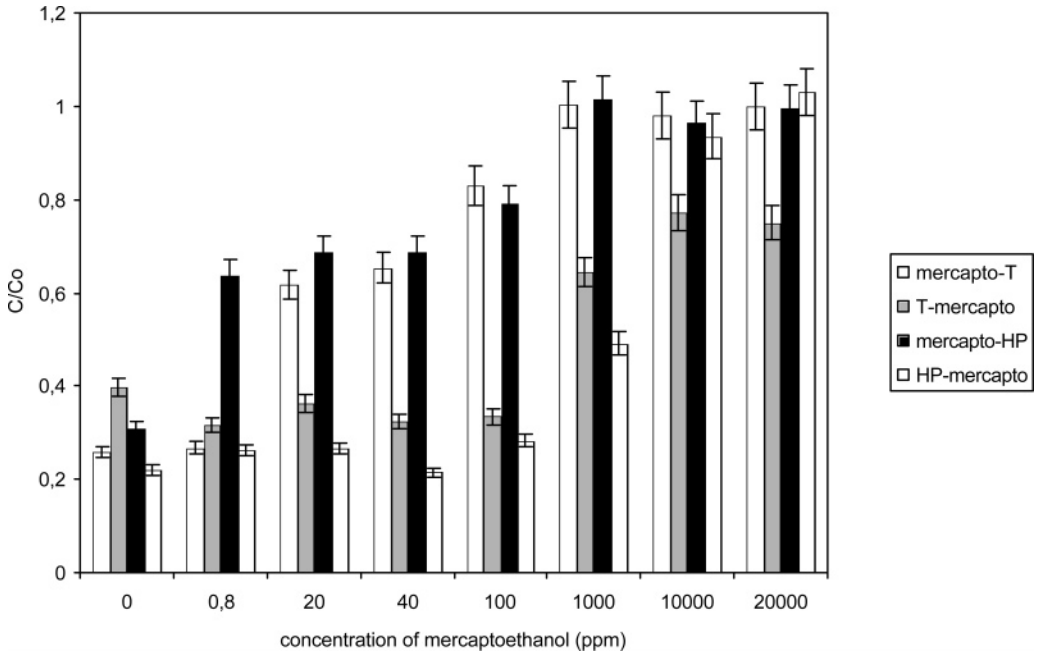

Figure 2. Effect of β-mercaptoethanol concentrations on [6*S*]-5-CH_3-H_4folate [0.2 μg/mL in phosphate buffer (0.1 M, pH 7)] before and after thermal (T, 80 °C/15 min) and high pressure (HP, 400 MPa/40 °C/15 min) treatments (mercapto-T and mercapto-HP indicate β-mercaptoethanol addition before the treatments whereas T-mercapto and HP-mercapto indicate β-mercaptoethanol addition after the treatments).

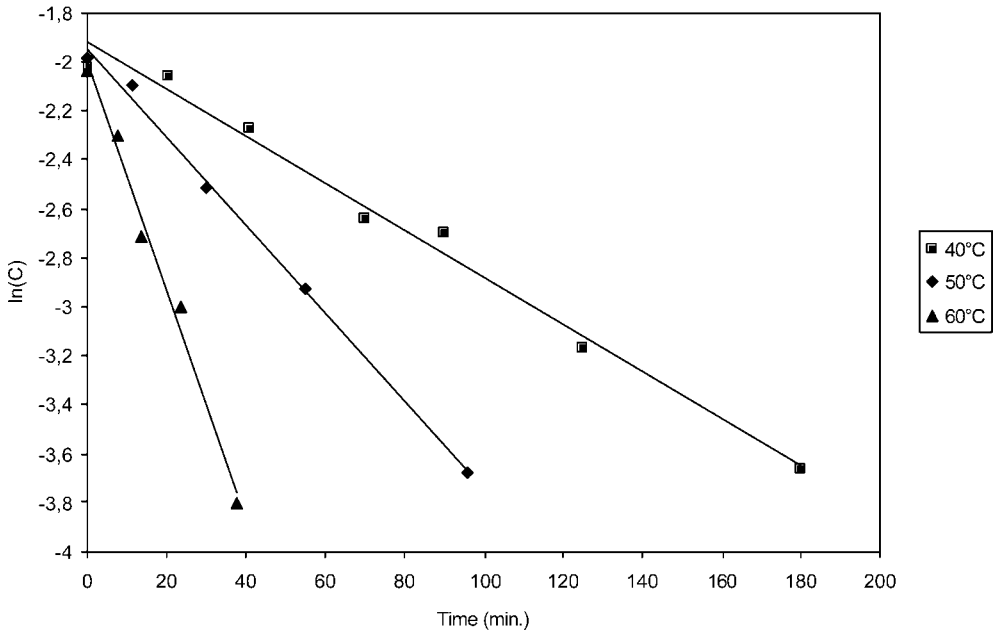

Figure 3. Effect of temperature on [6*S*]-5-CH_3-H_4folate stability.

conditions (e.g., longer treatment time, higher pressure level at high temperatures). When β-mercaptoethanol was added to the treated samples, the peak size of some oxidized folates decreased while the other peaks enlarged including the peak of [6*S*]-5-CH_3-H_4folate. This indicated that [6*S*]-5-CH_3-H_4folate oxidation occurred during thermal and high pressure treatments. In the case of samples with severe treatment (e.g., 90 °C), the peak size of some oxidized folates was unchanged by β-mercaptoethanol addition. It can be that the oxidized folates were resistant to β-mercaptoethanol or the covalent bond (such as C_9-N_{10} binding) was broken due to the given treatment resulting in an irreversible reaction as previously reported in the literature (*19*, *20*). However, further studies are still needed for identification of the oxidized folates and their sensitivities to a certain reductant such as β-mercaptoethanol including identification of their reduced products after reductant addition.

In this study, it was noticed that folate oxidation also occurred during pressure treatment and the treatment decreased the folate concentration. The evidence of folate degradation due to pressure can be explained by a pressure-enhanced oxidation reaction (Le Châtelier principle). Most publications have promoted that food quality characteristics such as flavor, pigment, and nutritional components can be well-maintained using high pressure treatment since pressure has no/limited effect on the covalent bonds. However, this statement must be interpreted with caution because pressure can either increase or decrease the chemical reaction rate. Previous studies (*24*, *27*) have reported negative activation volumes of folate degradation during high pressure

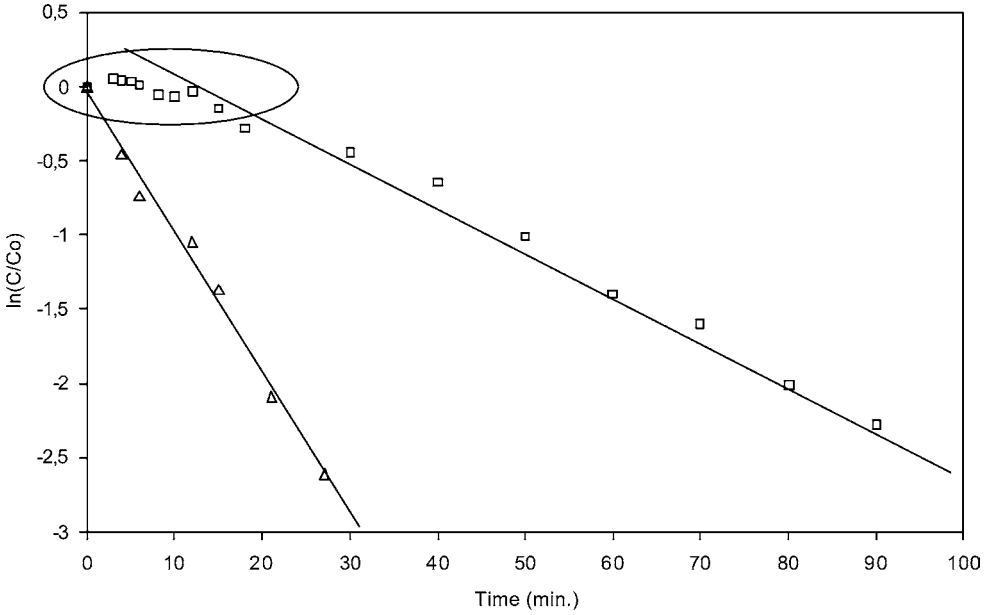

Figure 4. Effect of β-mercaptoethanol addition to the thermally treated [6*S*]-5-CH_3-H_4folate [0.2 μg/mL in phosphate buffer (0.1 M, pH 7)] on its stability and degradation kinetics (□, 50 °C; △, 90 °C).

treatments. It means that the reaction rate increases with pressure. This phenomenon is also noticed by Butz and co-workers (*15*).

Effect of β-Mercaptoethanol on Temperature and Pressure Stability of [6*S*]-5-CH_3-H_4folate. β-Mercaptoethanol and ascorbic acid are mostly used as antioxidants during extraction and purification to protect folates from oxidation. However, antioxidants such as β-mercaptoethanol can also act as a reductant to the oxidized folates. Hereto, its effect on evaluating folate stability and its degradation kinetics was studied in detail.

In the case of treated samples without β-mercaptoethanol addition, the relation between natural logarithm of the residual folate concentration and treatment time can be adequately described by first-order kinetics as depicted in **Figure 3**. This finding confirmed the results of previous studies (*12*, *14*, *15*, *24*, *25*, *27*). However, when β-mercaptoethanol was added to the treated samples, the folate degradation did not follow any more first-order kinetics. As shown in **Figure 4**, a plateau curve ("lag phase") is observed in the beginning of the degradation as a result of a (complete) reduction of the oxidized folates. This lag phase shortened when the treatment temperature was increased, and finally, it disappeared when applying a very high temperature level, e.g., 90 °C (**Figure 4**) (explanation cf. the previous discussion).

The natural logarithm of the residual folate concentration as a function of time after the lag phase could be described by first-order kinetics (**Figure 4**). Hereto, to evaluate the impact of β-mercaptoethanol addition on the kinetic information (*k* and E_a values) of folate degradation, the slope of the linear part after the lag phase was estimated (**Table 1**). **Table 1** shows that the calculated *k* values of samples with β-mercaptoethanol are lower (indicating higher folate stability) than samples without antioxidant, for example, at 50 °C. The *k* value of the sample without β-mercaptoethanol was four times higher than that with the reductant. It means that the "actual" degradation occurred faster.

In addition, the effect of β-mercaptoethanol on the estimated E_a values was evaluated. The E_a value was estimated using both a two step analysis (i.e., the *k* values estimated with eq 2 were inserted into eq 4 to estimate the E_a value) and a one step analysis using eq 5. On the basis of the estimated E_a values, it can be concluded that adding β-mercaptoethanol to the treated samples has no significant (95% confidence interval) effect on estimating the temperature dependence of the *k* values (i.e., E_a value).

Table 1. Effect of β-Mercaptoethanol (2%) Addition on the Kinetic Data of [6*S*]-5-CH_3-H_4folate[a] Thermal Degradation

	k (10^{-3} min^{-1})[a]	
T (°C)	without β-mercaptoethanol addition	with 2% β-mercaptoethanol addition after treatment
25	0.96 ± 0.02[b] (r^2 = 0.997)	
40	9.93 ± 0.52 (r^2 = 0.984)	
50	17.97 ± 0.52 (r^2 = 0.998)	3.95 ± 0.32[b] (r^2 = 0.969)
60	46.76 ± 3.15 (r^2 = 0.987)	
70		31.40 ± 1.06 (r^2 = 0.994)
80		
90		89.85 ± 5.31 (r^2 = 0.973)
Ea (kJ mol^{-1})[c]	89.89 ± 10.56[b] (r^2 = 0.973)	76.52 ± 11.79[b] (r^2 = 0.977)
Ea (kJ mol^{-1})[d]	87.05 ± 5.30[e]	82.67 ± 1.91[e]

[a] 0.2 μg/mL [6*S*]-5-CH_3-H_4folate in phosphate buffer (0.1 M; pH 7; 8.11 ppm initial O_2 concentration). [b] Standard error of linear regression analysis. [c] The value was estimated using linear regression analysis based on eq 4. [d] The value was estimated using nonlinear regression analysis based on eq 5. [e] Asymptotic standard error of nonlinear regression analysis.

As for thermal treatment, the oxidized folates due to pressure treatment could be reduced by β-mercaptoethanol addition. It can be observed in **Figure 5** that β-mercaptoethanol addition can deliver overestimation on pressure stability of [6*S*]-5-CH_3-H_4folate, i.e., higher than the "real" pressure stability. Hereto, adding β-mercaptoethanol to the treated samples can mislead the interpretation of folate stability and its degradation kinetics.

Effect of β-Mercaptoethanol in Extraction Buffer on Evaluating Stability of [6*S*]-5-CH_3-H_4folate. On the basis of the previous finding, the effect of using high antioxidant concentrations (especially β-mercaptoethanol and ascorbic acid) in the extraction buffer on folate quantification after thermal/pressure treatments was questioned. Hereto, in this study, the simulated extraction procedure using sample in buffer solution as described in **Figure 1** was conducted.

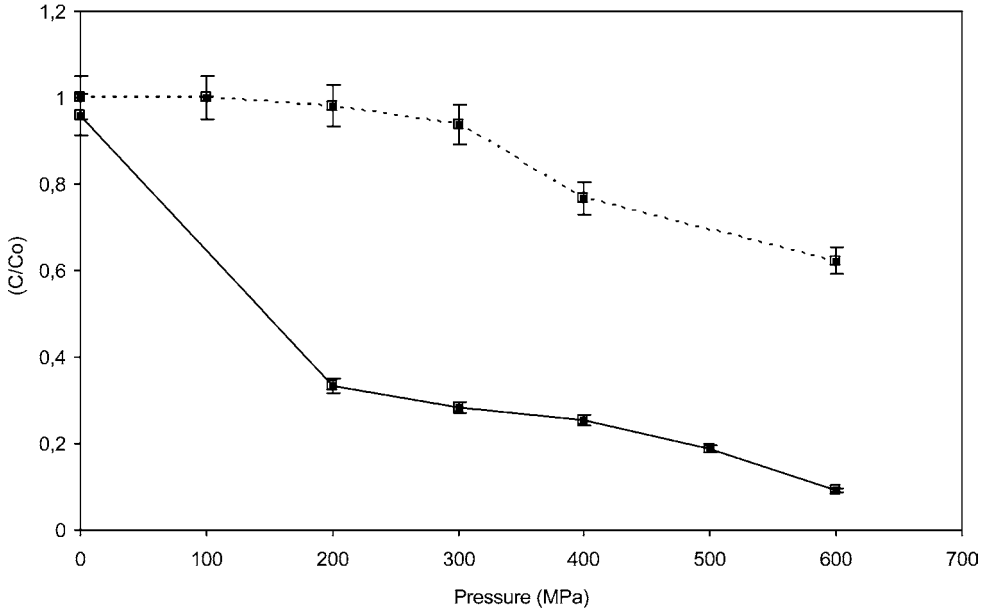

Figure 5. Effect of β-mercaptoethanol addition to the pressure-treated [6*S*]-5-CH_3-H_4folate [0.2 μg/mL in phosphate buffer (0.1 M, pH 7)] on its stability and degradation kinetics (dot and full lines indicate, respectively, with and without β-mercaptoethanol addition after pressure treatment at 40 °C for 15 min).

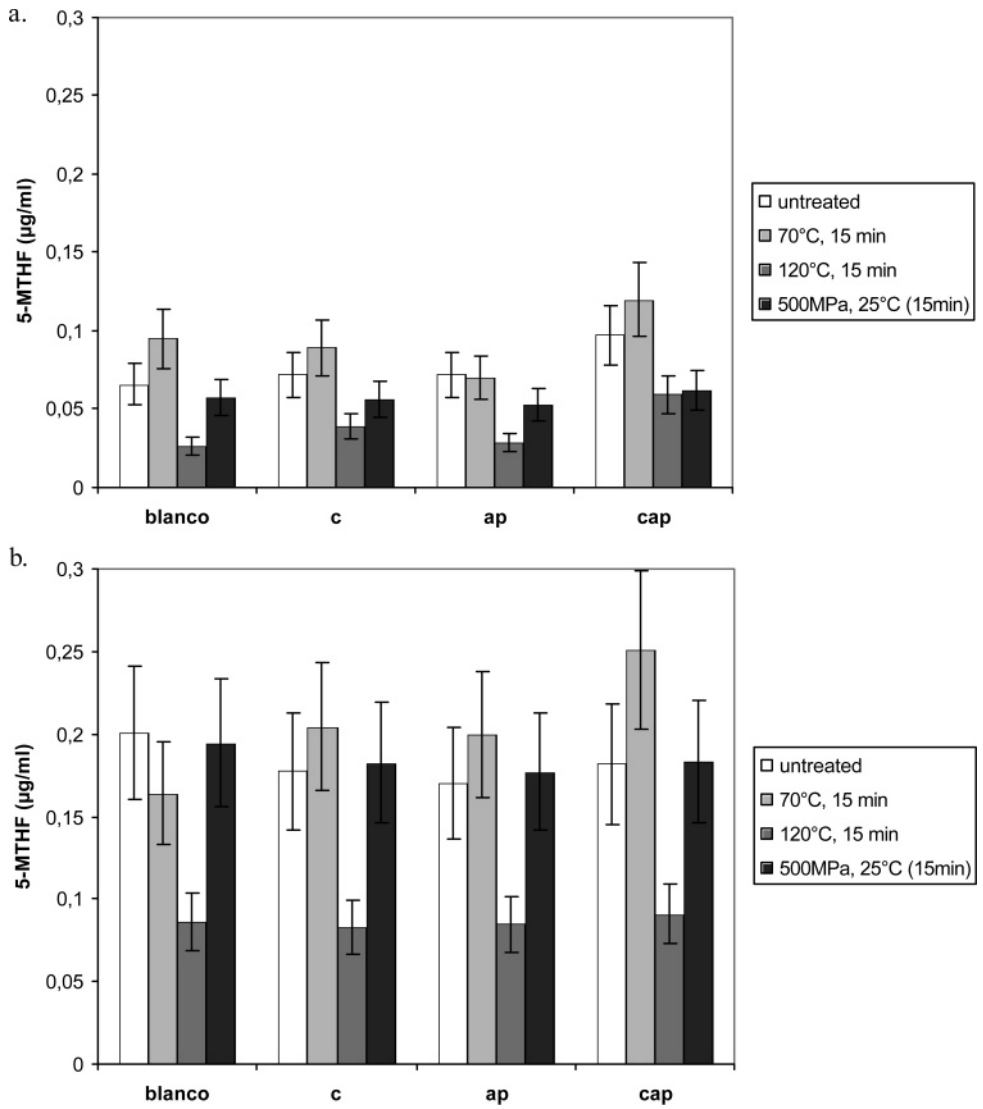

Figure 6. Concentration of unspiked (**a**) and spiked (**b**) 0.2 μg/mL [6*S*]-5-CH_3-H_4folate in carrot juice (c, after conjugase extraction; ap, after amylase and protease extraction; and cap, after conjugase, amylase, and protease extraction).

Table 2. Concentration of [6*S*]-5-CH_3-H_4folate during Extraction Procedure of Konings (*18*)

	untreated sample (μg/mL)		thermally treated sample (μg/mL)	phase during extraction
C_1	0.14	C_1	0.14	initial concentration
		C_2	0.03	thermal treatment
C_4	0.16	C_3	0.10	use of extraction buffer
C_6	0.15	C_5	0.09	first heating extraction
C_8	0.16	C_7	0.10	trienzyme extraction at 37 °C (4 h)
C_{10}	0.16	C_9	0.11	second heating extraction

As shown in **Table 2**, the residual folate concentration after thermal treatment (C_2) is around 20% of the initial concentration before treatment (C_1). When the treated sample was mixed with the extraction buffer, the estimated folate retention (C_3) increased up to 70% of C_1 or 60% of C_4. The increase in folate concentration of untreated sample after mixing with the extraction buffer (C_4) can be caused by the reduction of the oxidized folates, which were formed during sample storage or preparation. During the heating and trienzyme extraction, folate in the extraction buffer was stable. On the basis of these results, the use of antioxidant could overestimate the folate concentration in treated samples leading to an apparent higher folate stability.

To illustrate this problem, a case study on (spiked and unspiked) 5-CH_3-H_4folate stability in carrot juice was additionally carried out (**Figure 6**). The results showed that (i) the [6*S*]-5-CH_3-H_4folate stability was almost unaffected at 70 °C for 15 min and its concentration was decreased up to 50% during thermal treatment at 120 °C for 15 min; (ii) folates in carrot juice were completely destroyed at 500 MPa and 65 °C for 15 min (the folate concentration could not be determined because it was beyond the detection limit); and (iii) pressure treatment at 500 MPa and 25 °C for the same treatment time had little/no effect on folate stability. Moreover, the ascorbic acid content in carrot juice was also estimated, i.e., approximately 82 μg/mL (using the HPLC assay). The latter indicates that the folate stability in carrot juice can be enhanced by the presence of endogenous antioxidant. However, concerning the previous findings, it is difficult to conclude the real folate stability in food products and the role of endogenous factors (e.g., food matrix, endogenous antioxidant) in the folate stability during processing since the oxidized folates can also be reduced by antioxidant in the extraction buffer.

However, if all oxidized forms of the natural folates have a high bioavailability in humans, the use of reductant in the extraction buffer becomes interesting because the overestimation of folate stability can reflect the residual folate bioavailability in vitro after treatment. Gregory and co-workers (*26*) have shown that 10-formylfolic acid (i.e., the oxidized form of 10-formyltetrahydrofolic acid) and 5-methyldihydrofolic acid (i.e., the oxidized form of 5-methyltetrahydrofolic acid) exhibit high folacin activity for *Lactobacillus casei* in microbiological assays. Unfortunately, limited in vivo study in human has been investigated for other oxidized folates, which parentally come from the natural folates, and up to now, numerous contradictions still exist in the literature concerning the biological activity of these oxidized folates. For this purpose, further investigations are highly needed to study the bioavailability of these oxidized folates in human. Oxidation is the major cause of folate degradation, but these oxidized products can be diverse depending on the intensity of the treatments (e.g., thermal and pressure treatments). Hereto, the mechanisms of folate degradation and identification of these oxidized folates during thermal and pressure treatment must be initially studied and afterward, the oxidized folates of interest for intervention studies can be defined.

In the current situation, the extraction procedures described in the literature seem suitable to quantify total folate concentration for untreated food products. All oxidized folates formed during sample preparation can be partly or completely reduced. However, the use of antioxidant during extraction becomes a dilemma since food samples also contain folates with different stabilities. For example, tetrahydrofolic acid is very sensitive and it would be totally lost without antioxidant addition. Therefore, the current extraction procedures must be revised or adapted if they are to be used for evaluating the effect of certain treatments or processing on folate stability in food products. This conclusion calls for further analytical studies to overcome or to eliminate the overestimation of folate stability after processing or certain treatments due to the antioxidant addition. To face this problem, a direct assay procedure (i.e., without use of antioxidant) is preferable.

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