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Original Research Article

Folate monoglutamate in cereal grains: Evaluation of extraction techniques and determination by LC-MS/MS

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ABSTRACT

Folates are essential micronutrients for human health. To determine the total folate content, the extraction and quantification of seven monoglutamate folate derivatives in cereals (maize, rice, and wheat) were optimised and validated in this study. Di-enzyme treatment with α-amylase and rat conjugase was proved ideal for folate extraction from the cereal grains. The quantification method by liquid chromatography-tandem mass spectrometry was validated based on its matrix effect, linearity, sensitivity, recovery, inter-day and intra-day precision. The limits of detection and quantification of folate derivatives ranged from 0.03–0.88 and 0.1–1.0 μg/100 g among the three cereal samples. The absolute recoveries of most folate derivatives were 72–96 % for these cereal samples, with the exception of dihydrofolate, tetrahydrofolate, and folic acid (44–65 %). The inter-day and intra-day precisions were < 12 % for the three cereals. Analysis of folate content and composition in several cereal grains showed that the total folate levels were approximately 26 − 37 μg/100 g, with 5-methyl-tetrahydrofolate and 5-methyl-tetrahydrofolate as the dominant. MeFox, an oxidation product of 5-methyltetrahydrofolate, was detected at concentrations 20–39-fold higher than those of total folates in rice and wheat grains. This validated method is an efficient approach for folate determination in cereal crops.

1. Introduction

Folates, a generic term for tetrahydrofolate (H_4PteGlu) and its derivatives, are water-soluble vitamin B (B9) and play important roles in one-carbon metabolism. The H_4PteGlu molecule consists of a pteridine ring, a para-aminobenzoate ring, and L-glutamate moieties. Folate derivatives have distinct one-carbon units attached to the N5 and/or N10 positions of H_4PteGlu and exhibit various degrees of polyglutamation (1–6) (Hanson and Gregory III, 2011). As donors and acceptors of one-carbon units, folates are involved in multiple metabolic processes including the biosynthesis of nucleotides, thymidylate, methionine, serine, pantothenate, and formylmethionyl-transfer RNA, and provide methyl groups for most cellular methylation reactions (Hanson et al., 2000; Scott et al., 2000).

Microorganisms and plants are able to synthesise folates. However, humans cannot produce folates *de novo*; hence, humans depend on plant-derived foods as the primary source of folates (Blancquaert et al., 2010). Folate deficiency causes several human diseases. For example, folate malnutrition in pregnant women leads to growth retardation and neural tube defects of the foetus (Rader and Schneeman, 2006). The health impact of inadequate folate intake onadults includes megaloblastic anaemia, high homocysteine essential hypertension, and increased risks of cancer, cardiovascular disease, Alzheimer disease, coronary atherosclerosis, and other diseases (Blancquaert et al., 2010; Guo et al., 2017). Cereals such as wheat and rice are staple foods, but are low in folates. Folate biofortification by metabolic engineering and molecular breeding to increase the folate content of food crops has attracted great attentions (Strobbe and Van Der Straeten, 2017). An accurate folate determination method is needed and beneficial for folate improvement in food crops.

Folate derivatives are present at low concentrations in plant materials, and are sensitive to degradation induced by heat, light, oxidation, and pH, which makes folate detection facing great challenge (Zhang et al., 2005; De Brouwer et al., 2008). Several methods have been reported for folate extraction and determination from various plants and foodstuffs (Zhang et al., 2005; De Brouwer et al., 2008; Wang et al., 2010; Tyagi et al., 2015; Shohag et al., 2017; Wan et al., 2019; Zhang et al., 2019). Quantification of total folates typically involves the conversion of polyglutamated folates into monoglutamates by conjugase treatment. Three types of conjugases were report in previous research:

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plasma conjugase from human or rat serum (to form monoglutamate folates), chicken pancreas conjugase (to form diglutamate folates), and hog kidney conjugase (to form monoglutamate folates). Rat serum was shown to have a higher activity for folate extraction from spinach (Zhang et al., 2005). The traditional folate extraction procedure for total folate profiling includes two main steps: i) heating in a 100 °C water bath to release folates from bound proteins and deactivate the endogenous conjugase, thus interrupting folate conversion; and ii) conjugase treatment to hydrolyse complex polyglutamated folates into monoglutamate forms (Hyun and Tamura, 2005). This heating/boiling treatment coupled with deconjugation enabled extraction of folates from plant leaf samples (Zhang et al., 2005; Shohag et al., 2017). In addition, the other widely applied method was a tri-enzyme digestion procedure (α-amylase, protease, and conjugase), which help to release folates from complex carbohydrates and protein structures. This method was developed in the mid 1990s, and was particularly effective on cereal-based products such as rice seeds (De Brouwer et al., 2008; Dong et al., 2011). The method used for folate extraction is not uniform among various investigators. It has been reported that the enzyme-free heat treatment was more compatible than the tri-enzyme treatment for folate determination of infant milk formulae (Chandra-Hioe et al., 2017). An optimised one-step extraction with elimination of α-amylase and protease provides a more accurate and simplified method for folate analysis in pulse seeds (Zhang et al., 2018). Thus, other factors, such as the food matrix type and the effect of enzymes on the sensitivity of subsequent folate analysis, may affect analyte yields. Optimisation of folate extraction procedures from different food matrices is thus important for accurate folate identification and quantification.

For folate quantification, the microbiological assay is the most commonly used method for total folate content measurement (Scott et al., 2000), but it could not distinguish diverse folate derivatives present in plant samples. In the last two decades, the high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) (Freisleben et al., 2003; Smith et al., 2006) has been developed for separation and quantification of individual folate derivatives. LC–MS/MS showed great advantages due to its high sensitivity and accuracy, and it has been successfully applied for folate detection in several plant matrices (De Brouwer et al., 2008; Chandra-Hioe et al., 2017; Upadhyaya et al., 2017; Shohag et al., 2017).

Wheat, maize, and rice are the three major cereal crops worldwide. The prime objective of this study was to develop a cost-effective, sensitive, and reproducible method for quantitative analysis of folates in cereal grains. Here, evaluation of the effect of different folate extraction techniques, validation of a LC–MS/MS based quantification method, and application of them for folate analysis in wheat, maize, and rice cultivars were performed. This information can be used to determine folate levels in cereal grains and will be helpful for further folate biofortification by conventional or molecular breeding approaches.

2. Materials and methods

2.1. Materials and chemicals

Maize (*Zea mays* L.) seeds of inbred lines B73, GEMS9, and DAN3130 were harvested from the experimental station of the Chinese Academy of Agricultural Science in September 2018 at Langfang, Hebei Province, China. Rice seeds of the Daohuaxiang 2, Nipponbare, and Kitaake cultivars and wheat (*Triticum aestivum* L.) grains of Han6172, Liangxing 99, and Jimai 19 cultivars were obtained from a 2018 field trial (at Beijing) of the Institute of Crop Science, Chinese Academy of Agricultural Sciences.

The folate standards—10-formyl-folic acid (10 − CHO-PteGlu), 5,10-methenyl-5,6,7,8-tetrahydrofolate (5,10 − CH = H_4PteGlu), 5-formyl-tetrahydrofolate (5 − CHO-H_4PteGlu), 5-methyl-tetrahydrofolate (5 − CH_3-H_4PteGlu), dihydrofolate (H_2PteGlu), folic acid (PteGlu), tetrahydrofolate (H_4PteGlu), and methotrexate (MTX)—were purchased from Schircks Laboratories (Jona, Switzerland) and MeFox was obtained from Toronto Research Chemicals (Toronto, Canade). The purity of all folate standards were > 95 %. Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium ascorbate, β-mercaptoethanol, α-amylase (from *Aspergillus oryzae*, ~ 30 units/mg), and protease (Type XIV, from *Streptomyces griseus*, ≥ 3.5 units/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was purified on a Heal Force ultra-pure water system (Shanghai, China). Acetonitrile and formic acid (LC–MS grade) were purchased from Fisher Scientific (Geel, Belgium). The HPLC analytical column (Kromasil 100 − 5 C18, 2.1 × 50 mm, 2.5 μM particle size) was purchased from Akzo Nobel (Stockholm, Sweden), and an Agilent SB-C18 pre-column (2.1 × 5 mm, 2.7 μM particle size) was purchased from Agilent Technologies (California, USA). Rat serum was purchased from Solarbio.com (Beijing, China), and was aliquoted in 1 mL portions into 1.5 mL tubes upon arrival, then stored at −80 °C freezer before use. α-Amylase and protease were freshly prepared in water with the concentration of 40 mg/mL and 2 mg/mL, respectively. The endogenous folates in rat serum and protease were removed by incubation with one-tenth (*w/w*) of activated charcoal for 1 h on ice, followed by centrifuged at 13,000 rpm at 4 °C for 30 min (Sigma 3K15, Osterode am Harz, Germany), and the supernatant was used for the following incubation experiment.

2.2. Folate standard solutions

The folate stock solutions were prepared as previously described (Wan et al., 2019). The chemical powder of folate standards were dissolved as 0.1 mg/mL in a solution of 20 mM ammonium acetate in methanol and water (1:1, *v/v*) containing 1% (*w/v*) L-ascorbic acid, and 0.5 % (*v/v*) β-mercaptoethanol (pH6.2). 5,10 − CH = H_4PteGlu standard was prepared in a pH4.5 buffer. All standard solutions were stored at −80 °C before use. Working solutions were diluted with folate extraction buffer for spiking and calibration. The folate extraction buffer containing 50 mM phosphate buffer (pH 7.0), 0.5 % (*w/v*) sodium ascorbate, 0.2 % β-mercaptoethanol, was freshly prepared and used on the same day.

2.3. HPLC-MS/MS instrumentation

Folate separation and quantification was performed using an Agilent 1260 HPLC system coupled with an Agilent 6420 triple-quadrupole tandem MS operated in positive electrospray ionisation (ESI) mode (Palo Alto, CA, USA). A Kromasil 100 − 5 C18 column (50 × 2.1 mm, 2.5 μM particle size) with an Agilent SB-C18 pre-column (2.1 × 5 mm, 2.7 μM particle size) was used. The injection volume was 15 μL. The temperatures of the injector and column oven were maintained at 4 °C and 25 °C, respectively. The mobile phases were 0.1 % (v/v) formic acid in water (phase A) and 0.1 % (v/v) formic acid in acetonitrile (phase B). The gradient program was a total of 16.5 min. The initial mobile phase B was set at 5% at a flow rate of 0.3 mL/min. The proportion of mobile phase B increased linearly from 5 to 9% over 2 min. In the following 5.9 min, phase B increased to 9.5 %, then sharply increased to 20 % over 0.3 min. After holding at 20 % for 3 min at a flow rate of 0.6 mL/min, the proportion of phase B decreased to 5% in 0.2 min and holding on for 3 min. Subsequently, the flow rate decreased into 0.3 mL/min in 0.1 min followed by an equilibration time of 2 min. The mass spectrometer parameters were optimised for fragmentation of each folate standards with a gas temperature of 320 °C, drying gas flow at 11 L/min, nebuliser pressure at 35 psi, and capillary voltage at 3500 V. The precursor ion, the product ion and collision energy (eV) for each folate standard were *m/z* 470→295, 20 eV for 10 − CHO-PteGlu; *m/z* 456→412, 30 eV for 5,10 − CH = H_4PteGlu; *m/z* 460→313, 20 eV for 5 − CH_3-H_4PteGlu; *m/z* 474→327, 20 eV for 5 − CHO-H_4PteGlu; *m/z* 444→178, 20 eV for H_2PteGlu; *m/z* 446→299, 20 eV for H_4PteGlu; *m/z*

442→295, 20 eV for PteGlu; and *m/z* 455→308, 30 eV for the internal standard MTX. System operation, data acquisition, and data analyses were performed with Mass Hunter software.

2.4. Extraction of folates

Folate extraction was performed under subdued light to minimise light-induced degradation. The cereal grains were separately ground to fine powder using a grinder (Geno/Grinder 2010, Boston, USA), and the ground powder was filtered through the screen mesh (150 μm). Next, 50 mg of each powder were transferred to 1.5-mL screw-cap tubes (ST-150, Axygen, Union City, CA, USA). Four folate extraction methods—mono-enzyme treatment (rat serum), di-enzyme treatment A (α-amylase + rat serum), di-enzyme treatment B (protease + rat serum), and tri-enzyme treatment (α-amylase + protease + rat serum)—were performed, and three technique repeats were used for each sample. The heating treatment of the four approaches was operated in consistency: 1 mL extraction buffer was added to 50 mg of fine powder, after mixed homogeneously, the mixture was immediately boiled for 10 min in a 100 °C water bath, cooled on ice; then,

I) for mono-enzyme extraction, 30 μL of rat serum was added and the mixture was incubated at 37°C for 4 h to deconjugate polyglutamylated tails.

II)for di-enzyme extraction A, 20 μL α-amylase (40 mg/mL) was added, mixed well, and incubated at 37°C for 30 min; the mixture was boiled for 10 min to deactivate the α-amylase, cooled on ice, then 30 μL of rat serum were added, incubated at 37°C for 4 h.

III) for di-enzyme extraction B, 15 μL protease (2 mg/mL) was added, mixed well, and incubated at 37°C for 1h; the mixture was boiled for 10 min to deactivate the protease, cooled on ice, then 30 μL of rat serum were added and incubated at 37°C for 4 h.

IV) for tri-enzyme extraction, 20 μL of α-amylase (40 mg/mL) was added, mixed well, and incubated at 37°C for 30 min; next, 15 μL of protease (2 mg/mL) was added, incubated at 37°C for 1 h; subsequently, the sample was boiled for 10 min, cooled on ice, then 30 μL of rat serum was added and incubated at 37°C for 4 h.

The following procedure was same for the four methods: the sample was boiled for 10 min, cooled on ice for 10 min, and centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was transferred to a 3-kDa ultra-filtration tube (Millipore, Billerica, USA) for clean-up and centrifuged at 13,000 rpm at 4 °C for 20 min. Finally, the resulting solution was used for direct folate detection. MTX at a final concentration of 20 ng/mL was used as the internal standard and added to the extraction buffer at the beginning of the extraction procedure.

2.5. Matrix effect

The matrix effect (ME) was determined using the method of standard addition. Standard solutions of folates and MTX were added to the same volume of folate extraction buffer or blank cereal extracts. An absolute blank matrix is difficult to be obtained. Here, three cultivars of each cereal crops (mentioned in 2.1) were used for preparation of blank matrix. 50 mg of ground powder of each cereal grains was mixed with 1 mL of distilled ultra-pure water, and was heated for 30 min in a boiling water bath, and then 0.1 *g* of activated charcoal was added, followed by incubation for 1 h exposed to light. The ground suspension was centrifuged at 13,000 rpm for 30 min, and the resulting supernatants were filtered through 3 kDa filtration tubes. The filtrate of the three cultivars of each cereal were combined and used as matrices, respectively. The combined filtrate was stored at −20 °C and mixed with same volume of 2X extraction buffer A before use. The endogenous folate contents in the treated matrix were confirmed below the limit of detection. The ME (%) was calculated as B /A × 100 as previously described (Matuszewski et al., 2003), where A is the peak area of folate standards in extraction buffer, B is the peak area of the same amount of standards in blank crop extracts (n = 5). Folate standards of 10 μL (100 ng/mL) were mixed with 90 μL of extraction buffer A or 90 μL of blank l crop solution, and were directly subjected to LC–MS/MS after preparation.

2.6. Linearity and sensitivity

The linearity of the HPLC-MS/MS method was evaluated by preparing nine-point (1, 2, 5, 10, 50, 100, 200, 500, 1000 ng/mL) calibration curves with blank extract solution for MTX and each folate derivative (5,10 − CH = H_4PteGlu, 5 − CHO-H_4PteGlu, 5 − CH_3-H_4PteGlu, H_2PteGlu, PteGlu, 10 − CHO-PteGlu, MeFox and H_4PteGlu); linearity was evaluated by plotting the peak area at different concentrations and calculating the correlation coefficients (R^2). Sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ). A series of dilution of folate standards was prepared in the blank crop solutions. The LOD was defined as the amounts of analyte at which the analytical process could reliably differentiate from background levels and was accepted when the intensity of the signal was threefold the background noise (S/N > 3). The LOQ was defined as the amounts of analyte at which quantitative results can be reported with a high degree of confidence, which was accepted when the intensity of the signal was tenfold the background noise (S/N > 10).

2.7. Recovery and precision

Recovery indicates the extraction efficiency of the method. The recovery experiment was performed by spiking cereal samples with folate standards at high (50 ng/mL), medium (20 ng/mL), and low (5 ng/mL) concentrations at the beginning of folate extraction. The absolute recovery was calculated using the formula: R (%) = C/B × 100 (Matuszewski et al., 2003), where B is the peak area of folate standard in spiked blank matrix solution, C is the peak area of folate standard in spiked blank matrix solution, which went through the entire extraction procedure (n = 5).

To assess inter-day precision, samples of each cereal crop were prepared (n = 6) and extracted simultaneously, then analysed on three different days. The intra-day precision was determined by preparing and extracting samples (n = 6) simultaneously, then analysing them in one batch.

2.8. Application of the method

This method was applied to determine folate concentration in maize, rice and wheat grains. For each cereal, three different cultivars were selected (details mentioned in 2.1). After harvest, the whole grains of maize and wheat, and unpolished rice seeds were stored at −80 °C until analysis. About 10 grains of maize, 20 seeds of rice and wheat were grounded into fine powder. Extraction buffer (1 mL) was added to 50 mg of fine powder and mixed homogenously, and then the sample preparation was continued following the di-enzyme extraction A with α-amylase and rat serum treatment. Three biological replicates were used for each cereal cultivars.

2.9. Statistical analysis

The data of folate derivatives in cereal samples were shown as means ± standard deviations of three biological replicates.A student *t*-test was used to indicate the significance of folate concentrations between di-enzyme treatment and mono-enzyme treatment, and between tri-enzyme and mono-enzyme treatment in rice samples.

3. Results and discussion

3.1. Enzymatic folate extraction

Complete release of folates from plant and food matrices is vital for accurate determination of folate content. Here, to evaluate the effects of

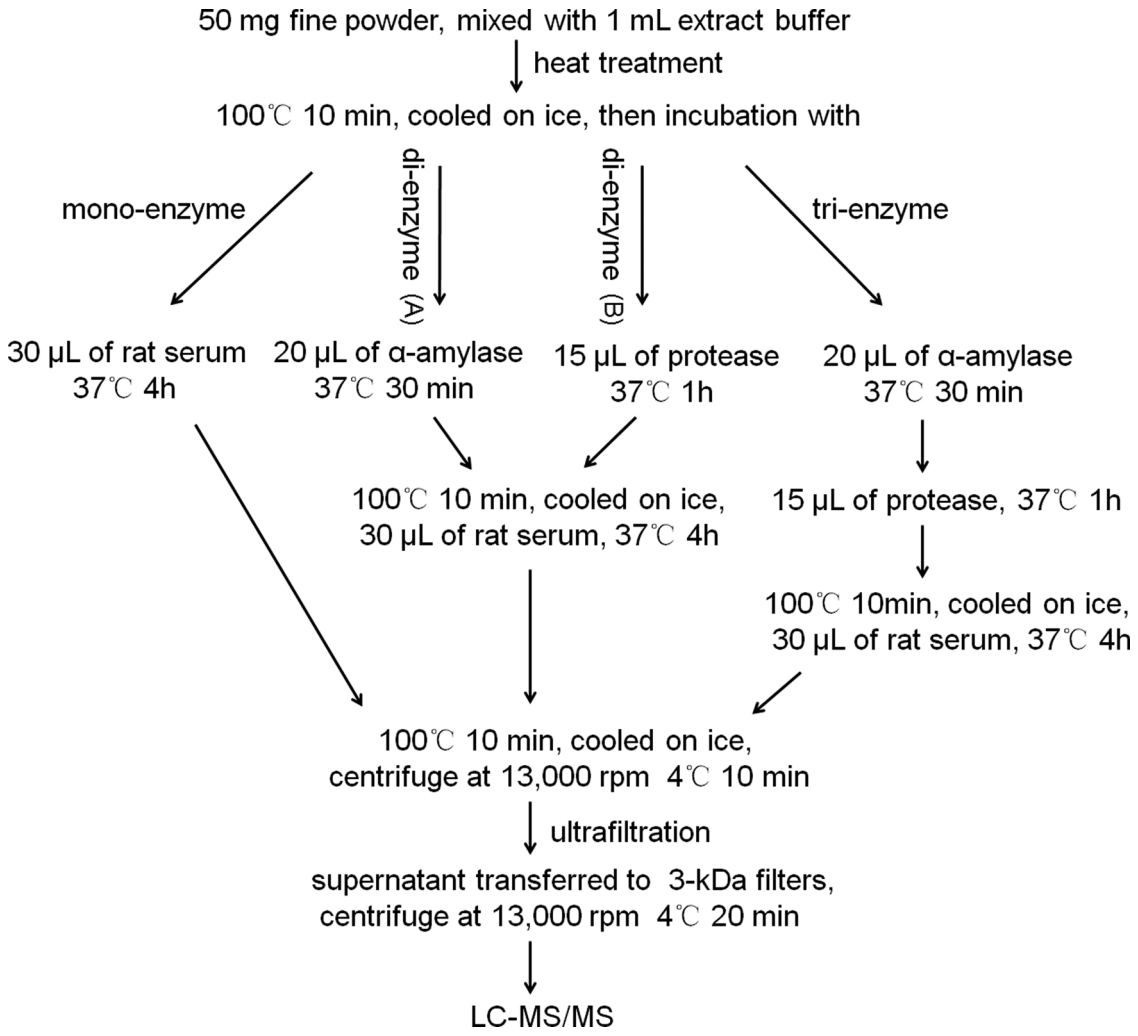

Fig. 1. Procedure diagram of mono-/di-/tri-enzyme extraction of folates from cereal grains.

Table 1
Folate content (μg/100 g) in rice, maize, and wheat assessed with four extraction methods.

Crops	Methods	Folate content (μg/100 g grains) (mean ± standard deviation)								
		10-CHO- PteGlu	5,10-CH= H_4PteGlu	5-CHO- H_4PteGlu	5-CH_3-H_4PteGlu	H_2PteGlu	PteGlu	H_4PteGlu	MeFox	Total folates
Rice	Mono-enzyme	1.22 ± 0.4	0.99 ± 0.1	6.17 ± 0.8	7.89 ± 0.7	0.45 ± 0.2	1.04 ± 0.2	1.07 ± 0.1	753 ± 58	18.8 ± 3.1
	Di-enzyme (A)	1.68 ± 0.2	1.41 ± 0.4	11.5 ± 1.6	10.3 ± 0.6	1.72 ± 0.3	1.43 ± 0.3	2.09 ± 0.1	732 ± 27	30.1 ± 3.6*
	Di-enzyme (B)	1.16 ± 0.4	1.63 ± 0.3	6.95 ± 0.9	6.15 ± 0.3	0.92 ± 0.5	1.18 ± 0.1	1.09 ± 0.4	815 ± 9.4	18.1 ± 2.0
	Tri-enzyme	1.24 ± 0.3	1.56 ± 0.2	9.85 ± 1.6	8.40 ± 0.1	1.66 ± 0.2	1.17 ± 0.1	2.04 ± 0.5	600 ± 37	25.9 ± 4.6*
Maize	Mono enzyme	1.36 ± 0.1	1.49 ± 0.2	13.2 ± 0.3	15.1 ± 0.3	0.75 ± 0.1	0.89 ± 0.1	1.49 ± 0.1	33.5 ± 2.2	34.3 ± 1.2
	Di-enzyme (A)	1.47 ± 0.1	1.52 ± 0.4	13.7 ± 1.9	17.0 ± 0.8	0.80 ± 0.2	1.02 ± 0.3	1.51 ± 0.8	36.6 ± 1.4	37.1 ± 4.3
	Di-enzyme (B)	1.09 ± 0.5	1.46 ± 1.1	12.5 ± 1.1	14.1 ± 1.5	0.76 ± 0.1	0.76 ± 0.1	1.27 ± 0.4	31.32 ± 2.1	31.8 ± 2.6
	Tri-enzyme	1.29 ± 0.5	1.51 ± 0.3	12.1 ± 0.8	15.0 ± 0.4	0.77 ± 0.1	0.80 ± 0.2	1.52 ± 0.3	31.56 ± 3.1	32.9 ± 1.4
Wheat	Mono-enzyme	1.32 ± 0.2	2.46 ± 0.5	17.7 ± 1.4	6.81 ± 0.3	0.51 ± 0.3	0.88 ± 0.2	1.55 ± 0.2	1263 ± 26	31.2 ± 1.5
	Di-enzyme (A)	1.65 ± 0.5	2.81 ± 0.4	18.7 ± 1.7	10.3 ± 0.9	0.84 ± 0.1	1.09 ± 0.1	1.62 ± 0.5	1136 ± 82	37.0 ± 1.4
	Di-enzyme (B)	1.06 ± 0.4	2.21 ± 0.6	16.0 ± 1.0	6.93 ± 1.1	0.47 ± 0.1	1.06 ± 0.2	1.30 ± 0.3	1220 ± 142	29.1 ± 2.7
	Tri-enzyme	1.18 ± 0.3	2.01 ± 0.4	17.3 ± 1.6	6.41 ± 1.2	0.81 ± 0.3	1.03 ± 0.2	1.63 ± 0.4	974 ± 105	30.4 ± 4.2

Mean comparisons of total folate data of Di-Enzyme (A) and Tri-Enzyme with Mono-Enzyme were analysed. *$p < 0.05$.

amylase and protease treatment on folate extraction from cereal grains, four methods including mono-enzyme (rat serum) treatment; di-enzyme (A, α-amylase + rat serum; B, protease + rat serum), and tri-enzyme (α-amylase, protease, and rat serum) treatment were assessed (Fig. 1), and harvest grains of rice (Daohuaxiang 2), maize (B73) and wheat cultivars (Han6172) were used for this purpose. Compared to mono-enzyme treatment, the total folate content of rice grains was significantly elevated by di-enzyme (A) treatment and tri-enzyme treatment to 1.6- and 1.37-fold ($P < 0.05$) that of mono-enzyme treatment, respectively (Table 1). The release of 5,10 − CH = H_4PteGlu, 5 − CHO-H_4PteGlu, 5 − CH_3-H_4PteGlu, H_2PteGlu, PteGlu, H_4PteGlu, and 10 − CHO-PteGlu was increased by α-amylase treatment. Compared to mono-enzyme treatment, 1.3- and 1.86-fold ($p < 0.05$) elevation of the concentrations of the two major folate derivatives, 5 − CH_3-H_4PteGlu and 5 − CHO-H_4PteGlu, were observed in di-enzyme (A) treatment. Protease treatment had a weaker effect on folate release from rice,

suggesting that heat treatment was able to denatured folate-bound proteins. These results suggested that α-amylase digestion was necessary for folate extraction from rice matrix, consistent with the findings of previous reports (De Brouwer et al., 2008). Here, di-enzyme (A) treatment with the combination of α-amylase and rat serum was found more efficient for folate extraction from rice grains than the tri-enzyme treatment, and the application of di-enzyme method showed great advantages with simplified extraction steps.

Compared to the mono-enzyme treatment, the application of α-amylase in the di-enzyme treatment had a slight effect on folate release from maize and wheat samples; 1.08- and 1.18-fold increases were observed, respectively (Table 1). No significant effect was observed in di-enzyme (B) and tri-enzyme treatments. For maize and wheat grains, mono-enzyme treatment could yield approximately 85–93 % of the folate data of the di-enzyme (A) treatment, and would be applied for high-throughput analysis of large numbers of samples, given the simplified extraction procedure. In addition, the α-amylase and protease in the tri-enzyme method did not further increase the folate yield, possibly due to folate degradation during additional enzyme incubation and deactivation steps. On the whole, the application of di-enzyme treatment with α-amylase and rat serum could produce the most optimal folate data among the three crops, thus, it was used for folate extraction in the subsequent experiments.

3.2. Optimisation of the HPLC and MS/MS conditions

The HPLC-MS/MS method has been used for analysis of folates in food crops and showed great advantages in accuracy and high resolution for folate derivatives (Upadhyaya et al., 2017). Use of a stepwise gradient of 0.1 % (v/v) of formic acid in water (solvent A) and acetonitrile (solvent B) on a C18 reverse-phase HPLC column reportedly enables resolution of folate derivatives and the folate analogue MTX (Tyagi et al., 2015). The gradient of the organic solvent and the column length should be optimised to retain folate compounds and reduce the total gradient time (Shahog et al., 2017). In this study, an Akzo Nobel analytical column (Kromasil 100 − 5 C18, 50 × 2.1 mm) was used, and the gradient of organic solvent were modified (details in material and methods 2.3). The total gradient time was 16.5 min, and the retention time of H_4PteGlu, 5 − CH_3-H_4PteGlu, 5,10 − CH = H_4PteGlu, 10 − CHO-PteGlu, 5 − CHO-H_4PteGlu, H_2PteGlu, PteGlu, and MTX were 1.99, 2.53, 5.08, 5.85, 6.25, 6.79, 7.13, and 8.62 min, respectively (Table 2). This chromatographic condition could achieve a good resolution of the identified eight folate derivatives and analogs. Folate detection was performed with an Agilent 6420 triple-quadrupole tandem MS operated in positive electrospray ionisation (ESI) mode. The multiple reaction monitoring (MRM) parameters including the precursor ion, the product ion and collision energy (eV) was optimised for fragmentation of folate standards, and one major product ion for each folate was selected for the subsequent analysis. The MRM parameters were listed in Table 2. For most folate standards, the major product ions were formed by a neutral loss of the glutamate residue (-147 loss); except for 5,10 − CH = H_4PteGlu, a loss of CO_2 (-44) was observed, for 10 − CHO-PteGlu, combined loss of a glutamate and a CO moiety, and for H_2PteGlu, loss of both glutamate and *p*-aminobenzoate. Previously, an additional peak was detected in the MRM chromatogram of the 5-F-THF trace (474→327) in several foodstuffs; the compound was assumed to be an oxidation product of 5-M-THF, a pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF, also known as MeFox (Ringling and Rychlik, 2017a). In this study, a large peak with similar behaviour to that of 5-F-THF was also detected in cereal grains, and this compound was confirmed to be MeFox with the commercial standard. Using the optimised HPLC conditions, the retention time of MeFox was 4.1 min; good separation of MeFox and 5-F-THF was vital to prevent misidentification and guarantee accuracy.

Table 2
Retention times and MRM parameters of folate derivatives.

Folates	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
10-CHO-PteGlu	5.85	470	295	20
5,10-CH = H_4PteGlu	5.08	456	412	30
5-CH_3-H_4PteGlu	2.53	460	313	20
5-CHO-H_4PteGlu	6.25	474	327	20
PteGlu	7.13	442	295	20
H_2PteGlu	6.79	444	178	20
H_4PteGlu	1.99	446	299	20
MTX	8.62	455	308	30
MeFox	4.1	474	327	20

3.3. Matrix effect (ME)

The ME is one of the limitations of atmospheric pressure ionisation interfaces, but soft ionisation enhances the success of LC–MS (Dams et al., 2003). Assessment of the ME is necessary for the development of LC–MS/MS methods. Co-elution of undetected matrix compounds could result in improvement or suppression of the signal, thus affecting the reproducibility, sensitivity, or accuracy of the assay (Matuszewski et al., 2003; Smith et al., 2006). In this study, standard solutions of folates and MTX were added to the same volume of folate extraction buffer or blank cereal extracts to 10 ng/mL. The MEs of the folate derivatives 5,10 − CH = H_4PteGlu, 5 − CHO-H_4PteGlu, 10 − CHO-PteGlu, 5 − CH_3-H_4PteGlu, PteGlu, and MeFox and the folate analogue MTX were 84.6–107 %, 85.1–119 %, and 81.7–112 % in rice, maize, and wheat, respectively (Table 3). H_4PteGlu and H_2PteGlu had ME values of 63.94–76.4 % in rice and wheat samples, respectively. On the whole, the MEs of the nine analytes in this study were within acceptable ranges.

3.4. Linearity and sensitivity

The linearity of the LC–MS/MS method was evaluated by using the multi-point standard calibration curve of folate derivatives in triplicate. The correlation coefficients (R^2) for all these folates were approximately > 0.99 in the three cereal matrices (Table 4), demonstrating good linearity within a specific concentration range. The sensitivity of the method was evaluated by determining the LOD and LOQ using the standard calibration curve of the folate derivatives in blank matrix. The LODs were 0.03–0.88, 0.05–0.4, and 0.07–0.25 μg/100 g in rice, maize, and wheat, respectively; the LOQs were 0.1–1.0, 0.4–0.75, and 0.2–0.85 μg/100 g in rice, maize, and wheat, respectively. The R^2, LOD, and LOQ values of this method were within the verified range (Table 4).

3.5. Recovery and internal standard

The absolute recovery of each folate derivative was determined by adding standard solutions of low (5 ng/mL), medium (20 ng/mL), and

Table 3
Matrix effects of folate derivatives and folate analogues.

Folates	Matrix effect (%)		
	Rice	Maize	Wheat
10-CHO-PteGlu	103	98.6	105
5,10-CH = H_4PteGlu	89.2	85.1	84.6
5-CHO-H_4PteGlu	107	119	112
5-CH_3-H_4PteGlu	84.6	87.5	81.7
H_2PteGlu	73.6	76.4	69.8
PteGlu	105	106	109
MTX	101	95.8	105
H_4PteGlu	67.1	63.9	65.4
MeFox	106	97.5	102

Table 4
Linearity and sensitivity measurements of folate derivatives and folate analogues.

Crops	Folates	LOD (μg/100 g)	LOQ (μg/100 g)	Slope (mean ± standard deviation, n = 3) (x 10^{-3})	R^2	Concentration range (μg/100 g)
Rice	10-CHO-PteGlu	0.25	0.75	267 ± 3	0.9985	0.75–400
	5,10-CH = H_4PteGlu	0.75	1.0	506 ± 7	0.9987	1.0–400
	5-CHO-H_4PteGlu	0.76	1.0	482 ± 5	0.9991	1.0–400
	5-CH_3-H_4PteGlu	0.88	1.0	247 ± 4	0.9993	1.0–400
	H_2PteGlu	0.5	0.93	82.7 ± 3	0.9991	0.93–400
	PteGlu	0.25	0.85	420 ± 5	0.9948	0.85–400
	MTX	0.15	0.3	193 ± 3	0.9987	0.3 – 400
	H_4PteGlu	0.03	0.1	925 ± 5	0.9988	0.1–400
	MeFox	0.22	0.5	208 ± 6	0.9982	0.5–1500
Maize	10-CHO-PteGlu	0.3	0.45	230 ± 5	0.9995	0.45–400
	5,10-CH = H_4PteGlu	0.1	0.5	191 ± 4	0.9996	0.5–400
	5-CHO-H_4PteGlu	0.1	0.5	312 ± 4	0.9994	0.5–400
	5-CH_3-H_4PteGlu	0.2	0.4	265 ± 5	0.9997	0.4–400
	H_2PteGlu	0.4	0.7	112 ± 10	0.9991	0.7–400
	PteGlu	0.3	0.5	225 ± 6	0.9988	0.5–400
	MTX	0.1	0.5	100 ± 4	0.9996	0.5–400
	H_4PteGlu	0.05	0.75	139 ± 3	0.9984	0.75–400
	MeFox	0.2	0.4	210 ± 6	0.9978	0.4–1500
Wheat	10-CHO-PteGlu	0.08	0.2	109 ± 4	0.9966	0.2–400
	5,10-CH = H_4PteGlu	0.12	0.3	207 ± 6	0.9982	0.3–400
	5-CHO-H_4PteGlu	0.08	0.22	290 ± 5	0.9983	0.22–400
	5-CH_3-H_4PteGlu	0.07	0.15	288 ± 10	0.9977	0.15–400
	H_2PteGlu	0.15	0.5	116 ± 9	0.9984	0.5–400
	PteGlu	0.25	0.85	210 ± 5	0.9981	0.85–400
	MTX	0.1	0.5	111 ± 3	0.9985	0.5–400
	H_4PteGlu	0.2	0.8	142 ± 7	0.9992	0.8–400
	MeFox	0.2	0.4	212 ± 8	0.9965	0.4–1500

Table 5
Absolute recoveries (%) of folate derivatives and folate analogues.

Folates	Absolute recovery (%)								
	Rice			Maize			Wheat		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
10-CHO-PteGlu	85.2	89.1	84.7	83.1	87.4	90.3	89.6	92.5	86.8
5,10-CH = H_4PteGlu	78.6	76.5	81.7	82.1	79.6	75.2	72.1	76.2	79.7
5-CHO-H_4PteGlu	90.2	89.5	92.7	91.4	86.3	85.6	88.2	92.4	87.6
5-CH_3-H_4PteGlu	84.2	82.7	80.1	83.5	81.2	85.9	83.5	87.3	81.6
H_2PteGlu	48.4	58.5	55.7	54.3	56.4	61.7	63.7	65.2	61.5
PteGlu	43.6	52.6	55.3	51.4	52.8	54.8	53.6	60.7	58.2
MTX	92.6	86.5	96.3	86.3	87.2	83.1	85.3	88.3	83.9
H_4PteGlu	56.3	57.9	52.7	48.2	52.7	54.6	49.7	58.2	56.3
MeFox	92.4	91.7	89.8	93.2	94.7	96.2	91.3	94.5	93.8

high (50 ng/mL) concentrations of the folate derivatives to the blank sample homogenate at the initiation of the extraction procedure. The recoveries of 10 – CHO-PteGlu, 5,10 – CH = H_4PteGlu, 5 – CHO-H_4PteGlu, 5 – CH_3-H_4PteGlu, MeFox, and MTX were in the ranges of 76.5–96.3 %, 75.2–91.4 %, and72.1–92.5 % in rice, maize, and wheat samples, respectively (Table 5). Recovery of H_2PteGlu, PteGlu, and H_4PteGlu was within the range of 43.6–65.2 %.

An internal standard is necessary for accurate and reproducible quantification to compensate for the loss of target analytes during sample preparation, as well as to correct for the ME and instrumental sensitivity in LC–MS (Rychlik, 2004). Stable isotope-labelled folate standards are ideal, but have limited commercial availability and are costly. Another option is the use of analogues, which have chemical and chromatographic properties similar to those of folates; therefore, MTX has been used for folate detection in plants (Zhang et al., 2005; Tyagi et al., 2015). In this study, MTX showed good ME and recovery values; thus, it was used as the internal standard for folate detection.

3.6. Inter-day and intra-day precision

The precision of the method was evaluated by performing inter- and intra-day runs of the three cereal folate extracts. The intra-day precision was calculated in a single batch with six replicates for each cereal sample; the intra-day precision was calculated in three separate batches, one on each of 3 days. The relative standard deviation (RSD) for inter-day precision and intra-day precision of the folate derivatives were 1.56–7.74 % and 1.61–6.13 % for rice, 1.4–7.77 % and 1.88–8.64 % for maize, and 1.7–11.3 % and 2.9–10.6 % for wheat samples (Table 6). These results demonstrated that the method had good reproducibility and repeatability.

3.7. Application of the method

The di-enzyme (A) treatment and HPLC-MS/MS method was used for folate extraction and determination in several cereal grains (rice cultivars Daohuaxiang2, Nipponbare, and Kitaake; maize inbred lines B73, GEMS9, and DAN3130; and wheat cultivars Han6172, Liangxing99, and Jimai19) to assess its efficiency. The total folate content was 30.2–33.7, 33.4–37.1, and 26.2–37.0 μg/100 g in rice, maize, and wheat samples, respectively (Fig. 2). 5 – CHO-H_4PteGlu and 5 – CH_3-H_4PteGlu were the major folate derivatives, constituting 38 % and 34 %, 36 % and 45 %, and 46 % and 27 % of the total folates in rice

Table 6
Inter-day and intra-day precision measurements.

Folates	Rice		Maize		Wheat	
	intra-day precision RSD (%)	inter-day precision RSD (%)	intra-day precision RSD (%)	inter-day precision RSD (%)	intra-day precision RSD (%)	inter-day precision RSD (%)
10-CHO-PteGlu	6.3	5.7	1.4	6.5	11.3	6.1
5,10-CH=H_4PteGlu	5.5	6.2	1.6	1.9	10.5	5.4
5-CHO-H_4PteGlu	1.6	4.5	7.8	4.3	1.7	5.5
5-CH_3-H_4PteGlu	4.6	2.1	6.3	5.7	5.5	8.3
H_2PteGlu	2.8	1.6	4.2	6.8	1.7	5.4
PteGlu	1.8	5.7	6.7	8.6	8.8	10.6
MTX	1.5	3.6	2.6	1.9	6.5	6.5
H_4PteGlu	7.7	6.1	4.5	4.5	7.4	2.9
MeFox	3.6	2.2	3.2	6.4	3.2	4.7

cultivar Daohuaxiang2, maize inbred line DAN3130, and wheat cultivar Liangxing 99, respectively. Minimal amounts of 5,10−CH=H_4PteGlu, H_2PteGlu, and PteGlu were found. PteGlu and 10−CHO-H_4PteGlu are not natural folate derivatives biosynthesised by plants, and they are oxidation products generated by degradation (De Brouwera et al., 2010). The folate content of rice samples determined in the present study (approximately 30 μg/100 g) was within the range of folate contents in rice cultivars 13−111 μg/100 g (Dong et al., 2011) and 11−51 μg/100 g (Ashokkumar et al., 2018). The folate levels of wheat grains detected in this study (approximately 26–37 μg/100 g) were in agreement with the values previously reported in wheat samples (ranged from of 10−91 μg/100 g with an average of 36.7 μg/100 g) (Riaz et al., 2019). Considering that the crop variety, harvest stage, growth environment, storage condition and time, analysis procedure may impact folate content (Iniesta et al., 2009; Jha et al., 2015), thus it was hard to do accurate comparison among different research.

Here, a large amount of MeFox was detected in the three cereal grains (Table7). In maize, the content of MeFox was comparable to the total folate level. In rice and wheat, the MeFox levels were 20–39-fold higher than the total folate content. MeFox has been found as an autoxidation product of 5−CH_3-H_4PteGlu: 5−CH_3-H_4PteGlu is autoxidised into 5-methyl-5,6-H_2PteGlu in the presence of O_2; 5−CH_3-H_4PteGlu and 5-methyl-5,6-H_2PteGlu are oxidised by hydrogen peroxide to 4a-hydroxy-5-methyl-4a,5,6,7-H_4PteGlu; in the absence of a reducing agent, 4a-hydroxy-5-methyl-4a,5,6,7-H_4PteGluundergoes structural rearrangement to a pyrazino-s-triazine derivative, also known as MeFox (Fazili and Pfeiffer, 2013). MeFox is present in long-term frozen-stored serum and plasma specimens and in fresh-frozen specimens (Fazili and Pfeiffer, 2013). MeFox has been found in foodstuffs including fresh vegetables, dried legumes, grain and grain products, and nuts; it was detected at high levels in wheat germs, oat flakes, and walnuts (Ringling and Rychlik, 2017a). However, MeFox is biologically inactive in microbiological assays. The adding of MeFox to 5−CH_3-H_4PteGlu would result in overestimation of both the plasma folate level and folate bioavailability (Ringling and Rychlik, 2017b). The mechanism underlying the accumulation of large amounts of MeFox in rice and wheat seeds, as well as the function of MeFox in plants, need further exploration.

Table 7
Amounts of MeFox in crop grains and MeFox-to-total-folate ratios.

Crops	MeFox (μg /100 g)	Total folates (μg /100 g)	Ratio of MeFox to total folates
Daohuaxiang2	724	30.1	24
Nipponbare	1186	34.7	34
Kitaake	1238	31.7	39
B73	36.6	37.1	0.99
GEMS9	46.4	36.7	1.3
DAN3130	43.9	33.4	1.3
Han6172	1123	37.0	30
Liangxing99	912	26.2	35
Jimai19	1015	26.3	39

4. Conclusion

We developed an improved method for folate extraction and

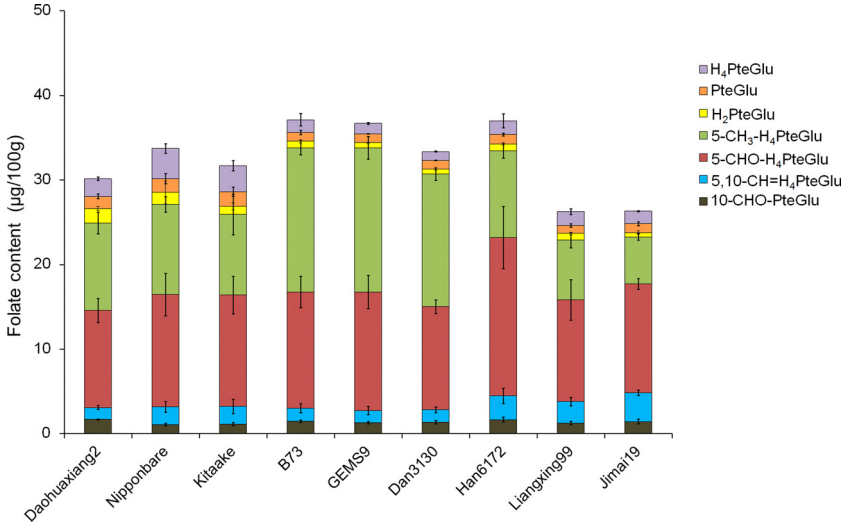

Fig. 2. Folate content and composition of cereal grains measured by the optimised di-enzyme extraction and LC–MS/MS method. Rice cultivars, Daohuaxiang2, Nipponbare, and Kitaake; maize inbred lines, B73, GEMS9, and DAN3130; and wheat cultivars, Han6172, Liangxing99, and Jimai19.

quantification in cereal grains. By use of different extraction methods with mono/di/tri-enzyme treatment, we found that di-enzyme treatment with α-amylase and rat serum was able to accomplish an effective extraction of folates from rice, maize, and wheat grains. The quantification method by LC–MS/MS developed in this study showed great reproducibility and sensitivity. These results will enable investigation of the folate levels in cereal cultivars and screening of materials rich in folates, thus promoting breeding of crops with enhanced folate levels. In this work, the effects of different combination of enzyme treatments on the final results was investigated, which could be suitable for analysis of folates in a wide range of complex matrices, and the validated quantification method for folate determinations can be applied to other crop and plant materials.

Authors' contributions

Q.L. and C.Z. designed and supervised the study. M.S. and Q.L. performed the experiments. M.S., Q.L., T.L., X.W., J.L., and L.H. analysed the data. M.S., Q.L., and C.Z. prepared the manuscript. All of the authors have read the final version of the manuscript and approved its submission for publication.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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