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A liquid chromatography–tandem mass spectrometric method for quantitative determination of native 5-methyltetrahydrofolate and its polyglutamyl derivatives in raw vegetables

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ABSTRACT

Folate deficiency is a prevalent phenomenon worldwide especially in underprivileged countries. Polyglutamyl 5-methyltetrahydrofolate (5MTHF) species are the naturally occurring principle folate in store-bought vegetables. Here we report a simple and complete extraction method for the determination of native polyglutamyl 5-methyltetrahydrofolate in vegetables using high performance liquid chromatography with tandem mass spectrometric detection (HPLC–MS/MS). Coarsely chopped samples (18 different vegetables) were steamed to inactivate glutamylase enzymes and liberate folate from binding proteins and extracted in a reducing buffer with $^{13}C_5$ 5MTHF stable isotope added as internal standard. The polyglutamyl 5-methyltetrahydrofolate species were separated in 9 min on a C_{18} column using a reversed phase system. HPLC eluate was interfaced with a triple quadrupole mass spectrometer operated in electrospray positive mode. The respective pseudomolecular cation of each polyglutamyl 5-methyltetrahydrofolate species was selected for fragmentation to a common daughter ion for detection. We quantitated polyglutamyl 5-methyltetrahydrofolate in store-bought vegetables from families *Brassicaceae*, *Asteraceae* and *Amaranthaceae* (including mustard greens, romaine lettuce and Swiss chard) of which most have not been quantitated previously. Most vegetables from *Asteraceae* and those from *Amaranthaceae* contained similar amounts of monoglutamyl 5MTHF and polyglutamyl 5MTHF while *Brassicaceae* were dominated by polyglutamyls and endive species (*Asteraceae*) contained mainly monoglutamyl 5MTHF. The precision of the method for the various polyglutamyl 5-methyltetrahydrofolate forms was 1–9% RSD, recovery 84–91%, limit of detection 64–658 fmol and limit of quantitation 193–1994 fmol. Herein we describe a rapid, sensitive and selective HPLC–MS/MS technique to quantitate polyglutamyl 5-methyltetrahydrofolate species. This method may be suitable for analyzing the polyglutamyl 5-methyltetrahydrofolate profile of inherent folates in a wide range of leafy green vegetables.

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1. Introduction

Folate belongs to the water-soluble B group vitamins. Anemia and neural tube defects in infants caused by folate deficiency are prevalent phenomena worldwide, especially in developing countries. Even in developed countries, there is rising concern about folic acid fortification for its possible antagonism against anticancer drugs, masking of B_{12} deficiency [1] and promoting existing tumors [2]. Thus, European governments recommend intake of native folate supplements, folate-fortified foods or foods naturally high in folate [1].

Cellular folate species are differentiated by the reduction state of the pteridine ring, one-carbon substitution at the N5 and/or N10 positions (formyl, methyl, methlene, and methenyl), and the length of the γ-glutamyls [3]. Using HPLC or HPLC/MS methods, it was found that the 5MTHF species occurring as polyglutamyl derivatives were the major folate form in store-bought vegetables and account for 50–90% of total folate [4–7].

5MTHF and its polyglutamate derivatives consist of three moieties: pteridine, *p*-aminobenzoate and a glutamyl chain (Fig. 1). In fact, the pentaglutamyl form of tetrahydrofolate is the central folate acceptor molecule in the one-carbon cycle in plants and humans [8,9]. Previous studies have shown the polyglutamyl chain of folate affects its biological activity both as a cofactor and the efficiency with which it is transported [10,11]. Polyglutamyl folates bind to specific protein with higher affinity than do corresponding monoglutamate forms which can protect folate from oxidation [12,13].

Several studies have also investigated the effect of polyglutamyl chain length on folate bioavailability in humans. Clinical studies have found conflicting results where monoglutamyl folate has

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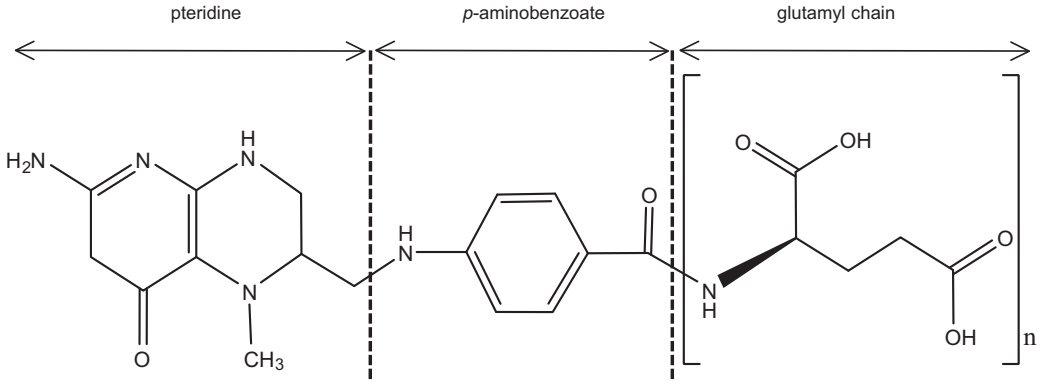

Fig. 1. Chemical structure of polyglutamyl 5-methyltetrahydrofolate.

greater bioavailability than polyglutamyl folate [14] or that there was no difference [15,16]. From Konings et al. [16] study on the absorption of folate, the food matrix, folate entrapment and the presence of γ-glutamyl hydrolase (GGH) inhibitors appeared to be more determinant than polyglutamylation. Unfortunately the exact quantity of each polyglutamate is often unknown and questions of bioavailability and bioactivity spur scientists to accurately characterize folates as they occur in food.

Quantitative determination of inherent folate polyglutamyl forms involves many challenges for detection. HPLC coupled with ultraviolet absorption, fluorescence detection [17] or electrochemical detection [18] offer the possibility for profiling inherent polyglutamyl folates, but these methods are limited by sensitivity or lack of selectivity and require complicated cleanup procedures as well as expensive affinity columns for purification and concentration. There is a need for a method providing rapid, simple, sensitive, and accurate quantitation of all the polyglutamyl 5-methyltetrahydrofolate species in vegetables. The application of high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) and the use of a stable isotope as internal standard (IS) to the determination of polyglutamyl 5MTHF species is a logical solution. This approach offers both high sensitivity and selectivity for the unambiguous identification and quantification of trace-level analyte in complex samples in a short time.

Another significant challenge for quantitating intact polyglutamyl folates in vegetables is preventing inadvertent hydrolysis of polyglutamyl folates during sample preparation. GGH is a ubiquitous enzyme in plants that can hydrolyze polyglutamyl into monoglutamyl or short-chain glutamyl forms during extraction [19]. Samples are usually homogenized at room temperature or in liquid nitrogen followed by boiling extraction [17,18,20]. However, cell disruption establishes contact between polyglutamyl folates and GGH [20] and the delay before the sample reaches 100 °C may allow for partial conversion by GGH. Indeed, previous researchers have reported different polyglutamyl folate profiles in the same vegetables possibly as an artifact of sample handling and workup [9,17,21–23].

Here we report a protocol involving rapid steam-inactivation of GGH followed by a high efficiency single step extraction without enzymatic digestion. Samples are immediately analyzed by HPLC–MS/MS to quantify 5MTHF species including their polyglutamyl forms using a stable isotope IS. Quantitative polyglutamyl 5-methyltetrahydrofolate profiles were determined for 18 vegetables from *Brassicaceae*, *Asteraceae* and *Amaranthaceae* of which most have not been analyzed previously and are considered good to excellent sources of dietary folate according to the USDA database [24]. To the best of our knowledge, the HPLC–MS/MS method reported herein is unique in its application to monitor modifications in vegetable polyglutamyl 5-methyltetrahydrofolate.

2. Materials and methods

2.1. Chemicals and reagents

All folate standards employed in this study are listed in Table 1. 5MTHF [$^{13}C_5$] Glu·Ca was used as an IS for both recovery and response of 5MTHF-Glu_{1-8} based on similar chemical characteristics. LC/MS-grade water and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA), formic acid (99%, purity) (Acros, Morris Plains, NJ, USA); glacial acetic acid (Acros, Morris Plains, NJ, USA); ammonium acetate from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA); ascorbic acid (99%, crystalline) from Sigma (St. Louis, MO,

Table 1
Folate standards used in this study.

	Standard	Abbreviated name	Formula
1‡	(6R,S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt	5MTHFGlu·Na_2	$C_{20}H_{25}N_7O_6·Na_2$
1*	(6S)-5-methyl-5,6,7,8-tetrahydrofolate-[$^{13}C_5$] Glu, calcium salt	5MTHF [$^{13}C_5$] Glu·Ca	$C_{15}{}^{13}C_5H_{25}N_7O_6·Ca$
2	(6R,S)-5-methyl-5,6,7,8-tetrahydropteroyldi-γ-L-glutamate	5MTHF-Glu_2	$C_{25}H_{32}N_8O_9$
3	(6R,S)-5-methyl-5,6,7,8-tetrahydropteroyltri-γ-L-glutamate	5MTHF-Glu_3	$C_{30}H_{39}N_9O_{12}$
4	(6R,S)-5-methyl-5, 6,7,8-tetrahydropteroyltetra-γ-L-glutamate	5MTHF-Glu_4	$C_{35}H_{46}N_{10}O_{15}$
5	(6R,S)-5-methyl-5,6,7,8-tetrahydropteroylpenta-γ-L-glutamate	5MTHF-Glu_5	$C_{40}H_{53}N_{11}O_{18}$
6‡	(6S)-5-formyltetrahydrofolate, sodium salt	5CHOTHF·Na_2	$C_{20}H_{23}N_7O_7·Na_2$
7‡	(6R)-10-formyltetrahydrofolate, sodium salt	10CHOTHF·Na_2	$C_{20}H_{23}N_7O_7·Na_2$
8‡	(6R)-5,10-methenyltetrahydrofolate, –Cl × HCl	5,10-CH^+THF·Cl × HCl	$C_{20}H_{22}N_7O_6·Cl \times HCl$
9‡	(6R)-5,10-methylenetetrahydrofolate, sodium salt	5,10-CH_2THF·Na_2	$C_{20}H_{23}N_7O_6·Na_2$
10‡	(6S)-tetrahydrofolate, sodium salt	THF·Na_2	$C_{19}H_{23}N_7O_6·Na_2$
11‡	Pteroylglutamic acid, sodium salt	FA	$C_{19}H_{19}N_7O_6·Na_2$
12	Pteroylhexa-γ-L-glutamic acid	FA-Glu_6	$C_{44}H_{54}N_{12}O_{21}$
13	Pteroylhepta-γ-L-glutamic acid, ammonium salt	FA-$Glu_7·NH_4$	$C_{49}H_{61}N_{13}O_{24}·NH_4$

All were purchased from Schircks Laboratories (Jona, Switzerland) except ‡ were gifts from Merck Eprova AG (Schaffhausen, Switzerland), and * was a gift from Abbott Nutrition (Columbus, OH).

Table 2
Precision and accuracy data for polyglutamyl 5MTHF.

Compound	Nominal (pmol)	Intra-day (n = 4)			Inter-day (n = 12)		
		Mean (pmol)	Precision %	Accuracy %	Mean (pmol)	Precision %	Accuracy %
5MTHF	Low (0.39)	0.37	0.3	95	0.36	2.5	92
	Median (1.56)	1.54	0.5	99	1.50	0.1	96
	High (7.80)	7.80	0.1	100	7.10	0.7	91
5MTHF-Glu_2	Low (1.19)	1.14	4.2	96	1.11	4.3	93
	Median (4.76)	4.57	4.1	96	4.57	7.1	96
	High (23.8)	23.8	5.2	100	23.3	6.5	98
5MTHF-Glu_3	Low (1.23)	1.23	5.2	100	1.17	9.7	95
	Median (4.92)	4.48	8.5	91	4.53	3.5	92
	High (24.6)	22.6	1.4	92	24.1	3.3	98
5MTHF-Glu_4	Low (2.78)	2.75	7.5	99	2.67	10.6	96
	Median (11.1)	10.4	7.9	94	10.3	5.5	93
	High (55.6)	52.8	5.7	95	50.0	7.0	90
5MTHF-Glu_5	Low (1.58)	1.53	7.9	97	1.37	6.5	87
	Median (6.32)	5.81	5.2	92	5.75	7.3	91
	High (31.6)	30.3	9.0	96	29.1	6.5	92
5MTHF-Glu_6	Low (3.53)	3.35	7.9	95	3.35	6.7	95
	Median (14.1)	13.8	3.5	98	13.8	5.9	98
	High (70.6)	69.9	2.3	99	64.2	3.0	91
5MTHF-Glu_7	Low (3.39)	3.25	5.2	96	3.19	6.5	94
	Median (13.6)	13.1	5.9	96	12.9	9.2	95
	High (67.8)	63.7	3.3	94	63.7	6.9	94

USA); 2-mercaptoethanol was obtained from Bio-Rad (Hercules, CA, USA); amylase (Fluka, #1065, St. Louis, MO, USA); pronase (Calbiochem, #53702, San Diego, CA); rat serum (Sigma, #S9759, St. Louis, MO, USA); sodium borohydride (Sigma, #S9125, St. Louis, MO, USA).

2.2. Preparation of standard and quality control (QC) solutions

Since 5MTHF-Glu_{6-7} are not commercially available, 5MTHF-Glu_6 and 5MTHF-Glu_7 stock solutions were prepared following Ndaw et al. [25] with minor modifications. Briefly, 2 mg FA-Glu_6 and FA-Glu_7 was sonicated in 1 mL reducing buffer (0.1 M ammonium acetate, 1% ascorbic acid, pH = 7.9) and 4 mL 3.2 M sodium borohydride ($NaBH_4$) was added. After shaking, the solution was left to stand for 10 min and adjusted to pH 7.4 with 5 M acetic acid. A 30 μL volume of 37% formaldehyde was added and after shaking for 30 s, 4 mL $NaBH_4$ was added and the solution was left to stand for 20 min at room temperature. The solution made to 25 mL with reducing buffer and filtered through a 0.22 μm nylon filter. The concentrations of 5MTHF-Glu_{6-7} were determined by HPLC-PDA from the standard curve of 5MTHF (mol/L versus HPLC peak area) since 5MTHF shares the same extinction coefficient.

All folate stock solutions (except 5MTHF-Glu_{6-7}): approximately 50 μg of each unlabeled folate standard and 90 μg labeled standard were separately transferred into 10 mL vials. Each standard was dissolved in 3 mL HPLC grade water and sonicated for 2 min. Concentrations were determined by UV–vis spectrometry using the molar extinction coefficient for 5MTHF at pH 7 reported by Blakley [26]. The molar extinction coefficients of 5MTHF-Glu_{2-7} are based on 5MTHF since they share the same chromophore.

Working solutions: appropriate aliquots of each unlabeled standard solution were mixed for a combined working solution of 5MTHF (1 μM), 5MTHF-Glu_2 (3 μM), 5MTHF-Glu_3 (3 μM), 5MTHF-Glu_4 (7 μM), 5MTHF-Glu_5 (4 μM), 5MTHF-Glu_6 (9 μM) and 5MTHF-Glu_7 (9 μM). The stock solution of IS was added to a final concentration of approximately 50 nM in each dilution of mixed standard and in samples.

QC solutions: prepared by mixing working solutions to low, medium and high concentrations. These solutions were used to confirm stabilities of the LC–MS/MS instruments and demonstrate the intra- and inter-day precision and accuracy of the method. Table 2 shows the concentration of each compound used. All standard solutions were prepared fresh daily.

2.3. Calibration and quantitation

For each folate, solutions of unlabeled and labeled compound were mixed in seven molar ratios where labeled IS was held constant. The concentration of each compound was chosen to ensure a linear dynamic range. Calibration curves revealed a linear response of the peak area ratios of unlabeled to labeled compound versus the corresponding moles injected for each unlabeled folate. The 5MTHF-Glu_8 species for which authentic standard was unavailable was determined by extrapolation of the best fit line ($y = -26.5x + 241$, $R^2 = 0.9989$) for response of Glu_5, Glu_6 and Glu_7 where y represents the MS/MS response of 5MTHF-Glu_n, and x represents the glutamyl length. The identity of 5MTHF-Glu_8 was based on appropriate parent > daughter MS/MS transitions and elution order. The calibration curve data for each folate is presented in Table 3.

Table 3
Calibration and sensitivity data for 5MTHF and its polyglutamyl derivatives.

Compounds	LOD (fmol)	LOQ (fmol)	Slope (peak area/pmol injected) mean ± SD (n = 4)	R^2	Linear range (pmol injected)
5MTHF	156	472	4333 ± 225	0.999	0.3–11
5MTHF-Glu_2	64	193	2886 ± 187	0.999	1–60
5MTHF-Glu_3	301	911	943 ± 51	0.999	1–63
5MTHF-Glu_4	261	792	286 ± 9	0.999	1–48
5MTHF-Glu_5	425	1290	115 ± 7	0.999	1–117
5MTHF-Glu_6	584	1770	81 ± 3	0.999	2–187
5MTHF-Glu_7	658	1994	56 ± 2	0.999	2–165

2.4. Vegetables

The vegetables investigated were spinach, Swiss chard, collard greens, kale, romaine lettuce, bok choy, turnip greens, Brussels sprouts, mustard greens, dandelion greens, broccoli rabe (also known as broccoli raab), broccoli and cauliflower. All vegetables were purchased from Whole Foods, a local supermarket in Columbus, OH. Escarole, frisée, curly endive, Belgian endive and radicchio were purchased from Meijer supermarket (Columbus, OH) and kept refrigerated until same-day analysis.

2.5. Steaming pre-treatment and extraction

The main challenge of preserving 5MTHF-Glu$_n$ speciation during extraction is inactivating GGH. Steaming prior to boiling to facilitate extraction was investigated as a means to this end due to the large latent heat of fusion for steam. This allows for more rapid heating which might inactivate GGH before significant conversion can take place. Steaming was applied by suspending 5 g fresh weight chopped vegetables as a single layer in a stainless steel wire mesh sieve over 1 L boiling water (2 L vessel) with a lid for 10 min. The pre-steaming period (0, 2, 5, 10 and 20 min) was optimized for cauliflower, spinach and curly endive (representative of each plant family) before extraction was applied for all vegetables. Satisfactory inactivation was recognized where extended steaming times had no effect on the 5MTHF-Glu$_n$ profile and total 5MTHF content.

Once steamed, the samples were flash-frozen and ground to a fine powder with ~250 mL liquid nitrogen in a 500 mL mortar and pestle for 2–3 min. The ground material was transferred to a 50 mL glass vial and 20 mL boiling extraction buffer added. Extraction buffer consisted of 0.1 M ammonium acetate, pH 7.9, 1% ascorbic acid (w/v), 0.2% (v/v) 2-mercaptoethanol and was prepared fresh each day. Subsequently, 20 μL IS (5MTHF [$^{13}C_5$]Glu) was added. Immediately after mixing, the vial (loosely capped) was placed in a boiling water bath for 10 min then cooled on ice. Suspension filtered using Whatman quantitative filter paper #1 and the filtrate transferred into a 25 mL volumetric flask. The filtrate was reconstituted to 25 mL with extraction buffer and the supernatant filtered through a 0.22 μm nylon syringe filter for HPLC–MS/MS analysis.

There was a concern that pre-steaming might degrade folate due to extra heat exposure. Thus, total folate was compared where steaming was applied prior to tri-enzyme versus tri-enzyme treatment alone (see Section 2.7). This method was applied for one vegetable (cauliflower) since the test is essentially for heat stability of folate. IS (20 μL of 62.5 mM 5MTHF [$^{13}C_5$]Glu) and 20 mL reducing buffer were added into fresh vegetables (5 g) then homogenized at room temperature and 10,000 rpm for 10 min (Polytron, PT 3100, Kinematica, Switzerland) until finely suspended particles remained. Samples were boiled immediately for 10 min to extract folates, cooled and reconstituted to 25 mL without filtering before tri-enzyme treatment was applied.

2.6. Di-enzyme treatment and polyglutamyl 5MTHF species determination

Di-enzyme treatment normally allows for a more complete liberation of folates from starchy and protein-rich matrices. In order to test whether di-enzyme treatment improved recovery beyond steaming alone, a di-enzyme treatment was applied after steaming. Vegetables were first steamed for 10 min as described above, boil-extracted for 10 min, and then treated with the first two enzyme treatments described by Chen and Eitenmiller [27] in their tri-enzyme method, i.e. omitting conjugase. This method was applied for representative vegetables from each plant family: cauliflower, spinach and curly endive. Adult carrot was used as a positive control for di-enzyme treatment since it has high starch content (1.43 g/100 g F.W. [24]).

2.7. Folate species determination

Although the folate speciation of some of the vegetables in this investigation has been described, we determined those and in addition the folate species for vegetables that had not been characterized. A tri-enzyme (pronase, amylase and conjugase) treatment [27] was carried out for all vegetables analyzed here with one modification—rat serum replaced chicken pancreas as conjugase. The completeness of 5MTHF species deconjugation was confirmed by absence of polyglutamyls in the LC/MS/MS-5MTHF-Glu$_n$ method. Background folate in the conjugase preparation was subtracted. During sample preparation, all manipulations were carried out under subdued light as folates are photosensitive.

2.8. HPLC–MS/MS

Chromatographic separation of 5MTHF and 5MTHF polyglutamates was achieved by reversed phase on a Waters Acquity UPLC, equipped with a binary pump, autosampler, column oven and degasser (Acquity, Waters Corp., MA, USA). A Sunfire C_{18} column (4.6 mm × 150 mm, 5 μm, Waters Corp., Milford, MA, USA) was held at 40 °C and a solvent system of aqueous formic acid 0.1% (v/v) (A) and acetonitrile (B) delivered at a flow rate of 1.8 mL/min. The gradient elution was applied as follows: 0–4 min, 0–20% B; 4–5 min, 20–95% B; 5–6.5 min, 95% B; 6.5–9 min, re-equilibration to initial conditions. The injection volume was 50 μL and the autosampler kept at 25 °C.

HPLC eluate was split approximately 1:10 and interfaced with a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) operated in positive ion electrospray mode. Acquisitions were performed by selected reaction monitoring (SRM) wherein the respective pseudomolecular cation of each 5MTHF species was fragmented by collisionally induced dissociation to a (m/z 313.1$^+$) fragment for detection. This fragment was the optimal daughter ion common to all 5MTHF compounds studied. Since the IS was labeled on the glutamyl moiety and the fragment was the pteridine portion, it also shared the unlabeled m/z 313.1$^+$ daughter ion. Source conditions were as follows: capillary voltage, 3.2 kV; source temperature, 110 °C; desolvation temperature, 400 °C; cone gas flow rate, 110 L/h; desolvation gas flow rate 800 L/h and cone voltage, 35 V. High purity nitrogen was used as desolvation and nebulizing gas and high purity argon was used as collision gas (3×10^{-3} mbar). Radio frequency 1 (RF1), SRM transitions, and collision energy voltages were optimized for each analyte, and the values are displayed in Table 4. Two HPLC–MS/MS runs were employed, one for 5MTHF and 5MTHF-Glu$_2$ and a second run for 5MTHF-Glu$_3$ through 5MTHF-Glu$_8$. This was necessary as the RF 1 optimum could not be specified for each SRM within a run yet was vastly different for the compounds 10 V versus 40 V. Despite the analysis time penalty, the added sensitivity allowed us to avoid a concentration step. Stable isotope 5MTHF was used as IS for both runs. Data acquisition was carried out using SRM windows (Table 4) with dwell times of 80 ms, an interscan delay time of 20 ms and inter-channel delay of 20 ms. All data were acquired and peak areas integrated using Masslynx 4.1 software (Waters Corp., Beverly, MA).

2.9. Method validation

The following criteria were used to evaluate the method: sensitivity, linearity (R^2), intra- and inter-day precision, accuracy,

Table 4
MS/MS parameters used during HPLC–MS/MS quantitation.

Elution time (min)	Compound	Precursor ion $[M+H]^+$ (m/z)	Product ion $[M+H]^+$ (m/z)	CE[a] (V)	RF1[b] (V)
2.80	5MTHFGlu	460.2	313.1	20	10
2.80	5MTHF [$^{13}C_5$] Glu	465.2	313.1	20	10
2.87	5MTHF-Glu_2	589.2	313.1	20	10
3.02	5MTHF-Glu_3	718.2	313.1	20	40
3.20	5MTHF-Glu_4	847.3	313.1	25	40
3.35	5MTHF-Glu_5	976.4	313.1	28	40
3.48	5MTHF-Glu_6	1105.4	313.1	28	40
3.63	5MTHF-Glu_7	1234.4	313.1	28	40
3.78	5MTHF-Glu_8	1363.4	313.1	28	40

[a] Collision energy (CE).
[b] Radio frequency via voltage on ion tunnel (RF1).

extraction efficiency and matrix effect. Sensitivity was assessed by evaluating the limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ for 5MTHF, 5MTHF-Glu_2, 5MTHF-Glu_3, 5MTHF-Glu_4, 5MTHF-Glu_5, 5MTHF-Glu_6 and 5MTHF-Glu_7 are based on definitions in FDA Guidance, 1999 [28]. The LOD is defined as $LOD = 3.3\sigma/S$ (where σ is the standard deviation of the blank response and S is the slope of the calibration curve). The LOQ is defined here as $LOQ = 10\sigma/S$. Intra- and inter-day precision and accuracy of the method was determined at three concentrations of QC (low, median and high concentration) run intermittently over intra-day and inter-day of analyses. The recovery experiment was carried out by repeat extraction of samples until no further analyte was detected. The percent recovery at each step was then calculated.

Evaluating matrix effects is essential when developing an HPLC–MS/MS method as ion suppression or enhancement can lead to erroneous results [29]. The matrix effect was calculated as $B/A \times 100$ where A is peak area of the stable isotope IS in extraction buffer and B is the peak area for IS in vegetable extract spiked with the same amount of IS. The matrix effect for 5MTHF Glu_2–Glu_7 was evaluated as $(B - C)/A \times 100$ where A is peak area of the 5MTHF Glu_2–Glu_7 standards in extraction buffer, B is the peak area for 5MTHF Glu_2–Glu_7 in vegetable extract spiked with the same amount of standard and C is the peak area of inherent 5MTHF Glu_2–Glu_7 in vegetable extract.

3. Results and discussion

3.1. HPLC–MS/MS optimization

Evaluation criteria for chromatographic performance included the requirement to retain polar folate compounds and accommodate large injection volumes to detect compounds present in trace amounts. A UPLC C_{18} column, UPLC hybrid C_{18} column and conventional C_{18} column were tested. The three columns gave similar full width at half maximum (2 s) and resolution. However, equilibration time was found to be much longer for the UPLC columns than for the conventional C_{18} column. This seemed to be related to the lower loading capacity of the narrower UPLC columns with a crude extract such as we had prepared. The conventional C_{18} column could also easily accommodate 50 μL injections and was selected for future experiments.

All folates and stable isotope IS showed better sensitivity in positive ion than in negative ion mode in our system. A representative HPLC–MS/MS chromatogram of broccoli rabe extract is shown in Fig. 2 showing the orderly elution of 5MTHF-Glu_n with increasing glutamate chain length. Commonly used buffer additives that could influence MS sensitivity were evaluated, including ammonium acetate and formic acid. Results showed that 0.1% (v/v) formic acid in the eluent yielded the best chromatographic resolution and ionization of folates.

3.2. Sample preparation

3.2.1. Pre-steaming time optimization

As indicated above, the main challenge of profiling intact polyglutamyl folates in vegetables is to completely disable GGH during extraction. Generally, boiling can quickly inactivate enzymes only if samples reach 100 °C soon after homogenization. During the delay before the sample reaches boiling, there is still the possibility for enzyme action. We initially tried boiling frozen ground tissue for extraction but experienced problems with repeatability of the polyglutamate profiles. Furthermore, the profiles suggested shorter chain 5MTHF species predominated although we expected penta- and hexa-glutamyl species to be major. Steaming was attempted as heat transfer rates are much higher and might more quickly inactivate endogenous enzymes. Indeed we found much more repeatable results and longer average chain lengths of polyglutamyl derivatives with this approach.

Steaming time was optimized according to inhibition of GGH allowing for accurate profiling of polyglutamates. Steaming times of 0, 2, 5, 10, 20 min were compared for the polyglutamates in cauliflower, spinach and curly endive which represent the three different families under study: *Brassicaceae*, *Amaranthaceae* and *Asteraceae*, respectively. Success was indicated as a plateau of maximum polyglutamates where the polyglutamyl profiles remained the same. Fig. 3A shows that 5MTHF-Glu_6 and 5MTHF-Glu_7 are enhanced with increasing steaming time in cauliflower. Comparing no steaming (Fig. 3B) to a 10 min steaming (Fig. 3C), the relative contribution of 5MTHF through 5MTHF-Glu_4 decreased by about 44%, while the percentage of 5MTHF-Glu_6 increased by 25%, 5MTHF-Glu_7 17% and 5MTHF-Glu_5 by 1%. From Fig. 3A, when 5MTHF through 5MTHF-Glu_8 species in cauliflower were summed, the folate recovery increased approximately 63% after pre-steaming for 10 or 20 min compared to no steaming.

No significant change of polyglutamyl folate profiles and total folate in spinach and curly endive was observed with steaming. This may reflect more rapid heat penetration for leafy vegetables with high surface area compared to cauliflower. Interestingly, when steaming was not applied, 5MTHF-Glu_6 and 5MTHF-Glu_7 in *Brassicaceae* vegetables appeared to hydrolyze to 5MTHF-Glu_3 then to 5MTHF with no accumulation of 5MTHF-Glu_4 and 5MTHF-Glu_2. In contrast, homogenates of several *Amaranthaceae* and *Asteraceae* species showed polyglutamates decreased as monoglutamate directly increased with no accumulation of 5MTHFGlu_{2-4} forms. Vegetables from the same family may share similar glutamylase isoforms. Orsomando et al. [21] has described multiple GGH isoforms in plants: AtGGH1 and AtGGH2. AtGGH1 cleaves polyglutamates, mainly to di- and tri-glutamates, whereas AtGGH2 yielded mainly monoglutamate.

Folates are labile compounds and one might suspect additional heat from steaming causes degradation. From our analysis, there was no apparent difference ($\alpha = 0.05$, df = 4, two samples pooled

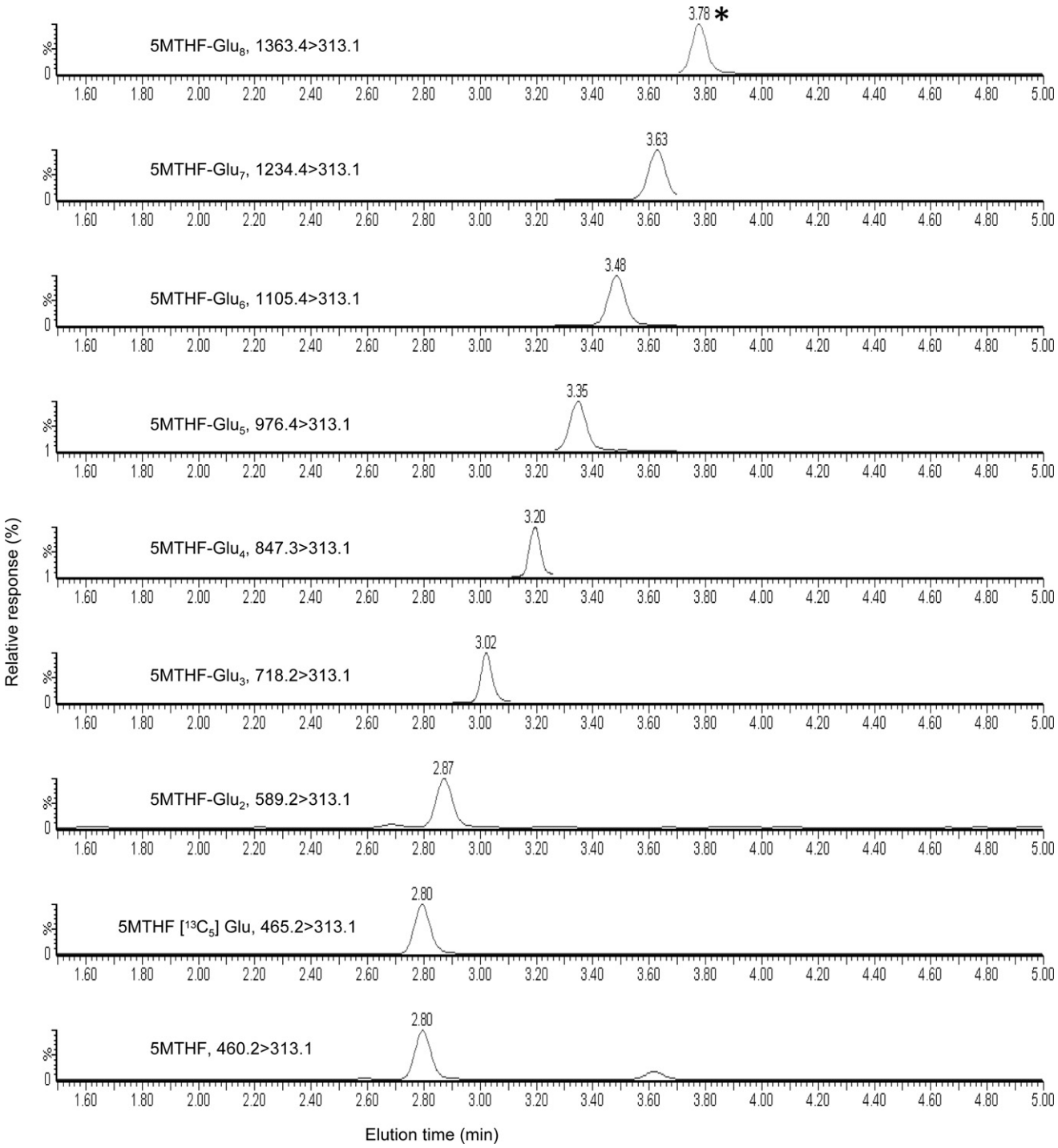

Fig. 2. HPLC–MS/MS chromatogram of polyglutamyl 5-methyltetrahydrofolate (5MTHF-Glu$_n$) in broccoli rabe extract. Steamed broccoli rabe (*Brassica rapa*) was freeze-ground in liquid nitrogen and extracted in boiling reducing buffer for 10 min. *5MTHF-Glu$_8$ for which authentic standard was unavailable was quantified by parent > daughter MS/MS transition and elution order consistent with species.

t-test, Minitab 15, Minitab Inc., State College, PA, USA) between triplicate steamed/boiled/tri-enzyme sample (77 μg/100 g F.W.±6 (SD)) and boiled/tri-enzyme samples (81 μg/100 g F.W. ±3 (SD)). Our finding is consistent with McKillop et al. who found no significant loss of folate after steaming spinach and broccoli for 4.5 and 15.0 min, respectively [30].

3.2.2. Steaming versus steaming plus di-enzyme treatment

Di-enzyme (pronase and amylase) treatment was investigated for possible improvement of recovery of polyglutamyl 5MTHF after steaming. Adult carrot was used as a positive control for di-enzyme treatment since it has high starch content (1.43 g/100 g F.W. [24]). We found a 25% increase for total 5MTHF although the polyglutamyl profile was unaltered. However, we found no improvement of di-enzyme treatment after steaming compared to steaming alone (three representative vegetables). All of the vegetables in our list have no starch according to the USDA database [24]. Although the vegetables in our list have protein, we did not observe any improvement with di-enzyme treatment after steaming versus steaming alone. When di-enzyme treatment was applied without

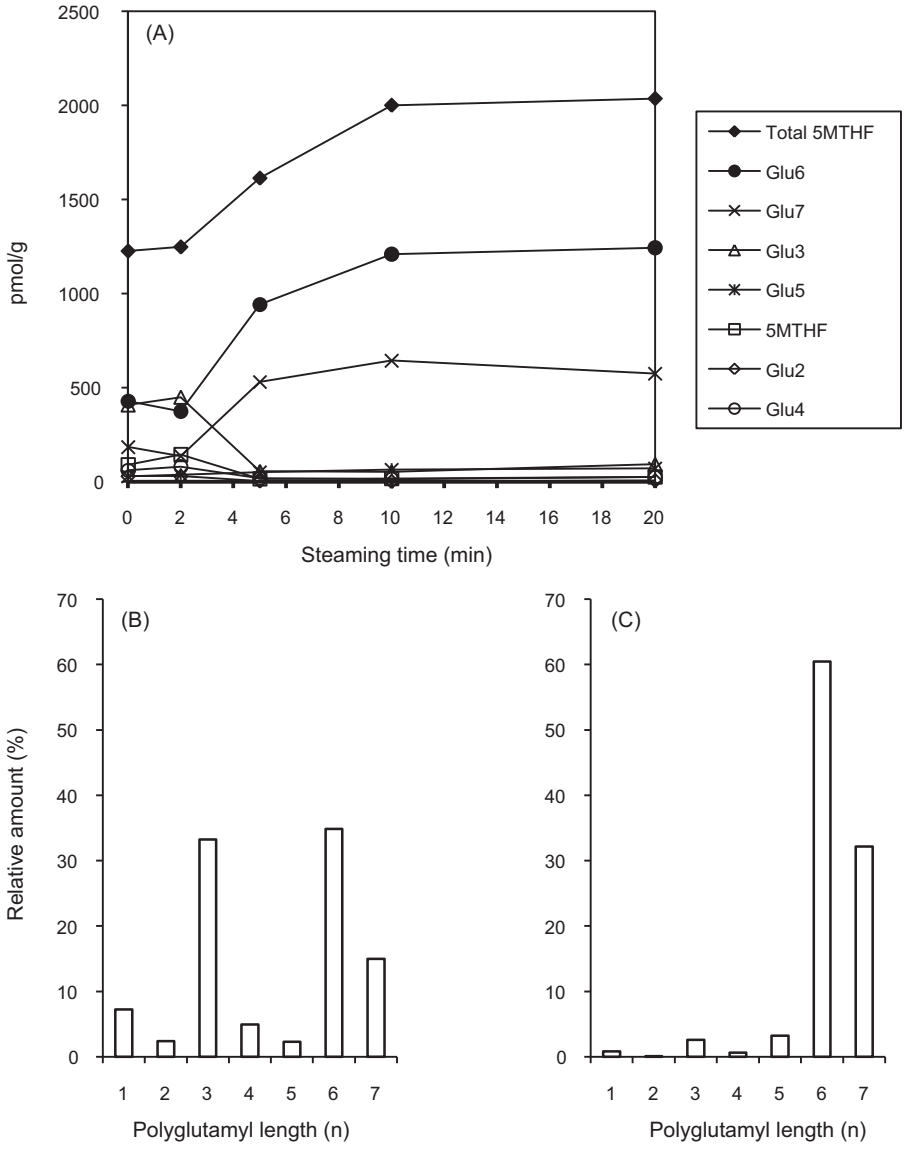

Fig. 3. Effect of steaming time on the polyglutamyl 5-methyltetrahydrofolate (5MTHF-Glu$_n$) species of cauliflower (*Brassica oleracea*). Cauliflower was steamed prior to grinding in liquid nitrogen and then extracted in boiling reducing buffer for 10 min. The values were determined according to individual calibration curves for 5MTHF-$(Glu)_{1-7}$ while the MS/MS response of 5MTHF-$(Glu)_8$ was estimated by extrapolation of the trend in response for 5MTHF-$(Glu)_{5-7}$. Data represented the mean of two replicates. (A) Absolute amount of polyglutamyl 5MTHF species and total 5MTHF as a function of steaming time. (B) Relative profile (% relative to total) of polyglutamyl 5MTHF species after boiling (no steaming). (C) Profile of polyglutamyl 5MTHF species after steaming for 10 min followed by boiling extraction.

pre-steaming, the same folate recovery was achieved as with steaming. The pre-steaming may have already liberated folates by denaturing the folate-binding protein. However, compared to the steaming result, di-enzyme alone gave a polyglutamyl profile skewed towards shorter chains (Fig. 3B). In addition, when 5MTHF polyglutamyl species were deconjugated (tri-enzyme treatment), 5MTHF matched the sum of the polyglutamyl species ($\alpha = 0.05$, df = 4, two samples pooled *t*-test). From our findings, pre-steaming may replace di-enzyme treatment for polyglutamyl 5MTHF determination in leafy green vegetables.

3.3. 5MTHF polyglutamyl species method validation

The extraction and HPLC–MS/MS methods were validated according to recovery, LOD and LOQ, precision, accuracy and performance of the IS for recovery and matrix effects. Recovery ranged from 84% to 91% for the various 5MTHF species with the first extraction and thus, one extraction was used throughout. The LOD we observe is similar to Lu et al. [38] and the LOQ of polyglutamyl 5MTHF species is also listed in Table 3. The intra-assay precision for the various polyglutamyl folates ranged from 1% to 9% RSD and inter-day assay precision from 1% to 11% RSD. Accuracy as indicated by intermittent quantitation of QC samples varied from 87% to 100% over intra- and inter-day analysis. Accuracy and precision data are shown in Table 2. Small losses observed for triplicate analyses were caused by degradation during heating according to spike recovery experiments and use of an IS accounted for this loss. No significant MS matrix effect was observed for 5MTHF-Glu_{1-7} ($0.088 < p < 0.611$, df = 4, $\alpha = 0.05$, two samples pooled *t*-test). The high extraction efficiency allowed for nearly complete recovery in a single step and HPLC–MS/MS detection provided sufficient sensitivities obviating the need for a concentration step.

3.4. Intact 5MTHF polyglutamate profiles of analyzed vegetables

Polyglutamyl profiles of the eighteen vegetables are presented in Table 5. It is clear that vegetables from the same family display similar polyglutamate profiles. In spinach and Swiss chard

Table 5
Intact 5MTHF monoglutamate and polyglutamate quantitative profile on a wet weight basis (pmol/g).

Vegetable common names	Glu_1	Glu_2	Glu_3	Glu_4	Glu_5	Glu_6	Glu_7	‡Glu_8	Sum (Glu-Glu_8)	Family	Species
Mustard greens	175	2	33	33	348	1443	1499	45	3577	*Brassicaceae*	*Brassica juncea*
Collard greens	110	2	13	16	193	669	459	132	1594	*Brassicaceae*	*Brassica oleracea*
Brussels sprouts	178	0	96	81	568	1291	496	71	2781	*Brassicaceae*	*Brassica oleracea*
Cauliflower	17	2	52	13	64	1208	644	0	2001	*Brassicaceae*	*Brassica oleracea*
Kale	31	0	19	9	48	423	428	226	1183	*Brassicaceae*	*Brassica oleracea*
Broccoli	72	0	72	36	54	388	360	1218	2199	*Brassicaceae*	*Brassica oleracea*
Broccoli rabe	474	6	47	23	223	937	856	233	2799	*Brassicaceae*	*Brassica rapa*
Turnip greens	225	15	60	15	165	565	818	677	2539	*Brassicaceae*	*Brassica rapa*
Bok choy	110	0	40	15	70	248	380	331	1193	*Brassicaceae*	*Brassica rapa*
Spinach	925	6	12	52	4901	330	18	34	6277	*Amaranthaceae*	*Spinacia oleracea*
Swiss chard	2191	11	23	114	2736	151	18	15	5258	*Amaranthaceae*	*Beta vulgaris*
Dandelion greens	1213	25	30	56	786	828	31	53	3021	*Asteraceae*	*Taraxacum officinale Wigg*
Romaine lettuce	853	13	7	12	881	343	21	0	2131	*Asteraceae*	*Lactuca sativa L.*
Escarole	2168	2	0	2	99	266	0	0	2537	*Asteraceae*	*Cichorium endivia*
Frisée	1676	0	0	0	36	147	0	0	1859	*Asteraceae*	*Cichorium endivia*
Belgian endive	2001	25	0	51	177	340	49	0	2643	*Asteraceae*	*Cichorium intybus*
Radicchio	1799	0	0	22	110	354	0	0	2285	*Asteraceae*	*Cichorium intybus*
Curly endive	2129	21	18	0	133	375	543	0	3219	*Asteraceae*	*Cichorium endivia*

The data was acquired according to individual calibration curves for 5MTHF-$(Glu)_{1-7}$ while the response of ‡ is calculated from the extrapolation of the best fit line ($y = -26.5x + 241$, $R^2 = 0.9989$) for response of Glu_5, Glu_6 and Glu_7 where y represents the MS/MS response of 5MTHF-Glu_n, and x represents the glutamyl length. All data were presented as the mean of 5 replicate analyses from different day. RSD (%) of 5 replicates were below 12% for all 5MTHF-Glu_n species in all vegetables analyzed.

(*Amaranthaceae*), we observed the predominant forms as 5MTHF-Glu_5 and 5MTHF. Collard greens, kale, Brussels sprouts, cauliflower and broccoli (species *Brassica oleracea*) had as predominant forms 5MTHF-Glu_6, 5MTHF-Glu_7, 5MTHF-Glu_8 and 5MTHF-Glu_5. The major folate species in turnip greens, broccoli rabe and bok choy (species *Brassica rapa*) were 5MTHF-Glu_6, 5MTHF-Glu_7 and 5MTHF-Glu_8 with high levels of 5MTHF. Dandelion greens and romaine lettuce (*Asteraceae*) showed predominantly 5MTHF, 5MTHF-Glu_5 and 5MTHF-Glu_6. The exception to these family trends was curly endive for which we found 5MTHF as the predominant form. Curly endive is from the same family as dandelion greens and romaine lettuce which had much higher occurrence of 5MTHF-Glu_5 and 5MTHF-Glu_6. We investigated closely related species to curly endive such as frisée, escarole, Belgian endive and radicchio and found all folate pools were primarily 5MTHF. The polyglutamyl folate distributions for the different families or species are illustrated in Fig. 4.

We are only aware of a few earlier studies that have reported quantitative polyglutamyl folate profiles in vegetables. Garratt et al. [22] have published information on polyglutamyl folates in spinach. The authors reported the predominant form in spinach as 5MTHF (74%), followed by 5MTHF-Glu_5 (10%) and 5MTHF-Glu_6 (10%) in

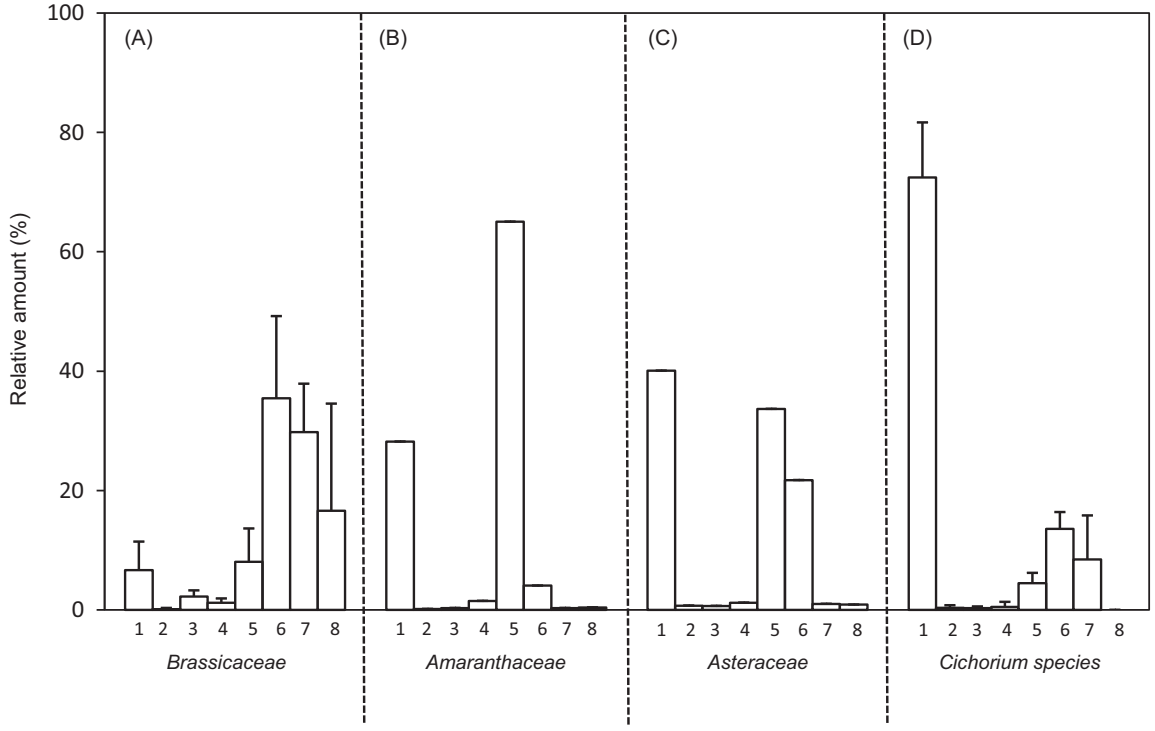

Fig. 4. Polyglutamyl 5-methyltetrahydrofolate (5MTHF-Glu_n) profiles in vegetables from various plant families. Levels determined by HPLC–MS/MS using authentic standards for 5MTHF-$(Glu)_{1-7}$ while the MS/MS response of 5MTHF-$(Glu)_8$ was estimated by extrapolation of the trend in response for 5MTHF-$(Glu)_{5-7}$. Values as x-axis represent polyglutamyl chain length. (A) Average profile (% relative to total) of nine vegetables from family *Brassicaceae*. (B) Average of two members of family *Amaranthaceae*: *Spinacia oleracea* (spinach) and *Beta vulgaris* (Swiss chard). (C) Average of two members of family *Asteraceae*: *Taraxacum officinale Wigg* (dandelion greens) and *Lactuca sativa L* (romaine lettuce). (D) Average of five members of family *Asteraceae*: *Cichorium endivia* (curly endive, frisée and escarole), *Cichorium intybus* (radicchio and Belgian endive) [39].

contrast to our results where we observed the predominant form as 5MTHF-Glu_5 (78%), with 5MTHF at 15% and 5MTHF-Glu_6 at 5%. In contrast to other folate extraction methods, Garratt et al. [22] used ice-cold 95% methanol, 5% phosphate buffer for extraction without a boiling step from general folate extraction methods which heat at 100 °C to inactivate GGH. The action of glutamylase is likely to blame for the lower polyglutamate levels reported by Garratt et al. In addition to glutamylase inactivation, heating can denature binding proteins and further release folates improving extraction efficiency [31]. Our results agree with Konings et al. [4], who found polyglutamyl folates in spinach accounts for 80%. We observed folate polyglutamyl species of broccoli similar to that reported by Verlinde et al. [17]. However the authors observed the predominant form as 5MTHF-Glu_3 while our study found 5MTHF-Glu_8 to predominate. Verlinde et al. [17] prepared samples for analysis by adding boiling extraction buffer into a frozen sample followed by homogenization in a blender. In our experience, the glutamyl hydrolase is likely active during this procedure. Indeed, the profile of intact folates in broccoli reported by these investigators is very similar to our results without steaming to inactivate GGH resulting in 5MTHF-Glu_3 as the predominant form. Zheng et al. [23] profiled polyglutamyl folates where they found diglutamyl folate in broccoli (48%) and in cauliflower (23%) while we only found trace amounts of diglutamyl form in these vegetables, but a very high level of 5MTHF-Glu_8 in broccoli (55%) and 5MTHF-Glu_6 in cauliflower (60%). Zheng et al. [23] acknowledge that high diglutamyl folate may be due to enzymic hydrolysis during extraction as they observed only traces of diglutamyl folate when the first extracts were analyzed. We observed a similar finding before we applied the steaming to inactivate the enzyme.

3.5. Folate speciation

The total 5MTHF folate contents we found are in line with literature values [4,5,32–37] although our polyglutamate profiles are in most comparisons distinctly shifted towards longer chain species. Readers should keep in mind that total 5MTHF is not equal to total folate. From our analysis, 5MTHF was the major form of folate in most of our vegetables (67–77% for non-leafy *Brassicaceae*, 56% for *Amaranthaceae* and 51–64% for *Asteraceae*) with 5-formyltetrahydrofolate (5CHOTHF) as the second major form (16–27% for non-leafy *Brassicaceae*, 21–30% for *Amaranthaceae* and 20–22% for *Asteraceae*). However, broccoli rabe had a similar proportion of 5MTHF (47%) and 5CHOTHF (43%) while unexpectedly the four leafy green vegetables belonging to the *Brassicaceae* family (collard greens, kale, bok choy and mustard greens) had a predominance of 5CHOTHF at 42–59%. For these four vegetables, 5MTHF species were secondary at 28–37%. Also of note, Swiss chard and escarole had 20% and 26%, respectively as 10-formyldihydrofolate (10CHODHF) and dandelion greens had 22% as 10-formylfolic acid (10CHOFA). Konings et al. also found significant contribution of 10CHOFA and 10CHODHF to total folate in many vegetables [4]. As folate species in these vegetables had not been characterized previously, this information expands the folate knowledge base.

4. Conclusion

The method reported herein illustrates linear MS/MS response for intact 5MTHF and polyglutamyl of each folate species tested, with acceptable precision and accuracy. The absolute recovery was reproducible at approximate 90%. Extraction loss and matrix effects were compensated for by using an isotopically labeled IS. By adapting this extraction procedure, our HPLC–MS/MS method may be suitable for analyzing native 5MTHF distribution in a wide range of complex food matrices.

The extraction method utilizing steaming before extraction showed potent inactivation of endogenous enzyme and stabilization of long chain polyglutamates with high extraction efficiency. This is a very important improvement compared to the existing methods as the *in vivo* profile is more weighted towards polyglutamates than previous reports suggested. In addition, steaming seems to have liberated folate from our vegetable matrices as efficiently as protease/amylase. Thus, steaming could obviate the need for the di-enzyme treatment, leaving only the conjugase step for folate species determination.

5MTHF and 5MTHF-Glu_n distribution of the same family or species were similar. Vegetables from *Asteraceae* and *Amaranthaceae* contained similar amounts of monoglutamyl and polyglutamyl species, *Brassicaceae* was dominated by polyglutamyls while *Cichorium endivia* was found to contain mainly monoglutamyl 5MTHF. Most vegetables involved in this experiment have not been analyzed previously for 5MTHF polyglutamyls. It is crucial to accurately determine the 5MTHF polyglutamate profiles as they exist in store-bought plants. Employing the method described in this report provides a reference point to fairly compare different plant 5MTHF profiles as well as extraction methods and processing treatments. Such fundamental information can aid researchers in several fields including plant biochemists, nutritionists and food scientists aiming to modulate polyglutamyl folate profiles.

References

[1] M. Rychlik, K. Englert, S. Kapfer, E. Kirchhoff, J. Food Compos. Anal. 20 (2007) 411.
[2] Y.I. Kim, Am. J. Clin. Nutr. 80 (2004) 1123.
[3] M.S. Field, D.M.E. Szebenyi, P.J. Stover, J. Biol. Chem. 281 (2006) 4215.
[4] E.J.M. Konings, H.H.S. Roomans, E. Dorant, R.A. Goldbohm, W.H.M. Saris, P.A. van den Brandt, Am. J. Clin. Nutr. 73 (2001) 765.
[5] L. Vahteristo, K. Lehikoinen, V. Ollilainen, P. Varo, Food Chem. 59 (1997) 589.
[6] P.M. Finglas, K. Wigertz, L. Vahteristo, C. Witthöft, S. Southon, I. Froidmont-Görtz, Food Chem. 64 (1999) 245.
[7] H. Müller, Z. Lebensm. Unters. Forsch. 196 (1993) 137.
[8] C. Wagner, in: L.B. Bailey (Ed.), Folate in Health and Disease, Marcel Dekker, New York, 1995, p. 23.
[9] E.A. Cossins, Can. J. Bot. 78 (2000) 691.
[10] B. Shane, Vitam. Horm. 45 (1989) 263.
[11] A. Brzezińska, P. Wińska, M. Balińska, Acta Biochim. Pol. 47 (2000) 735.
[12] J. Scott, F. Rébeillé, J. Fletcher, J. Sci. Food Agric. 80 (2000) 795.
[13] V. Schirch, W.B. Strong, Arch. Biochem. Biophys. 269 (1989) 371.
[14] A. Melse-Boonstra, P. Verhoef, C.E. West, J.A. van Rhijn, R.B. van Breemen, J.J.P. Lasaroms, S.D. Garbis, M.B. Katan, F.J. Kok, Am. J. Clin. Nutr. 84 (2006) 1128.
[15] D.J. McKillop, H. McNulty, J.M. Scott, J.M. McPartlin, J.J. Strain, I. Bradbury, J. Girvan, L. Hoey, R. McCreedy, J. Alexander, B.K. Patterson, M. Hannon-Fletcher, K. Pentieva, Am. J. Clin. Nutr. 84 (2006) 167.
[16] E.J.M. Konings, F.J. Troost, J.J.M. Castenmiller, H.H.S. Roomans, P.A. van den Brandt, W.H.M. Saris, Br. J. Nutr. 88 (2002) 235.
[17] P. Verlinde, I. Oey, M. Hendrickx, A. Van Loey, Food Chem. 111 (2008) 220.
[18] R.D. de la Garza, E.P. Quinlivan, S.M.J. Klaus, G.J.C. Basset, J.F. Gregory, A.D. Hanson, Natl. Acad. Sci. U.S.A. 101 (2004) 13720.
[19] E.P. Quinlivan, A.D. Hanson, J.F. Gregory, Anal. Biochem. 348 (2006) 163.
[20] A. Melse-boonstra, P. Verhoef, E.J.M. Konings, M. van Dusseldorp, A. Matser, P.C.H. Hollman, S. Meyboom, F.J. Kok, C.E. West, J. Agric. Food Chem. 50 (2002) 3473.
[21] G. Orsomando, R.D. De la Garza, B.J. Green, M. Peng, P.A. Rea, T.J. Ryan, J.F. Gregory, A.D. Hanson, J. Biol. Chem. 280 (2005) 28877.
[22] L.C. Garratt, C.A. Ortori, G.A. Tucker, F. Sablitzky, M.J. Bennett, D.A. Barrett, Rapid Commun. Mass Spectrom. 19 (2005) 2390.
[23] L. Zheng, Y. Lin, S. Lin, E.A. Cossins, Phytochemistry 31 (1992) 2277.
[24] USDA National Nutrient Database for Standard Reference-Release 22.
[25] S. Ndaw, M. Bergaentzle, D. Aoudé-Werner, S. Lahély, C. hasselmann, J. Chromatogr. A 928 (2001) 77.
[26] R.L. Blakley, The Biochemistry of Folic acid and Related Pteridines, North-Holland, London, 1969, pp. 94.
[27] L. Chen, R.R. Eitenmiller, J. Agric. Food Chem. 55 (2007) 3884.
[28] FDA, Guidance for Industry Validation of Analytical Procedures: Methodology, FDA, 1999.
[29] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. B 877 (2009) 2198.
[30] D.J. McKillop, K. Pentieva, D. Daly, J.M. McPartlin, J. Hughes, J.J. Strain, J.M. Scott, H. McNulty, Br. J. Nutr. 88 (2002) 681.

[31] G. Zhang, S. Storozhenko, D. Van Der Straeten, W.E. Lambert, J. Chromatogr. A 1078 (2005) 59.
[32] P. Lawrance, Individual folates in food stuffs: MAFF project 2B033. Report no. AS20/96/88, Laboratory of the Government Chemist, UK, 1996.
[33] M. Holasová, V. Fiedlerová, S. Vavreinová, Czech J. Food Sci. 26 (2008) 31.
[34] C.M. Witthöft, K. Forssén, L. Johannesson, M. Jägerstad, Scand. J. Food Nutr. 43 (1999) 138.
[35] D.J. McKillop, K.D. Pentieva, J.M. Scott, J.J. Strain, R. McCreedy, J. Alexander, K. Patterson, J. Hughes, H. McNulty, J. Agric. Food Chem. 51 (2003) 4382.
[36] A. Freisleben, P. Schieberle, M. Rychlik, Anal. Bioanal. Chem. 376 (2003) 149.
[37] M. Johansson, M. Jägerstad, W. Frølich, Scand. J. Food Nutr. 51 (2007) 22.
[38] W. Lu, Y.K. Kwon, J.D. Rabinowitz, J. Am. Soc. Mass Spectrom. 18 (2007) 898.
[39] S.J. Kays, J.C.S. Dias, Econ. Bot. 49 (1995) 115.