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STUDY ON FOLATE STABILITY DURING THERMAL PROCESSING

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ABSTRACT

Stability of folate at different pH (3 to 9) toward thermal (65 to 140°C) treatments was studied on a kinetic basis. Residual folate concentration after thermal treatment was measured using reverse phase liquid chromatography. A model to describe the temperature effect on folate degradation rate constant is presented with the degradation of folates followed first-order reaction kinetics. The estimated Arrhenius activation energy (E_a) values of folate thermal degradation were determined.

*At all pH values studied, it was found that **(i)** folic acid and 5-formyltetrahydrofolic acid were more thermo stable than tetrahydrofolic acid and 5-methyltetrahydrofolic acid and **(ii)** all folates studied had the highest stability at neutral pH (pH 7.0) or alkaline pH (pH 9.2).*

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1 INTRODUCTION

Folates represent a member of the water-soluble B vitamin family. This generic name refers to a number of chemical compounds, which are structurally related and have a biological activity similar to that of folic acid. Folates have come into focus due to their protective role against birth defects *e.g.* neural tube defects (MRC Vitamin Study Research Group, 1991; Czeizel *et al.*, 1992), cardiovascular diseases (Boushey *et al.*, 1995; Scott and Weir, 1996; Wald *et al.*, 1998), stroke (Perry *et al.*, 1995), cancer (Giovannucci *et al.*, 1998), Alzheimer's disease (Wang, 2002) and others. The chemical reactivity of some important folate compounds determines folate stability during food processing. Considerable losses are mostly caused by oxidation and can be enhanced by light, heat, pressure etc. (William, 1993; Gregory, 1996; Gregory, 1998; Butz *et al.*, 2004; Indrawati *et al.*, 2005).

Processing stability of folates in model systems or in food systems can be affected by several intrinsic (chemical structure, pH, oxygen) and extrinsic (temperature) factors. Thermal degradation of folates has been reported in literature (O'Broin *et al.*, 1975; Chen and Cooper, 1979; Paine-Wilson and Chen, 1979; Ruddick *et al.*, 1980; Day and Gregory, 1983; Mnkeni and Beveridge, 1983; Saxby *et al.*, 1983; Barrett and Lund, 1989; Hawkes and Villota, 1989; Viberg *et al.*, 1997). However, limited information on the effects of thermal treatments on folate (folic acid and different folate derivatives) stability in model system is available. This paper describes a screening study on temperature stability of different folate derivatives. Secondly, the effect of pH on the stability of folates in model systems during thermal treatments is discussed.

2 MATERIALS AND METHODS

2.1 Sample preparation

PteGlu, H_4PteGlu, 5-CHOH_4PteGlu and 5-CH_3H_4PteGlu were obtained from Schricks Laboratory (Jona, Switzerland). The stock solutions (1.0 mg/mL) were prepared by dissolving 10 mg folates in 10 mL sodium borate buffer (0.05 M, pH 9.2) containing 0.4% β-mercaptoethanol. The stock solutions were stored at –80°C. Working standard

solutions were daily prepared (5 µg/mL for PteGlu and 10 µg/mL for other folate derivatives) and different types of buffer solutions were used for thermal treatments, *i.e.* sodium acetate buffer (0.2 M, pH 3.4), sodium citrate buffer (0.1 M, pH 4.0), sodium acetate buffer (0.2 M, pH 5.0), sodium phosphate buffer (0.1 M, pH 7.0), sodium borate solution (0.05 M, pH 9.2). All organic solvents (acetonitrile) were obtained from Merck (Darmstadt, Germany). Deionized water was used to prepare all solutions. All procedures of sample preparation and treatment were carried out under subdued light by covering samples with aluminum foil.

In this study, the folate concentrations were spectrophotometrically determined (Konings, 1999). One mililiter of each stock solution (10 mg/10 mL) of H_4PteGlu, PteGlu, 5-CH_3H_4PteGlu and 5-$CHOH_4$PteGlu was diluted to 100 mL with phosphate buffer (0.1 M, pH 7.0). For each standard working solution, the absorbance different (A-A_o) was determined within 5 min, using a spectrophotometer at suitable wavelengths (setting given in Table 1).

A is the absorbance of the standard solution and A_o is the absorbance of the blank. 5-CH_3H_4PteGlu was determined based on the absorbance at 245 nm and the calculated absorbance ratio (290 nm/245 nm) had to be above 3.3 to eliminate the contamination of dihydro derivatives. The concentration for each standard working solution was calculated using equation 1.

Table 1: Molar absorption coefficients (ε)[a], molar mass (M) and maximum wavelength (λ_{max}) for folates

Folate derivative	pH	M	λ_{max} (nm)	ε (mL/µmol.cm)
Folic acid	7.0	441.4	283	27.6
5-CH_3H_4folate	7.0	457.4	290	31.7
H_4folate	7.0	445.4	297	29.1
5-HCO-H_4folate	7.0	473.5	285	37.2
10-HCO-folic acid	7.0	469.4	269	20.9
5,10-CH^+-H_4folate	2.0	456.4	352	25.0

[a]*Data from Blakley (1969)*

$$\text{Concentration (µg/mL)} = \frac{AM}{\varepsilon 1} \quad (1)$$

2.2 Isothermal treatment

Thermal experiments were performed in a water bath (T < 90°C) or in an oil bath (T ≥ 90°C). To ensure isothermal heating, the sample solutions were filled in capillary tubes (Hirchmann, 1.15 mm i. d. x 150mm length). After preset time intervals, the capillaries were withdrawn from the water bath or the oil bath and immediately cooled in ice water to stop the thermal degradation. The samples were stored in ice water until the HPLC assay. The blank (C_o) was defined as the concentration of the untreated sample. Each heat treatment was performed twice.

2.3 Data analysis

2.3.1 Primary model to describe time dependent vitamin degradation

The degradation rate of quality attributes can be described by a n^{th} order kinetic model (equation 2).

$$\frac{dC}{dt} = -kC^n \quad (2)$$

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 folates, *i.e.* PteGlu, 5-CH_3H_4PteGlu, 5-$CHOH_4$PteGlu and H_4PteGlu 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 3.

$$\ln (C) = \ln (C_o) - kt \quad (3)$$

Where C_o is the initial concentration of the folate or its derivatives, 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) and it is derived from the slope of the regression line.

2.3.2 Secondary model to describe temperature dependence of the degradation rate constant

The temperature dependence of the degradation rate constant (k) at a constant pressure is expressed

in terms of the activation energy (E_a) and estimated using the Arrhenius equation (equation 4).

$$k = k_{ref} \exp\left[\frac{E_a}{R_T}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (4)$$

Where k is the degradation rate constant (min^{-1}), k_{ref} is the degradation rate constant at T_{ref}, E_a is the activation energy (kJ/mol), R_T is the universal gas constant (8.314 $Jmol^{-1}K^{-1}$), T is the absolute temperature (K) and T_{ref} is the absolute reference temperature (K).

2.4 HPLC analysis for folate identification and quantification

In our study, a reverse phase HPLC (AKTA purifier, GE Health Amersham Biosciences, Uppsala, Sweden) using a Prevail C_{18} column (250 mm x 4.6 mm, 5 µm particle size, Alltech, Deerfield, IL) and Unicorn 4.0 data analysis software was used to identify and quantify folates. The column temperature was maintained at 25°C. Folates were detected using a UV (290 nm, AKTA purifier, Amersham Biosciences) detector. A linear gradient was performed using a mixture of acetonitrile-phosphate buffer (0.033 M, pH 2.15) with a flow rate of 1 mL/min. The gradient started at 5.0 % (v/v) acetonitrile, which was maintained isocratically for the first 4 min and afterwards, increased to 60 % (v/v) within 12 min. In this study, the injection volume was 100 µL. The folate peak was found at a retention time between 10 and 12 min. The concentration of folates was calculated based on their peak area and peak height in comparison to the external standard solutions (in the range of 0-15 µg/mL) of the same components. The correlation coefficient (r^2) of the standard curves in this study exceeded 0.99.

2.5 Standard curve of HPLC analysis

A chromatogram of folate is presented in Figure 1. PteGlu, H_4PteGlu, 5-CH_3H_4PteGlu and 5-$CHOH_4$PteGlu were detected by UV at 290 nm. The elution times of H_4PteGlu, 5-CH_3H_4PteGlu and 5-$CHOH_4$PteGlu and PteGlu were 10.50 min, 10.69 min, 11.47 min and 11.56 min, respectively. Vitamin concentration was quantified based on external standards. Standard curves showing a linear response of peak area or peak height versus folate concentration (UV detection at 290 nm) are given for example in Figure 2. The regression correlation (r^2) of the standard curves in this study were at least 0.99.

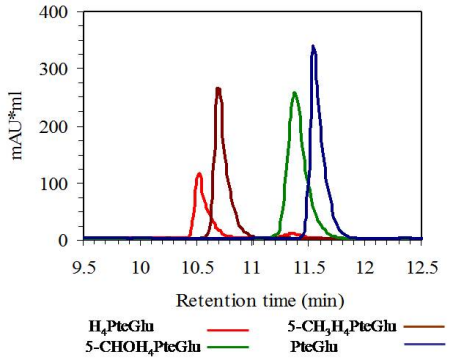

Fig. 1: Typical HPLC chromatogram of folates

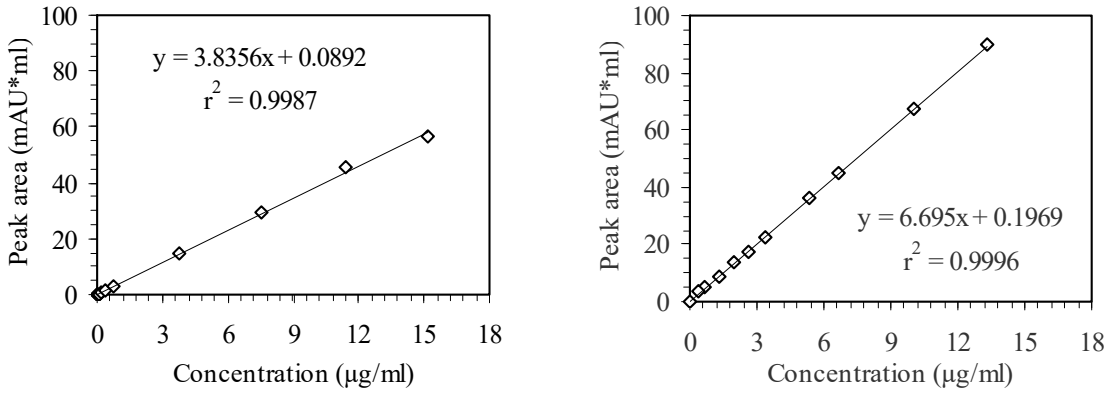

Fig. 2: Standard curves for (a) 5-CH_3H_4PteGlu, (b) 5-$CHOH_4$PteGlu at 290 nm

3 RESULTS AND DISCUSSION

3.1 Temperature stability of H_4PteGlu

H_4PteGlu was extremely labile towards temperature. The concentration of H_4PteGlu decreased during sample preparation. The molecule degraded very fast during treatment for example at 60°C. As reported by Paine-Wilson and Chen (1979), H_4PteGlu is rapidly oxidized by air in neutral solution (phosphate buffer, pH 7.0) with the formation of pABG and a number of pterins. Reed and Archer (1980) have reported that the rate of H_4PteGlu loss at pH 4 was equal to the rate of pABG formation, and 6-formylpterin was the major product at pH 7.0, with smaller levels of pterin and xanthopterin.

3.2 Temperature stability of PteGlu

PteGlu was very stable at temperatures lower than 120°C. A slight degradation was observed at temperatures higher than 120°C (Figure 3). The effect of pH on PteGlu stability at 160°C is shown in Figure 4. PteGlu was quite stable at pH 5.0 to 9.2, but unstable at more acidic pH (pH 3.4). At all pH values studied (3.4, 5.0, 7.0, 9.2), thermal degradation could be described by a pseudo first order kinetic model. This is in agreement with literature findings (Paine-Wilson and Chen, 1979; Mnkeni and Beveridge, 1983).

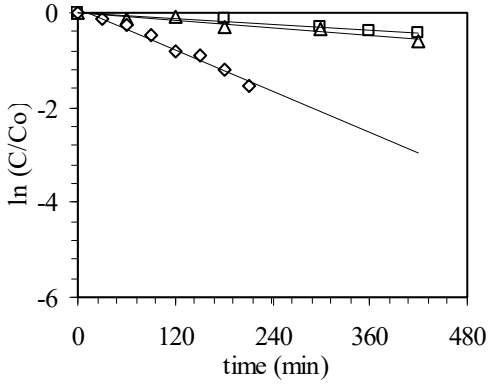

Fig. 3 :Logarithm of the ratio of concentration to initial concentration of PteGlu (5 µg/mL) as a function of treatment time [in acetate buffer (0.2 M, pH 5.0) at 120°C (□), 140°C (Δ) and 160°C (◊)]

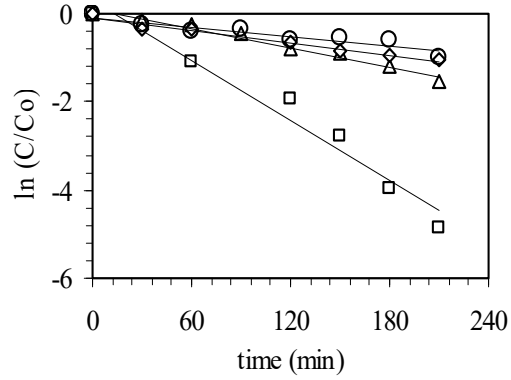

Fig. 4: Logarithm of the ratio of concentration to initial concentration of PteGlu (5 µg/mL) as a function of treatment time [at 160°C and pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)]

The estimated k and E_a values are summarised in Table 2. It can be stated that thermal stability of PteGlu is largely affected by pH. PteGlu is more thermostable at higher pH value and the stability decreased rapidly at low pH. PteGlu is stable up to 7 h heating at temperatures 120-140°C and pH 5.0 to 9.2. Acidification of the medium decreased the thermal stability of PteGlu.

The estimated E_a values are comparable to those obtained by Mnkeni and Beveridge (1982, 1983). They found that the E_a values of PteGlu degradation at pH 3 and 6 were 94.47 and 70.22 kJ/mol, respectively.

Temperature dependency of the rate constants for PteGlu degradation in the temperature range studied could be described by the Arrhenius equation (Eq. 4). The relation between natural logarithm of the degradation rate constants versus the reciprocal of absolute temperature is shown in Figure 5.

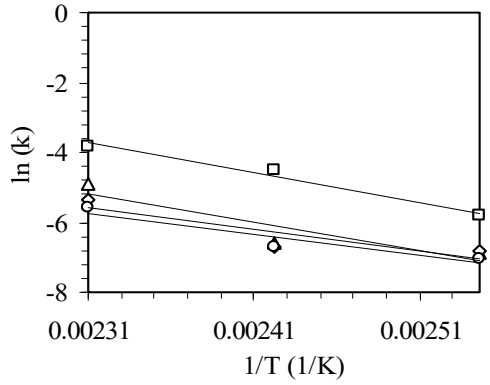

Fig. 5: Temperature dependence of the *k*-values for the thermal degradation of PteGlu at pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)

Table 2: k (x 10^{-3}) (min^{-1}) and E_a values for the thermal degradation of PteGlu (5 µg/mL) in buffer at pH 3.4, pH 5.0, pH 7.0 and pH 9.2

T (°C)	pH 3.4	pH 5.0	pH 7.0	pH 9.2
120	2.99±0.25*	1.06±0.10	1.08±0.19	0.86±0.22
140	11.31±0.64	1.36±0.17	1.26±0.20	1.18±0.14
160	22.24±3.06	7.31±0.45	4.74±0.36	3.77±0.64
E_a (kJ/mol)	71.22±11.36	67.50±31.17	51.67±25.40	51.46±16.31
r^2	0.98	0.82	0.81	0.91

**Standard error of regression*

3.3 Temperature stability of 5-CH_3H_4PteGlu

The effects of temperature on 5-CH_3H_4PteGlu stability were investigated at temperatures from 65 to 90°C. When the logarithm of the concentration was plotted as a function of time, thermal degradation of 5-CH_3H_4PteGlu could be described by a pseudo first order model (Figure 6). After 15 min treatments from 65°C up to 90°C, the folate degradation in sodium phosphate buffer was ranging from 14 % to 65 %. This folate derivative degraded very fast at 90°C, particular at low pH. The residual concentration of 5-CH_3H_4PteGlu in acetate buffer pH 3.4 was about 15% after heating for 15 min at 90°C. To investigate the influence of pH on the thermal degradation of 5-CH_3H_4PteGlu, different buffer solutions (pH ranging from 3.4 to 9.2) were used. The results are shown in Figure 7 at 80°C. The estimated kinetic parameters are summarized in Table 3.

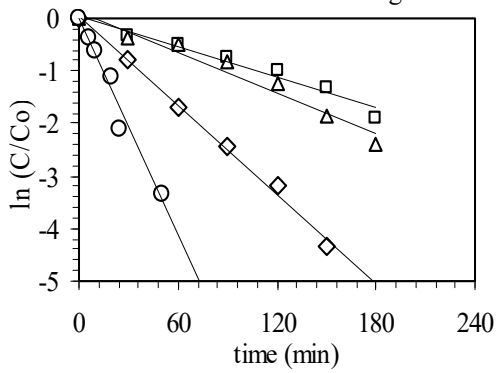

Fig. 6: Logarithm of the ratio of concentration to initial concentration of 5-CH_3H_4PteGlu (10 µg/mL) as a function of treatment time [in phosphate buffer (0.1 M, pH 7.0) at 65°C (□), 70°C (Δ), 80°C (◊) and 90°C (o)]

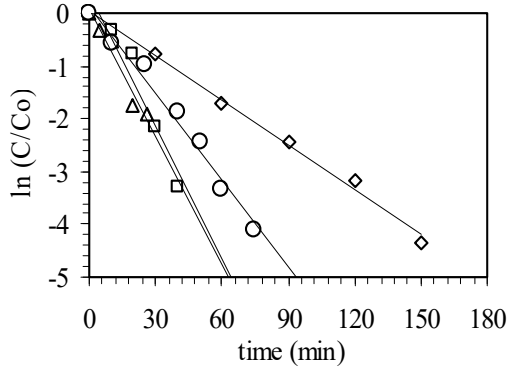

Fig. 7: Logarithm of the ratio of concentration to initial concentration of 5-CH_3H_4PteGlu (10 µg/mL) as a function of treatment time [at 80°C in buffer pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)]

Table 3: k (x 10^{-3}) (min^{-1}) and E_a values for the thermal degradation of 5-CH_3H_4PteGlu (10 µg/mL) in buffer pH 3.4, pH 5.0, pH 7.0 and pH 9.2

T (°C)	pH 3.4	pH 5.0	pH 7.0	pH 9.2
65	10.18±1.21[a]	14.41±2.25	9.73±0.83	9.61±0.81
70	22.45±2.55	31.27±4.67	13.06±1.12	15.22±1.03
80	79.98±14.57	80.79±8.72	28.14±0.93	55.29±3.28
90	165.62±23.66	105.86±11.06	68.31±5.86	112.36±11.81
E_a (kJ/mol)	114.18±10.82	80.83±16.32	79.98±4.88	103.94±8.04
r^2	0.98	0.93	0.99	0.99

[a]Standard error of regression

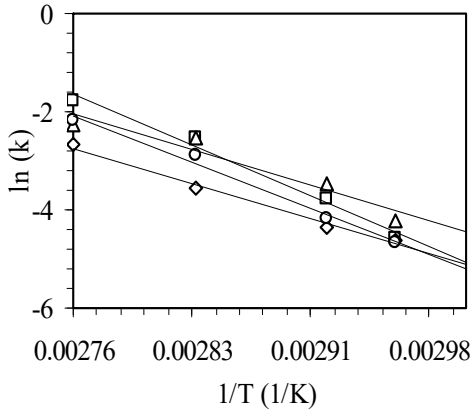

Fig. 8: Temperature dependence of the degradation rate constant for the thermal degradation of 5-CH_3H_4PteGlu in buffer at pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)

The stability of 5-CH_3H_4PteGlu in buffer solution depends on the pH. The highest stability at all temperature tested was in phosphate buffer pH 7.0, and the stability rapidly decreased with increasing alkalinity (pH 9.2) or acidity (pH 3.4–pH 5.0). Comparable results were reported by Paine-Wilson and Chen (1979). O'Broin *et al.* (1975) also investigated the effect of pH and buffer ions on the stability of several folates. This study showed the highest stability of 5-CH_3H_4PteGlu at pH 9.0 with a 0.1M Tris/HCl buffer. At 65°C and 70°C, the degradation rate of 5-CH_3H_4PteGlu at pH 5.0 was the highest compared to pH 3.4, 9.2 and 7.0. The degradation was enhanced by lowering the pH. The degradation of 5-CH_3H_4PteGlu at alkaline pH in this study could be explained by an oxidative mechanism involving the formation of 5-CH_3H_2PteGlu (Paine-Wilson and Chen, 1979). At acidic pH, the loss of 5-CH_3H_4PteGlu during heating may be due to formation of an unidentified nutritionally inactive derivate as suggested by O'Broin *et al.* (1975) rather than by oxidative cleavage at the C9-N10 bond resulting in the separation of the pteridine and p-aminobenzoyl ring. The substitution in the 5-position would be expected to be resistant to such a cleavage reaction (Rabinowitz, 1960).

The temperature dependence of the degradation rate constants of 5-CH_3H_4PteGlu was described by the Arrhenius equation (Figure 8).

It can be seen that 5-CH_3H_4PteGlu has the highest thermostability at pH 7.0, while the lowest thermostability is found at pH 3.4. Comparing the E_a values, the lowest temperature sensitivity of the *k*-values were observed for the degradation of 5-CH_3H_4PteGlu at pH 5.0 and 7.0.

3.4 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 (Figure 9).

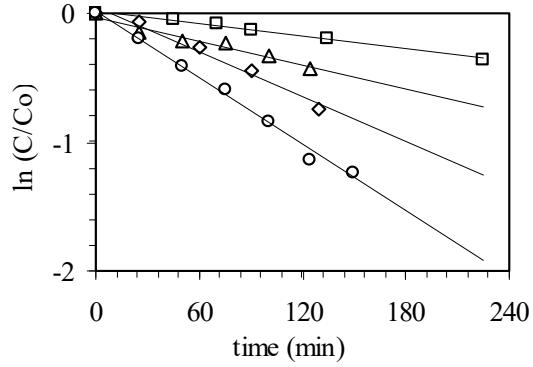

Fig. 9: Logarithm of the ratio of concentration to initial concentration of 5-$CHOH_4$PteGlu (10 µg/mL) as a function of treatment time [in acetate buffer (0.2 M, pH 5.0) at 80°C (□), 90°C (Δ), 100°C (◊) and 110°C (o)]

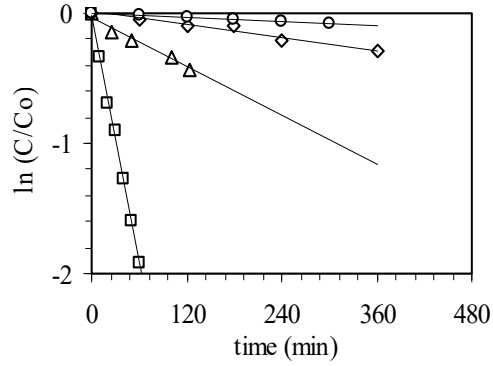

Fig. 10: Logarithm of the ratio of concentration to initial concentration of 5-$CHOH_4$PteGlu (10 µg/mL) as a function of treatment time [at 90°C in buffer pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)]

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 2) as shown in Figure 10. 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 (Table 4). 5-$CHOH_4$PteGlu was stable for 7 hrs

at 80°C at 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). Under acidic conditions, 5-$CHOH_4$PteGlu loses a molecule of water to form 5,10-$CH{=}H_4$PteGlu, especially at elevated temperature (May *et al.*, 1951). Paine-Wilson and Chen (1979) found this vitamin to be stable for 10 hrs at 100°C in the pH range from 4.0 to 12.0, and below pH 4.0 the stability rapidly decreased with pH. The results on thermal stability of 5-$CHOH_4$PteGlu are in line with previous reports (May *et al.*, 1951; Paine-Wilson and Chen, 1979).

Table 4: k (x 10^{-3}) (min^{-1}) and E_a values for the thermal degradation of 5-$CHOH_4$PteGlu in buffer pH 3.4, pH 5.0, pH 7.0 and pH 9.2

T (°C)	pH 3.4	pH 5.0	pH 7.0	pH 9.2
80	18.38±1.02[a]	1.65±0.08	0.32±0.02	0.17±0.02
90	31.57±0.70	3.10±0.33	0.82±0.06	0.26±0.01
100	61.22±2.82	5.78±0.45	1.52±0.12	0.29±0.04
110	98.81±3.05	8.59±0.33	2.19±0.05	0.65±0.04
E_a (kJ/mol)	64.17±2.45 0.99	62.81±3.56 0.99	71.78±8.96	47.18±11.13
r^2			0.97	0.90

[a]*Standard error of regression*

The temperature dependence of the rate constant for the thermal degradation of 5-$CHOH_4$PteGlu in buffer systems in the temperature range studied could be adequately described by the Arrhenius equation (Figure 11). The estimated activation energy ranged from 47.18 to 71.78 kJ/mol depending on the pH value. 5-$CHOH_4$PteGlu has the highest thermostability at pH 9.2 and the lowest thermostability was found at pH 3.4.

By comparing the thermostability of 5-$CHOH_4$PteGlu, PteGlu and 5-CH_3H_4PteGlu in all buffer systems were studied, it can be stated that all folates have a high thermostability at neutral or alkaline pH. The estimated E_a-values of folates at pH 3.4 and pH 9.2 were respectively 71.22 and 51.46 kJ/mol for PteGlu, 64.17 and 47.18 kJ/mol for 5-$CHOH_4$PteGlu and 114.18 and 103.94 kJ/mol for 5-CH_3H_4PteGlu. Moreover, it was observed that PteGlu is more thermostable than 5-$CHOH_4$PteGlu and 5-CH_3H_4PteGlu as shown in Figure 12.

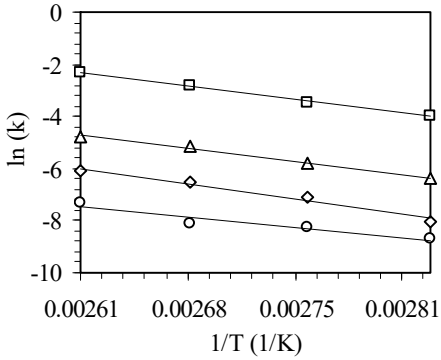

Fig. 11: Temperature dependence of the degradation rate constant for the thermal degradation of 5-$CHOH_4$PteGlu in buffer pH 3.4 (□), pH 5.0 (Δ), pH 7.0 (◊), pH 9.2 (o)

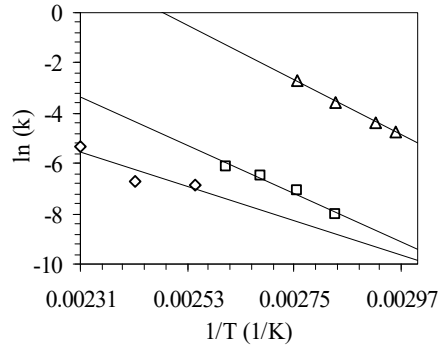

Fig. 12: Temperature dependence of the degradation rate constant of 5 µg/mL PteGlu (◊), 10 µg/mL 5-$CHOH_4$PteGlu (□) and 10 µg/mL 5-CH_3H_4PteGlu (Δ) in phosphate buffer (0.1 M, pH 7.0)

4 CONCLUSIONS

The thermal degradation of all folate derivatives studied in different buffer systems at temperatures from 65°C to 160°C followed pseudo first order kinetics. It was found that PteGlu and 5-$CHOH_4$PteGlu were quite stable whereas H_4PteGlu and 5-CH_3H_4PteGlu were less thermostable.

During thermal treatment, PteGlu, 5-CH_3H_4PteGlu and 5-$CHOH_4$PteGlu had the highest stability at neutral pH (pH 7.0) or alkaline pH (pH 9.2). PteGlu showed to be very stable up to temperatures of 120°C. This is in line with the studies on thermostability of folates published in literature.

The pH was found to have a pronounced influence on the thermal and pressure stability of folates. The largest stability of both PteGlu and 5-$CHOH_4$PteGlu was found at pH 9.2 for thermal

treatment and the stability decreased with increasing acidity.

H_4PteGlu was unstable during thermal treatment. 5-CH_3H_4PteGlu showed the greatest stability at pH 7.0 and exhibited parallel patterns of decreasing stability with increasing acidity and alkalinity during heat treatment.

By mutually comparing the different buffered systems, PteGlu acid showed the highest stability compared to 5-$CHOH_4$PteGlu, 5-CH_3H_4PteGlu and H_4PteGlu with respect to pH and temperature.

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