



## Analytical Methods

# High-pressure treatments induce folate polyglutamate profile changes in intact broccoli (*Brassica oleraceae* L. cv. *Italica*) tissue <sup>☆</sup>

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## ABSTRACT

In plant matrices, folates exist largely as folylpoly- $\gamma$ -glutamates requiring deglutamylation to monoglutamates prior to absorption, which might impair dietary folate bioavailability. This study investigated folylpoly- $\gamma$ -glutamate stability and conversions in broccoli tissue during thermal (25–90 °C, 30 min) and high-pressure treatments (0.1–600 MPa, 25–45 °C, 30 min) after vacuum packaging. Folates were analyzed based on poly- $\gamma$ -glutamate side chain length by RP-HPLC. During thermal treatments, folates were stable up to 90 °C, whereas differences in folylpoly- $\gamma$ -glutamate profiles towards higher conjugated folylpoly- $\gamma$ -glutamates were observed at elevated temperatures (70–90 °C). High-pressure treatments resulted in significant folate losses (48–78%). Depending on the pressure–temperature combinations studied, folylpoly- $\gamma$ -glutamates were converted to folylmono- and folyl-di- $\gamma$ -glutamates, which was shown to occur mainly during the initial stages of the high-pressure treatments, i.e. during pressure build-up and subsequent equilibration. Targeted application of high-pressure treatments can hence be applied to obtain broccoli with higher monoglutamate folate content. Implications towards folate bioavailability in relation to the observed folate degradation, however, requires further investigation.

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## 1. Introduction

Tetrahydrofolate (H<sub>4</sub>PteGlu) and its derivatives, collectively termed folates, differ in oxidation state, one-carbon substitutions and the number of  $\gamma$ -linked glutamate residues attached to the pteroyl moiety. Folates are essential cofactors in one-carbon transfer reactions in key biosynthetic and epigenetic processes (Stover, 2004). Folate deficiency has been shown to be prevalent in underdeveloped countries, and even in the Western world, subtle deficiency is a public health problem most notable in its association with the prevention of occurrence and reoccurrence of neural tube defects (Czeizel & Dudas, 1992). Optimal folate status has been considered to be protective against development of certain types of cancer, anemia, atherosclerosis, neural tube defects (NTDs), adverse pregnancy outcomes, and neuropsychiatric disorders (Kim, 2007). Folates in human diets mainly come from plant foods where green leafy vegetables are considered as best folate source (Scott, Rebeille, & Fletcher, 2000). As a means to enhance folate status, the consumption of folate-rich foods was shown to be relatively ineffective compared to either taking supplements or consuming foods fortified with folic acid (PteGlu), the synthetic form of the vitamin that does not naturally occur in foods (Cuskelly, McNulty, & Scott, 1996).

This finding has been assumed to be the result of the poor bioavailability of dietary folate, which consists of approximately 75% folylpoly- $\gamma$ -glutamates in a mixed diet (Melse-Boonstra et al., 2002), as compared with the high bioavailability of PteGlu (Brouwer, van Dusseldorp, West, & Steegers-Theunissen, 2001). Therefore, mandatory fortification of staple foods with PteGlu was introduced e.g. in the US, Canada and Chile and resulted in a significantly decreased prevalence of NTDs plus a reduction of total plasma homocysteine levels in the US population (Honein, Paulozzi, Mathews, Erickson, & Wong, 2001). Despite these results, mandatory fortification has not yet been implemented in many other countries because of concerns that chronic exposure to excessive doses of PteGlu may be mischievous to overall human health (Kim, 2007; Lucock & Yates, 2005). Consequently, dietary folates are predicted to have a higher margin of safety and are currently considered as an alternative for synthetic PteGlu (de la Garza, Gregory, & Hanson, 2007). In this context, synthetic L-5-methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu), the predominantly occurring natural folate derivative, was recently allowed as food fortificant in the European Union and also biofortification of vegetables is considered as a possible strategy (de la Garza et al., 2007; Finglas et al., 2006).

Intestinal folate absorption is achieved by a two-step process at the jejunal brush-border membrane by hydrolysis of the  $\gamma$ -linked glutamyl residues by folate  $\gamma$ -glutamyl hydrolase (FGGH) (Halsted et al., 1998), releasing monoglutamyl folate derivatives for subsequent membrane transport by the reduced folate carrier system.

<sup>☆</sup> High-pressure treatments influence folylpoly- $\gamma$ -glutamates in broccoli.

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Results of clinical studies on the effect of the extent of conjugation on folate bioavailability are, however, unequivocal as some have shown that, at physiological doses, synthetic folic acid exhibits a higher bioavailability than its pteroylpolyglutamates (PteGlu<sub>n</sub>) when administered as supplements (Melse-Boonstra, West, Katan, Kok, & Verhoef, 2004) or added to some foods (Wei, Bailey, Toth, & Gregory, 1996), in contrast to others showing that the degree of polymerisation of the  $\gamma$ -L-glutamate tail *per se* does not limit the short-term bioavailability of folates in some foods (Konings et al., 2002; McKillop et al., 2006). In general, however, it was often found that absorption of folylpolyglutamates is less than that of the monoglutamate (Brouwer et al., 2001). Among the critical factors that can explain the incomplete bioavailability of dietary folates are variations in intestinal pH, leaching and instability of reduced folates during food processing, folate instability in the digestive system before absorption, occurrence of folate binding proteins or FGGH inhibitors (i.e. some organic acids) in some foods, entrapment of folates in the food matrix and fiber content of foods (Brouwer et al., 2001). FGGH is, however, apparently ubiquitous in plants (Orsomando et al., 2005) and disruption of cellular compartmentation due to effects of food processing methods such as mixing, cutting, freezing and high hydrostatic pressure treatments can result in depolymerisation of folylpoly- $\gamma$ -glutamates *in situ* probably by endogenous plant folate  $\gamma$ -glutamyl hydrolase activity, leading to an improved bioaccessibility of folates with short  $\gamma$ -glutamate side chains in some vegetables (Melse-Boonstra et al., 2002). Bioaccessibility is defined as the proportion of an ingested nutrient which is in the absorbable form presented to the intestinal brush-border for absorption (Melse-Boonstra et al., 2002). However, only few data are available on the issue of processing vegetables with the aim of converting folylpolyglutamates to folylmonoglutamates.

In the late 1980s, the potential of high hydrostatic pressure processing for food industry application has attracted considerable research interest as this “non-thermal” technology enables some novel and diverse uses with some key advantages over traditional (i.e. thermal) food processing techniques. Among these promising applications, high hydrostatic pressure technology has been shown to induce destruction of microorganisms at moderate temperatures (Cheftel, 1995) and has hence been applied as a pasteurisation technique for a broad range of food products, including fruits and vegetables, using pressure–temperature–time combinations of 100–800 MPa, 5–60 °C (1–60 min) depending on the food product. The key idea behind high hydrostatic pressure processing is the principle of le Chatelier, according to which any phenomenon (phase transition, chemical reactivity, change in molecular configuration, chemical reaction) accompanied by a decrease in volume will be enhanced by pressure and vice versa. Consequently, pressure has a limited effect on the disruption of covalent bonds (Tauscher, 1995). Small molecules such as vitamins, pigments and volatile compounds are less affected by high hydrostatic pressure processing compared to proteins/enzymes which are often characterized by a complex three-dimensional structure stabilized by various covalent and non-covalent interactions. Research on high-pressure processing in the pressure domain from 100 to 1000 MPa combined with subzero to moderate temperatures (–20 up to 60 °C) has demonstrated effects on (i) phase transitions of water and lipids, (ii) cell wall and -membrane disruption processes, (iii) inactivation of vegetative microorganisms, (iv) toxins, (v) modification of biopolymers including both enzyme activation and enzyme inactivation, protein denaturation and gel formation, (vi) enzyme catalyzed conversion processes and, (vii) chemical (e.g. oxidation) reactions (Oey et al., 2008). Since the mechanisms of (bio)chemical reactions under pressure cannot always be anticipated by direct extrapolation of the reaction mechanism occurring at atmospheric pressure, further research is still required to elucidate the effect of high-pressure on food products. Despite that fo-

late  $\gamma$ -glutamyl hydrolase is apparently ubiquitous in plants, only few data are available on the influence of high hydrostatic pressure processing of vegetables regarding this enzyme. In literature, folylpolyglutamate hydrolysis was reported in green beans, leeks and cauliflower after high hydrostatic pressure treatments at 200 MPa (22 °C, 5 min) (Melse-Boonstra et al., 2002) but this was limited to a single observation. This study, however, suggested that high hydrostatic pressure could be applied to pasteurize vegetables with the aim of converting folylpoly- $\gamma$ -glutamates to folylmonoglutamates. Hereto, the goal of the current investigation was to study the influence of temperature and pressure on enzymatic folylpoly- $\gamma$ -glutamate conversions by endogenous folate  $\gamma$ -glutamyl hydrolase activity at atmospheric pressure (25–90 °C) and under high-pressure pasteurisation conditions (100–600 MPa, 25–45 °C) by analyzing folates based on the degree of polymerisation (DP) of the poly- $\gamma$ -glutamate side chain. Preliminary, experiments were conducted to investigate whether folylpoly- $\gamma$ -glutamate conversions in model systems occur by non-enzymatic hydrolysis. The effect of pressure and temperature treatments on folylpoly- $\gamma$ -glutamate profiles and folate stability was subsequently investigated as a case study in broccoli, which is a rich source of folylpoly- $\gamma$ -glutamates.

## 2. Materials and methods

### 2.1. Materials

All chemicals and reagents used were of analytical or HPLC grade. 5-Methyl-tetrahydro-folylpoly- $\gamma$ -glutamate standards (5-CH<sub>3</sub>H<sub>4</sub>-PteGlu<sub>2–5</sub>) and pteroyl-poly- $\gamma$ -glutamate standards (PteGlu<sub>6–8</sub>) were purchased from Schircks Laboratories (Jona, Switzerland) and were, respectively, 85–95% and 98.0–99.5% pure according to HPLC-analysis by the manufacturer. Also 5-formyl-tetrahydrofolic acid (5-CHO–H<sub>4</sub>PteGlu), tetrahydrofolic acid (H<sub>4</sub>PteGlu) and 5-10-methylene-tetrahydrofolic acid (5-10-CH<sub>2</sub>H<sub>4</sub>PteGlu) were obtained from Schircks Laboratories. 5-Methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu) was donated as Ca-salt by Eprova AG (Schaffhausen, Switzerland). Folate standards were stored in ampoules under argon atmosphere at –80 °C. Samples of raw broccoli (*Brassica oleracea* L. *botrytis* cv. *Italica*) from the same batch were purchased from a local distributor (var. Milady, Greenpartners, Sint-Katelijne-Waver, Belgium) or local supermarket (unknown var., Delhaize, Leuven, Belgium) and were stored at 4 °C for maximally one week. To study the influence of temperature and pressure on folate stability and folylpoly- $\gamma$ -glutamate distribution in intact broccoli tissue, inflorescence and some edible parts of the stems were double-vacuum packed in polyethylene bags using a vacuum sealing machine (Multivac A300/16, Wolfertschwenden, Germany) up to 1.1 Pa and thermostated for 5 min at 25 °C in a waterbath prior to treatments. Moisture content of broccoli was determined by drying samples ( $\pm$ 2 g FW) at 105 °C for 24 h.

### 2.2. Treatments

For thermal treatments (25–90 °C) at atmospheric pressure, samples were immersed in a waterbath during a preset time interval of 0–30 min. Combined pressure–temperature treatments were conducted under isothermal–isobaric conditions (25–45 °C, 0.1–600 MPa) using a laboratory pilot scale, multivessel, high-pressure apparatus (Resato, Roden, Netherlands) consisting of six cylindrical (6 × 43 mL) thermostated pressure vessels, with a TR15 oil/glycol mixture (Resato, Roden, Netherlands) as pressure transmitting medium. Before pressurization, the time needed to insert the samples and close the vessels was standardized at 45 s. The pressure was subsequently built up to the desired values at a constant rate

of 100 MPa/min. When the desired pressure values were reached, the pressure was isolated in the vessels and an equilibration period of 5 min was taken into account to allow the temperature inside the vessels to evolve back to the desired value after adiabatic heating (=time zero for isothermal–isobaric conditions). Pressure vessels were then decompressed after a preset time interval of 5–25 min. Samples were removed from the pressure vessels 45 s after decompression and immediately immersed in liquid nitrogen for storage at  $-80^{\circ}\text{C}$  before extraction of folates. For thermal experiments, samples consisted of  $\pm 20$  g vacuum packed broccoli per treatment. Also for combined pressure–temperature treatments,  $\pm 20$  g vacuum packed broccoli was inserted in each pressure vessel. Most treatments were conducted in duplicate.

### 2.3. Folylpolylglutamate extraction

A simultaneous di-enzyme extraction procedure was used for folylpoly- $\gamma$ -glutamate analysis based on the procedure described by Indrawati et al. (2004) with few modifications. Frozen broccoli samples ( $\pm 20$  g) were mixed with 140 mg L-ascorbic acid and 50 mL of boiling extraction buffer (phosphate buffer (0.1 M, pH 7.0)) in opaque polypropylene tubes (250 mL, Nalgene Labware, NY). Samples were homogenized at 11 000–13 000 rpm for 30 s using a mixer (IKA Ultraturrax T25, Janke & Kunkel GmbH, Staufen, Germany) and subsequently heated for 15 min in boiling water. After heat extraction, crude sample extracts were cooled in ice water and the pH was adjusted to pH 7.0 using HCl (37%) and NaOH (5 M). To extract matrix and protein bound folates, crude extracts were incubated at  $37.5^{\circ}\text{C}$  with 50  $\mu\text{L}$  amylase (Termamyl 120L, Novo Nordisk, Bagsværd, Denmark) and 50  $\mu\text{L}$  of a 1 mg/mL *Bacillus subtilisin* protease (Sigma–Aldrich, Steinheim, Germany) solution for 4 h in a shaking water bath (WB, Memmert, Schwabach, Germany) at a shaking speed of 2.5 Hz. Enzymes were inactivated by heating the digested samples for 5 min in boiling water and afterwards the extract was cooled in an ice water bath. The sample pH was adjusted to pH 7.0. Afterwards, samples were filtered, centrifuged (20 000g,  $4^{\circ}\text{C}$ , 30 min) and the collected supernatant was stored at  $-80^{\circ}\text{C}$  prior to conversion and purification of folates.

### 2.4. Folylpolylglutamate conversion to 5-methyltetrahydrofolic acid derivatives

Folylpolylglutamates of different folate derivatives were chemically converted to the according 5-methyltetrahydrofolylpolylglutamates ( $5\text{-CH}_3\text{H}_4\text{PteGlu}_n$ ) following the procedure described by Ndaw, Bergaentzle, Werner, Lahely, and Hasselmann (2001) with minor modifications. Briefly, 5 mL of the extraction supernatant was added to 15 mL Tris buffer (66 mM, pH 7.8) with 2.0 g Na-ascorbate and 1 mL 2-octanol. Thereafter, 10 mL of  $\text{NaBH}_4$  (3.2 M) was added and the mixture was incubated for 10 min. Then the pH was adjusted to pH 7.4 using  $\text{CH}_3\text{COOH}$  (5 M) and 80  $\mu\text{L}$  of HCHO (37%, approximately 1 mmol) was added. Next, the mixture was vigorously shaken for 30 s and within the following 30 s addition of  $\text{NaBH}_4$  (3.2 M, 10 mL) was started. Subsequently, the pH of the conversion solution was adjusted to approximately pH 0.85 using HCl (37%) and the mixture was incubated for 10 min. After incubation, the pH was changed to pH 5 with NaOH (5 M) and another 10 mL of  $\text{NaBH}_4$  (3.2 M) was added. Thereon, the conversion solution was incubated for 20 min and finally the volume was adjusted to 100 mL. 2-Octanol was discarded from the converted extract and aliquots were stored at  $-80^{\circ}\text{C}$  until purification. Samples were wrapped in aluminium foil to prevent UV-degradation of folates during conversion.

### 2.5. Folate isolation and purification

Folates were purified from thawed and filtered conversion mixtures using folate binding protein affinity chromatography (FBP-AC) columns. Purified bovine FBP's were obtained from Scripps Laboratories (San Diego, CA, USA) and immobilised on an *N*-hydroxysuccinimide ester resin (Affi-gel 10, BioRad Laboratories, Hercules, CA, USA) as described earlier (Indrawati et al., 2004). FBP columns were equilibrated with 5 mL phosphate buffer (0.1 M, pH 7.0) prior to application of the sample (5 mL) and subsequently washed using 5 mL phosphate buffer (25 mM, pH 7.0) containing NaCl (1 M). Columns were then further rinsed with 5 mL phosphate buffer (25 mM, pH 7.0). Thereafter, folates were eluted using 5 mL of a  $\text{CF}_3\text{COOH}$  (20 mM) with 1,4-dithioerythritol (20 mM) solution (pH 6.35) and were collected in a 5 mL volumetric flask containing 5  $\mu\text{L}$   $\beta$ -mercaptoethanol, 200  $\mu\text{L}$  of an L-ascorbic acid solution (25%, w:v), and 40  $\mu\text{L}$  of a KOH solution (60%, w:v). After elution, the volume was adjusted to 5 mL with the elution. The final pH of the eluate was pH 5.4. Subsequently 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma 1-7}$  concentrations were determined using reversed-phase HPLC. The purification procedure was performed in a dark room at  $4^{\circ}\text{C}$  in order to prevent degradation of folates.

### 2.6. RP-HPLC determination of 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma n}$ derivatives

Determination of converted folylpoly- $\gamma$ -glutamate concentrations was performed by linear gradient elution reversed-phase HPLC according to Ndaw et al. (2001). The apparatus consisted of an Agilent 1200 Series HPLC system (Agilent Technologies, Diegem, Belgium) equipped with UV-DAD (G1315B, Agilent Technologies, Diegem, Belgium) and fluorescence (RF-10AxL, Shimadzu, Kyoto, Japan) detectors. Analytical HPLC was performed at  $25^{\circ}\text{C}$  using a Prevail  $\text{C}_{18}$  column (250  $\times$  4.6 mm, 5  $\mu\text{m}$  particle size, Alltech, Deerfield, IL, USA), protected with  $\text{C}_{18}$  guard cartridge (7.5  $\times$  4.6 mm, 5  $\mu\text{m}$  particle size). Fluorescence detection was applied at excitation and emission wavelengths of, respectively, 295 and 356 nm at 20 Hz at the highest sensitivity magnification factor. The eluents used were a  $\text{NaH}_2\text{PO}_4$  solution (50 mM, pH 4.5, solvent A) and a 20% (v:v) acetonitrile in  $\text{NaH}_2\text{PO}_4$  (50 mM) solution (pH 4.5, solvent B) at a flow rate of 0.8 mL/min. After injection (5–100  $\mu\text{L}$ ), the concentration of solvent B was raised to 50% in 23.40 min and subsequently to 100% in 13.00 min. Thereon, the column was washed for 10.40 min with 100% B, after which it was equilibrated for 10.40 min with 100% solvent A.

As 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma n}$  standards are commercially available up to the penta- $\gamma$ -glutamate form,  $\text{PteGlu}_{\gamma 6-8}$  derivatives were purchased from Schircks Laboratories (Jona, Switzerland) and 40 nmol of each component was converted to 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma 6-8}$  to allow quantification of 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma 1-8}$  using external calibration curves based on peak area. Purities of the converted 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma 7-8}$  standards were 90–95% according to HPLC-analysis of these samples. Due to the high fluorescence yield of 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma n}$ , detection limits ranged from 0.2–0.02 pmol folylpoly- $\gamma$ -glutamates per injection. Intra- and inter-assay precision of the HPLC method was controlled by repeated analysis of 5- $\text{CH}_3\text{H}_4\text{PteGlu}_{\gamma 1-7}$  standards.

### 2.7. Reproducibility and recovery experiments

Efficiency of the conversion procedure was assessed by converting monoglutamate standard mixtures of 20 nmol 5- $\text{CHO-H}_4\text{PteGlu}$  and 20 nmol 5- $\text{CH}_3\text{H}_4\text{PteGlu}$  in triplicate and by analyzing the folate content of these solutions with a fluorimetric HPLC method for monoglutamate folate quantification as previously described (Indrawati et al., 2004).

Quality control experiments regarding FBP column maximal binding capacity were performed by loading the FBP columns with an overload of [6S]-5-CH<sub>3</sub>H<sub>4</sub>PteGlu (40 nmol). Reproducibility of the FBP-AC procedure was assessed by analyzing a converted and purified control broccoli extract in duplicate on each column ( $n = 6$  columns).

To control the recovery of the whole folate analysis procedure, which is influenced by the food matrix, folate degradation during heat extraction and digestion, extraction and conversion yields, folate recovery during FBP purification and precision of the HPLC-assay, broccoli samples ( $n = 6$ ,  $\pm 20$  g FW/sample, var. Milady), were analyzed at the beginning of the study. Recoveries were calculated based on dry matter content of the broccoli samples. Three samples were spiked just prior to the heat extraction with a total of 20 nmol folate (i.e.  $\pm 4$  nmol of each of the following derivatives: H<sub>4</sub>PteGlu, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu, 5-CHO-H<sub>4</sub>PteGlu, and 5-10-CH<sub>2</sub>H<sub>4</sub>PteGlu). The remaining samples ( $n = 3$ ) were considered as blank samples to correct for endogenous folate content. The spiked amount of folylmonoglutamates was determined by analyzing the spiking mixtures for H<sub>4</sub>PteGlu, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu, 5-CHO-H<sub>4</sub>PteGlu, and 5-10-CH<sub>2</sub>H<sub>4</sub>PteGlu content by a fluorimetric HPLC method for monoglutamate folate quantification (Indrawati et al., 2004). Since the samples that were used for the recovery experiment originated from a different batch than those used for the pressure-temperature treatments, the reproducibility of the folylpolyglutamate analysis procedure was assessed by analyzing untreated extracted, converted and purified samples (var. Milady) by HPLC ( $n = 10$ ).

### 2.8. Stability of folylpoly- $\gamma$ -glutamates in model systems

Thermal and high-pressure stability of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> (1  $\mu$ M) in occurrence or absence of antioxidants (*L*-ascorbic acid, 56.8 mM) was investigated in phosphate (0.1 M, pH 7.0), citrate (0.1 M, pH 4.0) and borate buffer (0.1 M, pH 9.0) to determine the possible influence of pH and processing conditions on non-enzymatic hydrolysis of folylpoly- $\gamma$ -glutamates. 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> samples were filled in glass crimp cap vials with rubber septa (800  $\mu$ L, 8.2  $\times$  30 mm, Cleanpack, Belgium) or flexible polyethylene tubes (400  $\mu$ L, Bioplastic, Landgraaf, Netherlands) for respectively thermal and combined pressure treatments, avoiding the inclusion of air bubbles. To prevent contamination with the pressure transmitting medium during combined temperature-pressure experiments, samples were covered with a barrier film (Parafilm M) and double-vacuum packed in polyethylene plastic bags using a vacuum sealing machine (Multivac A300/16, Wolfertschwenden, Germany) up to 1.1 Pa. Folate solutions and samples were stored on ice (0–4 °C) for maximum 15 min to minimize oxidation before treatment or analysis. To avoid photodegradation of folate, all preparative and treatment procedures were carried out under subdued light and samples were wrapped in aluminium foil. Thermal (25–90 °C, 30 min) and combined pressure-temperature (40 °C, 100–600 MPa, 25 min) treatments were conducted in duplicate as described earlier for broccoli with minor modifications, i.e. treatments differed in the fact that samples were cooled in ice water (0–4 °C) immediately after the treatments and were not frozen in liquid N<sub>2</sub>. Samples were immediately analyzed for folylpoly- $\gamma$ -glutamate content using the aforementioned RP-HPLC method.

## 3. Results and discussion

### 3.1. Stability of folylpoly- $\gamma$ -glutamates in model systems

Thermal (25–90 °C, 30 min) and high-pressure (40 °C, 100–600 MPa, 25 min) stability of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> (1  $\mu$ M) in occurrence or absence of antioxidants (*L*-ascorbic acid) was investigated in phosphate (0.1 M, pH 7.0), citrate (0.1 M, pH 4.0) and borate buffer

(0.1 M, pH 9.0) to determine the possible influence of pH and processing conditions on non-enzymatic hydrolysis of folylpoly- $\gamma$ -glutamates. In this context, alkaline hydrolysis of poly- $\gamma$ -glutamic acid has previously been reported at 90 °C (Kubota, Nambu, & Endo, 1996). In the current investigation, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> losses of 20–99% were observed in absence of *L*-AA in citrate (0.1 M, pH 4.0) and borate (0.1 M, pH 9.0) buffers depending on the applied temperature (25–90 °C, 30 min) at atmospheric pressure. In addition, no significant 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> losses were observed at pH 7.0 in phosphate buffer (0.1 M) under those conditions. During high hydrostatic pressure treatments 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> losses (10–90%) were observed in all buffer systems and depended on the buffer type and the applied temperature-pressure combination. Under pressure, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> was most stable in citrate buffer. Addition of *L*-AA (56.8 mM) in the model systems resulted in stabilization of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> at all investigated pH and pressure-temperature conditions, indicating that the aforementioned 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> losses were caused by oxidative degradation in accordance with results for 5-CH<sub>3</sub>H<sub>4</sub>PteGlu (Indrawati et al., 2004; Nguyen, Indrawati, & Hendrickx, 2003). Since 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7.5</sub> was the only detectable signal during RP-HPLC for all experiments, it was concluded that occurrence of non-enzymatic hydrolysis of  $\gamma$ -glutamyl moieties in folylpoly- $\gamma$ -glutamates could be excluded for thermal and combined temperature-pressure treatments in the investigated model systems.

### 3.2. Folylpolyglutamate analysis of broccoli

Reproducibility and validity of the analysis methodology was controlled at the beginning of the study. Hereto, the efficiency of the conversion procedure was initially tested using a mixture of 5-CHO-H<sub>4</sub>PteGlu (20 nmol) and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu (20 nmol) in phosphate buffer (0.1 M, pH 7.0) and showed recoveries of  $99.5 \pm 6.8\%$  ( $n = 3$  conversions). It should be noted, however, that these results did not account for possible dissolved matrix components that occur in real broccoli extracts and could hence result in an overestimated conversion factor. Quality control experiments for the FBP-AC purification procedure showed a maximal binding capacity of the columns of  $1.66 \pm 0.22$  nmol/column ( $n = 12$  analyses) whereas CV's of the purification method ranged from 3.5% to 7.9% for the detected folylpoly- $\gamma$ -glutamates in the control broccoli sample ( $n = 12$  analyses). HPLC-analysis of converted and purified PteGlu<sub>7.6–8</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7</sub> standards showed that impurities formed during the conversion could be removed by FBP-AC and that this resulted in a stable baseline during the HPLC-analysis. These results also showed that no changes in degree of polymerisation (DP) of the folylpoly- $\gamma$ -glutamates occurred during conversion and FBP-AC purification. Sample clean-up by FBP-AC was previously used as a highly selective clean-up procedure for folates in other complex food matrices (Konings, 1999; Ndaw et al., 2001) and was supposed to prevent interference from broccoli matrix components with folate analysis in the current investigation. For further validation, the recovery of the di-enzyme folate analysis was determined by extraction, conversion and purification of unspiked ( $n = 3$ ) and spiked ( $n = 3$ ) broccoli samples. Based on the determined moisture content ( $88.6 \pm 0.7\%$ ,  $n = 6$ ), spiking the broccoli matrix with folylmonoglutamates at  $8.2 \pm 2.0$  nmol/g dry matter ( $n = 3$ ) resulted in a recovery of  $98.6 \pm 8.2\%$ . This high recovery value is in accordance with results from others that used an analogue conversion and purification methodology for the analysis of folates in spinach and fruit juice (Ndaw et al., 2001).

### 3.3. Folylpolyglutamate distribution in broccoli

The reference folylpoly- $\gamma$ -glutamate distribution profile of broccoli in this study was determined by repeated analysis of untreated

raw samples ( $n = 10$ , var. Milady). Results for the detected predominantly occurring folylpoly- $\gamma$ -glutamates are presented in Table 1. No 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>8</sub> was detected in the reference broccoli extracts. Identification of the converted folylpoly- $\gamma$ -glutamates was initially performed based on the retention times of 5-CH<sub>3</sub>H<sub>4</sub>-PteGlu<sub>1–8</sub> standards after spiking. The total folate content was calculated as the sum of the detected 5-CH<sub>3</sub>H<sub>4</sub>PteGlu <sub>$\gamma$  $n$</sub>  derivatives (expressed in nmol) corrected for the moisture content of broccoli. Total folate content of the investigated broccoli variety Milady and the unknown variety were 315 ± 67 nmol/100 g FW ( $\approx$ 139 ± 28  $\mu$ g

**Table 1**  
Total folate content (nmol/100 g fresh weight) in raw broccoli for cultivars Milady ( $n = 10$ ) and an unknown variety ( $n = 2$ ) after conversion of folates to 5-methyltetrahydrofolate poly- $\gamma$ -glutamate derivatives (5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub>)

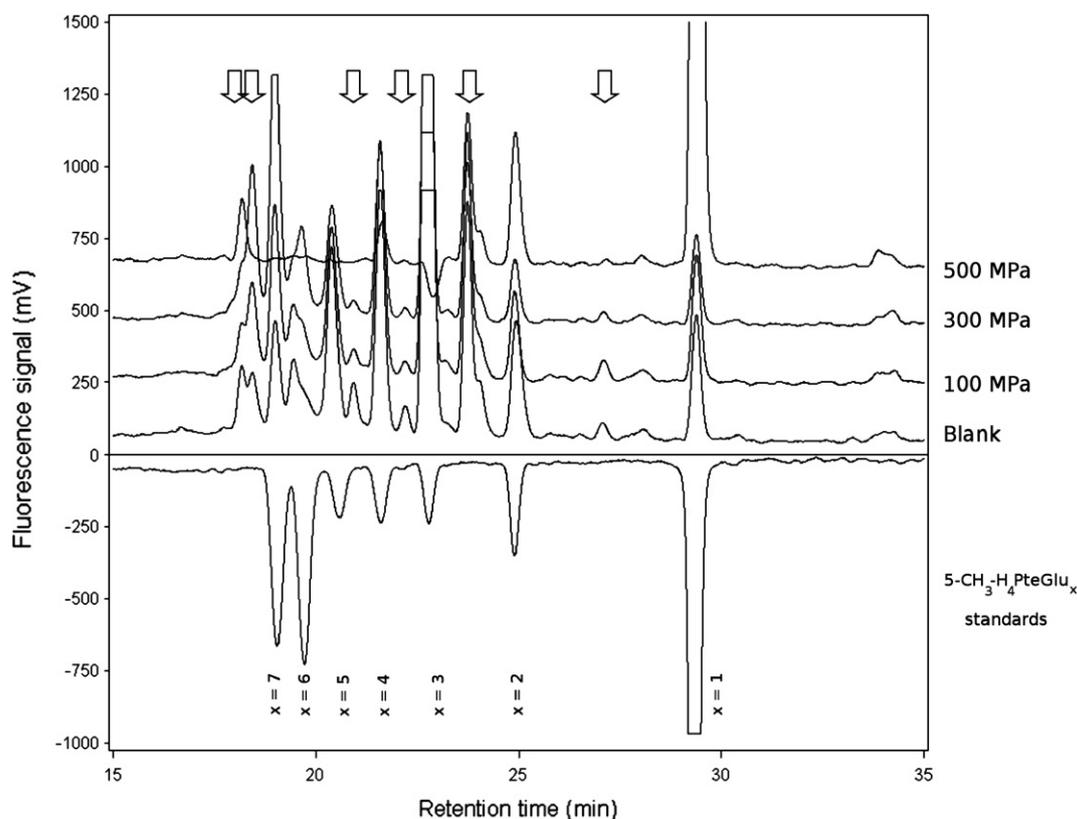
Folylpoly- $\gamma$ -glutamate	Milady		Unknown variety	
	nmol/g DM <sup>a</sup>	%	nmol/g DM <sup>a</sup>	%
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>1</sub>	0.29 ± 0.08	1.1 ± 0.2	0.72 ± 0.20	3.6 ± 1.0
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>2</sub>	1.39 ± 0.51	5.3 ± 1.3	1.45 ± 0.95	7.2 ± 4.7
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>3</sub>	14.38 ± 3.97	54.5 ± 7.2	11.63 ± 0.81	58.1 ± 4.0
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>4</sub>	2.90 ± 0.95	11.2 ± 3.8	1.32 ± 0.36	6.6 ± 1.8
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>5</sub>	2.49 ± 0.43	9.7 ± 1.7	1.82 ± 0.10	9.1 ± 0.5
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>6</sub>	1.80 ± 0.62	4.7 ± 3.6	1.39 ± 0.37	7.0 ± 1.9
5-CH <sub>3</sub> H <sub>4</sub> PteGlu <sub>7</sub>	3.51 ± 1.31	13.6 ± 4.5	1.69 ± 0.66	8.4 ± 3.3
Total folate content	Milady		Unknown variety	
	nmol/g DM <sup>a</sup>	nmol/100 g FW <sup>a</sup>	nmol/g DM <sup>a</sup>	nmol/100 g FW <sup>a</sup>
	26.2 ± 5.6	315 ± 76	20.01 ± 1.39	240 ± 17

Individual folylpoly- $\gamma$ -glutamate concentrations are expressed in nmol/g dry matter. Distribution profile is expressed relative to the total folate content (%). Values represent means ± SD.

<sup>a</sup> Moisture content 88.6 ± 0.7% ( $n = 6$ ).

PteGlu/100 g FW,  $n = 10$ ) and, respectively, 240 ± 17 nmol/100 g FW ( $\approx$ 106 ± 7  $\mu$ g PteGlu/100 g FW,  $n = 2$ ). These results are in accordance with the range of reported total folate values (100–394 nmol folates/100 g FW) for broccoli (Petersen, 1993; Stea, Johansson, Jagerstad, & Frölich, 2006; Zheng, Lin, Lin, & Cossins, 1992).

Based on the chromatograms it was found that folates in broccoli florets, analyzed with the aforementioned procedures, consisted of approximately 97–99% folylpoly- $\gamma$ -glutamates (mainly tri- $\gamma$ -glutamates) and very low concentrations of monoglutamyl folates (Table 1), which corresponds with available literature data (Zheng et al., 1992). In addition, it was observed that chromatographic peaks of penta-, tetra-, tri- and di- $\gamma$ -L-glutamates were followed by distinct signals which did not co-chromatograph with 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> standards (Fig. 1). This seems to be in accordance with observations from Zheng et al. (1992), detecting components that did not co-chromatograph with  $\gamma$ -carboxyl linked *p*-aminobenzoyl-poly-glutamate (*p*-ABGlu <sub>$\gamma$  $n$</sub> ) standards during anion exchange HPLC of hydrolyzed folylpolyglutamates from broccoli florets. It should be noted, however, that in contrast to the current study interference of matrix components could not be excluded by the latter and that analytical and chromatographic conditions were not comparable with the current analysis method. Within this context, however, it was shown by enzymatic analysis and tandem LC-MS (Ferone, Hanlon, & Singer, 1986) that azo-dyes of  $\alpha$ -carboxyl linked *p*-amino-benzoyl-poly-glutamates (*p*-ABGlu <sub>$\alpha$  $n$</sub> ), originating from hydrolyzed folylpoly- $\alpha$ -glutamates occurring in *E. coli*, elute consistently after their corresponding *p*-ABGlu <sub>$\gamma$  $n$</sub>  azo-dyes when analyzed by RP-HPLC on a C<sub>18</sub> column (i.e. *p*-ABGlu <sub>$\alpha$ 3</sub> after *p*-ABGlu <sub>$\gamma$ 3</sub>, etc.) and hence exert an analogue chromatographic behaviour similar to the unknown components in our study. Although further analysis is clearly necessary to identify the structure of these components, it might be possible that the current results indicate the occurrence



**Fig. 1.** Chromatograms of converted and purified folylpolyglutamate extracts of blank and high hydrostatic pressure treated (45 °C, 100–500 MPa, 30 min) broccoli (var. Milady). External 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> standards (0.1 pmol on column) are mirrored as reference. Unknown co-chromatographing compounds are indicated by an arrow.

of folylpoly- $\alpha$ -glutamates in broccoli as was previously shown by others for folylpolyglutamates in *E. coli* (Ferone et al., 1986) and hypothesized for broccoli (Zheng et al., 1992).

### 3.4. Influence of thermal treatments

Impact of thermal treatments on total folate content and folylpoly- $\gamma$ -glutamate distribution in broccoli var. Milady was investigated in a temperature range from 25 to 45 °C at atmospheric pressure. No significant changes in total folate content, calculated as the sum of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub>, were detected during 30 min treatments up to 45 °C. In addition, no significant differences in folylpoly- $\gamma$ -glutamate distribution were observed within the temperature range (Fig. 2), indicating that enzymatic  $\gamma$ -glutamate hydrolysis and folate degradation were negligible at the investigated conditions in this broccoli variety. Also for elevated temperatures, up to 90 °C, no reduction in total folate content was observed for the unknown variety. The retention of total folate content in broccoli during thermal processing seems to be in accordance with results from others (Stea et al., 2006), showing non-significant folate losses, based on FW, in blanched (5 s in boiling water) and subsequently *sous-vide* processed broccoli (90 °C, 7 min) relative to the folate content in raw samples. Remarkably, it was observed in the investigation of Stea et al. (2006) that the total folate content in broccoli increased by 31% on DM basis after blanching, whereas it was not changed on FW basis. These observations, remained, however, unexplained by the authors. Since subsequent thermal (100 °C, 5 min) or *sous-vide* (90 °C, 7 min) processing in that study resulted in non-significant folate losses relative to the blanched sample, i.e. both on DM (respectively, 15.7% and 11.5%) and FW basis (respectively, 13.2% and 16.8%), aforementioned findings could indicate that blanching either resulted in leakage of DM components to the brine and/or that the blanching step enhanced the extractability of folates from the broccoli matrix. Despite that broccoli samples were processed for a longer time period (30 min) without prior blanching in our study, none of the thermal treatments resulted in a significant increase or decrease of the total folate content on DM basis. It should be noted, however, that vacuum packaging of broccoli without brine in the current study prevented changes in moisture content and prevented folate leakage which has been reported to be the major cause for folate losses during vegetable processing (McKillop et al., 2002; Petersen, 1993; Stea et al., 2006). In addition, broccoli

florets contain relative high concentrations of L-ascorbic acid (Petersen, 1993), which has been related to enhanced folate stability during processing in other vegetables (Indrawati et al., 2004).

As depicted in Fig. 2, it was observed in the unknown broccoli variety that treatments at 70, 80 and 90 °C (30 min) resulted in apparently modified folylpoly- $\gamma$ -glutamate profiles with a shift of the DP to higher conjugated poly- $\gamma$ -L-glutamates relative to the untreated sample. In contrast to the predominant occurrence of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>3</sub> in the blank and 50 °C (30 min) treated broccoli samples, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>6</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>7</sub> were apparently prevalent after treatments at 70–90 °C (30 min), whereas no folate degradation seemed to occur during any of the treatments. The relative increase in folylpoly- $\gamma$ -glutamate content *in situ* might imply that thermal processing of vegetables could impair folate bioaccessibility. Since these results are based on limited observations in one broccoli variety, however, this hypothesis is rather tentative and weak and requires further investigation.

### 3.5. Influence of high-pressure thermal treatments

Impact of high-pressure thermal treatments on total folate content and folylpoly- $\gamma$ -glutamate distribution in intact broccoli tissue was investigated in a temperature–pressure range from 25–45 °C and 100–600 MPa for the Milady variety. It was observed that significant folate losses, ranging from 48–78% compared to the total folate content of raw broccoli ( $P < 0.05$ ), occurred during 25 °C/600 MPa, 30 °C/400–600 MPa, 35 °C/500–600 MPa, and 40–45 °C/300–600 MPa treatments (25 min) (Fig. 3). Knowledge on high-pressure stability of folates *in situ* in intact broccoli tissue has not been reported in literature, whereas it is limited for some other intact vegetables. Indrawati et al. (2004) reported that 5-CH<sub>3</sub>H<sub>4</sub>PteGlu was relatively stable in vacuum packed high-pressure/thermal treated (60 °C, 500 MPa, 5–100 min) asparagus and that stability of folates may be related to endogenous ascorbic acid concentrations. A previous study (Melse-Boonstra et al., 2002) also showed that high-pressure treatments at 200 MPa (22 °C, 5 min) resulted in severe folate losses of 43–81% for leeks, cauliflower and green beans when treated in direct contact with the pressure transmitting medium in contrast to minimal losses for vacuum packed high-pressure/thermal processed vegetables. Folate losses during high-pressure processing of intact vegetables in the previous studies (Indrawati et al., 2004; Melse-Boonstra et al., 2002) have therefore mainly been related to leaching. In the current

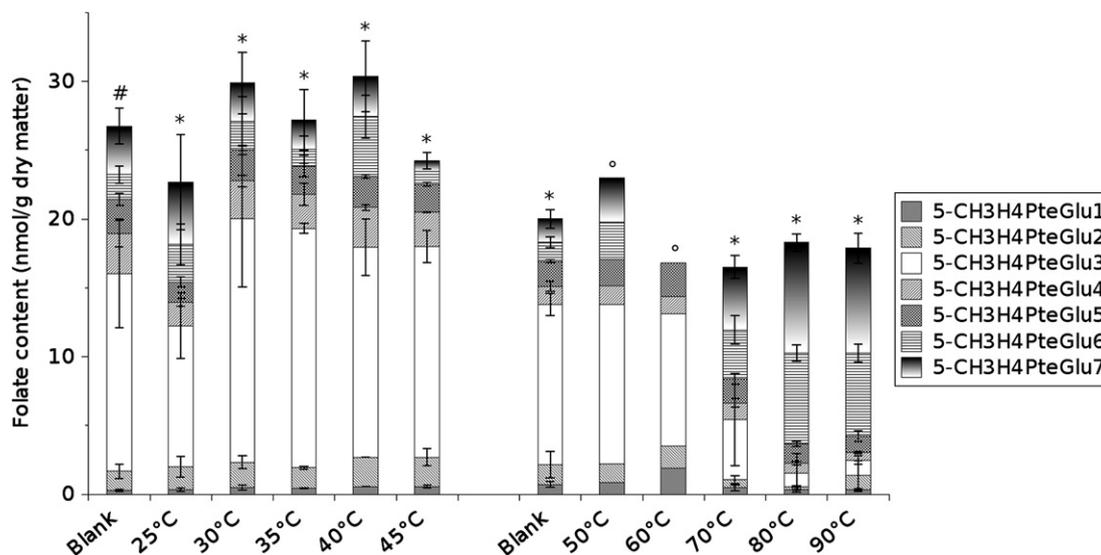
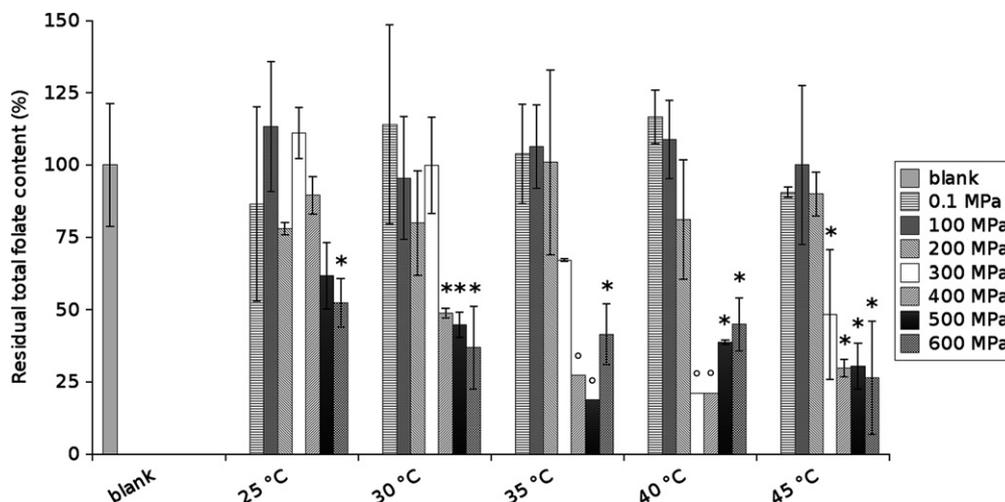


Fig. 2. Influence of thermal treatments (25–90 °C, 30 min) at atmospheric pressure on folylpoly- $\gamma$ -glutamate content and distribution in broccoli (var. Milady and unknown var.) in comparison with untreated samples (blank). Data represent mean residual 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> concentrations  $\pm$ SD based on DM. # $n = 10$ , \* $n = 2$ , ° $n = 1$ .



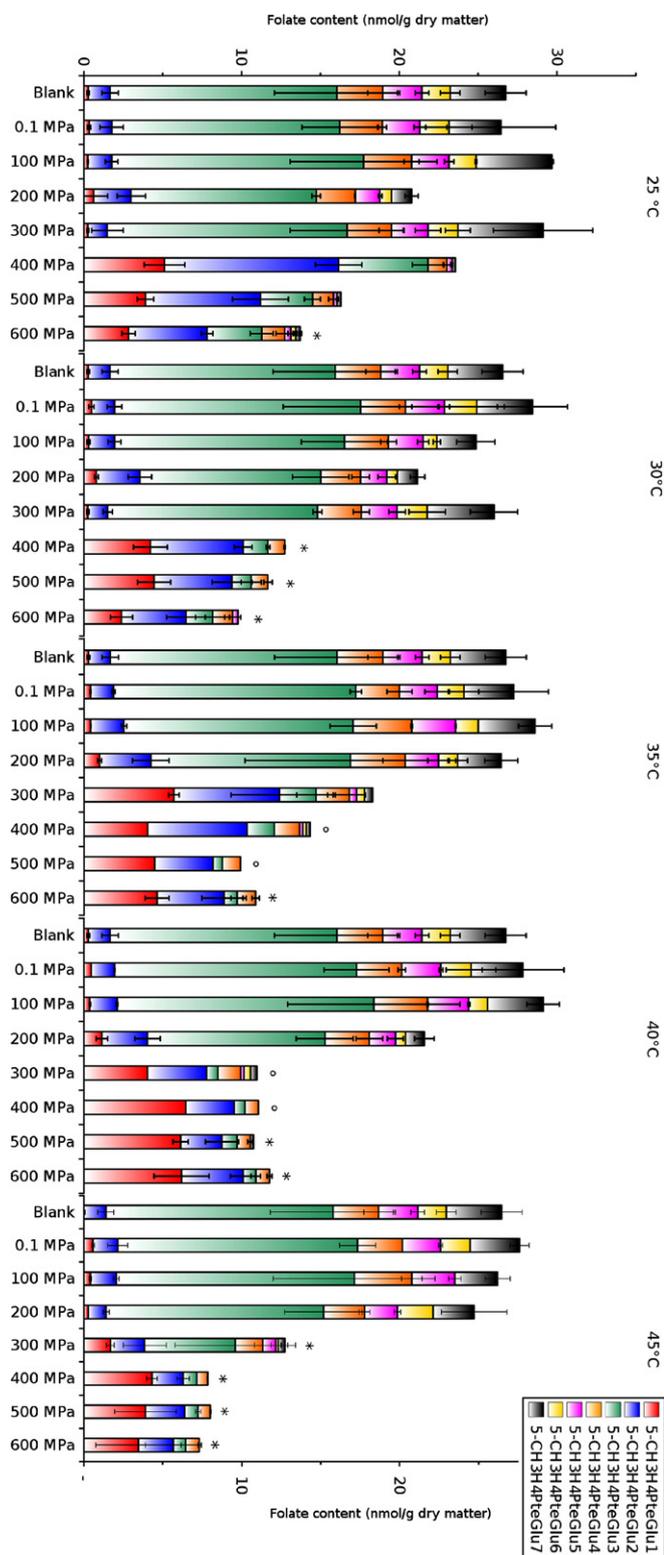
**Fig. 3.** Influence of thermal (25–45 °C, 0.1 MPa, 30 min) and isothermal–isobaric treatments (25–45 °C, 100–600 MPa, 25 min) on total folylpoly- $\gamma$ -glutamate content in broccoli (var. Milady), determined as the sum of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> concentrations, relative to the total folylpoly- $\gamma$ -glutamate content in untreated broccoli (blank). Data represent means  $\pm$  SD ( $n = 2$ ). \*Indicates significant difference with blanks ( $n = 10$ ) ( $P < 0.05$ , ANOVA with Tukey–Kramer *post hoc* test). °Indicates data for single measurements.

study, however, significant folate losses occurred in broccoli under the investigated isothermal–isobaric conditions despite prevention of leakage of folates through application of vacuum packaging. Since intermediate folate degradation products (i.e. dihydrofolate derivatives) would be converted to 5-CH<sub>3</sub>H<sub>4</sub>PteGlu during the conversion procedure, these results indicated that pressure treatments enhanced cleavage of the C<sub>9</sub>–N<sub>10</sub> covalent bond or formation of other folate degradation products, which is in accordance with negative activation volumes reported for high-pressure stability of folate vitamers in model systems (Nguyen et al., 2003).

It was previously reported (Melse-Boonstra et al., 2002) that high-pressure treatments (22 °C, 150–200 MPa, 5 min) resulted in an increased proportion of monoglutamate folates by 12–82% relative to the total residual folate content in processed leeks, cauliflower and green beans. In this study, no significant changes in folylpoly- $\gamma$ -glutamate profile were caused by high-pressure treatments at 25 °C, combined with pressures of 100 and 200 MPa (25 min) in broccoli, whereas differences in folyl- $\gamma$ -polyglutamate profile were observed at more severe pressure–temperature combinations. An overview of the results for 30 min treatments at 25–45 °C in the pressure range of 0.1–600 MPa is visualized in Fig. 4. Compared to broccoli treated at atmospheric pressure (25–45 °C, 25 min), amounts of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub> significantly increased from, respectively, 1.5–2.4% and 5.6–9.0% to, respectively, 15.3–60.4% and 16.4–47.0%, relative to the total residual folate content after 25 min treatments, depending on the applied temperature–pressure. Results for changes in 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–3</sub> concentrations in the investigated pressure–temperature domain are presented in Table 2. At 25 °C, e.g. amounts of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub> increased from respectively  $0.35 \pm 0.11$  and  $1.43 \pm 0.75$  nmol/g dry matter (after a 25 min treatment at 0.1 MPa), to, respectively,  $5.12 \pm 1.29$ , and  $11.05 \pm 1.46$  nmol/g dry matter (at 400 MPa), whereas amounts of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>3</sub> were reduced from  $14.45 \pm 2.39$  to  $5.67 \pm 1.00$  nmol/g dry matter. Also for treatments at 30 °C, a minimal pressure of 400 MPa was needed to obtain similar changes in the profiles of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub>, 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub>, and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>3</sub>, whereas at 35–45 °C similar results were obtained at lower pressure, starting at 300 MPa. Increases in 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub> concentrations coincided with decreased concentrations of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>3</sub> and decreased

concentrations of longer chain folylpoly- $\gamma$ -glutamates (Fig. 4) in accordance with the postulated influence of high-pressure on the conversion of the folylpoly- $\gamma$ -glutamates by endogenous plant folate  $\gamma$ -glutamyl hydrolase (FGGH) by Melse-Boonstra et al. (2002). It should be noticed, however, that the previous investigation was based on analysis of single samples and that the proportion of polyglutamate folates was previously indirectly measured as the difference between total and monoglutamate folate content using a tri-enzyme extraction folate assay. To our knowledge, no earlier studies have reported the influence of high-pressure treatments on folate conversions by measuring the distribution of specific folylpoly- $\gamma$ -glutamates. The relative accumulation of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub> by the postulated FGGH activity during high-pressure treatments in the current study is supported by a recent investigation (Orsomando et al., 2005) in which it was shown that endopeptidase activity of two *Arabidopsis* FGGH proteins resulted in cleavage of PteGlu<sub>5</sub> into PteGlu<sub>2</sub> plus PteGlu<sub>3</sub>, and into monoglutamates at atmospheric pressure *in vitro*. As depicted in Fig. 1, application of high-pressure did not only result in changes of folylpoly- $\gamma$ -glutamates since also the intensity of the signals for the unknown components decreased at higher pressure and temperature under the studied conditions. We were, however, not able to describe this effect quantitatively because no standards for the unknown components were available.

To investigate folate losses and the depoly- $\gamma$ -glutamylolation process as a function of treatment time, an isothermal–isobaric kinetic experiment (0–25 min) was performed at 35 °C and 400 MPa. It was observed that folate losses mainly took place during the dynamic conditions inherent to the pressure build-up (4 min) and the subsequent equilibration (5 min) phase before reaching isothermal–isobaric conditions (treatment time = 0) and during the first five minutes of the treatment. No long chain folylpoly- $\gamma$ -glutamates (5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>6–7</sub>), which account for approximately 17% of the total folate content in untreated broccoli, were detected after the dynamic pressure build-up and equilibrium phase (time = 0), whereas relative amounts of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub> had increased, respectively, to 6.0 and 32.2% at time zero. During treatment, concentrations of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>3</sub> decreased from 10.3 (time = 0) to 4.5 nmol/g dry matter after 5 min, however, this did not coincide with increases in 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1</sub> or 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>2</sub>. In addition, no further changes in folylpoly-



**Fig. 4.** Influence of thermal (25–45 °C, 0.1 MPa, 30 min) and isothermal-isobaric treatments (25–45 °C, 100–600 MPa, 25 min) on folylpoly- $\gamma$ -glutamate distribution in broccoli (var. Milady). Data represent mean residual 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> concentrations  $\pm$ SD based on DM for duplicate experiments. \* Indicates significant difference of total folate content, determined as the sum of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>1–7</sub> concentrations relative to untreated (blank) samples ( $n = 10$ ) ( $P < 0.05$ , ANOVA with Tukey-Kramer *post hoc* test).  $\circ$  Indicates data for single measurements.

$\gamma$ -glutamate profile were observed at other time points (10–25 min), indicating that FGGH activity mainly occurred during the initial stages of the high-pressure treatment.

It is well established that high hydrostatic pressure treatments can result in altered cell membrane permeability and in structural changes of intracellular organelles (Kato & Hayashi, 1999). The resulting solubilisation of intra-cellular substances (i.e. proteins, metal ions, etc.) and permeation of extra-cellular substances could enhance the contact between folylpoly- $\gamma$ -glutamates and FGGH. It should be noted that the subcellular location of plant FGGH's is still controversial since previously both extracellular (Huangpu, Pak, Graham, Rickle, & Graham, 1996) and cytosolic (Lin, Rogiers, & Cosins, 1993) sites have been proposed whereas vacuolar, mitochondrial and chloroplastic folylpoly- $\gamma$ -glutamates have recently been reported to comprise respectively, 20%, 50% and 10% of the total cellular folate in pea leaves (Orsomando et al., 2005). The latter, moreover, reported that both folylpoly- $\gamma$ -glutamates and FGGH's co-occur in beet root and pea leaf vacuoles, which strongly suggested the occurrence of potent endogenous FGGH inhibitors (i.e. competitive inhibition by folate binding proteins) as folylpoly- $\gamma$ -glutamate protectors in plants, which is in accordance with the occurrence of high-affinity FBP T-protein of glycine decarboxylase in pea leaf mitochondria (Rebeille, Neuburger, & Douce, 1994). Therefore, it can also be suggested from the current results that high hydrostatic pressure treatments could have influenced these proteins and/or their interactions, as was previously reported for e.g. plant pectinmethylesterase and its proteinaceous inhibitor from kiwi fruit (Nguyen et al., 2004). Release of folylpoly- $\gamma$ -glutamates from endogenous plant FBPs under pressure could have made them available for FGGH and could explain the observed folate losses during the experiments since it has been shown that protein binding in mitochondria protects folates from oxidative degradation (Rebeille et al., 1994; Scott et al., 2000). So far, there is no experimental evidence confirming these hypotheses and further research in this area is needed.

In conclusion, no non-enzymatic hydrolysis was observed for 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>5</sub> in model systems at pH 4.0, 7.0 and 9.0 during thermal (25–90 °C, 30 min) and combined temperature-pressure treatments (40 °C, 100–600 MPa, 25 min). No folylpoly- $\gamma$ -glutamate degradation was shown to occur in broccoli during thermal treatments (25–90 °C, 30 min). This was not the case during high-pressure treatments (25–45 °C, 100–600 MPa, 25 min) despite the use of vacuum packaging. In addition, this study demonstrated that high-pressure treatments above 200 MPa resulted in depoly- $\gamma$ -glutamylated folylpoly- $\gamma$ -glutamates at 25–45 °C with an accumulation of mono- and di- $\gamma$ -glutamate folates at most conditions, whereas this was not observed for the thermal treatments at the reference temperatures (25–45 °C, 0.1 MPa, 30 min). At elevated temperatures (70–90 °C) and atmospheric pressure, however, higher conjugated folylpoly- $\gamma$ -glutamates proportions were observed compared to the untreated sample of the unknown broccoli variety. The mechanism behind this observation remains unclear and it requires further investigation. Although pasteurisation of foods by thermal and high hydrostatic pressure treatments can be applied to achieve the same microbial destruction, the current findings showed that there are distinct differences between these technologies regarding effects on folate instability and folylpoly- $\gamma$ -glutamate profiles which could have its implications towards nutrient bioavailability. In this context, targeted application of mild high-pressure thermal processing can result in small folate losses that coincide with an accumulation of folylmonoglutamates in broccoli and hence enhance folate bioaccessibility, e.g. treatments at 25 °C, 400 MPa (25 min) resulted in  $\pm$ 10% total folate loss and 5-CH<sub>3</sub>H<sub>4</sub>PteGlu accumulation from 1.5% up to 21% of the total residual folate content. In addition, it should be stated that the underlying mechanism behind the influence of temperature and pressure on folate instability *in situ* and the enhanced enzymatic glutamate depolymerisation remains unknown and also calls for further research as enzyme targeted application

**Table 2**  
Influence of thermal/high-pressure processing (25–45 °C, 0.1–600 MPa, 30 min) on 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>γ1–3</sub> concentrations, expressed relative (%) to the total residual folate content in broccoli (var. Milady)

P (MPa)	Mono <sup>A</sup>	Di <sup>B</sup>	Tri <sup>C</sup>	Total <sup>a</sup>	mono <sup>A</sup>	di <sup>B</sup>	tri <sup>C</sup>	Total <sup>a</sup>
	<i>Untreated reference</i>							
Blank	1.1 ± 0.2	5.3 ± 1.3	54.5 ± 7.2	26.2 ± 5.6				
	25 °C				30 °C			
0.1	1.5 ± 0.1	7.3 ± 0.5	46.6 ± 7.5	25.9 ± 8.8	1.8 ± 1.1	6.7 ± 3.6	59.4 ± 1.4	27.8 ± 9.0
100	0.9 ± 0.1	5.0 ± 0.4	53.3 ± 5.2	29.7 ± 5.9	1.2 ± 0.1	6.6 ± 0.2	58.9 ± 2.1	25.0 ± 5.6
200	3.0 ± 4.3	11.7 ± 4.7	57.4 ± 2.9	20.4 ± 0.5	3.9 ± 0.3	13.2 ± 0.6	55.5 ± 3.9	21.0 ± 4.7
300	0.9 ± 0.1	4.1 ± 3.1	51.8 ± 8.3	29.1 ± 2.3	1.0 ± 0.2	4.8 ± 0.3	51.8 ± 7.6	26.2 ± 4.4
400	21.7 ± 3.9	47.0 ± 2.8	24.4 ± 6.0	23.5 ± 1.7	33.1 ± 7.3	46.2 ± 6.0	12.4 ± 1.3	12.8 ± 0.4
500	24.3 ± 1.3	44.8 ± 2.7	20.4 ± 0.6	16.2 ± 3.0	38.7 ± 12.7	42.0 ± 6.8	10.2 ± 4.5	11.8 ± 1.1
600	20.8 ± 0.3	36.5 ± 3.2	25.0 ± 1.3	13.7 ± 2.2	25.3 ± 2.6	43.4 ± 4.1	16.6 ± 4.7	9.7 ± 3.8
	35 °C				40 °C			
0.1	1.6 ± 0.2	5.6 ± 0.6	64.9 ± 12.2	26.6 ± 4.5	1.6 ± 0.1	5.7 ± 0.5	52.2 ± 10.9	27.1 ± 2.4
100	1.6 ± 0.2	7.6 ± 0.4	52.2 ± 1.8	27.9 ± 3.8	1.3 ± 0.0	6.1 ± 0.6	56.3 ± 12.1	28.5 ± 3.6
200	3.9 ± 0.7	12.2 ± 0.5	46.1 ± 10.6	26.5 ± 8.4	5.4 ± 0.3	13.6 ± 0.3	53.4 ± 5.0	21.3 ± 5.4
300	32.4 ± 1.7	38.0 ± 17.2	13.3 ± 7.0	17.6 ± 0.1	36.8 <sup>b</sup>	34.0 <sup>b</sup>	6.3 <sup>b</sup>	11.0 <sup>b</sup>
400	28.1 <sup>b</sup>	44.0 <sup>b</sup>	12.0 <sup>b</sup>	14.4 <sup>b</sup>	58.4 <sup>b</sup>	27.7 <sup>b</sup>	6.2 <sup>b</sup>	11.1 <sup>b</sup>
500	45.3 <sup>b</sup>	37.2 <sup>b</sup>	6.2 <sup>b</sup>	9.9 <sup>b</sup>	60.4 ± 6.0	25.6 ± 9.9	9.5 <sup>c</sup>	10.2 ± 0.2
600	43.1 ± 4.0	38.5 ± 3.1	7.5 ± 1.5	10.9 ± 2.8	52.2 ± 4.1	33.2 ± 0.1	7.1 ± 4.0	11.8 ± 2.4
	45 °C							
0.1	2.4 ± 0.3	9.0 ± 2.5	64.4 ± 3.6	26.6 ± 0.5				
100	1.6 ± 0.2	6.4 ± 1.0	57.0 ± 3.9	26.2 ± 0.0				
200	1.2 ± 0.0	4.7 ± 0.4	59.0 ± 15.4	23.6 ± 2.0				
300	15.3 ± 9.0	16.4 ± 3.2	43.0 ± 10.2	12.7 ± 5.9				
400	55.1 ± 1.3	25.1 ± 2.5	10.9 ± 1.0	7.8 ± 0.8				
500	47.2 ± 12.1	32.3 ± 8.4	10.5 ± 0.5	8.0 ± 2.1				
600	48.8 ± 3.0	30.8 ± 3.0	7.3 <sup>c</sup>	6.9 ± 5.1				

Residual folylpoly- $\gamma$ -glutamate concentrations were calculated in nmol/g dry matter. Values represent means  $\pm$  S.D. ( $n = 10$  for the untreated reference sample,  $n = 2$  for treated samples).

<sup>A</sup> 5-CH<sub>3</sub>H<sub>4</sub>PteGlu (%).

<sup>B</sup> 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>γ2</sub> (%).

<sup>C</sup> 5-CH<sub>3</sub>H<sub>4</sub>PteGlu<sub>γ3</sub> (%).

<sup>a</sup> Total residual folate content after 30 min treatment (nmol/g DM), broccoli moisture content is 88.6  $\pm$  0.7% ( $n = 6$ ).

<sup>b</sup> Data from single sample ( $n = 1$ ).

<sup>c</sup> Data from peak detected in only one of the duplicate samples.

of high-pressure treatments could be applied to engineer healthy processed foods.

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