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A rapid method for sensitive profiling of folates from plant leaf by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometer

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ABSTRACT

Previous published methods for the analysis of folates are time consuming because of lengthy sample extraction, clean-up and total running time. This study details the development and validation of a rapid, sensitive and robust method that combines a simple extraction step with ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometry. Here, we reported application of a tandem quadrupole mass spectrometer to analyze maximum seven vitamers of folate from plant origin. The analytical performance was evaluated by linearity, sensitivity, precision, recovery test and analysis of certified reference materials. The limit of detection and limit of quantification ranged between 0.003 and 0.021 μg/100 g FW and between 0.011 and 0.041 μg/100 g FW, respectively; the recovery and precession ranged from 71.27 to 99. 01% and from 1.7 to 7.8% RSD, respectively, depending upon folate vitamers. This newly developed and validated method is rapid (a chromatographic run time of 5 min), easy to be performed (no laborious and time consuming clean-up) and can be used to simultaneously analyze seven vitamers of folate from plant sources.

1. Introduction

Folates, the generic term for a group of water-soluble vitamin have attained great nutritional importance. Chemically, folates molecules are comprised of three parts: pteridine, *p*-aminobenzoate, and a glutamyl chain (from1 to 14 links) (Fig. 1) [1]. They participate in one-carbon transfer reactions in the biosynthesis of purine and pyrimidine as well as amino acid inter-conversions [2]. However, intake of this group of vitamins from natural food sources is considered to be below the dietary recommendations for human [3,4]. Low levels of plasma folate concentrations are associated with a number of impairments, including development of neural tube defects (NTDs) and other congenital defects, macrocytic anaemia, cardiovascular disease, and certain types of cancer [5,6]. Folate deficiency, characterized by a folate intake below the recommendations (<400 μg per day) [4], is an important type of micronutrient deficiency nearly all over the world. Humans and animals cannot synthesize folates by themselves, so plant food is a main source of this important group of vitamin. However, the naturally occurring folate content in most plant foods usually is quite low. Therefore, mandatory food crop fortification programs with folic acid are a common practice in many countries [7]. Another alternative is to increase the natural folate level in food crops either by plant breeding or by metabolic engineering. This strategy recently became an increasingly important topic in the area of plant and human nutrition [8]. However, the availability of an accurate method for analysis of various folate vitamers in food from the plant origin is a challenging issue for the researchers in plant and human nutrition. This is because of plant samples contain very minute amount of folate and complex food matrix.

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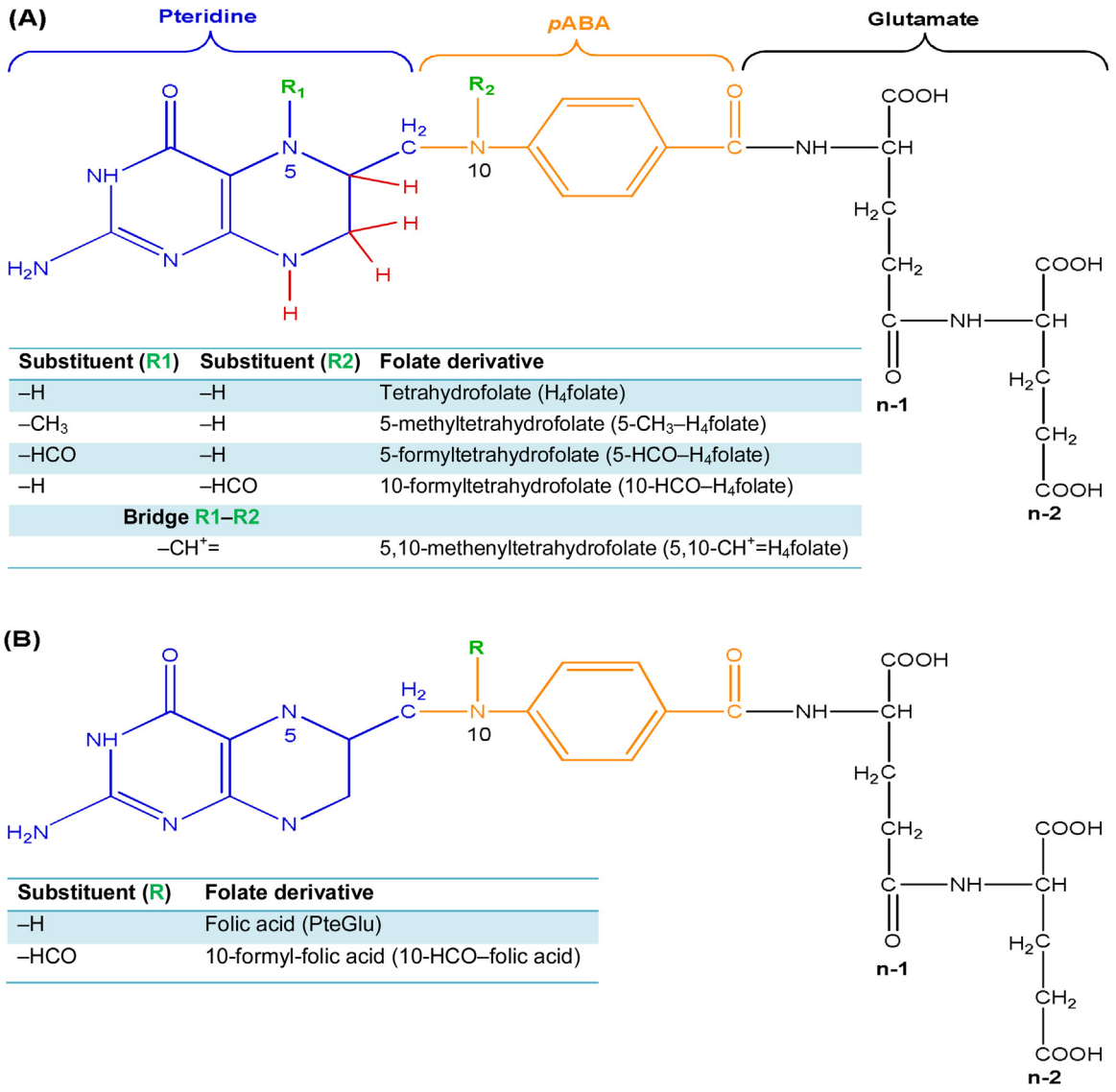

Substituent (R1)	Substituent (R2)	Folate derivative
–H	–H	Tetrahydrofolate (H_4folate)
$–CH_3$	–H	5-methyltetrahydrofolate (5-CH_3–H_4folate)
–HCO	–H	5-formyltetrahydrofolate (5-HCO–H_4folate)
–H	–HCO	10-formyltetrahydrofolate (10-HCO–H_4folate)
Bridge R1–R2		
$–CH^+=$		5,10-methenyltetrahydrofolate (5,10-CH^+=H_4folate)

Substituent (R)	Folate derivative
–H	Folic acid (PteGlu)
–HCO	10-formyl-folic acid (10-HCO–folic acid)

Fig. 1. Chemical structure of seven folates vitamers separated from the sample of plant origin. (A) Partially oxidized pteridine ring, and (B) Fully oxidized pteridine ring indicated in red color. Possible substitutions at the N5 and/or N10 positions by different C1 unite indicated in green color. Pterin, pABA, and glutamate moieties are depicted in blue, orange and black color, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Theoretically, more than 150 different vitamers of folates exist [9] although less than 50 are probably present in animal and plant tissue [10]. Most of the published chromatographic methods can only determine two to three vitamers, i.e. folic acid (PteGlu), tetrahydrofolate (H_4folate), and/or 5-methyltetrahydrofolate (5-CH_3-H_4folate), which is the main bottleneck when low folate amounts have to be analyzed. Therefore, it is important to develop a novel method that can determine more folate vitamers to obtain precise data of total folates in foods from plant origin [11]. Due to the large number of structural analogs, their low stability, and very low concentration in plant samples, folate analysis is an analytical challenge for the analytical food chemist [10]. The accuracy and sensitivity of folate analysis is highly dependent on the extraction method, the enzymatic deconjugation and detection techniques applied [12]. Additionally, folates are very sensitive to light, oxidants and pH of the medium. All these factors can significantly affect the accuracy of analysis. Furthermore, depending on the pH of the extraction buffer, heat treatment can affect the stability of folates and also causes non-enzymatic inter conversion [13]. Moreover, some protein and carbohydrate rich food require additional tri-enzyme treatment (α-amylase, protease and deconjugase) for the complete extraction of folates trapped in complex protein or carbohydrate structures [14]. This tri-enzyme digestion procedure is particularly effective for cereal-based and milk-based products. However, till now minimal information is available regarding the use of tri-enzyme digestion in analyzing foods of plant origin.

In the last 60 years, the most commonly used technique for quantitative analysis of food folates has been the microbiological assay (MA) that provides only a total folate content [15], associated with several drawbacks, for instance, it lacks information on specficity and vitamer distribution. Therefore, there is increasing application of chromatography based methods. Liquid chromatography (LC) [16] and Gas chromatography (GC) [17] are currently available for analysis of folate in both biological and food samples. The bottleneck of the GC method, similar to MA method, is the lack of capability to differentiate different folate vitamers in the sample. MA and GC method reduce their applicability because information of folate vitamers composition is important, as the different vitamers differ in their bioavailability [18]. In contrast to this, LC methods enable the determination of individual folate forms. In

general, folate determination has been performed using LC coupled with ultra-violet [19], fluorescence [20], diode array [21], electrochemical [22], or mass spectrometric (MS) [16], including tandem MS detection. However, additional purification of food extracts prior to LC analysis is necessary because of interferences from the matrix, which increases the analysis time and makes these methods more complicated [23]. Nevertheless, the LC methods have a great potential due to rapid progress of both separation and detection techniques as well as the possibility of automatizing the purification step.

Here, we report on the development and validation of a rapid, simple, accurate, quantitative, reproducible ultra-performance liquid chromatography tandem quadrupole mass spectrometry method for high throughput screening of large sample number of different cultivars, accessions, inbreed line, and mutants, capable of providing a folate profile, starting from only 100 mg of a flesh plant leaf sample. Single calibration curves, covering a wide dynamic range expand the use of our method for sensitive quantification of folate vitamers from plant samples of various origins. The method was used to investigate the differences in total folate and folate vitamer distribution in leaf samples of different vegetables and plants. This is the first successful implementation of a chromatographic method for the rapid and sensitive quantitative determination of seven folates vitamers in plant material within five minutes' running time. Parallel acquisition of multiple reaction monitoring (MRM) and full scan MS data allows both separation of the targeted folate vitamers and internal standard, and also the matrix background at the same time. Moreover, we investigate the effect of extraction parameters on the data of individual and total folates to simplify and reduce the extraction time.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from the following sources: UPLC–MS/MS grade 1% formic acid in acetonitrile, 1% formic acid in water, methanol, L (+)-ascorbic acid (99%, crystalline), 2, 3-dimercapto-1-propanol (BAL), α-amylase (Type I-A, from porcine pancreas, 23.5 units/μL, EC no 232-565-6) sera of rat (EC no 3–4–22–12), protease (Type XIV, from *Streptomyces griseus*, ≥3.5 units/mg EC no 232–909–5) and activated charcoal was from Sigma-Aldrich (St Louis, O.M., U.S.A.). Chicken pancreas (EC no 3–4–22–12) was purchased from Shanghai Wingch Chemical Technology Co. Ltd., (Shanghai, China). Monobasic potassium phosphate (≥99%, purity), dibasic potassium phosphate (≥99%, purity), sodium acetate (≥99%, purity) and sodium chloride (≥99%, purity) were purchased from Merck (Darmstadt, Germany). Mixed vegetables (BCR-485) were purchased from the Institute for Reference Material and Measurements (Geel, Belgium) and stored as vacuum-packed subsamples (2 g) at −80 °C until analysis. All other chemicals were of analytical grade commercially obtained from local chemical suppliers. Ultra-pure water (≤0.1 μS cm^{-1}) was obtained from Milli-Q system (Millipore, U.S.A.).

2.2. Folate standards and internal standard

Folic acid and the reduced forms of monoglutamyl folates (6,S)-5,6,7,8-tetrahydrofolate sodium salt (H_4folate), (6,S)-5-methyl-5,6,7,8-tetrahydrofolate sodium salt (5-CH_3–H_4folate) (6,S)-5-formyl-5,6,7,8-tetrahydrofolate sodium salt (5-HCO–H_4folate), 5, 10 −Methenyltetrahydrofolate (5,10 CH^+–H_4folate) and pteroyltri-γ-L-glutamic acid (PteGlu) were a kind gift from Merck & Cie (Schaffhausen, Switzerland). Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-formylfolic acid, sodium salt (10-CHO-folic acid), were purchased from Schirck's Laboratories (Jona, Switzerland). Methotrexate (MTX) was purchased from Sigma-Aldrich (St Louis, O.M., U.S.A.). The folate standards were stored at −80 °C under argon atmosphere until use. The standard stock solutions (200 μg mL^{-1}) and calibration solutions were prepared according to our previous report [24] with minor modifications, using 50 mM phosphate buffer containing 1% L(+)-ascorbic acid (w/v) and 0.1% 2,3-dimercapto-1-propanol (BAL) (v/v), pH 6.7 or 4 for 5,10-CH^+-H_4 folate (as this compound is more stable at acidic pH), under yellow fluorescent light. The standard stock solutions were placed in 1 mL tubes, flushed with argon gas, and stored under an argon atmosphere at −80 °C maximum for 3 months. The calibration solutions were prepared immediately before use by 1/10 dilution (v/v) of the stock solution. The concentration of the working solutions was checked by LC-DAD using PteGlu as internal standard (ISTD) as described previously [11] using following formula:

$$C\,(Analyte) = \frac{Area\,(Analyte) \times C\,(ISTD)}{Area\,(ISTD) \times ResponseFactor}$$

The wavelengths and response factors are provided in the Supplementary materials (see Table S1 in the online version at DOI: 10.1016/j.jchromb.2016.11.033).

2.3. Enzyme preparation

Enzyme preparation was performed as described in a previous study [25] with minor modifications. Briefly, protease was dissolve in distilled water (5 mg mL^{-1}), and α-amylase was used directly without any pretreatment. Protease and rat serum were mixed with one-tenth volume of activated charcoal to remove endogenous folates, stirred for 1 h on ice, centrifuged, filtered through 0.20 μm sterile syringe filter (Nalgene, Rochester, N.Y., U.S.A.) and divided into portions in 0.5 mL sterile tubes. Chicken pancreas solution was prepared by dissolving 5 mg of chicken pancreas in phosphate buffer (30 mL, 50 mM, pH 7.0) containing 1% ascorbic acid. Folate conjugase activity was confirmed using $PteGlu_3$ as described in our previous study [26]. Protease was kept at −20 °C and α-amylase at 4 °C for a maximum of 3 months.

2.4. Collection of plant materials

Different vegetables and leaf tissue of pak-choi (*Brassica rapa*, Chinensis, Jingfukuai), spinach (*Spinacia oleracea*, Dayuanye Bocai), lettuce (*Lactuca sativa,* Victoria lettuce), and rice leaf (*Oriza sativa,* Indica variety) were selected for tests of extraction procedure, method development, and validation experiments. All the vegetable samples were grown in the greenhouse of Zhejiang University under ambient temperature and light condition in March 2015 except rice. Rice leaf samples were collected from Zhejiang University farm in the same year. Tissue samples were weighed and, snap frozen in liquid nitrogen prior to use.

2.5. Sample extraction

Samples were protected against folate oxidation by argon gas, yellow fluorescence light, cooling on ice after heating and using amber glassware. Several extraction parameters were evaluated to optimize the best recovery of folates from the leaf samples. These extraction parameters included amount of leaf sample, extraction buffer volume, antioxidant type, amount and type of enzyme, pH of the extraction buffer and different boiling time to ensure best recovery of folates from a minimum amount of sample. The basic extraction procedure for folates from the leaf sample was adopted from our previous report [25–27] with several modifications. In brief, 100 mg of composite sample (fresh weight) was frozen and smashed in liquid nitrogen, immediately put in to

Table 1
Selected multiple reaction monitoring (MRM) transitions and compound parameters for seven folates vitamers and internal standard.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Retention time (t_r)	Dwell time (S)	Cone voltage (V)	Collision energy (V)
H_4folate	446.2	166.4	0.48	0.019	22	42
		299.3		0.019	22	13
5-CH_3–H_4folate	460.0	194.0	0.51	0.019	22	35
		313.0		0.019	22	20
5-HCO–H_4folate	474.3	327.1	0.68	0.019	32	22
		166.1		0.019	32	44
		299.1		0.019	32	32
		120.0		0.019	32	36
5, 10 CH^+–H_4folate	456.2	282.0	0.53	0.019	27	30
		412.0		0.019	27	30
10-HCO–H_4folate	474.3	412.2	0.67	0.025	60	34
		165.9		0.025	60	54
		327.1		0.025	60	28
10-CHO–PteGlu	470.0	175.9	0.64	0.019	27	30
		275.9		0.019	27	22
		295.2		0.019	27	28
PteGlu	442.0	176.0	0.75	0.019	22	27
		295.0		0.020	22	22
MTX	455.3	308.1	1.77	0.020	12	20
		175.1		0.020	12	38
		134.0		0.020	12	30
		106.0		0.020	12	72

1.25 mL graduated screw capped micro-tubes (Sangon Biotech Co., Ltd., Shanghai, China) and then 400 μL extraction buffer [50 mM phosphate buffer containing 1.0% of L (+)-ascorbic acid (w/v) and 0.1% 2, 3-dimercapto-1-propanol (BAL) (v/v) at pH 6.7, containing internal standard (MTX concentration 0.03 μg mL^{-1}), freshly prepared] were added. The sample were flushed with argon gas and capped before homogenized on a bench-top ball-mill (Scientz-48, Ningbo Scientz Biotechnology Co., Ltd, Ningbo, China) at 70 hz for 4 min. The capped tube was placed on a water bath at 100° C for 12 min and then rapidly cooled on ice. The tubes were then ultra-centrifuged at 27000 × *g* for 20 min at 4 °C and the supernatant was transferred to new graduated screw capped micro-tubes. For checking whether an additional enzyme treatment was necessary, 6 μL of α-amylase was added and incubated at room temperature for 10 min before extraction stapes. Thereafter, 15 μL of protease was added and tubes were incubated at 37 °C for 60 min. For deconjugation of polyglutamylated folates, 40 μL of chicken pancreas solution and 75 μL of rat serum were added, and the tubes were filled to an exact volume of 500 μL with extraction buffer and flushed with argon gas before capping, which was then incubated on a shaking water bath at 37 °C for 2 h. An additional treatment of 5 min at 100 °C was carried out to inactivate the enzyme, again followed by cooling on ice. The samples were then centrifuged again at 18000 × *g* for 15 min at 4 °C. The supernatant in the centrifuge tube was then subjected to sample clean-up.

2.6. Sample cleanup

Several sample cleanup protocols were evaluated to reduce the laborious time and to improve the detection limit and separation of the folates from interfering materials. Sample cleanup techniques includes solid phase extraction (SPE) with strong anion-exchange (SAX) isolute cartridges (3 mL/500 mg of quaternary amine N^+, counter ion Cl^-, Supelco, Bellefonte, PA, U.S.A.), ultra-filtration using different molecular weight (5 kDa and 10 kDa) cut-off membrane filter (Millipore, Carrigtwohill, Co. Cork, Ireland) and PVDF hydrophilic membrane filter (0.22 μm pore size, Millipore, Carrigtwohill, Co. Cork, Ireland). Solid phase extraction was performed using a visiprep SPE vacuum manifold (Supelco, Bellefonte, PA, USA) under reduced pressure as detailed in our previous report [25]. Membrane filters were preconditioned with 0.5 mL water for 5 min at 4 °C, removing water by hand shaking and kept for additional 5 min for dryness at 4 °C. For ultra-filtration, 0.5 mL supernatant solution was added to the upper chamber of the filter, centrifuged at 12,000 × *g* for 12 min at 4 °C. For general filtration, 0.5 mL supernatant solution was passed through a PVDF hydrophilic membrane filter using disposable syringe.

Table 2
Optimized conditions for tandem quadrupole (QqQ) mass detector for analysis of seven folates vitamers.

Source parameters	Value
Capillary voltage	3.12 kV
Source offset	25 V
Source temperature	150 °C
Desolvation gas temperature	400 °C
Desolvation gas flow	1000 L hr^{-1}
Cone gas flow	150 L hr^{-1}
Nebuliser gas flow	7.0 bar
Collision gas flow	0.15 mL/min

2.7. Tandem quadrupole mass spectrometer instrumentation

Considering the chemical and ionic properties of folate vitamers, several parameters were evaluated automatically and manually to optimize mass spectrometry. The detection was performed by a tandem quadrupole mass spectrometer (Waters Xevo TQ-S, Waters, Milford, MA, USA) operated in the positive electrospray ionization (ESI) mode. Prior to sample analysis Waters IntelliStart Technology (combining internal calibration fluidics and diagnostics software) integrated into MassLynx 4.1 Software, was used to automatically tune, calibrate, and conduct the systems performance checks prior to analysis. Further, IntelliStart was used to optimize all the MRM transitions for the seven folate vitamers and internal standard automatically. The automatically performed optimization was also confirmed manually, by a continually infused standard solution containing folates standards. The RADAR mode of the Xevo TQ-S was used, which enables both full scan data and MRM data to be acquired in parallel during the same sample analysis. Auto dwell time was used. System operation, data acquisition and data processing were controlled using MassLynx 4.1 software (Waters, Milford, MA, USA). Detailed MS/MS conditions were shown in Tables 1 and 2.

2.8. Ultra performance liquid chromatography (UPLC) instrumentation

Several parameters, including column type, column oven temperature, mobile phase composition, flow rate and injection volume were optimized to ensure the best performance. The chromatographic separation of individual folates was performed on a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) including a binary pump, a thermostatted autosampler, a thermostatted column compartment controlled by TargetLynx 4.1 (Waters, Milford, MA, USA) quantification application manager. The autosampler was maintained at 4 °C and the column oven temperature was tested from 20 to 45 °C. Four different types of column, ACQUITY UPLC HSS T3 column, dimension 2.1 mm × 100 mm, 1.8 μm particle size (Waters Corporation, Milford, USA), ACQUITY UPLC HSS T3 column, dimension 2.1 mm × 50 mm, 1.8 μm particle size (Waters Corporation, Milford, USA), ACQUITY UPLC BEH C18 column, dimension 2.1 mm × 100 mm, 1.7 μm particle size (Waters Corporation, Milford, USA), and ACQUITY UPLC BEH C18 column, dimension 2.1 mm × 50 mm, 1.7 μm particle size (Waters Corporation, Milford, USA) was tested to separation of folates. The flow rate was 0.4 mL/min; the injection volume was 2 μL and total running time was tested from 3 to 10 min. The mobile phase was a binary gradient mixture of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The needle wash solvent was a mixture of acetonitrile/water (50/50, v/v).

2.9. Validation procedure

Since blank matrix for analysis of folates from leaf sample was not available, a recombinant blank matrix was prepared from the reference material mixed vegetables BCR-485. For this purpose, sub-samples of BCR-485 were diluted by 1:20 (w/v) in ultrapure water and boiled in the presence of UV light to destroy most of endogenous folates. Thereafter, it was confirmed for absence of any measurable signal from folate vitamers, and ultrafiltrated to obtain a recombinant blank matrix. For matrix match calibration purposes, prepared standard and internal standard solutions were added to the recombinant blank matrix. The ratio of the peak areas of the folates and the internal standard was used to plot the calibration curve for each folate. These data were fit to a linear least-squares regression curve with a weighting factor of 1/x. The data were processed using the TargetLynx 4.1 (Waters, Milford, MA, USA) quantification application manager. TargetLynx automates data acquisition, processing, and reporting for quantitative results.

BCR-485 mixed vegetable reference material was used to confirm the validity of the newly developed method. Matrix effect (ME) was calculated as described previously [28] using the following formula, ME (%) = A/B × 100. Where, A is the peak area of spiked recombinant BCR-584 matrix and B is the peak area of standards in phosphate buffer. Sensitivity of the method was confirmed by evaluating the limit of detection (LOD; calculated as: 3.3σ/S, where σ is the standard deviation of the background noise of the blank and S is the slope of calibration curve) and limit of quantification (LOQ; calculated as: 10 σ/S). Recovery were determined from the pak-choi sample spiked at two different (low contained 7.45, 34.29, 31.81, 22.57, 0.415, 7.93, and 4.82 μg/100 g of H_4folate, 5-CH_3–H_4folate, 5-HCO–H_4folate, 10-CHO–H_4folate, 10-CHO–PteGlu, 5,10 CH^+–H_4folate and PteGlu, respectively; high contained 14.91, 68.59, 63.62, 45.15, 0.83, 15.87 and 9.65 μg/100 g of H_4folate, 5-CH_3–H_4folate, 5-HCO–H_4folate, 10-CHO–H_4folate, 10-CHO– PteGlu, 5,10 CH^+–H_4folate and PteGlu, respectively) concentration level before extraction according to the method described by [28,29]. The spiked pak-choi samples were then processed through the entire procedure. The recovery (R) was calculated from the following formula, R (%) = ((B-C)/A × 100, where A = peak area of the neat folate standards, B = peak area of the spiked sample with standard, C = peak area of extract. Intra and inter day precision were determined by calculating the RSD obtained on the same day and on different days at three determinations per concentration.

2.10. Statistical analysis

Statistical analysis of data was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The results are expressed as mean values (mean ± standard error), and the difference between the samples were compared by using a multiple-range test (least significance difference) at $P < 0.05$ probability level (Duncan's test).

3. Results and discussion

Folates, member of vitamin B family, essential cofactors for one-carbon metabolism, are essential for human health. Therefore, reliable data on folate levels in different plant materials are useful for various nutritional programs. Chemically folates are polar compounds with a large number of structural analogs, which makes them difficult to separate using conventional chromatography. Considering the unique physicochemical properties of different folates vitamers, we herein takes attempts to separate seven folate vitamers using reverse-phase chromatography.

3.1. Preliminary studies for method development

3.1.1. Optimization of sample extraction

Previous reports have shown that the combination of two antioxidants is necessary for the stability of the folates during the extraction [30]. The stability of different folate vitamers in the buffer solution has been investigated using the combinations of ascorbic acid and, 2, 3-dimercapto-1-propanol. Preliminary extraction tests confirm that best recoveries were obtained with a mixture of 1.0% of ascorbic acid and 0.1% of 2, 3-dimercapto-1-propanol, especially for H_4folate.

Heating in water bath at 100 °C denature the folate binding proteins and facilitates releasing the folates into the solution. The heating step also inactivates endogenous enzymes thus preventing further inter-conversion of the folate vitamers. Preliminary results showed that heating at 100 °C is necessary especially for 5-CH_3–H_4folate. The recovery for this vitamer from spinach and pak-choi was improved by 55% and 60%, respectively, by this procedure. Different heating periods, viz. 0, 8, 10, 12, 20 and 40 min at 100 °C were tested in our preliminary experiments. The duration of the heating above 12 and below 10 min did not improve recovery, but prolong heating step loss (~12%) of H_4folate as compared to 8 min. Therefore, 12 min heating period at 100 °C was chosen for further experiment. Different ratios of samples weight (g) versus the volume of the extraction buffer (mL) were evaluated to obtain the best recovery. Preliminary studies showed that the ratio of 1:5 (sample: buffer) resulted in the best recoveries. Higher ratios did not increase recoveries substantially, and therefore, the ratio of 1:5 was used in the subsequent experiments.

The pH of the extraction buffer during homogenization was also investigated to find the best recovery. These steps were tested at different pH values. Recovery of different folate vitamers varied with buffer pH during the extraction (Fig. 2a), with H_4folate being most sensitive to pH. The recovery of this folate was low at low pH, but could be doubled upon increasing the pH of extraction buffer. As no isotope-labelled standard for H_4folate was used to compensate for its loss [31], this aspect was critical and dominated the choice of pH. Similar results were also previously reported [32–35]. Recovery of 5-CHO—H_4folate was found to be optimal around pH

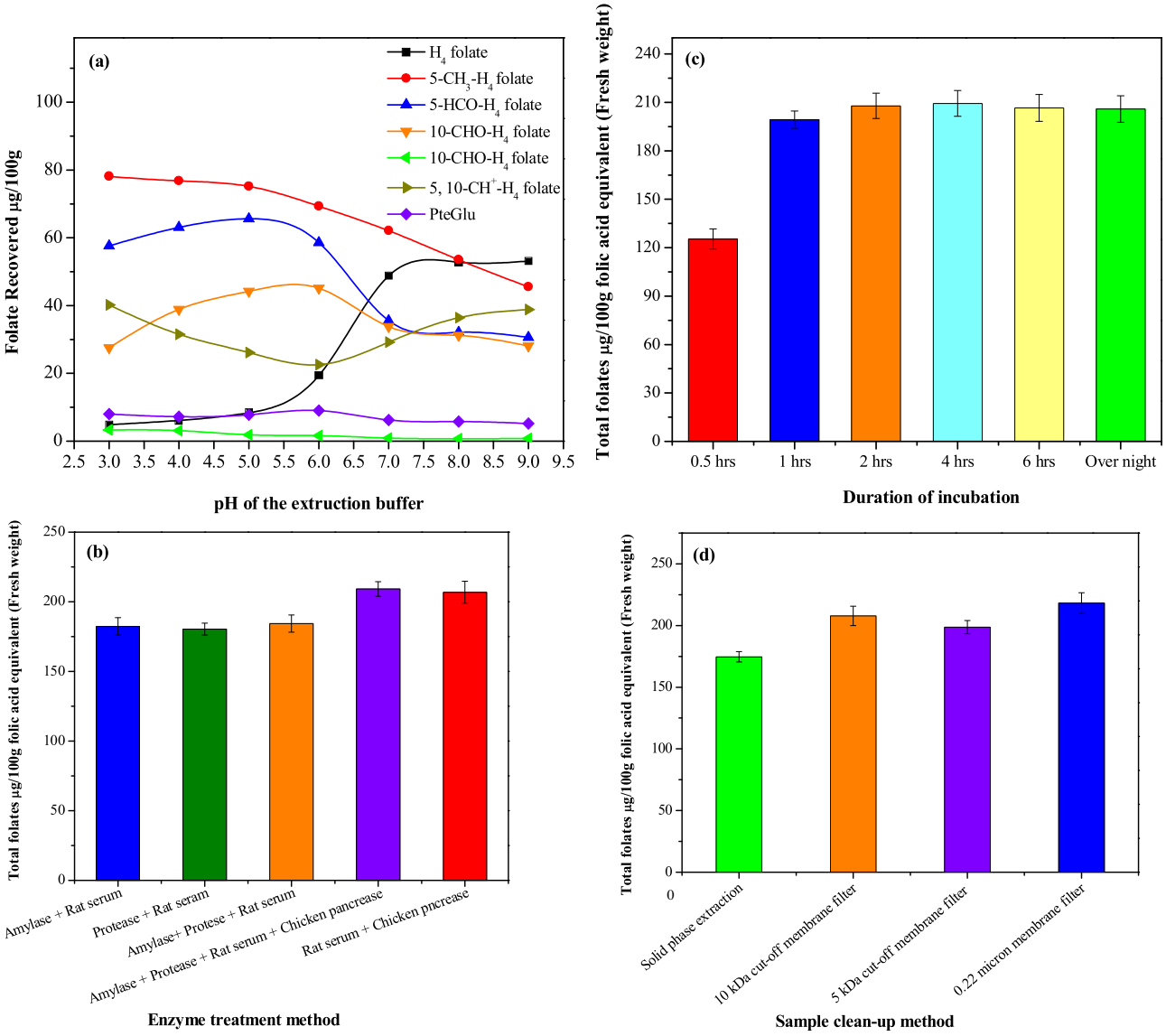

Fig. 2. Optimization of sample extraction parameters for folate from pak-choi (*Brassica rapa*, Chinensis). (a) Effect of pH of extraction buffer on the recovery of different folates vitamers. For this purpose, 50 mM phosphate buffer (pH from 3.0 to 9.0) containing 1.0% of L (+)-ascorbic acid (w/v) and 0.1% 2, 3-dimercapto-1-propanol (BAL) (v/v) was used during homogenization and heating. All the pak-choi (*B. rapa*, Chinensis) sample extract solutions were adjusted to pH 6.7 before deconjugation steps. (b) Effect of enzyme treatment on the recovery of total folate. For this purpose pak-choi (*B. rapa*, Chinensis) samples were treated with the combination of different enzymes during extraction, and analyzed for folates (n = 3). (c) Effect of incubation time on recovery of total folate. For this purpose pak-choi (*B. rapa*, Chinensis) samples were incubated for different duration times throughout the extraction process, and analyzed for folates (n = 3). (d) Evaluation of sample clean up techniques before chromatographic separation. For this purpose an extracted pak-choi sample (*B. rapa*, Chinensis) was cleaned up using different sample clean up techniques (Detailed in materials and method) and analyzed for folates (n = 3).

5. This compound was less stable at low pH, and the value of 5-CHO—H_4folate decreased at both low and high pH. The recovery of 10-CHO-PtGlu were higher at low pH, which agreed with previous report [36]. According to the results from the preliminary test, pH of the extraction buffer was adjusted to 6.7 for further experiments, as illustrated in Fig. 2.

Samples pretreated with α-amylase (6 μL) and protease (15 μL) after homogenization resulted in little or no significant change in total folate (Fig. 2b). This is in agreement with previous reports showing that monoenzyme treatment is suitable for vegetables [26,37]. Considering these results and the fact that plant leaves contain low amounts of carbohydrates and proteins compared to fruits and flower, therefore, we applied monoenzyme treatment for folate quantification from leaf samples in our further experiments. The efficiency with which folates are deconjugated may depend on folate species present, the sample matrix, the pH of the solutions, and the source of the conjugase (pteroylpoly-γ-glutamylcarboxypeptidase). The four main sources of conjugase used are hog kidney, human plasma, chicken pancreas, and rat serum. Combined use of rat serum and chicken pancreases significantly increased the recovery of total folate compare to rat serum alone (Fig. 2b), supported by the fact that chicken pancreas hydrolyse polyglutamyl folate to diglutamyl folate and rat serum hydrolyses diglutamyl folate to monoglutamyl folate, thereby increase the conjugase efficacy. In our previous study best result was obtained using a combination of both chicken pancreas and rat serum [27,31]. Furthermore, both chicken pancreas and rat serum works in the same pH ranges. Therefore, hydrolysis of polyglutamyl folate to diglutamyl folate is achieved by combined use of chicken pancreas and rat serum in the subsequent experiments.

Although a 1 h incubation with chicken pancreas and rat plasma folate conjugase is fully adequate for complete hydrolysis of

synthetic $PteGlu_3$ in the buffer solution alone, in some cases polyglutamyl folates in cereal-grain extracts were not fully hydrolyzed in 1 h [19]. This was also confirmed in the present experiments. Incubation times of 0.5, 1, 2, 4, 6 and over night were tested. The recovery of each folate increased with increasing duration of incubation over the first 2 h and reached a maximum at around 2 h incubation with rat serum and chicken pancreases (Fig. 2c). Prolonging the incubation to 6 h did not result in significant increase. This also means that the folates are stable under the conditions applied (0.05 M of phosphate buffer, pH 6.7, containing 1.0% of ascorbic acid and 0.1% 2, 3-dimercapto-1-propanol). However, long incubation time (>2 h) should be avoided because of the lability of H_4folate at this pH [38] and even at pH 6.7 longer incubation times should be avoided because there was a trend of lowering recoveries.

3.1.2. Optimization of purification

The presence of an endogenous matrix in food extracts interferes with folates and hampers the chromatographic separation of different folate vitamers. Considering that folate is also present at low level in most food matrices, removal of the interfering compounds through sample clean-up improves the detection limit and selectivity of folate detection. Three methods namely affinity chromatography using folate binding protein (FBP), solid phase extraction (SPE) using strong anion-exchange isolute cartridges and ultra-filtration using molecular weight cut off membrane filters are frequenty used for sample clean up prior to chromatographic separation [12]. Although FBP enables quantification at a ten-fold lower concentration than SPE [19], the lack of commercial availability of FBP columns precludes their routine use for folate analysis. Moreover, folate-binding protein exhibits low affinity to 5-HCO–H_4 folate, which may result in higher losses of this folate form during the purification step. Use of SPE for purification of sample extracts provides high recovery of different folate forms [16,26]; however, this method is laborious and the high concentration of salt used in elution step is likely to interfere ionization during MS analysis. Using a molecular weight cut-off membrane filter is efficient and also a cost effective for sample clean up as reported recently [35,39]. However, all these methods are time consuming, portions of folates are lost during clean up and are incapable of handling a large number of samples, for instance when screening inbreed lines for breeding purposes. To overcome the drawbacks with the existing methods for high throughout analysis of folate, the purification step was modified to a simple, efficient and cost-effective method. For quantification of folate from leaf sample we tested all the three methods as well as sample filtering by 0.22 μm PVDF hydrophilic membrane filters. The results show that folate recovery was higher when the samples were filtered through the 0.22 μm PVDF hydrophilic membrane filter (Fig. 2d). The average differences between folate contents in pak-choi determined with 10 kDa molecular weight cut-off membrane filter and 0.22 μm PVDF hydrophilic membrane filter were 4.7% in case of total folate content. In other words, it is possible to analyze folates in leaf without purification in screening studies when rapid determination of main folate forms is of greatest interest; otherwise more extensive purification may be necessary to quantify all folate forms.

3.1.3. Optimization of chromatography

The different folate vitamers exhibit small differences in their ionic character, which makes it difficult to separate them all by chromatographic methods. Prior to determining the optimal chromatographic conditions, individual folate standard and internal standards were directly infused to the atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and ESCi multi mode ionization source in both positive and negative ion mode. In comparison of all modes, the ESI^+ mode provided better sensitivity than any other modes tested in our system, which is also in conformity with earlier reports [40]. Therefore, ESI^+ mode was chosen for further experiments. The IntelliStart procedure (Waters) was used to optimize all MRM transitions for the seven folate vitamers automatically. The automatically performed optimization was also confirmed manually, by continually infused standard solution containing folates standards. For these analyses, two to four MRM transitions were used for each vitamer and the MRMs monitored are summarized in Table 1. The MS parameters were optimized to obtain the protonated molecule and most intense transitions as far as possible. Auto dwell time was used to ensure that approximately 15 data points were acquired for each chromatographic peak. The source parameters were optimized automatically with flow injection analysis and summarized in Table 2. For the sample analysis, full scan function used to assess background matrix during a standard MRM analysis. The additional functionality of full scan acquisition was acquired for each of the vitamers. This allowed us to search for other co-eluting compounds while monitoring the matrix background.

Table 3
Optimized five minutes gradient elution for UPLC–MS/MS analysis of seven folates vitamers.

Time (min)	Organic phase (%)	Aqueous phase (%)
Initial (0.00)	10.0	90.0
1.00	10.0	90.0
1.50	50.0	50.0
2.00	90.0	10.0
3.00	90.0	10.0
3.50	10.0	90.0
5.00	10.0	90.0

Evaluation criteria for optimization of UPLC performance included the organic solvents and column length to retain polar folate compounds and reduce total running time. The influences of temperature, pH, and mobile phase flow rate on retention time of folate vitamers were also evaluated. The best separation was observed at column oven temperature 40 °C. We evaluated four different chromatography columns (2 different phases at 2 different dimensions each) including ACQUITY UPLC HSS T3 column, dimension 2.1 mm × 100 mm, 1.8 μm particle size (Waters Corporation, Milford, USA), ACQUITY UPLC HSS T3 column, dimension 2.1 mm × 50 mm, 1.8 μm particle size (Waters Corporation, Milford, USA), ACQUITY UPLC BEH C18 column, dimension 2.1 mm × 100 mm, 1.7 μm particle size (Waters Corporation, Milford, USA), and ACQUITY UPLC BEH C18 column, dimension 2.1 mm × 50 mm, 1.7 μm particle size (Waters Corporation, Milford, USA). The best retention and separation was obtained with ACQUITY UPLC BEH, C_{18} column, dimension 2.1 mm × 50 mm, 1.7 μm particle size (Waters Corporation, Milford, USA), which is able to separate seven vitamers of folate within 5 min total running time. The optimized gradients are presented in Table 3. To minimize ion suppression from the matrix and co-eluted compounds, it is essential to increase chromatographic resolution. Therefore, reverse-phase column was chosen for its better adsorption capacity of polar compounds. Several organic solvents were tested to optimize the mobile phase. The mobile phases included organic solvents and volatile aqueous buffers at various pH values and ionic strengths. The results showed that usage of step wise increase in gradient consisting of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B) on a ACQUITY UPLC BEH, C_{18} column yielded good resolution of different folate vitamers and methotrexate as the internal standard.

3.2. Validation study

The analytical method was in-house validated and the following criteria were used to evaluate the method: detection and quan-

Table 4
Linearity and sensitivity of the method determination of seven folate vitamers.

Compound name	Limit of Detection (μg 100 g^{-1})	Limit of Quantification (μg 100 g^{-1})	Linearity range (μg 100 g^{-1}) (n = 8)	Slope (mean ± SD, n = 7 or 8)	Correlation of coefficient R^2
H_4folate	0.003	0.011	0.1–60	5996.89 ± 14.04	0.990
5-CH_3–H_4folate	0.006	0.022	0.1–60	5643.72 ± 9.81	0.993
5-HCO–H_4folate	0.012	0.034	0.1–60	3789.85 ± 10.61	0.999
10-HCO–H_4folate	0.021	0.041	0.1–60	3698.48 ± 9.35	0.999
5, 10 CH^+–H_4folate	0.017	0.032	0.005–20	3100.54 ± 8.79	0.999
10-HCO–PteGlu	0.007	0.023	0.005–20	3694.44 ± 85.92	0.998
PteGlu	0.011	0.035	0.005–20	646.86 ± 6.87	0.999

Table 5
Matrix effect, recovery and the precision of the method for determination of seven folate vitamers.

Folate vitamers	Matrix effect (%)		Recovery (%) ± SE (n = 3)		Precision (RSD%)	
	Without IS	With IS	Low	High	Intra-day assay	Inter-day assay
H_4folate	86.1	107.4	81.04 ± 2.8	89.14 ± 3.9	3.8	4.5
5-CH_3–H_4folate	92.8	110.5	98.22 ± 2.5	98.11 ± 1.2	1.7	2.7
5-HCO–H_4folate	102.2	118.3	98.23 ± 1.8	97.17 ± 3.1	2.3	3.1
10-HCO–H_4folate	97.5	102.4	69.75 ± 3.8	79.15 ± 6.24	3.8	6.7
5, 10 CH^+–H_4folate	75.1	116.3	76.50 ± 7.6	71.27 ± 8.23	4.9	7.8
10-HCO–PteGlu	92.1	102.1	98.11 ± 2.4	91.35 ± 2.14	2.8	5.2
PteGlu	110.3	108.7	92.77 ± 2.7	99.01 ± 1.8	5.9	7.6

Table 6
Precision of the method for folate determination in certified reference material BCR −485.

Folate vitamers	Mean content (μg 100 g^{-1}) (n = 3)	Certified/indicative value(μg 100 g^{-1})
H_4folate	28.78 ± 1.86	Not indicate
5-CH_3–H_4folate	249.0 ± 11.13	214 ± 42[a]
5-HCO–H_4folate	23.18 ± 1.63	Not indicate
Total folate as folic acid equivalent(FAE)	289.3 ± 14.22[A]	315 ± 28[b]

[a] Indicative HPLC-value for 5-CH_3–H_4folate in BCR − 485.
[b] Certified microbiological value for total folate in BCR − 485.
[A] Mean of triplicates, the sum of different folate forms is given in μg folic acid μg/100 g food after conversion using the molecular weight of 445.4 g/mol for H_4folate, 459.5 for 5-CH_3–H_4folate, 473.5 g/mol for 5-HCO–H_4folate and 441.4 g/mol for folic acid (PteGlu).

tification limits, the linearity, the recovery, intra-and inter-batch precision, accuracy and matrix effect.

3.2.1. Linearity

The linearity and sensitivity of the mass-spectrometric response was investigated by daily injecting standard solutions of seven folate vitamers (0.005–60 μg 100 g^{-1}) during the validation (Table 4). Prior to testing the linearity of a calibration model, several weighting factors and transformations were evaluated for homoscedasticity of the calibrators and subsequent performing of ANOVA tests on this model showed that there was a linear relationship between the peak area and the concentration of each folate form over the ranges tested as suggested by [41]. Linearity was also confirmed by plotting the peak area ratio of the folate standards to the internal standard methotrexate versus the concentration and expressed by the co-efficient of determination (R^2). The background of each matrix was subtracted for each individual point where necessary. The squared correlation coefficients for the eight-point calibration curves determined for the seven folate standards were in the range 0.990–0.999. The calibration curves relation to the internal standard methotrexate had a correlation coefficient higher than 0.990 for all folate forms.

3.2.2. Sensitivity

Sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ). The LOD is defined as the lowest concentration at which the analytical process can reliably differentiate from background levels, was accepted when the intensity of the signal is three times the background noise. The LOQ is defined as the lowest concentration at which quantitative results can be reported with a high degree of confidence, which was accepted when the intensity of the signal is ten times the background noise. For the folates derivatives, which were detected in different leaf samples, the limits of detection and quantitation were determined based on matrix match calibration curve, relating concentrations with signal to back ground noise ratios (Table 4). The LOD and LOQ ranged from 0.003 to 0.021 and 0.011 to 0.041 μg 100 g^{-1}, respectively.

3.2.3. Accuracy

Accuracy of the newly developed method was estimated by recovery tests and analysis of certified reference materials BCR-485. Pak-choi samples were spiked with a standard solution before extraction at two different concentration levels ((low contained 7.45, 34.29, 31.81, 22.57, 0.415, 7.93, and 4.82 μg/100 g of H_4folate, 5-CH_3–H_4folate, 5-HCO–H_4folate, 10-CHO–H_4folate, 10-CHO–PteGlu, 5,10 CH^+–H_4folate and PteGlu, respectively; high contained 14.91, 68.59, 63.62, 45.15, 0.83, 15.87 and 9.65 μg/100 g of H_4folate, 5-CH_3–H_4folate, 5-HCO—H_4folate, 10-CHO–H_4folate, 10-CHO–PteGlu, 5,10 CH^+–H_4folate and PteGlu, respectively). The mean recoveries ($n = 3$) of seven folates vitamers were in the range of 71.2 to 99.01% (Table 5), which indicates that the method is accurate considering the low concentrations. Fig. 3 illustrates the MRM detection of seven folate vitamers and internal standard with and without spiked, and using different sample clean up method. In both cases all vitamers and internal standard were baseline separated except 10-CHO–H_4folate, therefore pure standard was used to calculate the overlapped portion with 5 CHO–H_4folate.

Certified reference material mixed vegetables (BCR-485) were used to check the accuracy and as a quality control (Table 6). In BCR-485, only H_4folate, 5-CH_3–H_4folate and 5-CHO–H_4folate were quantified, and the sum of folates, expressed as folic acid, was 289.30 ± 14.22 μg 100 g^{-1} (n = 3). In our previous report using LC −UV/FLD we have detected 5-HCO–H_4-folate in BCR-485 but failed to quantify due to masking effect [25]. However, this time we have detected 5-HCO–H_4-folate in BCR-485 and was 23.18 ± 1.68 μg 100 g^{-1} (n = 3), and other folates vitamers were not detected, which was well in line with previous reports [42,43]. The amount and vita-

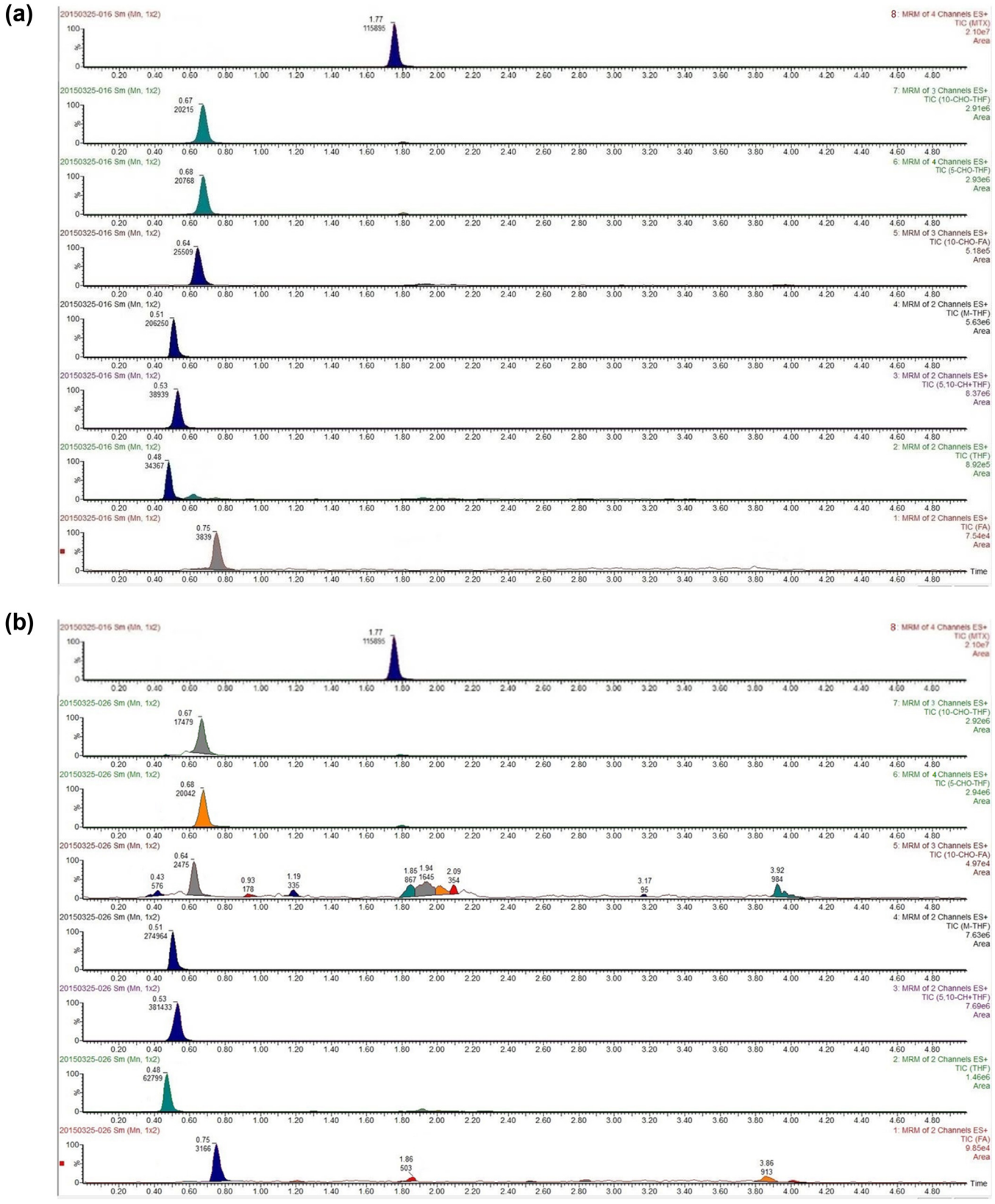

Fig. 3. Multiple reaction monitoring (MRM) of seven folate vitamers and internal standard. (a) Pak-choi (*Brassica rapa*, Chinensis) sample was spiked with 0.5 μg 100 g^{-1} of each folate vitamers after extraction. Samples were clean-up using 10 kDa cur-off membrane filters (details in materials and methods). (b) Pak-choi (*Brassica rapa*, Chinensis) without spike. Samples were clean-up using 0.22 μm PVDF hydrophilic membrane filters (details in materials and methods).

mer detected in this study was slight different form a recent report from Ringling and Rychlik [11], this is might be due to different batch of CRM, extraction and instrumental variation.

3.2.4. Precision and stability

The intra- and inter day precision of the entire analytical procedure was evaluated by analyzing six replicates of spiked pak-choi samples on three separate occasions (Table 5). The intraday precision varied between 1.6 to 4.1% RSD; inter-day precision varied between 2.8 to 7.8% RSD.

The stability of pak-choi extracts in the autosampler (+4 °C) was tested by re-injectiocn of triplicate samples. The difference between initial (0 h) and replicate (24 h) values of total folate was less than 4%, which indicating that the samples were stable for at least 24 h in the autosampler except more unstable folate vitamers H_4folate.

3.2.5. Internal standard and matrix effect

To compensate for losses during cleanup and for ionization interferences in the ion source, internal standards have been applied in several studies. Internal standardization is important for an accurate and reproducible quantification. Though the usage of stable isotope labelled folate standards is the best option [44,45], these were not used owing to their limited availability and high costs for analyzing large number of samples for screening experiments. Therefore, MTX, which has similar chemical and chromatographic properties as folates was used as internal standard in our experiment anaolgously to previous reports [30,35,39].

Evaluating matrix effects is essential when developing new MS method. Co-eluting, undetected matrix compounds can enhance or suppress the signal and, therefore, affects the reproducibility, sensitivity and accuracy of the method [28,46]. For assessing the matrix effect the internal standard was added before sample preparation. As can be deduced from the results in Table 5, suppression or enhancement of the signal was compensated for by use of the internal standard. Furthermore, during sample analysis, the RADAR mode was used, which enables us to screen for other co-eluting compounds, while monitoring the matrix background.

3.3. Folates in different leafy samples

This is the first report where we separate seven vitamers of folate using UPLC–MS/MS from the samples plant origin. The main folate vitamers found in lettuce, spinach, pak choi and rice leaf were H_4folate, 5-CH_3–H_4folate, 5-HCO–H_4folate, 10-HCO–H_4folate, 5,10 CH^+- H_4folate, 10-HCO-PteGlu and PteGlu (Fig. 4). Total folate content in lettuce, spinach, pak choi and rice leaf were 117.45, 223.74, 207.85, 118.3 μg/100 g, respectively. 5-CH_3–H_4folate was found to be the most abundant in all leaf sample analyzed. When all the seven analogs of folates are considered, these results are in agreement with those previously published in literature for similar plants analyzed using either microbiological assay or LC method [37,47,48].

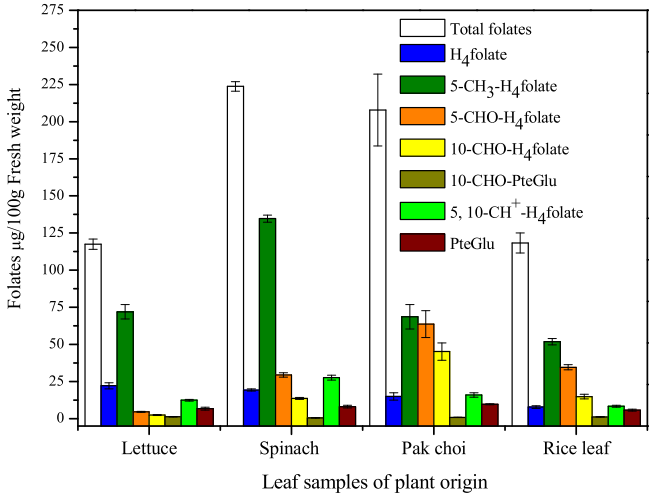

Fig. 4. Total folate and vitamers distribution in lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), pak-choi (*Brassica rapa*, Chinensis) and rice leaf (*Oriza sativa*) samples. Samples were extracted and analyzed using our newly developed and validated method. Samples were clean-up using 10 kDa cur-off membrane filters (details in materials and methods). Only total folate is given in μg folic acid equivalent μg 100 g^{-1} samples after conversion using the molecular weight of 445.44 g/mol for H_4folate, 459.56 g/mol for 5-CH_3–H_4folate, 473.44 g/mol for 5-HCO–H_4folate, 473.44 g/mol for 5-HCO–H_4folate, 457.1 g/mol for 5,10-CH^+-H_4folate, 469.4 g/mol for 10-HCO–PteGlu and 441.4 g/mol for PteGlu. Error bars indicate the standard error (n = 3).

4. Conclusions

An analytical method for quantifying the folates in plant leaf samples has been developed. This method is rapid (a chromatographic run time of 5 min) and easy to operate (no laborious clean-up). This is the first application of ultra-performance liquid chromatography tandem quadrupole mass spectrometer quantitatively analyze seven vitamers of folate from plant origin. By eliminating the lengthy clean-up optimum extraction condition makes this method more time efficient, and suitable for high throughput screening studies of different plant species. The validation procedures of this innovation show that the proposed method is selective, precise, accurate and sensitive. The advantage of using short analytical column is to reduce the total running time, and improve chromatographic efficiency and selectivity. The method has been successfully applied to the determination of folates in lettuce, spinach, pak-choi and rice leaf samples. This method should be applicable to vegetables and other food matrices of plant origin for the quantitative analysis of folates.

Safety consideration

General guidelines for work with organic solvents and acids were considered. 2,3-dimercapto-1-propanol (BAL) has been indicated to be toxic by European Union regulatory information. Solutions containing BAL should be handled with care in a fume hood due to its pungent odor and toxicity.

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References

[1] A.D. Hanson, J.F. Gregory III, Folate biosynthesis, turnover, and transport in plants, Ann. Rev. Plant Biol. 62 (2011) 105–125.
[2] J.R. Suh, A.K. Herbig, P.J. Stover, New perspectives on folate catabolism, Ann. Rev. Nutr. 21 (2001) 255–282.

[3] The Micronutrient Initiative (MI), Vitamin and Mineral Deficiency: A Global Damage Assessment Report, United Nations Children's Fund (UNICEF), New York, 2004.
[4] Office of Dietary Supplements (ODS), Dietary Supplement Fact Sheet, National Institutes of Health (NIH), Bethesda, MD, 2009.
[5] H.J. Blom, G.M. Shaw, M. den Heijer, R.H. Finnell, Neural tube defects and folate: case far from closed, Nat. Rev. Neurosci. 7 (2006) 724–731.
[6] P.J. Stover, Physiology of folate and vitamin B-12 in health and disease, Nutr. Rev. 62 (2004) S3–S12.
[7] C.M. Pfeiffer, Z. Fazili, L. McCoy, M. Zhang, E.W. Gunter, Determination of folate vitamers in human serum by stable-isotope-dilution tandem mass spectrometry and comparison with radioassay and microbiologic assay, Clin. Chem. 50 (2004) 423–432.
[8] T. Hossain, I. Rosenberg, J. Selhub, G. Kishore, R. Beachy, K. Schubert, Enhancement of folates in plants through metabolic engineering, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 5158–5163.
[9] C.M. Baugh, C.L. Krumdieck, Naturally occurring folates, Ann. New York Acad. Sci. 186 (1971) 7–28.
[10] J.F. Gregory 3rd, Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folatess, Adv. Food Nutr. Res. 33 (1989) 1–101.
[11] C. Ringling, M. Rychlik, Analysis of seven folates in food by LC–MS/MS to improve accuracy of total folate data, Euro. Food Res. Technol. 236 (2013) 17–28.
[12] J. Arcot, A. Shrestha, Folate: methods of analysis, Trends Food Sci. Technol. 16 (2005) 253–266.
[13] V. De Brouwer, G.F. Zhang, S. Storozhenko, D. Van Der Straeten, W.E. Lambert, pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates, Phytochem. Anal. 18 (2007) 496–508.
[14] T.H. Hyun, T. Tamura, Trienzyme extraction in combination with microbiologic assay in food folate analysis: an updated review, Exp. Biol. Med. 230 (2005) 444–454.
[15] J.W. DeVries, J.I. Rader, P.M. Keagy, C.A. Hudson, Microbiological assay-trienzyme procedure for total folates in cereals and cereal foods: collaborative study, J. AOAC Int. 88 (2005) 5–15.
[16] P. Stokes, K. Webb, Analysis of some folate monoglutamates by high-performance liquid chromatography–mass spectrometry. I, J. Chromatogr. A 864 (1999) 59–67.
[17] S.R. Dueker, Y. Lin, A.D. Jones, R. Mercer, E. Fabbro, J.W. Miller, R. Green, A.J. Clifford, Determination of blood folate using acid extraction and internally standardized gas chromatography–mass spectrometry detection, Anal. Biochem. 283 (2000) 266–275.
[18] J. Gregory, S.D. Bhandari, L.B. Bailey, J.P. Toth, T.G. Baumgartner, J.J. Cerda, Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects, Am. J. Clin. Nutr. 55 (1992) 1147–1153.
[19] C.M. Pfeiffer, L.M. Rogers, J.F. Gregory, Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography, J. Agric. Food Chem. 45 (1997) 407–413.
[20] S. Ndaw, M. Bergaentzlé, D. Aoudé-Werner, S. Lahély, C. Hasselmann, Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates, J. Chromatogr. A 928 (2001) 77–90.
[21] E. Konings, A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour, J. AOAC Int. 82 (1998) 119–127.
[22] P.J. Bagley, J. Selhub, Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrochemical detection, Clin. Chem. 46 (2000) 404–411.
[23] L. Vahteristo, P. Finglas, Chromatographic determination of folates, Chromatogr. Sci. Ser. 84 (2000) 301–324.
[24] J.D.M. Patring, J.A. Jastrebova, S.B. Hjortmo, T.A. Andlid, I.M. Jagerstad, Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection, J. Agric. Food Chem. 53 (2005) 2406–2411.
[25] M. Shohag, Y. Wei, N. Yu, J. Zhang, K. Wang, J. Patring, Z. He, X. Yang, Natural variation of folate content and composition in spinach (Spinacia oleracea) germplasm, J. Agric. Food Chem. 59 (2011) 12520–12526.
[26] M.J.I. Shohag, Yanyan Wei, Ning Yu, Lingli Lu, Jie Zhang, Zhenli He, Johan Patring, Xiaoe Yang, Folate content and composition of vegetables commonly consumed in China, J. Food Sci. 77 (2012) 239–245.
[27] M. Shohag, Y. Wei, X. Yang, Changes of folate and potential other health promoting phytochemicals in legume seeds as affected by germination, J. Agric. Food Chem. 60 (2012) 9137–9143.
[28] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[29] V. De Brouwer, S. Storozhenko, J.C. Van De Steene, S.M. Wille, C.P. Stove, D. Van Der Straeten, W.E. Lambert, Optimisation and validation of a liquid chromatography–tandem mass spectrometry method for folates in rice, J. Chromatogr. A 1215 (2008) 125–132.
[30] S.D. Garbis, A. Melse-Boonstra, C.E. West, R.B. van Breemen, Determination of folates in human plasma using hydrophilic interaction chromatography-tandem mass spectrometry, Anal. Chem. 73 (2001) 5358–5364.
[31] S. Mönch, M. Rychlik, Improved folate extraction and tracing deconjugation efficiency by dual label isotope dilution assays in foods, J. Agric. Food Chem. 60 (2012) 1363–1372.
[32] E. Konings, A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour, J. AOAC Int. 82 (1999) 119.
[33] L.T. Vahteristo, V. Ollilainen, P.E. Koivistoinen, P. Varo, Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid chromatography, J. Agric. Food Chem. 44 (1996) 477–482.
[34] L.T. Vahteristo, V. Ollilainen, P. Varo, Liquid chromatographic determination of folate monoglutamates in fish, meat, egg, and dairy products consumed in Finland, J. AOAC Int. 80 (1997) 373–378.
[35] G.F. Zhang, S. Storozhenko, D. Van Der Straeten, W.E. Lambert, Investigation of the extraction behavior of the main monoglutamate folates from spinach by liquid chromatography-electrospray ionization tandem mass spectrometry, J Chromatogr. A 1078 (2005) 59–66.
[36] L. Vahteristo, K. Lehikoinen, V. Ollilainen, P. Varo, Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland, Food Chem. 59 (1997) 589–597.
[37] M. Hefni, V. Ohrvik, M. Tabekha, C. Witthoft, Folate content in foods commonly consumed in Egypt, Food Chem. 121 (2010) 540–545.
[38] M. Yon, T.H. Hyun, Folate content of foods commonly consumed in Korea measured after trienzyme extraction, Nutr. Res. 23 (2003) 735–746.
[39] K. Tyagi, P. Upadhyaya, S. Sarma, V. Tamboli, Y. Sreelakshmi, R. Sharma, High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato, Food Chem. 179 (2015) 76–84.
[40] J.D. Patring, J.A. Jastrebova, Application of liquid chromatography–electrospray ionisation mass spectrometry for determination of dietary folates: effects of buffer nature and mobile phase composition on sensitivity and selectivity, J. Chromatogr. A 1143 (2007) 72–82.
[41] A.M.d. Almeida, M.M. Castel-Branco, A. Falcao, Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods, J. Chromatogr. B 774 (2002) 215–222.
[42] J. Jastrebova, C. Witthoft, A. Grahn, U. Svensson, M. Jagerstad, HPLC determination of folates in raw and processed beetroots, Food Chem. 80 (2003) 579–588.
[43] S. Kariluoto, Folate in Rye: Determination and Enhancement by Food Processing, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland, 2008, pp. 47–49.
[44] A. Freisleben, P. Schieberle, M. Rychlik, Comparison of folate quantification in foods by high-performance liquid chromatography-fluorescence detection to that by stable isotope dilution assays using high-performance liquid chromatography-tandem mass spectrometry, Anal. Biochem. 315 (2003) 247–255.
[45] M. Rychlik, Revised folate content of foods determined by stable isotope dilution assays, J. Food Compos. Anal. 17 (2004) 475–483.
[46] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1217 (2010) 3929–3937.
[47] U.S. Department of Agriculture (USDA)-Agricultural Research Service (ARS). USDA National Nutrient Database for Standard Reference, release 27; USDA-ARS: Beltsville, MD, Feb 2015.
[48] M.J.W.F. Madelene Johansson, Folates in lettuce: a pilot study, Scan. J. Food Nutr. 51 (22) (2007) 30.