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ARTICLE

Influence of Thermal Processing on Hydrolysis and Stability of Folate Poly-γ-glutamates in Broccoli (*Brassica oleracea var. italica*), Carrot (*Daucus carota*) and Tomato (*Lycopersicon esculentum*)

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The folate poly-γ-glutamate profile, their concentrations, and hydrolysis by endogenous γ-glutamyl hydrolase (GGH) were evaluated in broccoli, carrot and tomato. Further studies on the effect of time and temperature on folate poly-γ-glutamate hydrolysis and stability were carried out in broccoli since this vegetable showed the highest long-chain and total folate poly-γ-glutamate concentration. The evolution of L-ascorbic acid, total phenols and Trolox equivalent antioxidant capacity (TEAC) values was evaluated in parallel. Upon thermal inactivation of GGH prior to crushing, it was observed that broccoli, carrot and tomato contained poly-γ-glutamates with one to seven glutamate residues but differed in the predominant poly-γ-glutamates. Crushing of raw broccoli, carrot and tomato resulted in significant poly-γ-glutamate profile changes in broccoli and carrot (indicating GGH-catalyzed hydrolysis) but not in tomato. In this study, the actual crushing of raw broccoli matrix had a greater effect on folate poly-γ-glutamate hydrolysis than incubation conditions (0−30 min at 25−55 °C). During treatments at 25−140 °C, folate retention was higher at 80 and 100 °C than at the other temperatures. A similar trend in thermal stability was observed for folates, vitamin C, total phenols and TEAC value, an indication that conditions that result in endogenous antioxidants degradation might also result in folate degradation.

KEYWORDS: Antioxidants; crushing; broccoli; carrot; tomato; folate poly-γ-glutamates; temperature; time

INTRODUCTION

Folates play a key role in DNA synthesis and in the methylation cycle (*1*). In humans, folates play a well documented role in preventing neural tube defects and could possibly reduce the incidence of cancer and cardiovascular disease (*2*). Humans entirely depend on the diet for a sufficient folate status, with fruits and vegetables being the major sources (*1*). The folate content of fresh fruits and vegetables may vary depending on factors such as plant species, variety, plant organ considered, the stage of maturity at harvest and the climatic conditions during growing (*3*−*7*).

Food folates consist of a mixture of several derivatives which differ in one-carbon unit substitution, oxidation status and the γ-glutamate chain length. Plant-based food folates predominantly occur as 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu$_n$), whereas other derivatives such as 5-formyl- (5-CHO-H_4PteGlu$_n$), 10-formyl- (10-CHO-PteGlu$_n$), 5,10-methenyl (5,10-CH^+-H_4PteGlu$_n$) and unsubstituted tetrahydrofolate (H_4PteGlu$_n$) also occur in plants but in smaller amounts compared to 5-CH_3-H_4PteGlu$_n$ (*3*,*4*,*8*,*9*). In intact plant tissue, folates predominantly occur as poly-γ-glutamates with one to eight γ-linked glutamate residues (*10*−*15*). At the subcellular level, folate poly-γ-glutamates are distributed in different concentrations in the cytosolic fraction (consisting of cytosol and vacuole), mitochondria and chloroplasts. For pea leaf, it has been shown that the bulk of folates (60−80%) occur in the cytosolic fraction while 11−30 and 3−11% occur in the mitochondria and chloroplasts respectively (*4*, *16*, *17*). Recently, it was shown that cytosolic folates predominantly occur in vacuoles in pea leaves and beet roots (*8*). It is well established that hydrolysis of folate poly-γ-glutamates yielding shorter glutamate chain poly-γ-glutamates and monoglutamates is catalyzed by γ-glutamyl hydrolase (GGH) that has been located in vacuoles (*8*), in the cytosol (*18*) and in the extracellular matrix (*19*). Since folate poly-γ-glutamates and GGH have been found co-occurring, e.g. in

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vacuoles of pea leaves and beet roots, a mechanism protecting folate poly-γ-glutamates from GGH attack is thought to exist (*8*). Vegetable processing treatments resulting in cell membrane damage, e.g. crushing, freezing, thermal and high pressure treatments in the presence of active endogenous GGH, have been demonstrated to facilitate folate polyglutamate hydrolysis (*13*−*15*, *20*), and have therefore been suggested as strategies to increase the bioavailability of plant-based food folates (*13*). High folate losses have been reported in fruits and vegetables during postharvest storage and processing (*5*, *22*, *23*) and have been associated with leaching and/or oxidative cleavage of folates into biologically inactive pterin and *p*-aminobenzoylglutamate. During processing, the extent of folate oxidation is influenced by factors such as amount of oxygen present, temperature, pressure, pH, light, metal ions and the duration of exposure to oxidants (*14*, *15*, *23*). Regarding folate oxidation, the presence of exogenous antioxidants has been found to reduce the extent of folate oxidation during analysis and heating in model systems (*23*−*26*). In this respect, it has been hypothesized that endogenous food antioxidants such as ascorbic acid could stabilize folates during food processing (*23*). However, studies involving parallel evaluation of folates and endogenous antioxidants stability in foods during processing are still scarce. Hereto, the aims of this study were (i) to screen the native folate poly-γ-glutamate profile and activity of GGH in different vegetables by using broccoli, carrot and tomato as case studies to respectively represent green vegetables, root vegetables and fruit-based vegetables and (ii) to evaluate the effect of temperature and time on folate poly-γ-glutamate hydrolysis and stability in broccoli, because it is one of the vegetables known to contain a high concentration of folate poly-γ-glutamates. In this study, the effect of temperature and time on vitamin C concentration, i.e. L-ascorbic acid (L-AA) and dehydroascorbic acid (DHAA), total phenols and TEAC value was evaluated in parallel.

MATERIALS AND METHODS

Sample Preparation and Treatments. Two batches of fresh broccoli (unknown varieties) from two different origins (origin Spain, var. A; origin Italy, var. B) were purchased from a local supermarket and stored at 4 °C for less than 5 days before use. The florets together with about 1 cm of the stalks were cut from several broccoli heads and collected into one batch per variety. One batch of carrots (variety Nerac, origin Belgium) was obtained from a local supermarket and stored at 4 °C for less than 5 days before use. The carrots were washed, peeled, cut into pieces (approximately 1 cm) and combined into a single batch. One batch of red-ripe tomatoes (unknown variety, origin Belgium) was obtained from a local supermarket and stored at 4 °C for less than 5 days before use. All tomatoes were washed, cut into eight longitudinal slices per tomato and combined into a single batch. The dry matter (DM) content of fresh samples was determined after drying at 105 °C for 16 h. The DM content of the two batches of broccoli was 11.1 ± 0.3% (var. A) and 11.0 ± 0.2% (var. B). The carrot and tomato contained 8.0 ± 0.2 and 5.3 ± 0.1% DM respectively.

To determine the native folate poly-γ-glutamate profile, 60 g portions of broccoli (var. A), carrot and tomato were taken at random and vacuum-packed (Multivac A300/16, Germany) in polyethylene bags. The samples were then blanched for 10 min in boiling water to inactivate endogenous GGH and other enzymes. Subsequently, samples were immediately cooled in ice water, frozen in liquid nitrogen and crushed (Grindomix GM 200, Retsch, Germany) into a frozen powder, which was stored at −80 °C until folate analysis. Samples blanched prior to crushing are identified with the code blanched.

Starting material for thermal treatments was obtained by freezing in liquid nitrogen the aforementioned batches of raw broccoli (var. A and B) and crushing them into frozen powders, which were thoroughly mixed using a kitchen mixer (A 907D, Kenwood, U.K.). The frozen powder of each variety was packed in plastic bottles and stored at −80 °C prior to treatments. For thermal treatments at 25−55 °C (0−30 min), aliquots (60 g) of starting material of broccoli (var. A) were vacuum-packed in polyethylene bags and subsequently thawed for 10 min at 25 °C in a shaking water bath (WBU 45, Memmert, Germany). The samples were then heated (25−55 °C) for preset time intervals (0−30 min) in a thermostated water bath (W14 Grant Instruments, Cambridge, England), after which they were immediately immersed in boiling water for 10 min to inactivate enzymes. Subsequently, samples were cooled in ice water, frozen in liquid nitrogen and stored at −80 °C until further analysis. For thermal treatments at 25−140 °C (30 min), starting material for broccoli (var. B) was packed in stainless steel tubes (length 100 mm, diameter 12 mm) and thawed (25 °C, 5 min) in a shaking water bath. The samples were then heated for 30 min in a thermostated oil bath (Grant Instruments, Cambridge, U.K.) at 25−140 °C. After the thermal treatments, samples were immediately cooled in ice water, frozen in liquid nitrogen and stored at −80 °C until further analysis. A summary of the various treatments performed on broccoli, carrot and tomato is shown in **Figure 1**.

Analysis of Folate Poly-γ-glutamates. The folate content of broccoli, carrot and tomato was analyzed by folate extraction, conversion into 5-CH_3-H_4PteGlu$_n$, purification by affinity chromatography and reverse phase (RP)-HPLC quantification procedures (*12*), previously optimized for broccoli (*14*). For folate extraction, 20 g of broccoli, 50 g of carrot or 50 g of tomato was added with 0.5 g of L-ascorbic acid into a 250 mL opaque polypropylene tube (Nalgene Labware, Rochester, NY). Subsequently, 50 mL of boiling phosphate buffer (0.1 M, pH 7.0) was added and the mixture homogenized for 30 s (Ultra Turrax T20, IKA, Germany). The sample-containing tubes were then immersed in boiling water for 10 min and subsequently cooled in ice water, after which the pH was adjusted to 7.0 using KOH (6 M) or HCl (4 M). To release matrix bound folates, a dienzyme extraction procedure was performed by adding 50 μL of amylase (Termamyl type L, Novo Nordisk, Denmark) and 50 μL of protease (Subtilisin Carlsberg type VIII, Sigma-Aldrich, Germany) solution (1 mg/mL) to the samples followed by incubation for 4 h at 37 °C in a shaking water bath. Enzymes were then inactivated by immersing the samples in boiling water for 5 min followed by cooling in ice water. After centrifugation (20000*g*, 4 °C, 30 min), the supernatant was collected and filtered using sintered glass filters (pore size No. 4, Labo Service, Belgium), frozen in liquid nitrogen and stored at −80 °C until conversion.

Conversion of all folate derivatives to 5-CH_3-H_4PteGlu$_n$ was carried out as follows: 2.0 g of sodium ascorbate, 15 mL of Tris-HCl buffer (66 mM, pH 7.8) and 1 mL of 2-octanol were added in an Erlenmeyer flask and mixed with broccoli, tomato or carrot extract (5 mL). Subsequently 10 mL of sodium borohydride solution (3.2 M) was added and the solution stirred for 10 min. The pH was then adjusted to 7.4 using acetic acid (5 M) followed by addition of 80 μL of formaldehyde (37%). After shaking within less than a minute, 10 mL of sodium borohydride solution (3.2 M) was gently added and the pH of the solution adjusted to approximately 0.85 using HCl (37%). After stirring for 10 min, the pH was adjusted to 5.0 using sodium hydroxide (5 M). Subsequently 10 mL of sodium borohydride solution (3.2 M) was gently added and the solution stirred for another 20 min. The volume was finally adjusted to 100 mL using Tris-HCl buffer (66 mM, pH 7.8) followed by immediate freezing in liquid nitrogen and storage at −80 °C until folate purification.

Folate purification was performed by a bovine folate binding protein (Scripps Laboratories, San Diego, CA) based affinity chromatography (FBP-AC) procedure described by others (*27*). To minimize folate degradation, affinity chromatography was carried out under subdued light at 3−5 °C. Columns were conditioned with 5 mL of phosphate buffer (0.1 M, pH 7.0) prior to sample loading, i.e. 5 mL of converted broccoli, tomato or carrot solution, corresponding to maximally 20% of the maximal column binding capacity. The columns were subsequently rinsed with 5 mL of phosphate buffer (25 mM, 1 M NaCl, pH 7.0) and 5 mL of phosphate buffer (25 mM, pH 7.0). Elution of folates was performed using 4.6 mL of trifluoroacetic acid (20 mM) and 1,4-dithioerythritol (20 mM) solution. The eluate was collected in a recipient containing 5 μL of β-mercaptoethanol, 50 μL of KOH solution (6 M) and 200 μL of ascorbic acid solution (25% w:v). After elution, the volume was adjusted to 5 mL with the elution solution, after which samples were stored at −80 °C until HPLC analysis.

RP-HPLC analysis of 5-CH_3-H_4PteGlu$_n$ was performed as previously described (*14*) using a liquid chromatography system (1200 Series, Agilent Technologies, Diegem, Belgium) equipped with a fluorescence detector (RF-10AxL, Shimadzu, Kyoto, Japan). Gradient elution at a flow rate of 0.8 mL/min was performed at 25 °C for separation of polyglutamates, on a

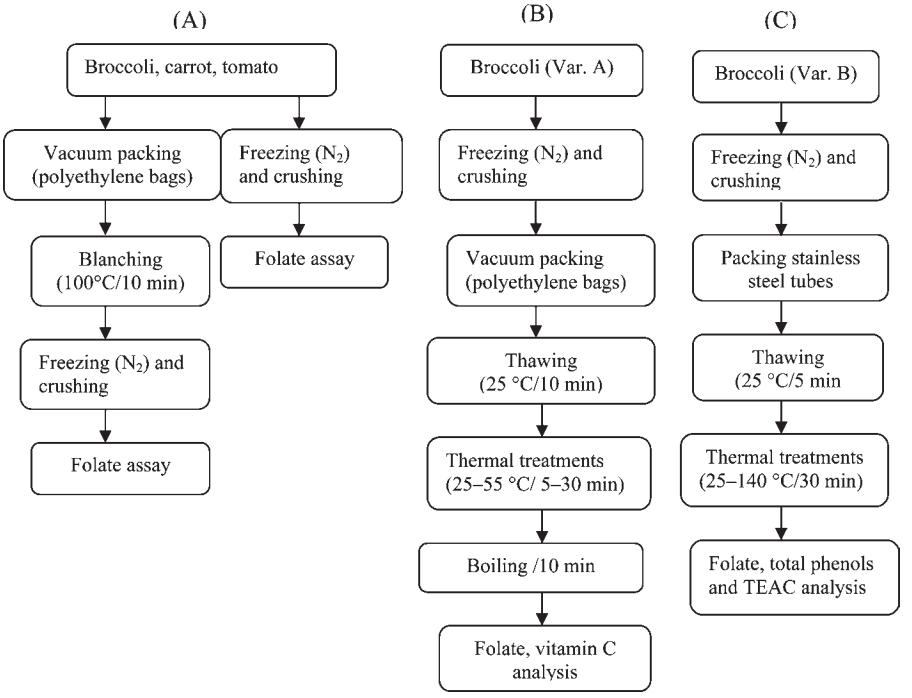

Figure 1. A scheme of the various steps involved in the determination of the (**A**) native folate poly-γ-glutamate profile and the influence of GGH activity on the native folate poly-γ-glutamate profile of broccoli, carrot and tomato; (**B**) influence of thermal treatments at mild temperatures (25−55 °C/0−30 min) on the total folate and vitamin C concentration and the folate poly-γ-glutamate profile of broccoli; (**C**) influence of thermal treatments at mild to high temperatures (25−140 °C/30 min) on the total folate and phenolic content, and total water-soluble antioxidant capacity (TEAC).

RP C18 column (Prevail C18, 250 mm × 4.6 mm, 5 μm particle size, Grace, Deerfield, IL). Elution was started with 100% NaH_2PO_4 (0.05 M, pH 4.5, solvent A). The concentration of solvent B, i.e. 20% (v:v) acetonitrile in NaH_2PO_4 (0.05 M, pH 4.5), was subsequently linearly increased to 50% (v:v) within 23 min, and then to 100% (v:v) in 13 min. The conditions were maintained at 100% solvent B for 10.40 min followed by column re-equilibration with 100% solvent A for 10.40 min prior to injection of the next sample. Fluorescence detection (FD) was applied at λ_{ex} = 295 nm and λ_{em} = 356 nm. Folate poly-γ-glutamates in samples were identified based on comparison with retention times of 5-CH_3-H_4PteGlu$_{1-7}$ standards. (6*R*,*S*)-5-Methyl-5,6,7,8-tetrahydrofolic acid poly-γ-glutamate standards (5-CH_3-H_4PteGlu$_{\gamma 2-5}$), pteroylhexa-γ-L-glutamic acid (PteGlu$_{\gamma 6}$) and pteroylhepta-γ-L-glutamic acid (PteGlu$_{\gamma 7}$) were obtained from Schircks Laboratories (Jona, Switzerland). 5-CH_3-H_4PteGlu$_{\gamma 6}$ and 5-CH_3-H_4PteGlu$_{\gamma 7}$ standards were prepared by conversion of PteGlu$_{\gamma 6}$ and PteGlu$_{\gamma 7}$ as outlined above. (6*S*)-5-methyl-5,6,7,8-tetrahydrofolic acid (5-CH_3-H_4PteGlu) was obtained from Merck Eprova AG (Schaffhausen, Switzerland). Quantification of folates was done using external calibration curves of folate standards (0.1 to 1.0 pmol of 5-CH_3-H_4PteGlu$_{1-7}$ on column) based on peak area with R^2 ranging from 0.981 to 0.997.

Analysis of L-AA and DHAA in Broccoli. L-AA and DHAA were extracted as described by others (*28*). Hereto, 10 g of broccoli was homogenized for 1 min with 50 mL of cold (4 °C) extraction solution (20 mM NaH_2PO_4, pH 2.1, 1 mM EDTA) using an ultrahigh speed mixer. The homogenate was centrifuged (27200*g*, 4 °C, 30 min). The supernatant was collected and stored at −80 °C until HPLC analysis. L-AA and DHAA were quantified as previously described (*29*). Prior to HPLC analysis, the supernatant was thawed at 25 °C for 5 min and its pH adjusted to 4.0 using NaOH (1 N). The supernatant was then placed in the autosampler at 4 °C for HPLC analysis of L-AA. DHAA was quantified after precolumn reduction to L-AA by a 30 min incubation at 23 °C of a mixture of 0.5 mL of supernatant and 1 mL of a reducing agent solution, i.e. 2.5 mM Tris(2-carboxy-ethyl)phosphine HCl (TCEP) in 20 mM NaH_2PO_4 (pH 3.5, 1 mM EDTA). After reduction, the samples were placed in the autosampler for HPLC analysis of L-AA. To quantify L-AA, isocratic elution for 30 min at a flow rate of 0.8 mL/min at 25 °C was performed on a Prevail C18 column (250 mm × 4.6 mm, 5 μm particle size, Grace) using a 1200 series liquid chromatography system (Agilent Technologies) equipped with DAD detector. L-AA was detected at 245 nm and eluted 6.4 min after injection (20 μL) using ammonium acetate solution (10 mM, pH 3.0, 1 mM EDTA) as eluent. LAA concentrations were quantified using external calibration curves (0.8−16.8 nmol L-AA on column) with R^2 above 0.999. The DHAA content of the samples was determined as the difference in L-AA content after and before reduction.

Determination of Water-Soluble Antioxidants in Broccoli. Extraction of water-soluble antioxidants was carried out by homogenizing 10 g of broccoli with 20 mL of phosphate buffer (0.1 M, pH 7.0) at 4 °C for 30 s. After centrifugation (11000*g*, 4 °C, 15 min), the supernatant was filtered (paper No. 597, Whatman), frozen in liquid nitrogen and stored at −80 °C until antioxidants measurement.

Total phenolics in broccoli were quantified using the Folin−Ciocalteu procedure as described by others (*30*) with some modifications. Gallic acid was used as a calibration standard (0 to 2.0 mM, R^2 > 0.99). Hereto, 250 μL of broccoli extract or gallic acid solution was added to 500 μL of double distilled water plus 1250 μL of Folin−Ciocalteu reagent (10%, w:v) and incubated for 1 h under subdued light at 25 °C. Subsequently, the absorbance was measured at 755 nm and 25 °C. The total phenolic content in broccoli was expressed as gallic acid equivalents (GAE), i.e. mg/100 g fresh weight (FW).

The total water-soluble antioxidant capacity was determined using the Trolox equivalent antioxidant capacity (TEAC) assay (*31*) using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) as radical source and Trolox (0.0 to 17.5 mM) in phosphate buffer (5 mM, pH 7.4) as calibrator. An ABTS stock solution (5 mM) was prepared in phosphate buffer (5 mM, pH 7.4). Prior to analysis, $ABTS^{\bullet+}$ radical cations were generated by addition of activated MnO_2 to a concentration of 0.5 g/100 mL and stirring for 30 min at room temperature. To prepare a working solution, the $ABTS^{\bullet+}$ radical cation solution was diluted in phosphate buffer (5 mM, pH 7.4) to approximately 0.15 mM. For TEAC measurements, 15 μL of broccoli extract or Trolox standard solution was added to 2.9 mL of the $ABTS^{\bullet+}$ radical cation solution and incubated for 30 min at 30 °C under subdued light. The absorbance was then measured at 738 nm and 30 °C. The total water-soluble antioxidant capacity of broccoli was expressed as TEAC value (mM/100 g FW). Trolox calibration curves were linear in the concentration range of 0.0 to 17.5 mM (R^2 0.997).

Data Analysis. Results for folates, total phenolics and TEAC value are reported as mean ± standard deviation (n = 3). Significance of differences for the means of total folate content, percent distribution of

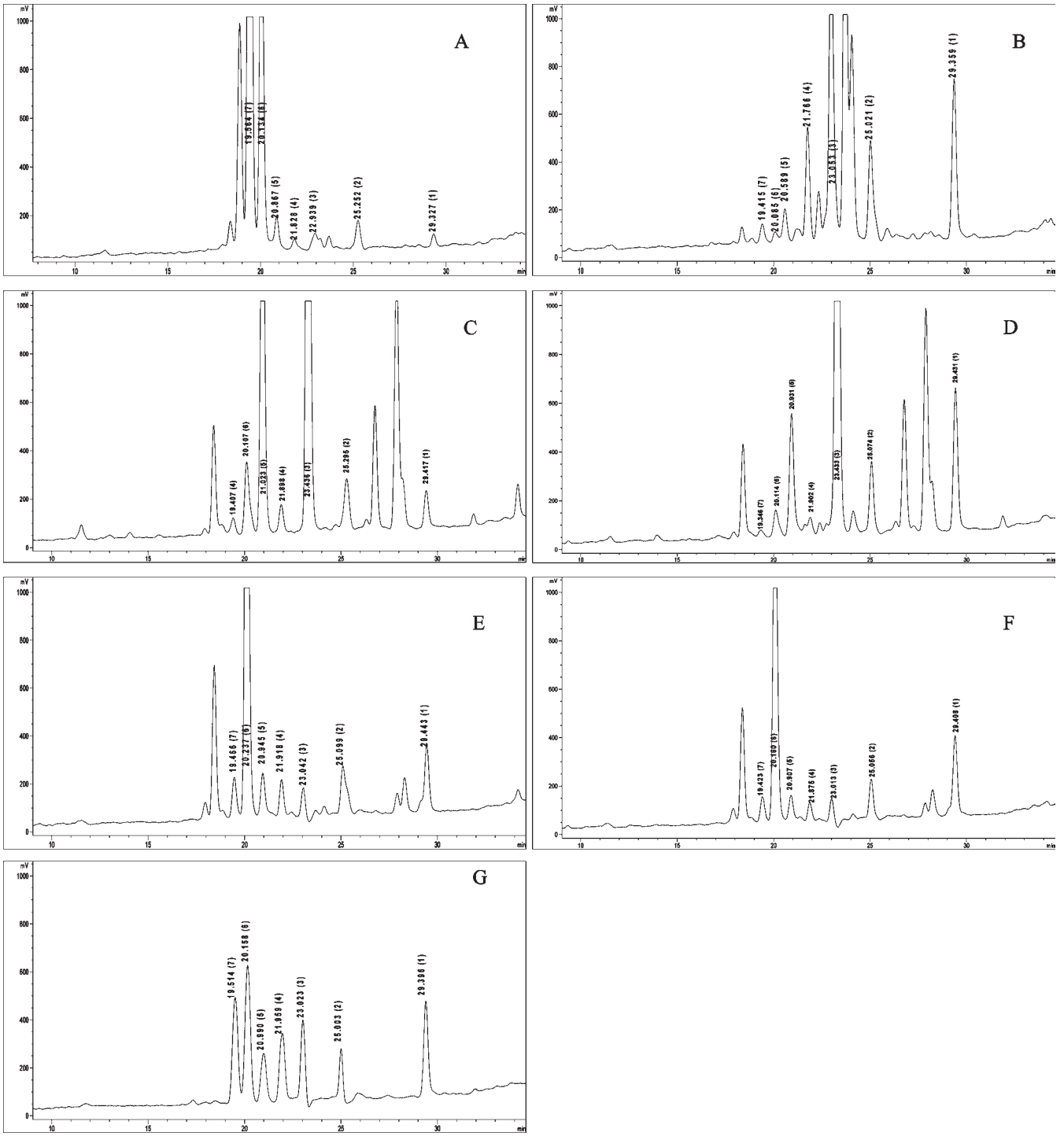

Figure 2. Chromatograms showing the separation and identification of folates in 100 μL injection volume of (**A**) blanched broccoli, (**B**) raw broccoli, (**C**) blanched carrot, (**D**) raw carrot, (**E**) blanched tomato, (**F**) raw tomato and (**G**) standard solution containing 0.5 pmol of 5-CH_3-H_4PteGlu$_{2-7}$ and 0.25 pmol of 5-CH_3-H_4PteGlu on column. Numbers next to peak apexes (outside parentheses) indicate the exact retention time while numbers inside parentheses indicate the number of glutamate units on the folate molecule. Peaks with a flat apex were quantified using 20 μL injections.

various folate derivatives, total phenols and TEAC values in treated samples was performed by one way ANOVA with Tukey's post hoc test at significance 0.05 (S-Plus 6.1.2, release 1 for Microsoft Windows: 2002). For vitamin C which was measured in duplicate, average values are reported and no further statistical analysis was performed.

RESULTS

Analysis of Folates in Broccoli, Carrot and Tomato. Folates in broccoli, carrot and tomato were analyzed after conversion to 5-CH_3-H_4PteGlu$_n$, which can be sensitively quantified using FD due to its high quantum yield (*12*, *32*). All the steps of folate analysis were conducted under subdued light and in the presence of antioxidants to minimize oxidation. Purification by FBP-AC, a method well recognized for its specific isolation and purification of folates thus excluding matrix interference during quantification (*12*, *27*, *33*), resulted in a satisfactory separation of 5-CH_3-H_4PteGlu$_{1-7}$ in standard solutions and samples (**Figure 2**). The entire folate analysis procedure was previously validated by analyzing a certified reference material (BCR 485) and broccoli spiked with 5-CH_3-H_4PteGlu$_{1-7}$ standards (*15*), whereby a

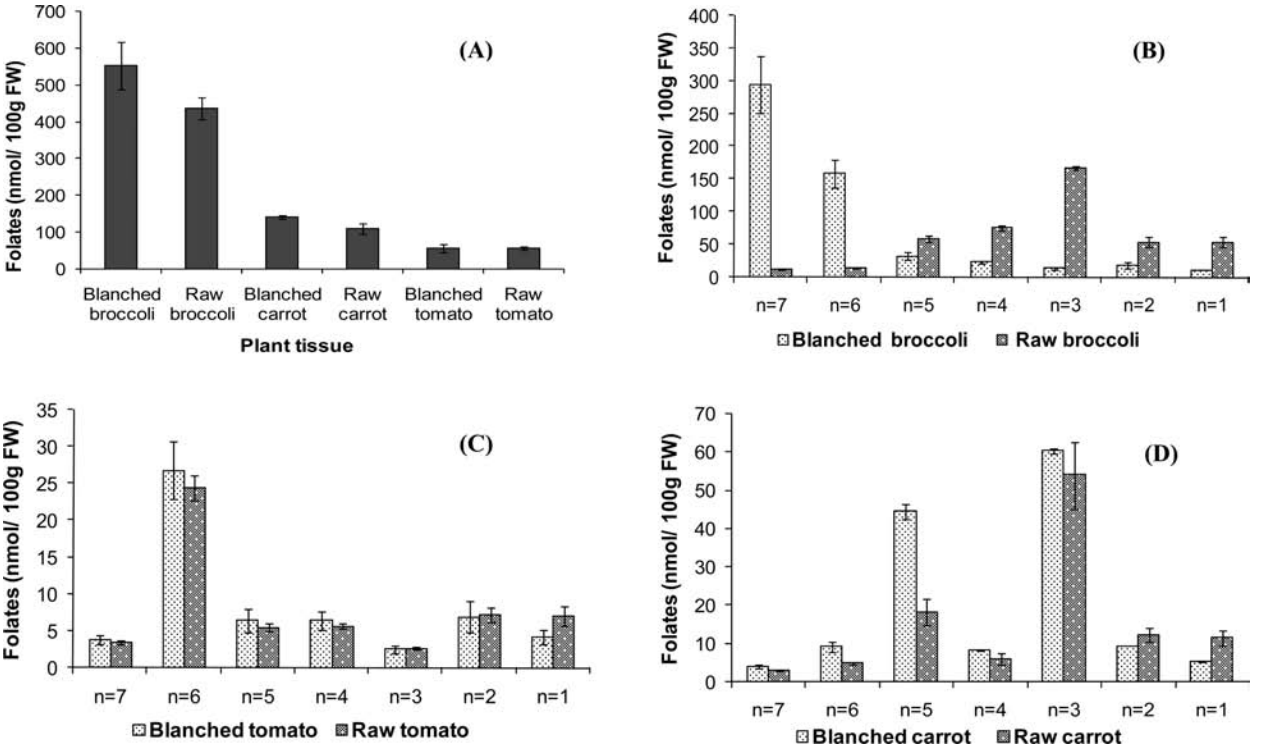

Figure 3. Total folate concentration in broccoli carrot and tomato (**A**); native folate poly-γ-glutamate profile (blanched samples) and the influence of endogenous GGH (raw samples) on the native folate poly-γ-glutamate profile of broccoli (**B**), tomato (**C**) and carrot (**D**).Values are mean ± standard deviation (n = 3). FW stands for fresh weight, and n = 1 to n = 7 indicate the number of glutamate residues on the folate molecule.

Table 1. Influence of Temperature on Individual Folate Derivative and Total Folate Concentration (nmol/100 g FW) in Broccoli (Var. B) Treated for 30 min[a]

folate derivative[d]	raw (untreated)	25 °C	40 °C	60 °C	80 °C	100 °C	120 °C	140 °C
hepta-γ-glutamate	133.68 ± 79.78	26.92 ± 2.33 a	23.12 ± 3.43 b	21.88 ± 3.77 b	26.13 ± 3.41 b	25.52 ± 1.99 b	24.61 ± 2.30 b	23.08 ± 0.06 b
hexa-γ-glutamate	77.81 ± 39.34	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	15.97 ± 1.27 b	6.08 ± 10.54 b	17.34 ± 2.12 b	16.51 ± 0.54 b
penta-γ-glutamate	29.29 ± 8.86	37.79 ± 0.75 a	37.18 ± 3.96 a	39.15 ± 9.89 a	43.52 ± 8.91 a	43.45 ± 4.75 a	34.76 ± 0.50 a	37.32 ± 0.07 a
tetra-γ-glutamate	80.89 ± 24.04	49.74 ± 24.99 a	27.20 ± 2.87 b	30.37 ± 1.18 b	35.90 ± 8.35 b	34.40 ± 4.92 b	25.01 ± 1.30 b	26.53 ± 2.13 b
tri-γ-glutamate	193.44 ± 13.90	287.24 ± 12.37 a	248.33 ± 22.82 a	236.77 ± 39.73 a	317.69 ± 60.38 a	327.70 ± 2.13 b	203.94 ± 2.40 a	235.38 ± 25.33 a
di-γ-glutamate	63.61 ± 9.21	36.00 ± 4.47 b	22.42 ± 2.80 b	22.56 ± 4.98 b	21.21 ± 6.25 b	33.99 ± 7.14 b	19.25 ± 0.93 b	22.50 ± 2.53 b
monoglutamate	22.74 ± 4.29	9.13 ± 0.16 b	5.47 ± 0.52 b	4.84 ± 1.14 b	8.32 ± 1.95 b	7.79 ± 0.92 b	5.79 ± 0.55 b	7.43 ± 0.70 b
total folates (nmol/100 g FW)[d]	601.46 ± 161.10 a	446.82 ± 43.58 a	363.71 ± 28.11 b	355.58 ± 57.41 b	446.83 ± 82.60 a	470.81 ± 18.28 a	306.09 ± 3.76 b	348.67 ± 30.23 b

[d] Mean ± standard deviation (n = 3); for 140 °C treatment, n = 2. [a] Small letter a after the values indicates values that were not significantly different, while b indicates values that were significantly different compared to the raw sample.

recovery of 91% was found. Recoveries around 100% for conversion of 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu, and spiking broccoli with a mixture consisting of H_4PteGlu, 5-CHO-H_4PteGlu, 5-CH_3-H_4PteGlu and 5,10-methylene- (5-10-CH_2H_4-PteGlu) were previously reported (*14*).

The total folate concentration in broccoli, carrot and tomato was determined as the sum of individual 5-CH_3-H_4PteGlu$_n$ derivatives. The total folate content in raw and thermally treated samples was based on the DM content of fresh samples i.e., 11.1, 11.0, 8.0 and 5.3% for broccoli var. A, broccoli var. B, carrot and tomato respectively, since thermal treatments were performed in closed systems (moisture content of treated samples did not change). Folate concentrations amounting to 552.4 ± 63.9 and 601.5 ± 161.1 nmol /100 g FW were found for broccoli var. A (**Figure 3A**, blanched broccoli) and var. B (**Table 1**, raw broccoli) respectively. Folate concentrations in blanched and raw tomato amounted to 57.1 ± 10.9 and 55.9 ± 4.9 nmol/100 g FW respectively (**Figure 3A**), while blanched and raw carrot were found to contain 140.8 ± 3.9 and 109.8 ± 12.8 nmol/100 g FW respectively (**Figure 3A**).

Influence of Processing on the Native Profile and Stability of Folates in Broccoli, Carrot and Tomato. The native folate poly-γ-glutamate profile was evaluated by extracting folates from broccoli, carrot and tomato samples blanched prior to crushing. Endogenous GGH activity was examined by extracting folates from broccoli, carrot and tomato samples crushed prior to blanching. This study showed that folates in broccoli, carrot and tomato exist as mono- to hepta-γ-glutamates (**Figure 3B−D**), and that the predominant folate poly-γ-glutamates are different for each plant matrix. The predominant folate poly-γ-glutamates in broccoli (**Figure 3B**) were hepta- (52.5 ± 2.9%) and hexa-γ-glutamates (28.3 ± 1.5%), in tomato (**Figure 3C**) these were hexa-γ-glutamates (47.2 ± 2.4%), while in carrot (**Figure 3D**) these were tri- (42.9 ± 1.0%) and penta-γ-glutamates (31.7 ± 1.2%). In blanched broccoli, tomato and carrot, the contribution of folate monoglutamates to the total folate pool was 0.9 ± 0.1, 7.2 ± 0.5 and 3.9 ± 0.3% respectively.

The influence of endogenous GGH activity on the native folate poly-γ-glutamate profile differed for each plant matrix as shown in **Figure 3B−D**. Crushing broccoli prior to blanching resulted in almost complete hydrolysis of the predominant hepta- and

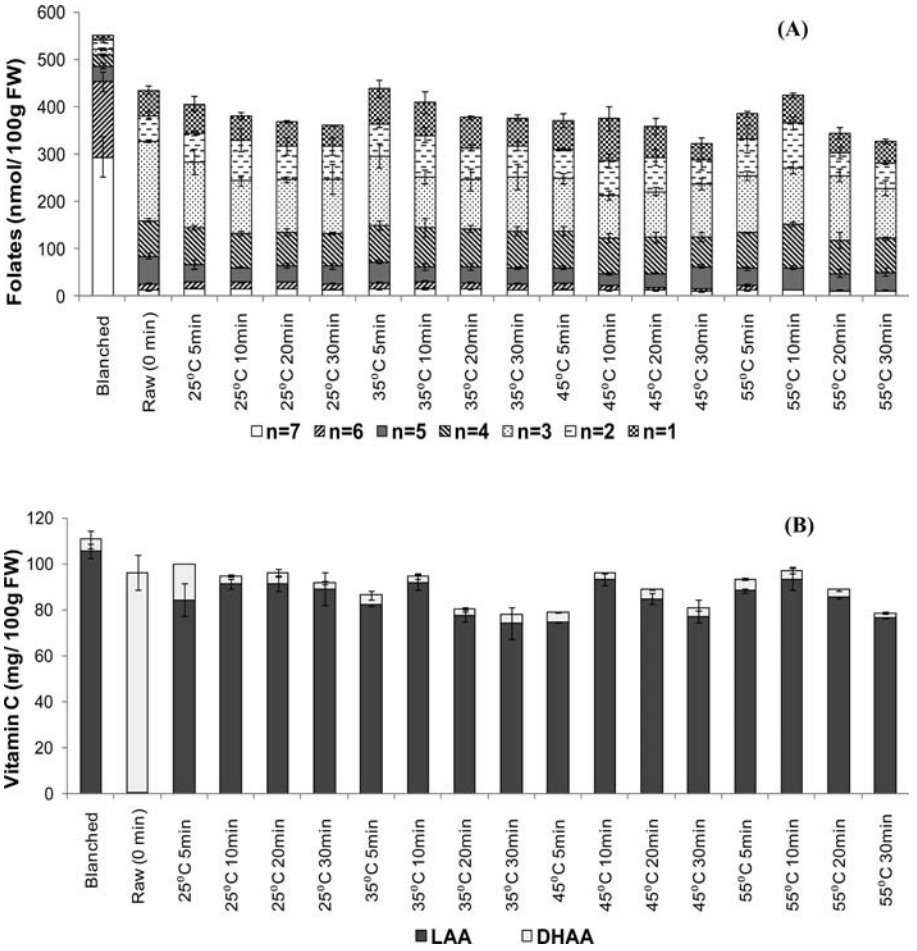

Figure 4. Effect of temperature and time on folate poly-γ-glutamate profile and stability (**A**) and vitamin C stability (**B**) in raw crushed broccoli; $n = 1$ to $n = 7$ indicate the number of glutamate residues on the folate molecule. Folate values are mean $\pm$ standard deviation ($n = 3$).

hexa-γ-glutamates to shorter chain poly-γ-glutamates (mainly tri-γ-glutamates) and monoglutamates (**Figure 3B**). In carrot (**Figure 3D**), the concentration of hexa-, tetra-, tri- and particularly penta-γ-glutamates decreased but only a limited increase in monoglutamates was observed. In tomato, however, crushing prior to blanching did not significantly influence the poly-γ-glutamate profile.

Broccoli was chosen as a case study for further experiments because it (i) contained higher total folate concentration, (ii) contained higher concentration of poly-γ-glutamates with the longest γ-glutamyl chain and (iii) showed higher extent of GGH-catalyzed hydrolysis of the predominant poly-γ-glutamates than tomato and carrot. The influence of thermal treatments at mild temperatures (25−55 °C, 0−30 min) on total folate and individual folate derivative concentration is shown in **Figure 4A**. Different thermal treatments did not significantly change the concentrations of various folate derivatives except that of hexa-γ-glutamate, which decreased to undetectable levels after incubating raw crushed broccoli at 45 °C (30 min) and 55 °C (10−30 min). Relative to blanched broccoli, raw crushed broccoli (0 min) contained 21% less total folate. Further and significant ($p < 0.05$) folate losses that increased with time, i.e. 26−34% (25 °C), 20−31% (35 °C), 32−41% (45 °C) and 30−41% (55 °C) were observed during treatments for 5−30 min. The influence of thermal treatments at mild to high temperatures (25−140 °C, 30 min) on folate stability in raw crushed broccoli is illustrated in **Table 1**. Relative to the raw sample, treatments at 40, 60, 120, and 140 °C resulted in significant ($p < 0.05$) folate losses amounting to 39, 41, 49 and 42% respectively while treatments at 25, 80, and 100 °C resulted in nonsignificant folate losses. Although no further studies were performed on the stability of folates in tomato and carrot, it was observed that relative to the blanched samples, raw tomato and carrot samples retained 98 and 78% folates respectively (**Figure 3A**).

To establish if endogenous antioxidants influenced the stability of folates, the evolution of water-soluble antioxidants during thermal treatments was evaluated in parallel. The concentrations of L-AA and DHAA were determined in broccoli (var. A) treated for 5−30 min at 25−55 °C. The method used to analyze L-AA in broccoli was previously (*29*) observed to result in 97% recovery of L-AA spiked into broccoli florets at the beginning of the extraction procedure. As shown in **Figure 4B**, the total vitamin C (i.e., L-AA + DHAA) concentration decreased as a function of time and temperature. Relative to blanched broccoli, treated samples (25−55 °C, 0−30 min) showed vitamin C losses ranging from 12 to 30%. A linear correlation was observed between the residual folate and vitamin C concentration for similar thermal treatments. For the different treatment times, linear correlation coefficients of 0.72, 0.83, 0.79, and 0.92 were found for treatments at 25, 35, 45, and 55 °C respectively.

The effect of thermal treatments (40−140 °C, 30 min) on the total phenol content and TEAC values was evaluated during treatments of raw crushed broccoli (var. B). Triplicate analysis of total phenols in raw and treated broccoli showed relative standard deviation (RSD) values in the range of 1.8 to 7.0%. The phenol contents of samples treated at 40, 60, 100, and 120 °C were either comparable or significantly lower than that in the raw sample (**Table 2**) whereas higher values were observed in the

Table 2. Effect of Temperature on Total Water-Soluble Phenols and Total Water-Soluble Antioxidant Capacity (TEAC Value) of Crushed Broccoli Treated for 30 min[a]

temp (°C)	total phenols (mg GAE/100 g FW)[b]	TEAC value (mM Trolox/100 g FW)[b]
raw (untreated)	9.19 ± 0.22	45.57 ± 2.22
40	7.25 ± 0.31 b	42.36 ± 4.04 a
60	5.21 ± 0.24 b	38.50 ± 3.75 a
80	9.43 ± 0.17 a	55.34 ± 1.25 a
100	7.91 ± 0.55 b	61.34 ± 11.90 b
120	8.98 ± 0.59 a	70.75 ± 2.94 b
140	15.32 ± 0.58 b	78.00 ± 1.27 b

[a] Small letter a after the values indicates values that were not significantly different, while b indicates values that were significantly different compared to the raw sample. [b] Mean ± standard deviation (n = 3).

sample treated at 140 °C. The total water-soluble antioxidant capacity was determined using the TEAC assay. To prevent the inhibition of its formation that could result in antioxidant capacity overestimation (*34*), the $ABTS^{\bullet+}$ radical cation used for the TEAC assay was generated prior to addition of broccoli extracts. The TEAC values (RSD: 1.63−9.7%) observed for broccoli samples are shown in **Table 2**. Treatments at 40−80 °C had no significant effect on the TEAC value while treatments at 100−140 °C significantly increased the TEAC value. No linear correlation was found between folate concentration and total phenols or TEAC values.

DISCUSSION

Folates in Broccoli, Carrot and Tomato. Differences in one carbon substitution, state of reduction at the pteridine moiety and number of glutamate residues in the poly-γ-glutamate chain result in the occurrence of a large number of folate derivatives in fruits and vegetables. In the majority of plants including broccoli, tomato and carrot, 5-CH_3-H_4PteGlu$_n$ are the predominant derivatives, which co-occur with smaller proportions of H_4PteGlu$_n$, 5-CHO-H_4PteGlu$_n$, 10-CHO-PteGlu$_n$, 10-formyldihydrofolate (10-CHO-H_2PteGlu$_n$) and 5,10-CH^+-H_4PteGlu$_n$ (*3*, *7*, *9*, *10*, *35*). In this study, all folate derivatives were converted to 5-CH_3-H_4PteGlu$_n$ resulting in total folate contents of broccoli, tomato and carrot that are in the range of values reported in literature (**Table 3**). Variations in reported values could be attributed to factors such as differences in analytical methods, varietal differences, maturity of the fruit/vegetable at harvest, postharvest handling practices and growing conditions. The total folate contents, i.e., 552.4 ± 63.9 and 601.5 ± 161.1 nmol/100 g FW, obtained in the current study for broccoli var. A and var. B respectively were higher than other values reported in literature but lower than 689.5 ± 29.3 nmol/100 g FW reported previously (*15*). The folate values obtained for tomato were in the range of values observed in a recent study (*7*) but lower than the values reported by others (*9*, *10*). For carrot, we determined a higher folate concentration than that reported by others (*3*, *35*−*38*). The folate analysis procedure used in the current study previously showed a satisfactory recovery (*15*). Selective FBP-AC prevented interference from matrix compounds. In this study, dienzyme extraction and freezing with liquid nitrogen followed by crushing could have facilitated better release of matrix bound folates from broccoli and carrot in accordance with a recent study (*5*), where lettuce and spinach samples subjected to similar treatments exhibited higher folate content than most literature data. Most folate data reported in the literature have been obtained with methods involving hydrolysis of folate poly-γ-glutamates to monoglutamate using exogenous GGH (from rat plasma, hog kidney or chicken pancreas) and mostly quantify the monoglutamate content of foods using HPLC-FD. It was previously demonstrated (*12*) that such measurements of folate monoglutamates result in folate underestimation since none of the aforementioned exogenous GGHs resulted in complete hydrolysis of folate poly-γ-glutamates to monoglutamates in plant tissues. Literature folate values obtained using the microbial assay are generally higher than those obtained using HPLC-FD (after poly-γ-glutamate hydrolysis with exogenous GGH) probably due to the ability of *Lactobacillus casei* to grow in the presence of folates containing up to 3 glutamate residues (*39*). Though the same folate analysis method as used previously (*14*) was used in the current study, the folate content obtained was 2-fold higher probably due to differences in variety or origin since such have been demonstrated to result in up to 6-fold differences in the folate content of vegetables (*5*, *40*). Other factors such as maturity at harvest, storage conditions such as temperature and duration, climatic conditions (e.g., light) during growing and the dry matter content of the fruit/vegetable could also contribute to differences in reported folate values (*4*, *5*, *40*).

Table 3. Folate Values (nmol/100 g FW) of Broccoli, Carrot and Tomato Reported in Literature: Comparison with Values Obtained in the Current Study

food/status during folate extraction	total folates (nmol/100 g FW)	anal. meth	ref
broccoli			
raw	77	HPLC/MS/MS	*38*
raw	113	HPLC-FD	*36*
raw	147	HPLC-FD	*3*
raw	232	MA (*L. casei*)	*21*
raw (unknown variety, Belgium)	240	HPLC-FD[a]	*14*
raw	258	HPLC-FD	*35*
raw (var. Milady, Belgium)	315	HPLC-FD[a]	*14*
raw	401	MA (*L. casei*)	*48*
boiled	428	MA (*L. casei*)	*20*
blanched (var. A: Spain)	552	HPLC-FD[a]	this study
raw (var. B: Italy)	602	HPLC-FD[a]	this study
raw (var. Gramel: Spain)	690	HPLC-FD[a]	*15*
carrot			
raw	30	HPLC-FD	*3*
raw	31	HPLC-FD	*37*
raw	32	HPLC/MS/MS	*38*
raw	36	HPLC-FD	*35*
cooked	36	HPLC-FD	*3*
raw	40	HPLC-FD	*36*
raw (var. Nerac, Belgium)	110	HPLC-FD[a]	this study
blanched (var. Nerac, Belgium)	141	HPLC-FD[a]	this study
tomato			
raw	9	HPLC-FD	*37*
raw	56	HPLC-FD[a]	this study
blanched (unknown variety, Belgium)	57	HPLC-FD[a]	this study
raw	11−63	HPLC-FD	*7*
raw	80−230	HPLC-ECD	*9*
raw	100	HPLC-ECD	*10*

[a] Hydrolysis of folate poly-γ-glutamates to monoglutamates not performed; MA, microbial assay; MS, mass spectrometry; FD, fluorescence detection; ECD, electrochemical detection.

Influence of Endogenous GGH on the Native Folate Poly-γ-glutamate Profile in Broccoli, Carrot and Tomato. Folates in plants occur predominantly as poly-γ-glutamates with the number of γ-linked glutamate units (one to eight) and the predominant folate derivatives being dependent on the plant species under consideration. Polyglutamation is thought to play a role in retaining folates

in various cell compartments in addition to being the preferred substrates for most folate-dependent enzymes (*1*). The poly-γ-glutamate chain is also reported to enhance the binding of folates to folate binding proteins thus rendering them less susceptible to degradation (*1*). In accordance with literature (*1*, *9*−*15*, *20*, *41*), folates in the three plant matrices investigated in the current study occurred predominantly as poly-γ-glutamates, and the predominant derivatives were different for each plant matrix. The predominant occurrence of hepta- and hexa-γ-glutamates in the native folate profile of broccoli was in agreement with our previous report (*15*) while the predominant occurrence of hexa-γ-glutamates in tomato was in accordance with recent literature data (*9*, *10*). In accordance with the current study, raw broccoli was previously reported to predominantly contain tri-γ-glutamate (*14*, *15*). However, other authors (*11*) reported the predominant occurrence of di- (about 40%) and tri-γ-glutamates (about 20%) in broccoli. The aforementioned authors also reported that di-γ-glutamate was predominant (69%) in carrot with only 19% tri-γ-glutamate being reported. Differences in reported folate poly-γ-glutamate profiles for a particular plant tissue could be attributed to differences in the ability of the analytical method to detect various polyglutamates and GGH-catalyzed hydrolysis of folate poly-γ-glutamates during analysis.

In the current study, poly-γ-glutamate profile changes attributed to enzymatic hydrolysis were observed after inducing cell and tissue damage through crushing raw samples of broccoli and carrot while no changes were observed in tomato. In raw crushed broccoli, hydrolysis of hepta- and hexa-γ-glutamates resulted in increased concentrations of shorter poly-γ-glutamates (mainly tri-γ-glutamates) and monoglutamates. In this context, it could be suggested that broccoli GGH could exhibit endopeptidase activity in accordance with a previous report (*8*) on the activity of two *Arabidopsis thaliana* GGH isozymes. This, however, requires further investigation. In tomato, the occurrence of two catalytically active GGH isozymes (LeGGH1 and LeGGH2) and one catalytically inactive isozyme (LeGGH3) was recently reported (*42*). Moreover, the latter reported that LeGGH1 exhibited exclusive endopeptidase activity while LeGGH2 exhibited endo- and exopeptidase activity. The current observation of limited changes in poly-γ-glutamate profile of tomatoes (red-ripe) is in agreement with the previous report (*42*) that red-ripe tomatoes only exhibited 5% GGH activity compared to mature-green tomatoes. There is no literature data on the existence of GGH isozymes in broccoli and carrot. However, the observation of differences in poly-γ-glutamate hydrolysis at the natural pH of broccoli (around pH 6.5) in the current study and pH 4.3 in a previous study (*15*) points to the importance of pH in GGH activity, in analogy with previous observation for cabbage GGH (*41*). The latter reported that, at pH 8.0,the hydrolysis of pteroylpenta-γ-glutamic acid ($PteGlu_5$) by cabbage GGH mainly resulted in pteroyltri-γ-glutamic acid ($PteGlu_3$) while at pH 5.0, $PteGlu_5$ was hydrolyzed into pteroyldi-γ-glutamic acid ($PteGlu_2$) and pteroylglutamic acid (PteGlu).

The current results suggest that a diet composed of thermally processed broccoli (thermal treatment prior to tissue disruption), carrot and tomato would mainly provide folates as hepta-, hexa-, penta- and tri-γ-glutamates. Since hydrolysis to monoglutamates is essential for absorption at the intestinal mucosa, the activity of brush border GGH is crucial. However, the aforementioned enzyme has been found to be inhibited by certain components in foods, e.g., legumes, tomatoes and orange juice (*43*). Such inhibition would result in low folate bioavailability from plant-based thermally processed foods predominantly containing folate poly-γ-glutamates. To address this concern, it has been suggested that increasing the monoglutamate concentration in foods prior to ingestion could increase the bioavailability of plant-based food folates (*13*). In this study, crushing raw broccoli and carrot prior to blanching facilitated folate poly-γ-glutamate hydrolysis resulting in an increase of monoglutamate content (relative to blanched samples) from 10.0 ± 0.3 to 53.8 ± 8.1 (5-fold), 5.4 ± 0.3 to 11.5 ± 2.4 (2-fold) nmol/100 g FW respectively (**Figure 3B**,**D**). However, a significant amount (about 90%) of the folates in the three plant tissue remained as poly-γ-glutamates. Incubating raw crushed broccoli for 5−30 min at 25−55 °C did not result in a further increase in monoglutamate concentration. Besides crushing, other processing parameters have been reported to increase the monoglutamate content of broccoli and other plant-based foods during processing. It was previously observed that folate poly-γ-glutamate hydrolysis by broccoli GGH resulted in a higher accumulation of folate monoglutamates at pH 4.3 than at pH 6.5 (*15*). In another study (*14*), high pressure processing (e.g., 400 MPa, 35 °C) was reported to significantly increase the folate monoglutamate content of intact broccoli. Considering the observations of the current study and the available literature on GGH-catalyzed hydrolysis of folate poly-γ-glutamates in vegetables, the possibility of further increasing the monoglutamate content of vegetables by combining different processing parameters such as temperature (≤60 °C), pH and pressure should be further investigated. In the context of increasing the monoglutamate content of plant-based foods, genetic engineering recently resulted in a transgenic tomato plant that accumulated six times more folate monoglutamate compared to the control plants (*10*). If such transgenic plant based foods are found to be safe and acceptable to consumers, it could be possible to obtain high proportions of folate monoglutamates from a mixed diet consisting of fruits and vegetables that inherently contain high levels of folate monoglutamates (e.g., such genetically modified tomato) and those whose monoglutamate content could be increased during processing (e.g., broccoli). Literature data on folate bioavailability indicates that the bioavailability of natural food folates relative to that of folic acid ranges from 30 to 98% (*44*−*46*) and attributes low folate bioavailability to the instability of reduced folates and the existence of natural food folates as poly-γ-glutamates. However, most of this data compares different foods which could differ in many aspects that can influence folate bioavailability (e.g., presence of GGH inhibiting components, pH, proportion of poly-γ-glutamates, presence of antioxidants, matrix effects). Although a few studies (*47*, *48*) suggested that natural food folate bioavailability is not affected by the proportion of poly-γ-glutamates, more studies comparing the bioavailability of folates from the same food matrix but with different proportions of monoglutamates and poly-γ-glutamates are needed. In this respect, it is important that the folate analytical procedure used in such studies can distinguish between folates with different poly-γ-glutamate chain lengths.

Thermal Stability of Folates and Water-Soluble Antioxidants. Regarding folate stability, treatments of raw crushed broccoli at 25−55 °C (5−30 min) and 40 and 60 °C (30 min) decreased the folate poly-γ-glutamate concentration. However, despite the decrease in poly-γ-glutamate concentration during treatments at temperatures up to 60 °C, no significant increase in folate monoglutamates was observed, an indication of simultaneous folate poly-γ-glutamate hydrolysis and degradation. Since no leaching occurred, the folate losses observed were mainly attributed to oxidation. The high folate losses observed at 120 and 140 °C were attributed to acceleration of oxidation by the intense heating. Contrary to our observation of significant folate losses in crushed broccoli treated for 30 min at 40 and 60 °C, it was previously reported (*14*) that treating broccoli for a similar duration at 25 to 90 °C resulted in nonsignificant folate losses

probably because intact broccoli was used as opposed to crushed broccoli in the current study. The observation in this study of nonsignificant folate losses in crushed broccoli heated for 30 min at 100 °C was in agreement with a recent report that steaming broccoli (100 °C/5 min) resulted in no folate losses (*49*) and indicates that exclusion of leaching could result in better folate retention. Since folates are water-soluble, heating fruits and vegetables while in contact with water could increase the rate of loss for this vitamin. For example, boiling (10 min) and blanching (in boiling water, 4 min) of broccoli caused 56 and 70% folate losses respectively (*21*, *50*). In this context, it has been demonstrated that a significant proportion of folates lost during soaking, and boiling of legumes and vegetables could be traced in the water (*21*, *51*).

Besides oxidation, other degradative reactions such as glycation of folates could contribute to the folate losses in plant-based foods. Recently, glycation in the presence of excess concentrations of fructose and glucose together with a limited concentration of 5-CH_3-H_4PteGlu was shown to result in the formation of N(2α)-[1-(carboxyethyl)]-5-CH_3-H_4PteGlu (*52*). Since broccoli contains high concentrations of glucose and fructose (*53*), glycation of folates with these sugars cannot be ruled out and should be investigated.

In this study, the 12−30% decrease in vitamin C content observed during treatments of raw crushed broccoli at 25−55 °C (0−30 min) was attributed to enzymatic oxidation of L-AA to DHAA in accordance with a previous report (*29*) that raw crushed broccoli retained less vitamin C and contained higher amounts of DHAA than broccoli blanched prior to crushing. L-AA was not detected in raw broccoli (0 min) due to enzymatic oxidation of this compound to DHAA, since this sample was not subjected to thermal treatment to inactivate enzymes. Although DHAA is reported to possess some antioxidant capacity (*54*), its formation in vegetables could result in further antioxidant capacity loss since it is susceptible to hydrolytic cleavage to 2,3-diketogulonic acid. During 30 min treatments of raw crushed broccoli at 40−140 °C, TEAC values decreased at 40 and 60 °C whereas increased values were observed at 80−140 °C. The increase in antioxidant capacity of vegetables during processing at elevated temperatures is reported elsewhere and has been attributed to the formation of novel antioxidant compounds, e.g., Maillard reaction products and increased antioxidants extractability from the vegetable matrix (*31*,*55*). On the other hand, the decrease in antioxidant capacity at temperatures below 80 °C has previously been attributed to the action of oxidative enzymes such as ascorbic acid oxidase, polyphenol oxidase and peroxidase (*56*). Studies relating the stability of folates to the presence of antioxidants have mostly been performed in model systems (*24*−*26*). One study showed that folates in fruits and vegetables with high L-AA content exhibited higher thermal and pressure stability in comparison to those with lower L-AA concentration (*23*). The existence of a correlation between folate and vitamin C concentration during treatments at 25−55 °C (5−30 min) of raw crushed broccoli suggested that enzymatic oxidation of endogenous antioxidants could lower folate stability in raw crushed vegetables such as broccoli. This calls for further investigation into the role of oxidative enzymes in influencing the stability of water-soluble antioxidant and folates.

The results of this study provide additional insight into the influence of processing on stability and profile of folate poly-γ-glutamates, which could influence the bioavailability of folates from fruits and vegetables. The results of the current study indicate that although some fruit and vegetable processing treatments result in increased folate monoglutamate concentration, such treatments could also decrease the total folate and antioxidant capacity of fruits and vegetables. Therefore studies evaluating whether natural food folate monoglutamates are more bioavailable than poly-γ-glutamates are urgently needed in order to evaluate if the moderate folate losses occurring during treatments that increase the monoglutamate content are justifiable. In the context of understanding folate poly-γ-glutamate hydrolysis, the simultaneous occurrence of degradation and hydrolysis could make it impossible to quantify the changes in folate profile. In order to perform proper kinetic studies on folate poly-γ-glutamate hydrolysis in vegetable matrices such as broccoli, the use of purified endogenous GGH might be an interesting initial approach.

ABBREVIATIONS USED

GGH, γ-glutamyl hydrolase; TEAC, Trolox equivalent antioxidant capacity; PteGlu, pteroylglutamic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolate; 5-CHO-H_4PteGlu, 5-formyltetrahydrofolate; 10-CHO-PteGlu, 10-formyltetrahydrofolate; 5,10-CH_2-H_4-PteGlu, 5,10-methylenetetrahydrofolate; 5,10-CH^+-H4PteGlu, 5,10-methenyltetrahydrofolate; $H_4PteGlu_n$, tetrahydrofolate; L-AA, L-ascorbic acid; DHAA, dehydroascorbic acid; DM, dry matter; RP, reverse phase; FD, fluorescence detection; FBP-AC, folate binding proteins affinity chromatography; GAE, gallic acid equivalents; FW, fresh weight; RSD, relative standard deviation; MA, microbial assay; MS, mass spectrometry; FD, fluorescence detection; ECD, electrochemical detection.

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