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THE KINETICS STUDY ON 5- FORMYLTETRAHYDROFOLIC ACID DEGRADATION AND 5,10- METHENYLTETRAHYDROFOLIC ACID FORMATION DURING THERMAL AND COMBINED HIGH PRESSURE THERMAL TREATMENTS

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ABSTRACT

The effect of different pH levels (3.4 to 7.0) on 5-formyltetrahydrofolic acid (5-CHOH4PteGlu) stability during thermal (80-110°C) and high pressure thermal (100-800 MPa/65-70°C) processing was studied. The results showed that (i) the degradation of 5-CHOH4PteGlu during treatments (in the presence of ascorbic acid) was due to not only oxidation but also a conversion to 5,10-methenyltetrahydrofolic acid (5,10-CH=H4PteGlu); and (ii) multiple response analysis was successfully applied to describe the kinetics of 5-CHOH4PteGlu degradation during thermal and/or high pressure processing.

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1 INTRODUCTION

Thermal and high pressure processing can affect the stability of folates. The chemical reactivity of some important folate compounds makes the vitamin vulnerable to degradation during food processing (Forssén *et al*., 2000). Whereas, 5,10-CH=H_4PteGlu is formed as the degradation product of 5-CHOH_4PteGlu (Hawkes and Vilotta, 1989; Butz *et al*., 2004). Considerable losses of folates are caused by oxidation which results in biologically inactive forms, namely pABG (Forssén *et al*., 2000). Evidence shows that folates can be protected towards oxidation by the presence of antioxidants such as vitamin C (Butz *et al*., 2004; Indrawati *et al*., 2004). Stralsjö *et al*. (2003) studied the stability of total folate in rosehips after drying and demonstrated a pronounced role of ascorbic acid a protective agent against oxidation. Butz *et al*. (2004) indicated that reducing agents such as ascorbic acid and thiols exert multiple protective effects on folates through their actions as oxygen scavengers, reducing agents and free radical scavengers.

Considerable losses of folates are primarily caused by oxidation resulting in the formation of biologically inactive forms, namely *p*-aminobenzoyl glutamate (pABG). The studies concerning the kinetic study of 5-CHOH_4PteGlu during temperature and high pressure processing are still limited. It has been observed that 5,10-CH=H_4PteGlu is formed as one of the degradation products of 5-CHOH_4PteGlu (Stokstad and Thenen, 1972).

Firstly, this paper is to describe the effect of pH on the stability of 5-CHOH_4PteGlu in model systems during thermal or high pressure treatments. Secondly, the basic insight (mechanistic and kinetic study) in the effect of ascorbic acid on 5-CHOH_4PteGlu stability during temperature and/or high pressure treatments is generated.

2 MATERIALS AND METHODS

2.1 Experimental set up

Stock solutions of 5-$CHOH_4$PteGlu (1 mg/mL) were prepared by dissolving 10 mg 5-$CHOH_4$PteGlu (>95% purity, Schricks Laboratory, Jona, Switzerland) in 10 ml sodium borate solution (50 mM; pH 9.2) and stored at -80°C. The working solution (10 µg/ml) was prepared on the day of use by diluting the 5-$CHOH_4$PteGlu stock solution in different buffer solutions, i.e. acetate buffer (0.2 M, pH 3.4 and pH 5.0) and phosphate buffer (0.1 M, pH 7.0). All procedures of sample preparation and all treatments were carried out under subdued light by covering samples with aluminum foil. 5-$CHOH_4$PteGlu and 5,10-$CH{=}H_4$PteGlu (Schricks Laboratory, Jona, Switzerland) were used as external standards.

2.2 Effect of ascorbic acid

To study the effect of ascorbic acid on 5-$CHOH_4$PteGlu stability, the working solutions were prepared daily by dissolving 5-$CHOH_4$PteGlu (10 µg/mL) in the thermostatic acetate buffer (0.2 M, pH 5.0) in presence (0.02%) and absence of ascorbic acid. After sample preparation and prior to thermal and pressure treatments, the solutions were flushed with humidified air (flow rate = 350 cc min^{-1}) for 20 min at 25°C to achieve ≈ 8 ppm (≈ 0.25 mM) oxygen in the samples. All procedures of sample preparation and all treatments were carried out under subdued light by covering samples with aluminum foil, avoided from direct contact with air.

2.3 Thermal treatment

Thermal experiments were performed in an oil bath. The samples were enclosed in glass vials with a rubber septum (800 µl, 30 mm length, 8.2 mm internal diameter, Cleanpack, Belgium). After treatments, the samples were kept in an ice bath to stop further degradation. Subsequently, the residual vitamin concentrations were measured. The blank (C_o) was defined as the concentration of thermally untreated samples.

2.4 Combined high pressure thermal treatment

Isobaric-isothermal treatments were conducted in a multivessel HP apparatus (Resato, Roden, Netherlands). In this study, kinetic experiments were performed in the pressure range from 100 to 800 MPa combined with temperatures in the range from 50 to 70°C. The samples were enclosed in 0.3 mL flexible micro tubes (Elkay, Leuven, Belgium) and brought into the P vessels which had already been equilibrated at the desired temperature. Pressure was built up slowly using a standardized pressurization rate of about 100 MPa/min to minimize adiabatic heating. After reaching the desired pressure, the individual vessels were closed at the same time. The starting time of the isobaric isothermal experiment (time = 0) is 2 min after reaching the desired pressure. At this moment, the 1st vessel was decompressed and the residual concentration of the corresponding sample was considered as blank (C_o). The other vessels were then decompressed as a function of time. After withdrawal, the samples were cooled in an ice bath and stored in liquid nitrogen until the measurement of the residual vitamin concentration using Reverse Phase HPLC analysis.

2.5 Identification and quantification of folate

A Reverse Phase HPLC (1200 Series Agilent Technology) analysis using a RP-C_{18} column (Prevail C_{18}, 5µm, 4.6 x 250 mm, Alltech (Grace), Deerfield, IL) and Chemstation software was used to identify and quantify 5-$CHOH_4$PteGlu and 5,10-$CH{=}H_4$PteGlu. 5-$CHOH_4$PteGlu and 5,10-$CH{=}H_4$PteGlu were detected using a UV/VIS detector respectively at 290 nm and 254 nm. Elution was carried out with a flow rate of 1 mL/min and the injection volume was 100 µL. The column was equilibrated for 4 minutes using phosphate buffer (33 mM, pH 2.1) containing 5% acetonitrile and afterwards, a linear gradient was built up from 5% to 60% acetonitrile within 12 min. In the last step, the column was washed using phosphate buffer (33 mM, pH 2.1) containing 60% acetonitrile for 4 minutes. In this study, 5,10-$CH{=}H_4$PteGlu and 5-$CHOH_4$PteGlu were respectively eluted at a retention time between 11.2-11.6 min and 12.0-12.1 min after injection. The concentrations of 5-$CHOH_4$PteGlu and 5,10-$CH{=}H_4$PteGlu were estimated based on the peak area in comparison to the external standard solutions of the same components which are dissolved together in the same working buffer solution. The correlation coefficient (r^2) of the standard curve was at least 0.99. The data were analyzed using SAS (USA, 2001) and Athena Visual software (USA, 2007).

2.6 Data analysis

The degradation rate of quality attributes can be described by an n^{th} order kinetic model (equation 1).

$$\frac{dC}{dt} = -kC^n \quad (1)$$

Where C is the concentration of the considered quality aspect at time t, k is the degradation rate

constant, n is the reaction order and t is the treatment time.

Previous studies have shown that the thermal destruction of 5-CHOH$_4$PteGlu in buffer systems followed first order reaction kinetics in a wide pH range (1 to 12) (Paine-Wilson and Chen, 1979; Barrett and Lund, 1989; Hawkes and Villota, 1989). For a first order reaction (n = 1) and under constant intrinsic and extrinsic (*e.g.* pressure, temperature) conditions, equation 1 was integrated to equation 2.

$$\ln (C) = \ln (C_o) - kt \quad (2)$$

Where C_o is the initial concentration of the quality aspect, k is the degradation rate constant and t is the treatment time. When the natural logarithm of the residual concentration is plotted as a function of treatment time, the degradation rate constant (k) can be estimated by linear regression analysis (SAS, 2001). The k value is derived from the slope of the regression line.

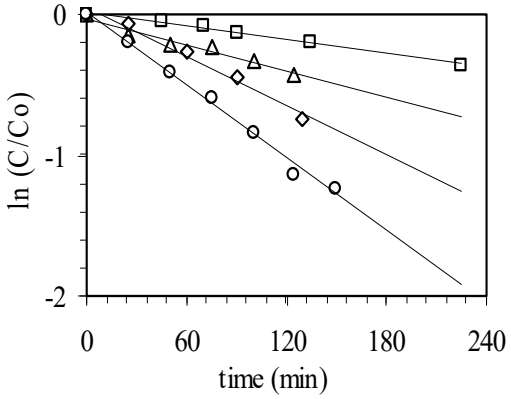

Fig. 1: Logarithm of the ratio of concentration to initial concentration of 5-CHOH$_4$PteGlu as a function of treatment time [in acetate buffer (0.2 M, pH 5.0) at 80°C (□), 90°C (Δ), 100°C (◊) and 110°C (o)]

3 RESULTS AND DISCUSSION

3.1 Temperature stability of 5-CHOH$_4$PteGlu

Thermal stability of 5-CHOH$_4$PteGlu was screened at temperatures above 60°C. At all pH values tested, 5-CHOH$_4$PteGlu showed a high stability at temperatures below 70°C. This vitamin was stable for 6 h at 60°C in the pH range from 3.4 to 9.2. As expected, increasing temperature (above 70°C) enhanced the degradation rate constant (Fig. 1).

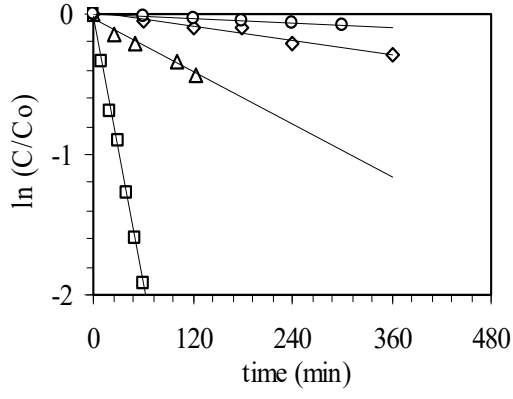

Fig. 2: Logarithm of the ratio of concentration to initial concentration of 5-CHOH$_4$PteGlu as a function of treatment time [at 90°C in buffer pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)]

The effect of pH on the rate of thermal 5-CHOH$_4$PteGlu degradation was investigated in different buffer solutions at pH values ranging from 3.4 to 9.2. It was observed that in all cases the thermal degradation of 5-CHOH$_4$PteGlu could be accurately described by pseudo first order kinetics (Equation 1 and 2) as shown in Figure 2. The thermal stability of 5-CHOH$_4$PteGlu increased in neutral or mildly alkaline solutions, as the degradation rate constants decreased when pH increased from 3.4 to 9.2. 5-CHOH$_4$PteGlu was stable for 7 hrs at 80°C and pH ranging from 7 to 9.2, and slightly degraded during heating at higher temperatures. 5-CHOH$_4$PteGlu was unstable at low pH (Hawkes and Villota, 1989).

3.2 Pressure stability of 5-CHOH$_4$PteGlu

To investigate the effect of temperature and pressure on 5-CHOH$_4$PteGlu (10 µg/mL) degradation, acetate buffer (0.2 M, pH 5.0) and phosphate buffer (0.1 M, pH 7.0) were used. The pressure treatments were carried out in a pressure range from 200 MPa to 800 MPa and temperatures from 20 to 75°C. 5-CHOH$_4$PteGlu in phosphate buffer at pH 7.0 was stable at all pressure and temperature combinations. However, it was degraded in acetate buffer at pH 5.0 and the degradation followed a first order kinetic reaction (Fig. 3). From this figure, one can observe that the stability of 5-CHOH$_4$PteGlu at pH 5.0 was also dependent on pressure. Figure 4 shows pressure degradation of 5-CHOH$_4$PteGlu at 60°C and 800 MPa in acetate buffer (0.2 M, pH 5.0) compared to that in phosphate buffer (0.1 M, pH 7.0). Increasing pH from 5 to 7 clearly retards pressure and temperature degradation of 5-CHOH$_4$PteGlu. After 2 hrs of treatment at 800 MPa and 60°C in phosphate buffer (0.1 M, pH 7.0), only 5% of 5-CHOH$_4$PteGlu was lost. The concentration of 5-CHOH$_4$PteGlu at high pH values can be better maintained than at low pH values.

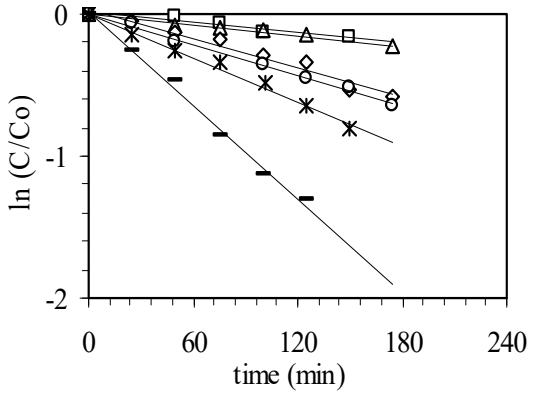

Fig. 3: Logarithm of the ratio of concentration to initial concentration of 5-CHOH$_4$PteGlu as a function of treatment time [in acetate buffer (0.2 M, pH 5.0) at 200 MPa (□), 300 MPa (Δ), 400 MPa (◊), 500 MPa (o), 600 MPa (*), 800 MPa (-)]

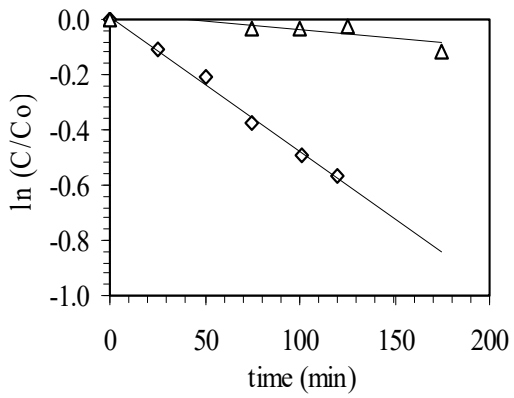

Fig. 4: Logarithm of the ratio of concentration to initial concentration of 5-CHOH$_4$PteGlu as a function of treatment time [at 800 MPa and 60°C in acetate buffer (0.2 M, pH 5.0) (◊) and phosphate buffer (0.1 M, pH 7.0) (Δ)]

3.3 Effect of ascorbic acid on 5-CHOH$_4$PteGlu stability during thermal and high pressure thermal treatments

In this study, the effect of ascorbic acid concentration (0.02%) on the stability of 5-CHOH$_4$PteGlu (10 µg/ml) was investigated in acetate buffer (0.2 M) at a certain pH, namely pH 5.0 and the initial oxygen concentration was also controlled before the treatment (0.25 mM). In presence of ascorbic acid, on the one hand, the thermal and pressure degradation of 5-CHOH$_4$PteGlu was highly retarded but on the other hand, the formation of 5,10-CH=H$_4$PteGlu was induced during thermal and high pressure thermal treatments as observed in Figure 5. The formation of 5,10-CH=H$_4$PteGlu was clearly observed during thermal treatment and high pressure temperature treatment at extreme pressure temperature combinations (>60°C; >600 MPa).

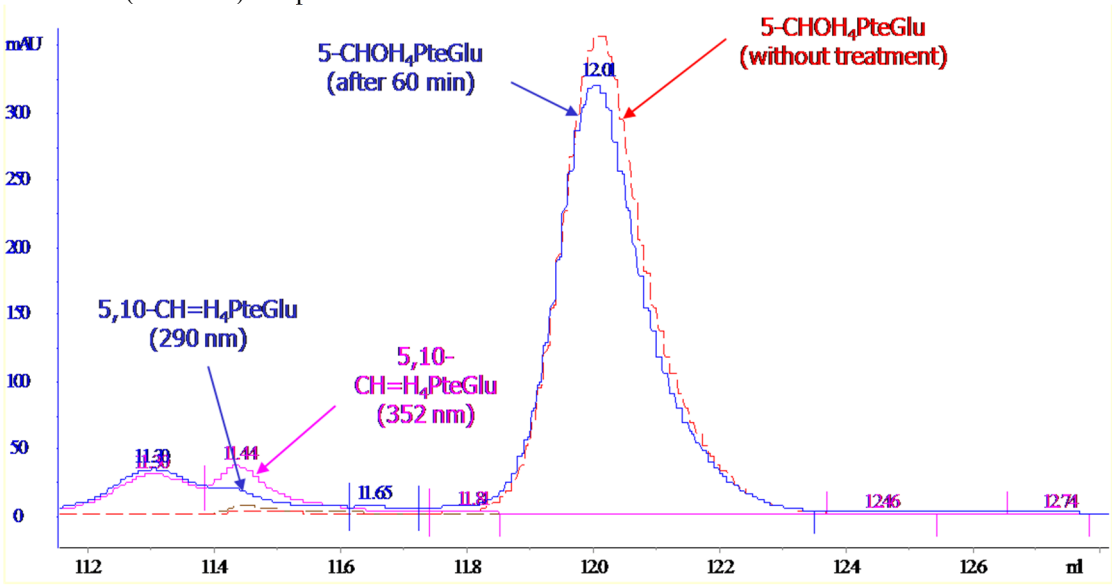

Fig. 5: The conversion of 5-CHOH$_4$PteGlu to 5,10-CH=H$_4$PteGlu [5-CHOH$_4$PteGlu (10 µg/ml) in acetetate buffer (0.2 M, pH 5) – T = 90°C, O_2 ~ 0.25 mM]

Furthermore, it was observed that prolonging the treatment time enhanced both the 5-CHOH$_4$PteGlu degradation and the 5,10-CH=H$_4$PteGlu formation (Fig. 6). The formation of 5,10-CH=H$_4$PteGlu was less than the 5-CHOH$_4$PteGlu degradation. It can be that 5-CHOH$_4$PteGlu was not totally converted

to 5,10-CH=H_4PteGlu, but it also gave an indication that a subsequent degradation of 5,10-CH=H_4PteGlu could occur during the treatments. Hereto, it is necessary to gain insight in the mechanism and kinetics of 5-CHOH_4PteGlu stability during thermal and high pressure thermal treatments. Based on these results, it is obvious that the use of ascorbic acid can enhance the conversion of 5-CHOH_4PteGlu to 5,10-CH=H_4PteGlu. Since β-mercaptoethanol has previously been used for the kinetic study on 5-CHOH_4PteGlu degradation (Nguyen *et al*., 2003; Nguyen *et al*., 2006), it is questioned whether β-mercaptoethanol induces the same conversion as ascorbic acid. Hereto, the same experimental set up as described by Nguyen *et al*. (2006) was applied in this investigation and the evolution of 5,10-CH=H_4PteGlu concentration was followed as an additional response.

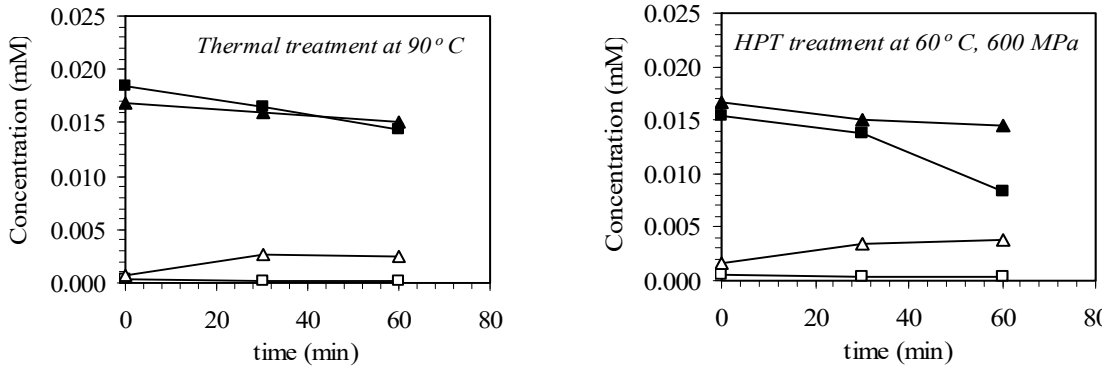

Fig. 6: Concentration of 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu as a function of treatment time [in acetate buffer (0.2 M, pH 5) in absence (■) and presence (▲) of ascorbic acid (0.02%) (closed symbols: 5-CHOH_4PteGlu and open symbols: 5,10-CH=H_4PteGlu)]

The effect of pH on 5-CHOH_4PteGlu stability towards temperature (in the absence and presence of ascorbic acid) was studied. The evolution of the 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu concentration as a function of time was followed under isothermal condition at various temperatures (80-110°C). The formation of 5,10-CH=H_4PteGlu was clearly noticed at pH 3.4 and pH 5.0 and not at pH 7.0. At acidic pH, 5-CHOH_4PteGlu loses one molecule of water to form 5,10-CH=H_4PteGlu due to an increase in electronegativity of the formyl group at the N_5 position, whereas, increasing the pH (more alkaline) reduces the electronegativity of this group. Consequently, the formation of 5,10-CH=H_4PteGlu is retarded at high pH as observed at pH 7.0. During thermal treatment of 5-CHOH_4PteGlu at pH 3.4 (Fig. 7), 5,10-CH=H_4PteGlu was initially formed and afterwards eliminated (Fig. 8). Increasing temperature enhanced the rate of (i) 5-CHOH_4PteGlu degradation, (ii) 5,10-CH=H_4PteGlu formation, and (iii) 5,10-CH=H_4PteGlu elimination.

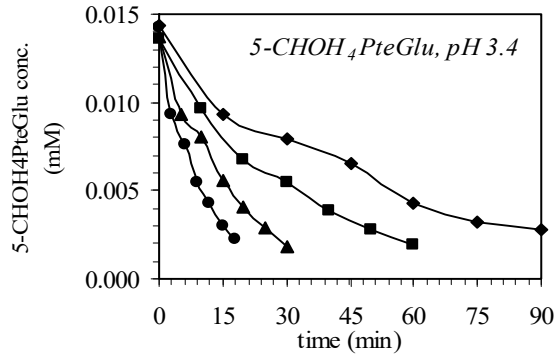

Fig. 7: Concentration of 5-CHOH_4PteGlu as a function of treatment time in buffer pH 3.4, treatment at 80°C (♦), 90°C (■), 100°C (▲), 110°C (●)

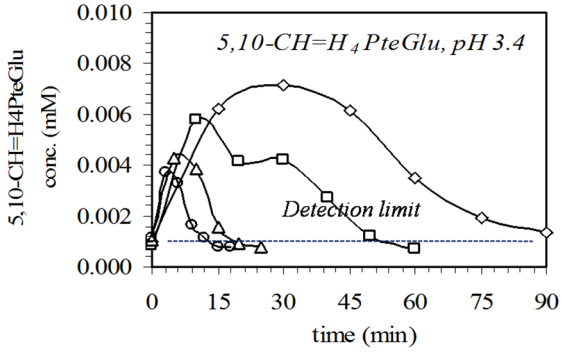

Fig. 8: Concentration of 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu as a function of treatment time in buffer pH 3.4, treatment at 80°C (◊), 90°C (□), 100°C (Δ), 110°C (o)

As reported by some authors (Ndaw *et al*., 2001; Stanger, 2002), 5- and 10-CHOH_4PteGlu can be converted to 5,10-CH=H_4PteGlu, 5,10-methylene-THF and in irreversible way, to 5-methyl-THF.

The formation of 5,10-CH=H_4PteGlu can be explained by the loss of water molecule of 5-CHOH_4PteGlu and this reaction was induced by increasing temperature. In this study, it was also noticed that the concentration of 5,10-CH=H_4PteGlu formed was less than the concentration of 5-CHOH_4PteGlu degraded.

3.4 The mechanism of 5-CHOH_4PteGlu degradation and 5,10-CH=H_4PteGlu information

Next to the 5-CHOH_4PteGlu conversion to 5,10-CH=H_4PteGlu, oxidation may also contribute to the 5-CHOH_4PteGlu degradation. Based on these evidences, the reaction pathway described in equation 1 is postulated to describe 5-CHOH_4PteGlu degradation during thermal treatment.

$$\begin{array}{l} 5-CHOH_4PteGlu \xrightarrow{k_1} 5,10-CH=H_4PteGlu + H_2O \xrightarrow{k_2} P \\ \downarrow k_3 \\ D \end{array} \quad (3)$$

In this reaction, k_1 is the rate constant of the conversion of 5-CHOH_4PteGlu to 5,10-CH=H_4PteGlu, k_2 is the degradation rate constant of 5,10-CH=H_4PteGlu, k_3 is the rate constant for oxidation of 5-CHOH_4PteGlu, P presents the degradation product(s) of 5,10-CH=H_4PteGlu and D presents the oxidation product(s) of 5-CHOH_4PteGlu.

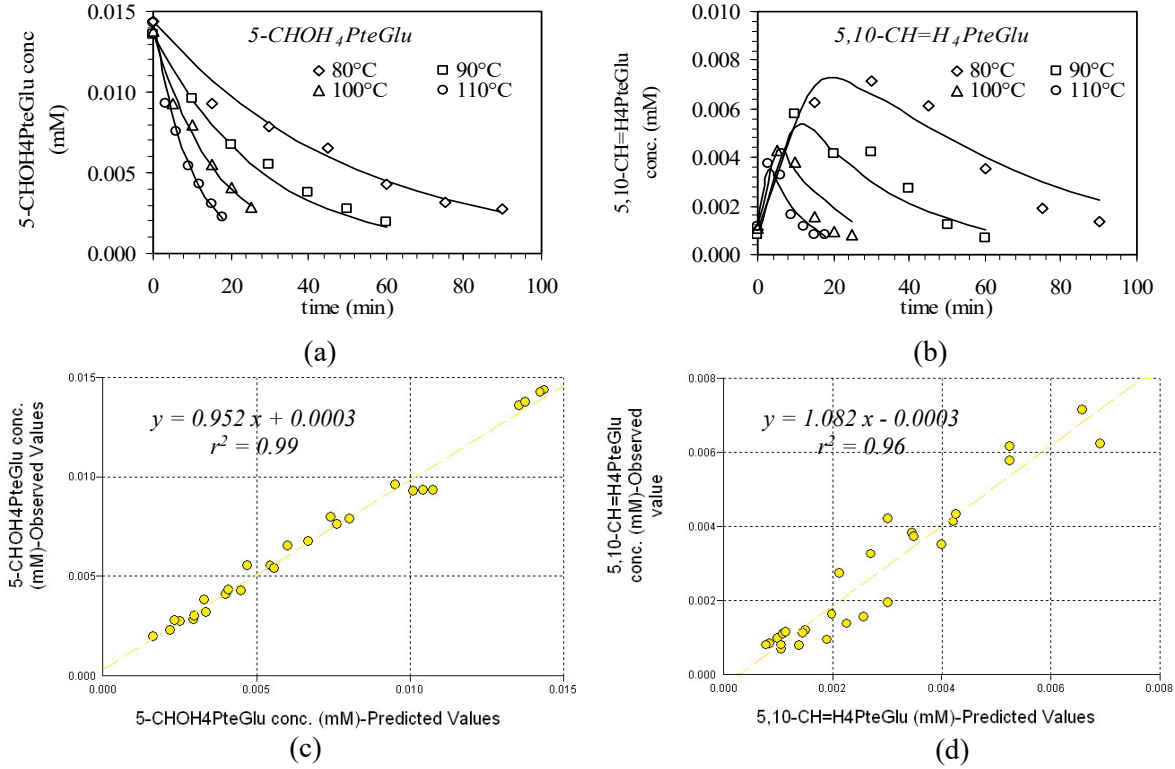

Fig. 9: (a and b): Folate concentration as a function of treatment time, symbols: experimental values, solid lines from model fitting of equation 1, (c and d): the correlation between the experimentally determined concentration values and the estimated concentration values of 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu

To validate the hypothesized mechanism of 5-CHOH_4PteGlu degradation (equation 3), multiresponse modelling was carried out using Athena Visual software. From Figure 9, it can be seen that (*i*) the quality of model fitting to the experimentally observed data of 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu is quite good, (*ii*) the relation between the experimentally observed data and the data predicted based on the suggested reaction pathway (equation 3) has a high correlation (≥0.96).

The estimated kinetic parameters for 5-CHOH_4PteGlu oxidation, the conversion of 5-CHOH_4PteGlu to 5,10-CH=H_4PteGlu and for the

elimination of 5,10-CH=H_4PteGlu are summarized in Table 1.

Table 1: Kinetic parameters for thermal degradation of 5-CHOH_4PteGlu in acetate buffer (0.2 M, pH 3.4) at a T_{ref} of 90°C

Reaction	Estimated kinetic parameters
5-CHOH_4PteGlu → 5,10-CH=H_4PteGlu	
$k_{ref1}(x10^{-3})$ (min^{-1})	E_{a1} (kJ/mol)
2.26 ± 1.02*	22.10 ± 0.67
5,10-CH=H_4PteGlu → other compounds	
$k_{ref2}(x10^{-3})$ (min^{-1})	E_{a2} (kJ/mol)
0.46 ± 0.33	34.48 ± 3.04
5-CHOH_4PteGlu → other compounds	
$k_{ref3}(x10^{-3})$ (min^{-1})	E_{a3} (kJ/mol)
2.07 ± 1.06	20.61 ± nd

**Standard error of the estimated value, nd: not determined*

The *k*-values at reference T (90°C) for 5-CHOH_4PteGlu degradation to component D (k_{ref3} value) and for 5-CHOH_4PteGlu conversion to 5,10-CH=H_4PteGlu (k_{ref1} value) are of similar magnitude. Both 5-CHOH_4PteGlu degradation to component D and 5-CHOH_4PteGlu conversion to 5,10-CH=H_4PteGlu occurred faster as compared to the elimination of 5,10-CH=H_4PteGlu. With regard to the T dependency of the rate constant (i.e. E_a value), the reaction rate constant of 5,10-CH=H_4PteGlu elimination is more sensitive towards T than both the conversion of 5-CHOH_4PteGlu to 5,10-CH=H_4PteGlu and 5-CHOH_4PteGlu oxidative degradation.

However, the latter conclusion must be further validated as the E_a value for the 5-CHOH_4PteGlu oxidative degradation was not properly estimated, probably due to the limited information that can be deduced from the data set available for this reaction pathway. Therefore, to improve and/or better validate the proposed reaction pathways, other response parameters such as oxidation products of 5-CHOH_4PteGlu degradation might be needed.

Based on the results obtained from the multiresponse analysis, it is concluded that the suggested reaction pathway (equation 3) can describe the 5-CHOH_4PteGlu degradation during thermal treatment at atmospheric pressure. Therefore, it is questioned whether the 5-CHOH_4PteGlu degradation during high pressure thermal treatment also follows the same reaction pathway.

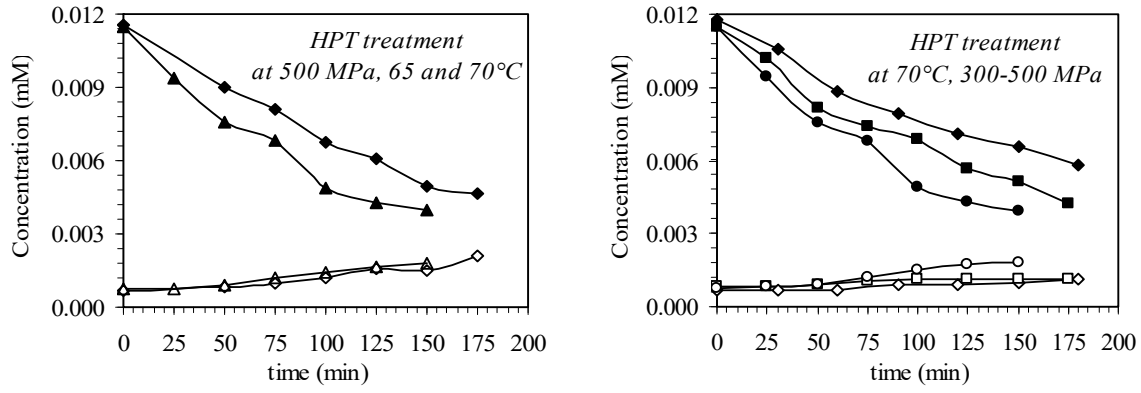

Fig. 10: Concentration of 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu as a function of treatment time [in acetate buffer (0.2 M, pH 5.0), (*a*) at 500 MPa combined with 65°C (♦) and 70°C (▲) and (*b*) at 70°C combined with 300 MPa (♦), 400 MPa (■) and 500 MPa (●) (closed symbols: 5-CHOH_4PteGlu and open symbols: 5,10-CH=H_4PteGlu)]

In this investigation, pressure degradation of 5-CHOH_4PteGlu under isobaric isothermal conditions was studied at pH 5.0 as previously carried out by Nguyen *et al*. (2006). The evolution of two response parameters, namely 5-CHOH_4PteGlu and 5,10-CH=H_4PteGlu concentrations was followed as a function of time. Figure 10 illustrates the evolution of both responses during HP-T treatments at constant P (*i.e.* 500 MPa) combined with different T (*i.e.* 65 and 70°C) and at constant T (*i.e.* 70°C) combined with various P levels (i.e. 300-500 MPa). As during pressure treatment, the elimination of 5,10-CH=H_4PteGlu was not noticed, only the reaction pathways of 5-CHOH_4PteGlu degradation to component D and the 5-CHOH_4PteGlu conversion to 5,10-CH=H_4PteGlu are considered in the multiresponse analysis.

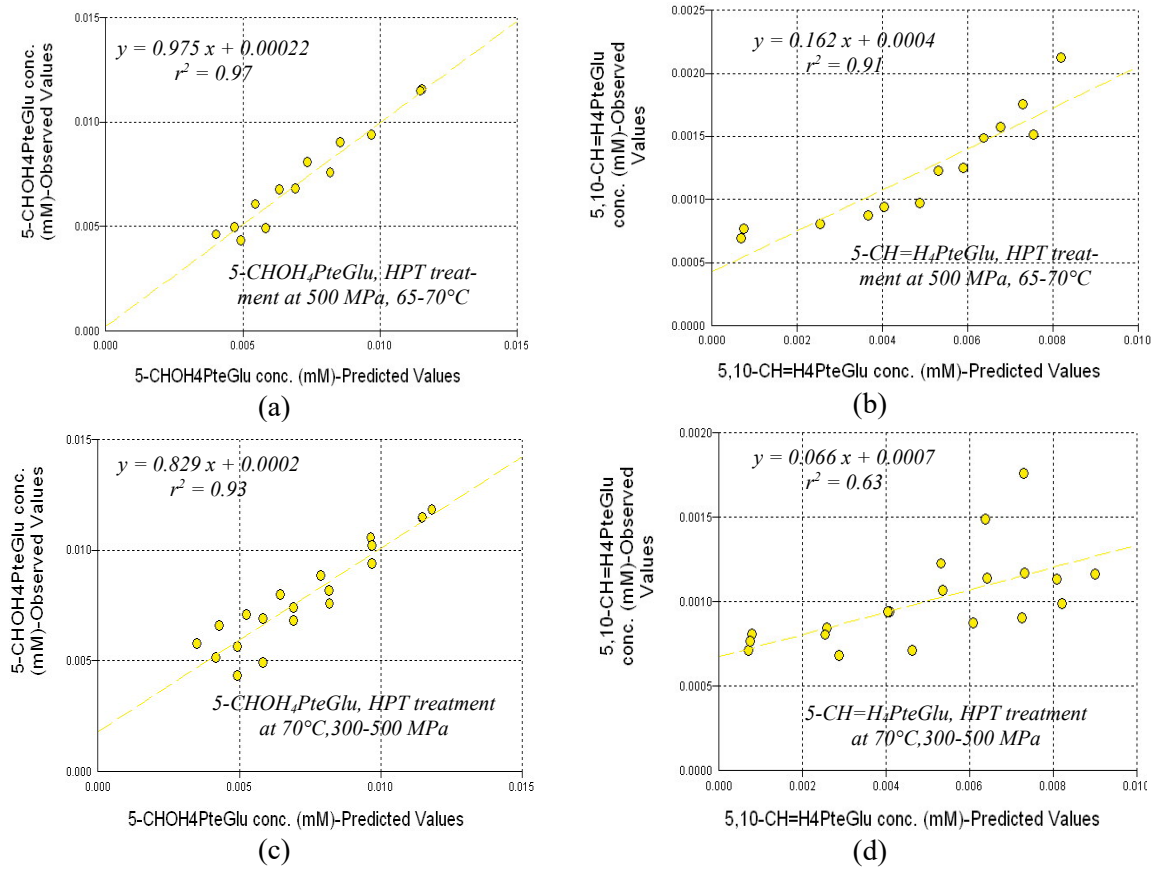

Fig. 11: Correlation between the experimentally determined concentration values and the concentration values estimated of (a) 5-CHOH$_4$PteGlu and (b) 5,10-CH=H$_4$PteGlu, at 500 MPa combined with 65°C and 70°C; (c) 5-CHOH$_4$PteGlu and (d) 5,10-CH=H$_4$PteGlu, at 70°C combined with 300 MPa-500 MPa in acetate buffer (0.2 M, pH 5.0)

As can be seen in Figure 11 the model fits the experimentally obtained 5-CHOH$_4$PteGlu data rather well but it overestimates the 5,10-CH=H$_4$PteGlu. The relation between experimentally observed value of both responses (i.e. 5-CHOH$_4$PteGlu and 5,10-CH=H$_4$PteGlu) and predicted value has a low correlation (in case of Figure 11d).

Based on the results obtained, it could not be concluded that the degradation reaction pathway of 5-CHOH$_4$PteGlu during pressure treatment is different from thermal treatment at atmospheric pressure. For pressure experiments, the folate concentration after equilibration time (2 min after pressure build up) is considered as C_o (folate concentration at time = 0 under isobaric isothermal condition). Under P, the reaction rate constants for each reaction pathway could also have different combined P-T dependencies and during pressure build up, different reactions might have already occurred to different extents. Consequently, the folate concentration after 2 min equilibration time might not correspond to the C_o for all reaction pathways.

Since the vitamin degradation has a complex pathway, modeling using multiresponse analysis can be useful to understand the mechanism and kinetics of vitamin degradation. It might be needed to monitor the pressure/temperature/time history during treatment. To allow an adequate study on the mechanism of vitamin degradation under pressure *e.g.* using multiresponse analysis, the experimental set up to achieve isobaric isothermal conditions and the identification/quantification of the breakdown products must be further investigated.

To have an idea about the effect of pressure and temperature on 5-CHOH$_4$PteGlu degradation and 5,10-CH=H$_4$PteGlu formation using the current experimental set up, apparent k values were estimated using pseudo first order reaction. Table 2 summarizes the apparent k values of 5-CHOH$_4$PteGlu degradation and the 5,10-CH=H$_4$PteGlu formation. It is clear that the rate of both reactions increased with elevating pressure at constant temperature.

Table 2: Apparent *k*-values (x 10^{-2} min^{-1}) for the degradation of 5-$CHOH_4$PteGlu (10 µg/mL ≈ 0.021 mM) and the formation of 5,10-$CH=H_4$PteGlu in acetate buffer (0.2 M, pH 5.0) at different pressure and temperature combinations

P (MPa)/T (°C)	5-$CHOH_4$PteGlu degradation	5,10-$CH=H_4$PteGlu formation
300/70	0.40 ± 0.02*	0.29 ± 0.03
	r^2 = 0.99	r^2 = 0.91
400/70	0.45 ± 0.02	0.22 ± 0.04
	r^2 = 0.98	r^2 = 0.97
500/70	0.79 ± 0.05	0.72 ± 0.06
	r^2 = 0.99	r^2 = 0.97
600/65	0.83 ± 0.03	0.62 ± 0.07
	r^2 = 0.99	r^2 = 0.94
800/65	1.20 ± 0.03	0.91 ± 0.09
	r^2 = 0.99	r^2 = 0.95

**Standard error of regression analysis*

4 CONCLUSION

Vitamin degradation may follow a complex pathway and multiresponse analysis can be useful to understand the mechanism and kinetics of vitamin degradation during food processing. Detailed further investigations in identification and quantification of breakdown products are required for detailed insight in the reaction mechanism involved. This study has clearly shown that (*i*) antioxidants do not only increase the stability of 5-$CHOH_4$PteGlu but also enhance the conversion of 5-$CHOH_4$PteGlu to 5,10-$CH=H_4$PteGlu either at atmospheric pressure combined with high temperatures (>90°C) or at high pressure combined with high temperatures (P>300 MPa, T>60°C), and (*ii*) both 5-$CHOH_4$PteGlu degradation and 5,10-$CH=H_4$PteGlu formation are also enhanced by decreasing pH. This finding implies that hot extraction and adding high concentrations of antioxidants during folate extraction do not only mislead the identification of 5,10-$CH=H_4$PteGlu but also underestimate the quantification of 5-$CHOH_4$PteGlu in food products. Moreover, the hypothesized mechanism and the kinetic information obtained in this study may contribute to a better understanding of the temperature and pressure stability of 5-$CHOH_4$PteGlu in antioxidant-rich food products such as in fruit juices.

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