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Investigation of the extraction behavior of the main monoglutamate folates from spinach by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

An LC–MS/MS method has been developed for the determination of main monoglutamate folates in spinach with folic acid as an internal standard. A sample preparation with ultrafiltration (molecular weight cut-off membrane, 5 kDa) was followed by a chromatographic run of 14.2 min, rendering the method very simple and fast. The LODs in diluted spinach matrix were 0.02, 0.09, 0.05 and 0.03 ng/mL (0.037, 0.17, 0.092 and 0.055 μg/100 g calculated according to the fresh weight of spinach) for 5-methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, and 10-formylfolic acid, respectively. Using this method, the extraction behaviour of the main naturally occurring monoglutamate folates has been investigated in detail. It is found that 10 min of heating at 100 °C, incubation with rat serum at 37 °C (0.05 M phosphate buffer, pH = 6.5) for 4 h and the ratio of 10 (volume of extraction buffer versus the weight of sample, mL/g) are the optimal parameters for folate extraction from spinach. The final quantitative result of the individual folates in spinach is highly influenced by the pH (from 2.9 to 8.6) of the extraction buffer.
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Keywords: Folates; Spinach; LC–MS/MS; Extraction

1. Introduction

Folic acid is a water-soluble vitamin (B9) that plays a key function in the methylation cycle and DNA biosynthesis. Humans and animals cannot synthesize folates by themselves, so plant food is the main source of this vitamin. However, the naturally occurring folate content in most plant foods usually is very low, particularly in cereals. Hence, fortification of cereal-grain products with folic acid is common practice in many countries [1]. Another option is to enhance the folate level in plants either by genetic engineering or by breeding techniques. This recently became an increasingly important topic [2–4]. However, the availability of reliable data about various folate derivatives in plants is one of key issues for researchers in this field.

Folate analysis has been an analytical challenge for a long time because of the large number of structural analogs, their lability, and low level in natural samples. The analytical methods included mainly microbiological methods [5] for total folate content measurement and high performance liquid chromatography (HPLC) for the individual folates. Among the HPLC methods, different detection modes have been applied, such as ultra-violet (UV), diode array and fluorescence [6–12] detection, electrochemistry [13], microbiology [14,15] and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [16–33]. Although very sensitive, fluorescence and electrochemical detection cannot be used for all folates because of the structural diversity of folates. UV-spectrophotometry is universally applicable to all folates, but is often not sensitive enough to quantify the low level of folates in samples. Hence, LC–MS shows great advantages because of its high sensitivity and selectivity. Successful results in fo-

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late analysis obtained with LC–MS are already numerous [16–33].

Since LC with tandem mass spectrometric detection (LC–MS/MS) offers both high sensitivity and much better selectivity for the unambiguous identification and quantification of trace-level analytes in complex samples, long sample preparation procedures can often be shortened. This is beneficial in folate analysis because time consuming sample preparations can cause the loss of individual folates (e.g. 69% recovery of tetrahydrofolate by affinity chromatography) [34]. Purification with solid phase extraction or affinity columns was replaced by a simple and fast ultrafiltration (membrane with molecular weight cut-off of 5 kDa) in the present work.

Matrix effect, however, is one of shortcomings of atmospheric pressure ionization (API) interfaces although this soft ionization is responsible for the increasing success of LC–MS during the last decades [35]. To overcome this problem, several strategies have been proposed: additional clean-up, better chromatographic separation, and appropriate internal standardization for compensation. For the internal standard, an isotopically labeled compound is the best choice because it has the same chromatographic and ionization characteristics as the target compound. However, isotopically labeled compounds are not always commercially available, or they are highly expensive. Thus, often another compound with similar chemical, chromatographic and ionization characteristics is used. In this work, two different candidate internal standards were compared.

Previous reports applied different sample preparation techniques for folate analysis and the data showed a large variation [36], e.g. 5-methyltetrahydrofolate in spinach ranged from 46 [7] to 137 μg/100 g [19]. Similar variations were observed for other folates, like 5-formyltetrahydrofolate and tetrahydrofolate. Sample preparation is very important to the final outcome with the extraction itself being the key step [37]. It is necessary to investigate all parameters of the sample preparation that can influence the final result. This can then be applied to interpret data obtained by other analytical methods.

The aim of this work was two-fold: first, to set up an LC–MS/MS method to analyze the main monoglutamate folates in plant material with spinach as a model, and second, to investigate the effect of extraction parameters on the data of individual folates in spinach.

2. Experimental

2.1. Chemicals and reagents

The following folates were purchased from Schirck's Laboratories (Jona, Switzerland): 10-formylfolic acid (10-CHO-PteGlu) and folic acid triglutamate ($PteGlu_3$). 5-Methyltetrahydrofolate (5-MTHF), tetrahydrofolic acid (THF), 5-formyltetrahydrofolate (5-CHO-THF), folic acid (PteGlu) and methotrexate (MTX) were from Sigma (Bornem, Belgium).

The purity of the folates and the preparation of stock solutions were the same as in our previous work except that now two antioxidants (1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol) were used in all folate stock and standard solutions (instead of only 1.0% of ascorbic acid). All stock solutions were distributed in small vials (1 mL) and were stored at −80 °C [38].

LC–MS grade water, acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid, acetic acid, ammonium formate, ammonium acetate, ascorbic acid, 2-mercaptoethanol and other reagents were of high purity grade and were either from Merck (Leuven, Belgium) or Sigma (Bornem, Belgium). Rat serum was from Harlan Netherlands (Horst, The Netherlands).

2.2. Mass spectrometric instrumentation and settings

All experiments were performed by electrospray ionization utilizing heated auxiliary gas in the multiple reaction monitoring (MRM) mode on an Applied Biosystems API 4000 tandem quadrupole mass spectrometer (Foster City, CA, USA), operated in the positive ionization mode with the Analyst 1.4 controlling software. Source conditions were set as follows: temperature at 750 °C, ionspray voltage at 5.5 kV, the resolution of Q1 and Q3 were unit, dwell time was 50 ms, interface heater was on, gas 1, gas 2, curtain gas, and collision activated dissociation (CAD) gas were 90, 90, 40, and 6.5 psig, respectively. The voltage applied to the detector (channel electron multiplier, CEM) is 2200 V. The compound parameters for the folates and MTX are listed in Table 1.

Y-axis probe position was set at 0 mm when the HPLC flow rate was higher than 0.5 mL/min (HPLC pump) while it was set at 5 mm when flow rate was 10 μL/min (syringe pump for continuous infusion). *X*-axis probe position was always kept at 5 mm. In view of eventual nonvolatile salts present in the samples, the mass spectrometer was set with a delay of 2 min.

To investigate the ionization of a compound and to optimize the compound parameters, the infusion mode was carried out at 10 μL/min with a syringe pump (Harvard Apparatus, Hollington, MA, USA). A 0.1 μg/mL folate

Table 1
Compound parameters for folates and MTX[a]

	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (V)
5-MTHF	460	313	51	15	10	29
THF	446	299	76	15	8	29
5-CHO-THF	474	327	71	15	20	29
10-CHO-PteGlu	470	295	76	15	8	37
PteGlu	442	295	51	15	6	27
MTX	455	308	81	15	8	29

[a] DP: declustering potential; EP: entrance potential; CXP: collision cell exit potential; CE: collision energy; V: volt.

solution in methanol/water (50/50, v/v) containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol was used for this purpose. The organic solvent and buffer effects were evaluated using flow injection analysis (FIA) with the HPLC system. A 5-μL aliquot of the mixture of 1 μg/mL (each folate) was injected for each determination at a flow rate of 1 mL/min, the source parameters were optimized automatically with FIA under conditions closely resembling the real chromatographic conditions.

2.3. HPLC conditions

The HPLC system is an Agilent 1100 (Palo Alto, CA, USA) including a quaternary pump (flow rate 1.0 mL/min), an autosampler, column oven, and degasser. The needle wash solvent was a mixture of methanol/water (50/50, v/v). A Purospher Star RP-18 end-capped column (150 mm × 4.6 mm I.D.; octadecylsilyl, 5-μm particle size from Merck, Darmstadt, Germany), and a guard column RP 18 (4 mm × 4 mm I.D.; octadecylsilyl, 5-μm particle size also from Merck, Darmstadt, Germany) were used for all analyses.

The mobile phase consisted of eluent A (0.1% of formic acid in water) and eluent B (0.1% of formic acid in acetonitrile). The starting eluent was 95% A/5% B. The proportion of B was increased linearly to 16% in 3 min and then to 17% in 4 min. The proportion of B was then increased immediately to 100% and kept for 3 min. Afterwards, the mobile phase was immediately adjusted to its initial composition and held for 4 min in order to re-equilibrate the column. The injection volume was 20 μL. The column was kept at 35 °C in a column oven. The autosampler (kept at 4 °C) was equipped with a black door avoiding samples to be exposed to light. Under these conditions, the retention times of THF, 5-MTHF, 10-CHO-PteGlu, 5-CHO-THF, PteGlu and MTX were 4.57, 4.78, 5.90, 6.04, 6.22 and 6.51 min, respectively.

2.4. Preparation of calibrators

A folate stock solution, containing 17.5, 49.7, 32.2 and 14.9 μg/mL of 5-TMHF, THF, 5-CHO-THF, and 10-CHO-THF in extraction buffer (0.05 M of phosphate pH 6.5 containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol), was serially diluted in extraction buffer to prepare the standard solutions ranging from 0.07 to 50 ng/mL. The 10 μg/mL solution of the IS was also prepared in extraction buffer. The stock solution, the standard solutions and the IS solution were all kept at −80 °C.

2.5. Sample preparation

For the plant samples, 0.5 g of plant material (fresh weight) was grinded to a fine powder in liquid nitrogen. The powder was transferred to a 15-mL tube, and 2.5 mL of extraction buffer (0.05 M of phosphate buffer containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol, pH 6.5, freshly prepared) was added. Subsequently, extraction buffer and 0.1 mL of the 10 μg/mL IS solution (folic acid) were added until a final volume of 5.0 mL. After mixing, 2 mL of this solution was transferred into another tube. The capped tube was placed at 100 °C for 10 min (inhibition of enzymatic interconversions) and flash-cooled on ice. For deconjugation of polyglutamylated folates, 100 μL of rat serum was added to the extraction solution, which was then incubated at 37 °C for 4 h. An additional treatment of 10 min at 100 °C was carried out, again followed by cooling on ice. Primary centrifugation was at 14 000 × g for 15 min before the ultrafiltration over a 5 kDa molecular weight cut-off membrane filter (Millipore, Brussels, Belgium) at 12 000 × g for 30 min. The final solution at the bottom of centrifugation tube was ready for LC–MS/MS analysis. During the sample preparation, all manipulations are carried out under subdued light.

2.6. Matrix matched calibration and data analysis

For calibration purposes, 0.2 mL of the standard solutions (see preparation of calibrators) were added to 0.4 mL of the 100-fold diluted spinach extract and the IS (final concentration: 0.19 μg/mL). The ratio of the peak areas of the folates and the internal standard was used to plot the calibration curve for each folate. For each calibration curve, five different concentrations were used. These data were fit to a linear least-squares regression curve with a weighting factor of $1/x$.

2.7. Validation of the method

The following criteria were used to evaluate the method: sensitivity, linearity (R^2), intra- and inter-batch precision, accuracy and matrix effect. Sensitivity was assessed by evaluating the LOD and LOQ values. Intra- and inter-batch precision were determined by the RSD obtained on 1 day and on different days at three levels. The accuracy of the method was assessed by comparing the folates found to the folates added. Matrix effect was investigated based on the method of Matuszewski et al. [39].

3. Results and discussion

3.1. LC–MS/MS optimization

All folates and MTX showed much better sensitivity in positive than in negative mode. Thus, the positive mode was chosen for all folate analyses, which is in contrast to Garbis et al. [24], but similar to Frisleben et al. and Rychlik et al. [16,18–20]. The organic solvents, methanol and acetonitrile, commonly used in reversed chromatography were tested. It was found that all folate responses increased with an increasing percentage of both organic solvents up to high concentrations (90%). Higher percentages resulted in a decreased response, which has also been observed and explained by Benijts et al. [40]. However, at lower percentages (5–30%), acetonitrile resulted in a higher signal to noise for the fo-

lates as compared to methanol. Acetonitrile was therefore adopted in our further experiments. Different buffer additives that could influence sensitivity were evaluated, such as ammonium formate, ammonium acetate, dimethylamine, acetic acid and formic acid. The results showed that 0.1% of formic acid in the eluent yielded the best results for ionization of the folates and MTX.

The most intense fragment from all folates analyzed results from the loss of the glutamate moiety (*m/z* 147), e.g. for PteGlu *m/z* 442/295, for THF *m/z* 446/299, for 5-MTHF *m/z* 460/313, for 5-CHO-THF *m/z* 474/327 and for MTX *m/z* 455/308. However, for 10-CHO-PteGlu the *m/z* 470/295 transition results from the loss of both the glutamate and the 10-formyl group (*m/z* 28), which is different from the report by Freisleben et al. [16]. The transition used in their work on 10-CHO-PteGlu is *m/z* 470/452 (loss of water). The fragment at *m/z* 452, however, is not significant in our experiments.

3.2. Internal standard selection and matrix effect

Internal standardization is important for an accurate and reproducible quantification. The IS not only compensates for the loss of analyte during the sample preparation, but is also crucial in LC–MS to compensate for matrix effects and the variation of instrumental sensitivity. The use of isotopically labeled folates is one option in LC–MS. Due to the cost and limited commercial availability, some researchers used one labeled folate as IS for all folates while others synthesized the labeled folates themselves [16,18–20]. Another option is to use a structurally related compound. In this way, Garbis et al. used MTX as an internal standard for the determination of folates in human plasma [24]. MTX has chemical and chromatographic properties related to folates. Thus, MTX was also evaluated as an internal standard in our work. In this way, the matrix effect on MTX and folates was examined according to the method of Matuszewski et al. [39]. The matrix effect (ME) is then calculated as follows: ME (%) = A/B × 100. Whereby A: the data obtained in the matrix; B: the data obtained without matrix. The standard solutions of folates and MTX were mixed with the same volume of extraction buffer or spinach extract solution to investigate the matrix effect. The results (Fig. 1) show that the matrix effect on PteGlu and on the other folates was similar and not really pronounced. On the contrary, the spinach matrix clearly suppressed the ionization of MTX for about 50%. This excluded the use of MTX as an IS. Since folic acid was expected to have similar characteristics as the other folates regarding to chemical, chromatographic, mass spectrometric and matrix effect properties, we evaluated its use as an IS for the developed procedure. The most important criterion is that the IS itself is not present in the samples to be analyzed. This has been confirmed by the following experiments. Firstly, no PteGlu has ever been found in spinach during our experiments, not even as a result of possible conversion from other folates during sample manipulation. Secondly, there was no PteGlu demonstrated in spinach in other reports based on LC-UV, fluorescence, or MS [7,11,16,18,19,36]. Konings et al. [7] made the survey on various foods possibly containing PteGlu, e.g. cereals, bread, rice and potatoes. However, none of the vegetables they analyzed contained folic acid. Thus, for the determination of folates in spinach and other vegetables not containing PteGlu there is no objection to use PteGlu as an IS. However, for the application of this method to other materials, such as cereal foods or other foods fortified with PteGlu, of course the choice of IS should be reconsidered. Isotopically labeled PteGlu is then more appropriate.

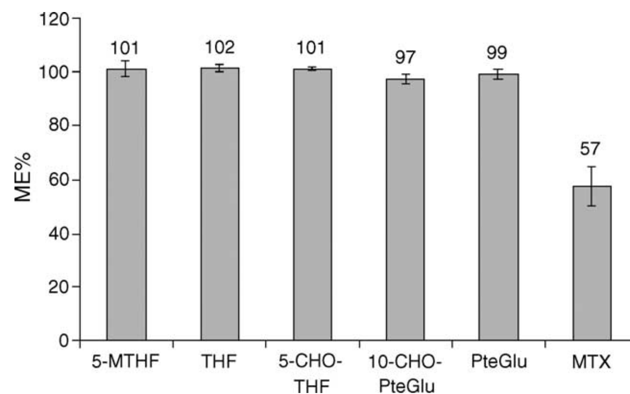

Fig. 1. The matrix effect on each folate and MTX ($n = 4$). Four hundred μL (10 ng/mL) of each folate and MTX were mixed with 400 μL of extraction buffer (0.05 M of phosphate buffer, pH 6.5, containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol) or with 400 μL of spinach extract solution. ME % = signal in spinach extraction solution/signal in extraction buffer × 100.

3.3. Sample preparation

Solid phase extraction or affinity chromatography was used in earlier folate analyses. The selectivity, however, of LC–MS and LC–MS/MS could allow a simpler sample preparation. Protein precipitation with acetonitrile followed by syringe filtration were used by Garbis et al. for the folate extraction from human plasma [24]. The main challenge with the complex plant matrices remains the matrix effect, which affects both the precision and accuracy. As discussed above, PteGlu has similar characteristics as the other folates and is considered as an ideal IS here. After homogenization in the extraction buffer, a centrifugation step followed by a ultrafiltration on a 5 kDa cut-off membrane successfully removed most of the polymers present in the plant extracts [41]. Folates were found not to be retained on the membrane, as demonstrated from the recovery experiment. The recovery ranges from 91.3 to 117.0% for low, middle and high levels within the calibration range of all target analytes. This method is much simpler as compared to the affinity chromatographic procedure which is rather time consuming [38].

3.4. Method validation

To determine the limit of detection (LOD) and quantitation (LOQ) for each folate in spinach, we serially diluted a

Table 2
LODs, LOQs and calibration characteristics

Folate	LOD (ng/mL)	LOQ (ng/mL)	Intercept (mean ± SD, $n=3$)	Slope (mean ± SD, $n=3$)	R^2	Linear range (ng/mL)
5-MTHF	0.02	0.088	0.0005 ± 0.0007	6.7 ± 0.5	0.9998	0.088–17.5
THF	0.09	0.25	−0.0005 ± 0.0002	2.7 ± 0.1	0.9999	0.25–49.7
5-CHO-THF	0.05	0.16	−0.0002 ± 0.0003	4.9 ± 0.2	0.9999	0.16–32.2
10-CHO-PteGlu	0.03	0.075	−0.0006 ± 0.0021	8.9 ± 0.3	0.9999	0.075–14.9

Table 3
Precision and accuracy data

Folate	Intrarun precision ($n=3$, % RSD)			Interrun precision ($n=3$, % RSD)			Accuracy ($n=3$)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
5-MTHF	8.8	1.7	1.8	25.0	2.4	2.2	94.0	128.5	128.2
THF	10.6	3.9	2.8	8.6	6.9	6.3	94.5	89.1	92.9
5-CHO-THF	7.4	1.5	2.1	6.1	2.7	3.0	97.3	109.8	106.5
10-CHO-PteGlu	8.7	1.7	2.4	7.6	3.6	3.1	99.3	113.4	108.7

spinach sample containing 0.17, 0.50, 0.32 and 0.15 ng/mL of 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu, respectively. Using a signal-to-noise ratio of 3 and 10, respectively, the LOD for 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu was 0.02, 0.09, 0.05 and 0.03 ng/mL (0.037, 0.17, 0.092 and 0.055 μg/100 g calculated according to the fresh weight of spinach) while LOQ values were 0.088, 0.25, 0.16 and 0.075 ng/mL (0.16, 0.46, 0.29 and 0.14 μg/100 g calculated according to the fresh weight of spinach), respectively. Because of possible matrix effects, the quantitation was based on standard series added to spinach. The calibration curve for each folate consisted of five points within their linear range concentration, and the background of the matrix was subtracted for each individual point. The calibration curve characteristics for each folate are listed in Table 2.

The precision and accuracy for intra- and inter-assay runs were evaluated using matrix-based quality control samples prepared at three levels (LQC contained 0.088, 0.25, 0.16 and 0.075 ng/mL of 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu, respectively; MQC contained 1.75, 4.97, 3.22 and 1.49 ng/mL of 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu, respectively; and HQC contained 17.53, 49.66, 32.19 and 14.90 ng/mL of 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu, respectively). Since no blank matrix free of folates could be obtained, 1000 times diluted spinach extracts were used here. The intra- and inter-assay precision and accuracy results are shown in Table 3.

The intra- and inter-assay precision ranged from 1.5 to 10.6% (RSD) and from 2.4 to 25.0% (RSD), respectively. Accuracy ranged between 89.1 and 128.5%. The small loss observed for THF (recovery 89.1 to 94.5%) was probably caused by degradation of this folate.

The quantitative results obtained for the four main folates in spinach are shown in Table 4. A representative chromatogram of a spinach extract is shown in Fig. 2.

3.5. Investigation of extraction conditions

3.5.1. Antioxidants

The folate stability has been investigated using different combinations of antioxidants of ascorbic acid and 2-mercaptoethanol. Earlier reports have already demonstrated that the combination of both antioxidants is necessary for the stability of the folates during the whole experiment [24]. A respective amount of 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol was confirmed to be necessary for stabilizing all folates in our experiments, especially for THF.

3.5.2. The effect duration of heating at 100 °C

The heating time at 100 °C was set at 0, 5, 10, 20, 40 and 60 min. The results showed that heating at 100 °C is necessary especially for 5-MTHF. The recovery for this folate from spinach was improved by 50% by this procedure. The heating step denaturates binding proteins and inactivates en-

Table 4
Quantitative results (μg/100 g ± SD; $n=3$) for the monoglutamate folates in spinach

	5-MTHF	THF	5-CHO-THF	10-CHO-PteGlu
Batch 1	77.9 ± 4.3	44.2 ± 5.6	30.4 ± 0.5	3.3 ± 0.5
Batch 2	72.7 ± 2.9	51.6 ± 4.9	29.6 ± 0.7	2.6 ± 0.5
Batch 3	90.3 ± 4.8	40.4 ± 0.2	30.4 ± 5.2	3.5 ± 0.3
Batch 4	96.1 ± 3.3	41.5 ± 1.3	37.9 ± 5.4	3.1 ± 0.2
Interrun mean	84.2	44.4	32.1	3.1
Interrun RSD %	12.8	11.4	12.1	11.6

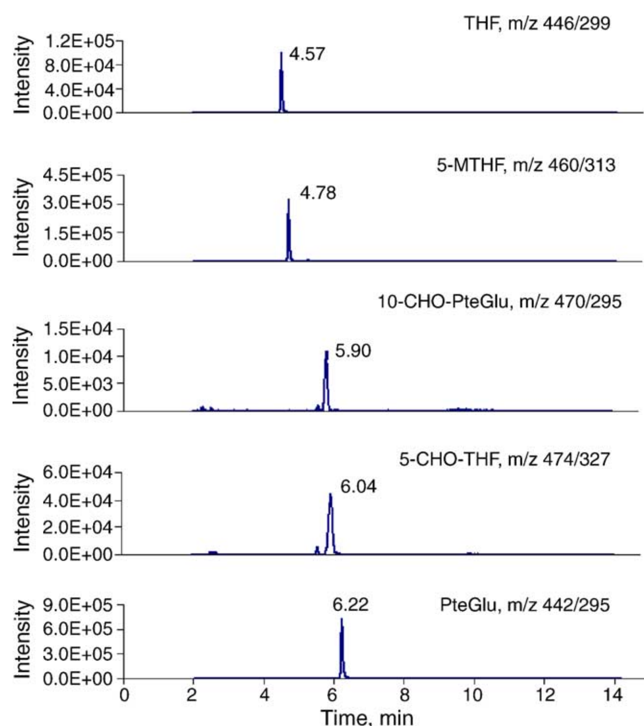

Fig. 2. LC–MS/MS chromatogram of the main naturally occurring monoglutamated folates and PteGlu (as IS) in a spinach extract. A 0.5-g of fresh spinach was used for each determination. Hundred μL of the 10-μg/mL IS solution and 2.5 mL of extraction buffer (0.05 M phosphate buffer, pH 6.5, containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol) were followed by addition of extraction buffer to form a final volume of 5.0 mL. A 2-mL aliquot of this spinach extract was then deconjugated with 0.1 mL of rat serum at 37 °C for 4 h. The delay time for the mass spectrometer was 2 min. The detected folates in spinach were 87.5, 43.9, 30.5 and 3.3 μg/100 g for 5-MTHF, THF, 5-CHO-THF and 10-CHO-PteGlu, respectively.

dogenous enzymes thus preventing further conversion of the folates. The duration of the heating between 5 and 60 min is indifferent except that a loss (∼10%) of THF was observed after 60 min as compared to 5 (or 10) min. Consequently, a 10-min heating period at 100 °C was preferred to prevent further interconversion.

3.5.3. *Time of incubation with folate conjugase*

Although a 1 h incubation with rat plasma folate conjugase is fully adequate for the complete hydrolysis of synthetic $PteGlu_3$ in buffer solution alone, polyglutamyl folates in cereal-grain extracts were not fully hydrolyzed in 1 h [37]. This was also confirmed in our experiments with spinach. Incubation times of 1, 2, 4, 8 and even 19 h were tested with spinach extract. The recovery of each folate increased with increasing duration of incubation over the first 4 h and reached a maximum at around 4 h incubation with rat serum. Prolonging the incubation to 8 h did not result in a substantial gain. This also means that the folates are stable under the conditions applied (0.05 M of phosphate buffer, pH 6.5, containing 1.0% of ascorbic acid and 0.1% 2-mercaptoethanol). This is in contrast to the low pH conditions (pH 4.8) with hog kidney (HK) conjugase. There, long incubation times (>3 h) should be avoided because of the lability of THF at this pH [42]. However, even at pH 6.5 longer incubation times (over night [16]) should be avoided because there was a trend to lower recoveries under these conditions.

3.5.4. *The volume of extraction buffer*

The effect of the ratio of the volume (mL) of the extraction buffer versus the weight (g) of sample on the recovery was also evaluated. A 2.5 g of spinach and different volumes of extraction buffers were taken for experiment. The ratios were 1, 2, 4, 10 and 40 by using 2.5, 5.0, 10, 25 and 100 mL of extraction buffer. Initial extraction yields were low and increased with an increase of the ratio up to a value of ten. Higher ratios did not enhance recoveries substantially, so a ratio of 10 was used in further experiments.

3.5.5. *Effect of pH*

The extraction buffers applied in other reports had different pH values, such as 4.1 [43,44], 4.9 [45], 6.0 [46], 7.0 [47] and 7.8 [7,16,19,48]. We investigated the effect of the pH on the final result. The most pH dependent steps in the sample preparation are the extraction and the deconjugation. These steps were investigated separately.

3.5.5.1. *Effect of pH on deconjugation.*

Earlier reports gave different results on the deconjugation with enzymes. Most authors confirmed that protease and α-amylase contributed to the folate extraction from starch rich foods, such as cereal. For vegetables, such as spinach, Martin et al. and Aiso and Tamura [49,50] found higher recoveries of folates after a trienzyme treatment. However, Ndaw et al. and Shrestha et al. [47,51] did not observe any effect of protease and amylase on folate recovery from spinach. The protease and amylase treatment was also tested in our experiments. It was found that both treatments had almost no effect on folate recovery from spinach, moreover, protease resulted in a very high background. Thus, only conjugase was adopted in our experiments. Three types of conjugase were reported in previous studies: plasma conjugase such as human or rat plasma conjugase (resulting in monoglutamate folates), chicken pancreas conjugase (to form diglutamate folates) and hog kidney conjugase (to form monoglutamate folates). Ndaw et al. compared the activity of rat plasma, chicken pancreas and hog kidney conjugase and the result showed that rat plasma had the best activity. However, hog kidney is usually used in folate analysis because of its common availability [52]. We compared human plasma and rat serum conjugase. Rat serum had a higher activity under the same conditions, which also was reported by Vahteristo et al. [42].

The effect of the pH on the deconjugation with rat serum was tested with $PteGlu_3$ as model compound in buffer solution. The deconjugation yields were 66, 67, 81, 97, 97, 96 and 41% at a pH of 2.9, 4.0, 5.0, 6.0, 6.5, 7.0 and 8.6, respectively. These results are similar to the ones reported by Doherty et al. [53], except that the reaction yield at pH 7 here (96%) is

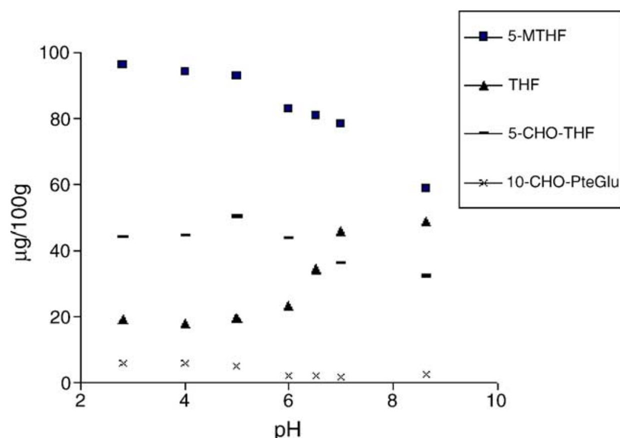

Fig. 3. The effect of pH on the recovery of main naturally occurring reduced monoglutamate folates from spinach. A 0.05 M phosphate buffer (pH from 2.9 to 8.6) containing 1.0% of ascorbic acid and 0.1% of 2-mercaptoethanol was used during homogenization and heating. All of the spinach extract solutions were adjusted to pH 6.5 before deconjugation. The other steps were the same as in Fig. 2.

higher than their results (72.3%). A pH of 6.5 was chosen for the deconjugation step in this work.

3.5.5.2. Effect of the pH on folate recovery during homogenization. The effect of the pH during treatments before the deconjugation step, e.g. homogenization, heating at 100 °C for 10 min and another 20 min for cooling down in ice was also evaluated. These steps were tested at different pH values. After this all extraction solutions were adjusted to the same pH (6.5) for deconjugation and further experiments. The final results are presented in Fig. 3.

The most pH dependent folate clearly is THF. The recovery of this folate was low at low pH, but could be doubled upon increasing the pH of the extraction buffer. This phenomenon was also observed by Vahteristo et al. [54]. This group used hog kidney conjugase at pH 4.9 for deconjugation. Consequently, they did not observe THF because THF degraded at this low pH. Vahteristo et al. also investigated the effect of pH (pH 4.9 and 6.0) on folate recovery from broccoli and green peas [36]. They found that THF recovery was much higher at high pH (pH 6.0). The same effect was also observed by Konings [34]. A possible cause was the degradation of THF under acidic conditions as confirmed by the experiment below. 5-MTHF seemed to be more stable under acidic conditions. For 5-CHO-THF, there was an optimum around pH 5. This compound was less stable at low pH, and at pH 6.5 or higher, interconversion of 10-formyltetrahydrofolate to 5-HCO-THF [55] was reduced. Hence the value of 5-CHO-THF decreased at both low pH and high pH. 10-CHO-PteGlu values was higher at low pH, this was also observed by Vahteristo et al. [36], but the absolute amounts of this folate are very small compared to other folates present in spinach.

3.5.5.3. The stability of folate standards at different pH. To understand the influence of the pH on the final data of folates in spinach, the stability of each folate standard has been examined at different pH values with and without the sample preparation. The results showed that all folates mentioned in this work were very stable at different pH values without the process of sample preparation. However, upon sample preparation, THF recovery showed a small decrease (around 10%) at high pH (between 6.0 and 8.6) and an additional decrease (around 20–30%) at low pH (between 2.8 and 5.0). This loss of stability at low pH explains the low THF values in spinach under those conditions. Likewise, 5-CHO-THF was not stable at low pH (from 2.8 to 4). However, above pH 5 it was stable, again explaining why the low pH was not optimal for this folate in spinach analysis. 5-MTHF showed higher stability at low pH, but degraded at high pH values (<10%). 10-CHO-PteGlu was very stable under all pH conditions examined here.

From these results, it is clear that the pH of extraction buffer directly influences the final data for each folate derivative. This undoubtedly is one of the main reasons why earlier data varied much.

4. Conclusions

A new and rapid LC–MS/MS method has been successfully developed for folate analysis in spinach. The method can be applied also to samples without endogenous or added folic acid, such as green vegetables. However, for samples rich in starch or eventually containing endogenous folic acid, such as cereal-foods, tri-enzyme treatments and another IS should be considered. This work investigated extensively all possible extraction conditions and their effects on the final result for each folate. The pH of the extraction buffer clearly has the most important relevance in the analysis of folates in spinach.

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References

[1] C.M. Pfeiffer, Z. Fazili, L. McCoy, M. Zhang, E.W. Gunter, Clin. Chem. 50 (2004) 423.
[2] G.J.C. Basset, E.P. Quinlivan, S. Ravanel, F. Rébeillé, B. Nichols, K. Shinozaki, M. Seki, L.C. Adams-Phillips, J.J. Giovannoni, J.F. Gregory III, A.D. Hanson, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1496.
[3] G.J.C. Basset, S. Ravanel, E.P. Quinlivan, R. White, J.J. Giovannoni, F. Rébeillé, B.P. Nichols, K. Shinozaki, M. Seki, J.F. Gregory III, A.D. Hanson, Plant J. 40 (2004) 453.
[4] T. Hossain, I. Rosenberg, J. Selhub, G. Kishore, R. Beachy, K. Schubert, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 5158.
[5] J. Scott, F. Rébeillé, J. Fletcher, J. Sci. Food Agric. 80 (2000) 795.

[6] W. Luo, H. Li, Y. Zhang, C.Y.W. Ang, J. Chromatogr. B 766 (2002) 331.
[7] 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.
[8] A.R.B. de Quiros, C.C. de Ron, J. Lopez-Hernandez, M.A. Lage-Yusty, J. Chromatogr. A 1032 (2004) 135.
[9] J. Jastrebova, C. Witthoft, A. Grahn, U. Svensson, M. Jagerstad, Food Chem. 80 (2003) 579.
[10] E.S. Osseyi, R.L. Wehling, J.A. Albrecht, J. Chromatogr. A 826 (1998) 235.
[11] R.F. Hoherty, G.R. Beecher, J. Agric. Food Chem. 51 (2003) 354.
[12] B.P. Day, J.F. Gregory III, J. Agric. Food Chem. 29 (1981) 374.
[13] P.J. Bagley, J. Selhub, Clin. Chem. 46 (2000) 404.
[14] P. Kelly, J. McPartlin, J. Scott, Anal. Biochem. 238 (1996) 179.
[15] M.S. Kariluoto, L.T. Vahteristo, V.I. Piironen, J. Sci. Food Agric. 81 (2001) 938.
[16] A. Freisleben, P. Schieberle, M. Rychlik, Anal. Bioanal. Chem. 376 (2003) 149.
[17] P. Stokes, K. Webb, J. Chromatogr. A 864 (1999) 59.
[18] M. Rychlik, M. Netzel, I. Pfannebecker, T. Frank, I. Bitsch, J. Chromatogr. B 792 (2003) 167.
[19] A. Freisleben, P. Schieberle, M. Rychlik, Anal. Biochem. 315 (2003) 247.
[20] M. Rychlik, J. Food Composit. Anal. 17 (2004) 475.
[21] B.C. Nelson, C.M. Pfeiffer, S.A. Margolis, C.P. Nelson, Anal. Biochem. 313 (2003) 117.
[22] B.C. Nelson, C.M. Pfeiffer, S.A. Margolis, C.P. Nelson, Anal. Biochem. 325 (2004) 41.
[23] B.C. Nelson, J.J. Dalluge, S.A. Margolis, J. Chromatogr. B 765 (2001) 141.
[24] S.D. Garbis, A. Melse-Boonstra, C.E. West, R.B. van Breemen, Anal. Chem. 73 (2001) 5358.
[25] R.M. Kok, D.E.C. Smith, J.R. Dainty, J.T. van den Akker, P.M. Finglas, Y.M. Smulders, C. Jakobs, K. de Meer, Anal. Biochem. 326 (2004) 129.
[26] P.M. Thomas, V.P. Flanagan, R.J. Pawlosky, J. Agric. Food Chem. 51 (2003) 1293.
[27] R.J. Pawlosky, V.P. Flanagan, J. Agric. Food Chem. 49 (2001) 1282.
[28] R.J. Pawlosky, V.P. Flanagan, R.F. Doherty, J. Agric. Food Chem. 51 (2003) 3726.
[29] R.J. Pawlosky, V.P. Flanagan, C.M. Pfeiffer, Anal. Biochem. 298 (2001) 299.
[30] S. Cha, H.J. Kim, Bull. Korean Chem. Soc. 24 (2003) 1308.
[31] Z. Fazili, C.M. Pfeiffer, Clin. Chem. 50 (2004) 2378.
[32] D.J. Hart, P.M. Finglas, C.A. Wolfe, F. Mellon, A.J.A. Wright, S. Southon, Anal. Biochem. 305 (2002) 206.
[33] R.J. Pawlosky, V.P. Flanagan, J. Agric. Food Chem. 49 (2000) 1282.
[34] E.J.M. Konings, J. AOAC Int. 82 (1999) 119.
[35] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.
[36] L. Vahteristo, K. Lehikoinen, V. Ollilainen, P. Varo, Food Chem. 59 (1997) 587.
[37] C.M. Pfeiffer, L.M. Rogers, J.F. Gregory III, J. Agric. Food Chem. 45 (1997) 407.
[38] G.F. Zhang, K.E. Maudens, S. Storozhenko, K.A. Mortier, D. Van Der Straeten, W.E. Lambert, J. Agric. Food Chem. 51 (2003) 7872.
[39] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[40] T. Benijts, R. Dams, W. Gunther, W. Lambert, A. De Leenheer, Rapid Commun. Mass Spectrom. 16 (2002) 1358.
[41] G.F. Zhang, K.A. Mortier, S. Storozhenko, J. Van De Steene, D. Van Der Straeten, W.E. Lambert, Rapid Commun. Mass Spectrom. 19 (2005) 963.
[42] L.T. Vahteristo, V. Ollilainen, P.E. Koivistoinen, P. Varo, J. Agric. Food Chem. 44 (1996) 477.
[43] M. Yon, T.H. Hyun, Nutr. Res. 23 (2003) 735.
[44] H.S. Lim, A.D. Mackey, T. Tamura, S.C. Wong, M.F. Picciano, Food Chem. 63 (1998) 401.
[45] K.E. McMartin, V. Virayotha, T.R. Tephly, Arch. Biochem. Biophys. 209 (1981) 127.
[46] J.F. Gregory, D.B. Sartain, B.P.F. Day, J. Nutr. 114 (1984) 341.
[47] S. Ndaw, M. Bergaentzlé, D. Aoudé-Werner, S. Lahély, C. Hasselmann, J. Chromatogr. A 928 (2001) 77.
[48] S.D. Wilson, D.W. Horne, Anal. Biochem. 142 (1984) 529.
[49] J.I. Martin, W.O. Landen, A.G.M. Soliman, R.R. Eitenmiller, J. Assoc. Off. Anal. Chem. 73 (1990) 805.
[50] K. Aiso, T. Tamura, J. Nutr. Sci. Vitaminol. 44 (1998) 361.
[51] A.K. Shrestha, J. Arcot, J. Paterson, Food Chem. 71 (2000) 545.
[52] R. Engelhardt, J.F. Gregory III, J. Agric. Food Chem. 38 (1990) 154.
[53] R.F. Doherty, G.R. Beecher, J. Agric. Food Chem. 51 (2003) 354.
[54] L.T. Vahteristo, V. Ollilainen, P.E. Koivistoinen, P. Varo, J. Agric. Food Chem. 44 (1996) 477.
[55] S.A. Kashani, B.A. Cooper, Anal. Biochem. 146 (1985) 40.