



The University of
Nottingham

UNITED KINGDOM • CHINA • MALAYSIA

Abilgos Ramos, Riza (2010) Folate profiling in wild and transgenic rice. PhD thesis, University of Nottingham.

Access from the University of Nottingham repository:

http://eprints.nottingham.ac.uk/12870/3/Chapter_2.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the University of Nottingham End User licence and may be reused according to the conditions of the licence. For more details see:
http://eprints.nottingham.ac.uk/end_user_agreement.pdf

For more information, please contact eprints@nottingham.ac.uk

2 DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR THE MEASUREMENT OF MONO- AND POLYGLUTAMATED FOLATES IN RICE

Abstract

A sensitive LC-MS/MS method has been developed and validated for the analysis of the mono- and polyglutamated folates in rice grain. Four extraction methods were evaluated in terms of recovery of all folate species. The combination of methanol and phosphate buffer (pH 6.0) extraction with single enzyme (α -amylase) treatment gave the best recovery for most of the folates found in rice grains. Under the optimised method of extraction, enzyme treatment and ultrafiltration, most of the folate forms in the rice matrix were successfully measured. The precision, accuracy and recovery of the method were generally within the accepted guidelines for a quantitative bioanalytical method. Intra- and inter-day precision varied from 0.5 to 16.6% RSD and 2.8 to 20.4% RSD, respectively. The intra- and inter-day accuracy ranged from 83.3 to 117.1% and 81.9 to 125.2%, respectively. This validated method offers a sensitive approach to determine the mono- and polyglutamate folates in a complex rice matrix.

2.1 Introduction

2.1.1 The complexity of folates

A key factor in the science of folate is access to reliable analytical tools. The theoretical number of all native folate metabolites or vitamers is over a hundred in the biological systems (Gregory, 1989; Rebeille *et al.*, 2006). Analysis of this vitamin has always been a complicated and troublesome task because folate exists in ppb-levels and in different forms (Seyoum and Selhub, 1998); many of them are sensitive to degradation and interconversions - during sample pre-treatment folates can easily interconvert i.e. some folate vitamers may convert to others depending on the pH – due to their state of reduction at the 5, 6, 7 and 8 positions of the pteridine moiety (McKillop *et al.*, 2002; Gregory, 1989). These factors make both sample preparation and analysis challenging. The most stable folate form, folic acid (PteGlu), does not exist naturally and is normally produced synthetically and used in pharmaceutical and fortified food products (Eitenmiller and Landen, 1999). The unstable nature of folate, sensitivity to light and heat, and low concentration levels in most cereals make reliable analysis challenging (Rychlik *et al.*, 2004; Munger *et al.*, 2004; Rebeille *et al.*, 2006). In addition, the properties of the cereal matrix with its high level of complex carbohydrates also need to be addressed.

2.1.2 Methods of folate measurement

The most commonly used method in folate analysis is microbiological assay (MA) which provides the single measurement of total folate representing the sum of all folate derivatives. MA is the officially recognised method for folate analysis using *Lactobacillus rhamnosus* ATCC 7469 as the test organism (AOAC, 2000). High-performance liquid chromatographic (HPLC) methods have been developed for the purpose of measuring the various forms of folate in the food matrices (Wigertz and Jagerstad, 1995; Vahteristo *et al.*, 1996; Vahteristo *et al.*, 1997; Pfeiffer *et al.*, 1997; Konings, 1999). Usually, HPLC methods are based on either reversed-phase or ion exchange separation. Polyglutamate folates can be separated and vitamers determined by an ion-pair HPLC method (Varela-Moreiras *et al.*, 1991; Seyoum and Selhub, 1998). The most common HPLC detection methods are UV, diode array, fluorescence, and electrochemical detection (Bagley and Selhub, 2000) and can be used in combinations as Vahteristo *et al.*, (1997) used UV and fluorescence dual detection in confirming peak identity and purity, for instance. In recent years, combining HPLC with mass spectrometry (MS) has gained popularity for its robustness of application and reliability in providing the analytical information about the various forms of folate in a number of matrices. Mass spectrometric detection has the advantage of being accurate and highly specific in measuring various metabolites in one time run. Folate in plant tissues is considered a primary metabolite directly involved in the normal growth and development. Folate

metabolites have more diversified chemical structures that require measurement by a sensitive and specific analytical method such as LC-MS/MS.

Since 1999 tremendous progress has been made using mass spectrometry as a detection technique with improved sensitivity and specificity (De Hoofman and Stroobant, 2002; Poletini, 2006) to measure the relatively low concentrations of folate vitamers in biological samples. It is now considered the method of choice for the quantitative determination of metabolites in biological samples (Dettmer *et al.*, 2007). When combined with the HPLC, MS/MS proved to be very promising in studying individual folate forms and their specificity and sensitivity in complex matrix such as rice. The technique permits the detection of very low levels of target analytes in the presence of the complex matrix background that explains the widespread acceptance of the method today.

2.1.3 Folate extraction and LC-MS/MS technique

The extraction of comprehensive folate populations from plant materials is particularly challenging due to the metabolic and structural characteristics of plant tissues, in addition to the relatively low concentration and instability of the naturally-existing metabolites.

The accuracy and sensitivity of folate analysis is highly dependent on the merits of the preparative methods made such as extraction, enzymatic treatment and

detection techniques. Sample quality, specifically the purity and absence of possible interfering compounds, is unquestionably a critical factor in obtaining adequate chromatographic separation for folate analysis; hence, the folate extraction procedure is of prime importance and sample handling is critical. Since cellular folates are trapped in the matrix of any biological or food material, suitable extraction protocols have to be optimised and/or developed to capture the greatest possible number of folates from a biological sample and it should also be compatible with folate analysis by liquid chromatography tandem mass spectrometry (Polettini, 2006).

The QTRAP™ (Applied Biosystems) which is the MS/MS system used in this study, is a hybrid between a quadrupole and a linear ion trap mass spectrometer enabling the functionality of both techniques in one instrument. Of particular use is precursor ion and neutral loss scans coupled with MS/MS enhanced product ion spectra (EPI). Such capability enables the identification of structurally similar analytes by using a common chemical 'signature' coupled with EPI spectra, providing confidence in the identification of known compounds or aid in the identification of unknowns (Ardrey, 2003; Smith *et al.*, 2006).

As discussed earlier, the primary advantage of chromatography is the ability to separate and quantify individual folate forms which is not possible with MA. Coupled with mass spectrometry (MS or MS/MS), it provides a reliable and robust folate identification and quantitation.

Although folate measurements of its monoglutamated forms have been successfully performed in rice for over a decade now using LC-MS/MS, there are not many existing methods for measurement of its polyglutamated forms that have been reported yet. To fully understand and have a clear target for folate biofortification of staple crops, there is a need of a sensitive and selective method for folate measurement to study not only the monoglutamated but also the various polyglutamated folate forms in cereals like rice.

Here the development of a sensitive and selective analytical method is presented for the quantitative determination of mono- and polyglutamated folate forms in rice by means of liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). Later, the methodology has been applied to the profiling of folate metabolites particularly the intact polyglutamate folates in various rice materials.

2.2 Aims and Objectives

The purpose of this study is to develop and optimise an appropriate folate extraction method in rice that would link with a previously developed LC-MS/MS method for the simultaneous measurement of mono- and polyglutamated folates in plant materials. The method is required to measure the expected low concentration of each folate analyte in rice to enable total folate concentration to be determined. Also, to further optimise the existing LC-MS/MS method in measuring low folate concentrations in rice and in similar food matrices.

2.3 Materials and Methods

2.3.1 Chemicals, reagents, standards and quality control

Solvents were all of HPLC grade. Analytical grade of sodium ascorbate, ammonium acetate, monobasic sodium monophosphate and methanol were purchased from Fisher Scientific (Loughborough, UK) while 2-mercaptoethanol was obtained from Sigma-Aldrich (Poole, UK). Deionized water was obtained from a Maxima USF Elga water system while N,N-dimethylhexylamine was from Acros Organics (Loughborough, UK). Mobile phases were filtered through 0.45 μm polypropylene Whatman[®] Vectaspin microcentrifuge filters (Whatman[®], Maidstone, UK) prior to use.

Folate standards pteroyl-glutamic acid (PteGlu) was obtained from Sigma Aldrich (Poole, UK). Methotrexate (MTX), methotrexate tri-glutamate (MTX-3), methotrexate hexa-glutamate (MTX-6), (6R,S)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CHO-H₄PteGlu), (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CH₃-H₄PteGlu), (6R,S)-5,6,7,8-tetrahydrofolic acid, trihydrochloride (H₄PteGlu), (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid, chloride (5,10-CH₂-H₄PteGlu), (6R,S)-5,6,7,8-tetrahydropteroyltri-L-glutamic acid, trihydrochloride (PteGlu₃), (6R,S)-5-methyl-5,6,7,8-tetrahydropteroyltri-L-glutamic acid, calcium salt (5-CH₃-H₄PteGlu₃), (6R,S)-5,6,7,8-tetrahydropteroyltetra-L-glutamic acid, trihydrochloride

(PteGlu₄), (6R,S)-5-methyl-5,6,7,8-tetrahydropteroyltetra-L-glutamic acid, calcium salt (5-CH₃-H₄PteGlu₄), (6R,S)-5,6,7,8-tetrahydropteroylpenta-L-glutamic acid, trihydrochloride (PteGlu₅), (6R,S)-5-formyl-5,6,7,8-tetrahydropteroylpenta-L-glutamic acid, calcium salt (5-CHO-H₄PteGlu₅), (6R,S)-5,6,7,8-tetrahydropteroylhexa-L-glutamic acid, trihydrochloride (PteGlu₆), (6R,S)-5-formyl-5,6,7,8-tetrahydropteroylhexa-L-glutamic acid, calcium salt (5-CHO-H₄PteGlu₆), (6R,S)-5,6,7,8-tetrahydropteroylocta-L-glutamic acid, trihydrochloride (PteGlu₈) were purchased from Dr B. Schircks Laboratories (Jona, Switzerland). The list of these standards is shown in Table 2.1.

Certified reference materials (CRMs) play a critical role in validating the accuracy of nutrient data for food samples. The CRM 121 used for the quality control of analytical measurement of total and forms of folate was obtained from the European Commission, Institute for Reference Materials and Measurement (Geel, Belgium).

CRM 121 is a whole meal flour which is packaged into food-grade, heat-sealed, aluminium laminate sachets and used as a quality control material to test the method. The indicative total folate content in CRM 121 was 51 µg/100g (Finglas *et al.*, 1999).

Table 2.1. Internal and Folate Standards Used for LC-MS/MS analysis

Form	Precursor Ion (Q1) (m/z)	Product Ion (Q3) (m/z)
PteGlu	440.1	310.8
5,10-CH ₂ -H ₄ PteGlu	456.2	327.0
5-CH ₃ -H ₄ PteGlu	458.4	329.0
5-CHO-H ₄ PteGlu	472.5	315.0
5-CH ₃ -H ₄ PteGlu ₂	587.5	329.0
PteGlu ₃	698.6	128.0
5-CH ₃ -H ₄ PteGlu ₃	716.6	128.0
PteGlu ₄	827.7	128.0
5-CH ₃ -H ₄ PteGlu ₄	845.7	128.0
PteGlu ₅	478.0	128.0
5-CH ₃ -H ₄ PteGlu ₅	487.0	128.0
5-CHO-H ₄ PteGlu ₅	493.8	128.0
PteGlu ₆	542.5	128.0
5-CH ₃ -H ₄ PteGlu ₆	551.5	128.0
PteGlu ₈	671.6	128.0
Methotrexate (MTX)	453.2	324.0
Tri-MTX	711.5	128.0
Hexa-MTX	549.1	128.0

2.3.1.1 Extraction buffer

The following buffers were prepared fresh and stored in 4°C for immediate use:

Aqueous-based – 0.075M KH₂PO₄, 0.052M sodium ascorbate (C₆H₈O₆), 0.1% (v/v) 2-mercaptoethanol, pH 6.0

Concentrated stock – 0.075M KH₂PO₄, 0.4M C₆H₈O₆, 0.8% (v/v) 2-mercaptoethanol (C₂H₆O₅), pH 6.0

Organic-based – concentrated stock: CH₃OH (5:95)

2.3.1.2 Stock solutions

Polyglutamate folate standards (1.0 mg each) were dissolved in 10 mL methanol and water (50:50) containing 20 mM (0.1%) ammonium acetate (3:1, v/v) and 0.1% sodium ascorbate (w/v) to a final concentration of 0.1 mg/ml (pH 6.0) with slight modification (addition of 0.1% 2- β -mercaptoethanol (v/v)). Monoglutamate folate standards were prepared spectrophotometrically (Perkin-Elmer Lambda 7 UV spectrophotometer) in 0.1M sodium phosphate buffer pH 7.0 based on Blakley (1969) method for correct concentrations (Table 2.2). Sonication was necessary to facilitate solubility of the standards. All stock solutions were subdivided into aliquots and stored in amber tubes at -80°C until required for use.

Table 2.2. Extinction coefficients of monoglutamated folates (Source: Blakley, 1969)

Folate Form	λ_{\max} (nm)	ϵ ($M^{-1}cm^{-1}$)	Molecular weight
Folic acid	282	27,600	441.4
5-CH ₃ -H ₄ PteGlu	290	31,700	459.4
5-CHO-H ₄ PteGlu	285	37,200	473.4

2.3.1 Method development and optimisation

Four extraction methods were evaluated with modifications to test the suitability of each to an existing LC-MS/MS method used with other plant materials like broccoli and spinach in a previous study (Garratt *et al.*, 2005; Santoyo Castelazo, 2009) in measurement of mono- and polyglutamated folate forms. Table 2.3 summarises the extraction methods applied to unpolished rice grain.

Table 2.3. Extraction methods used for LC-MS/MS folate measurement in rice grain

METHOD	Enzyme	Deconjugation	Heat treatment	Extraction	pH
Method 1	None	None	None	MeOH/ PO ₄	6.0
Method 2	A-amylase	None	none	MeOHAA/ PO ₄	6.0
Method 3	A-amylase	None	75°C	BAA (+)	6.0
Method 4	A-amylase	none	none	BAA (-)	7.2

MeOH – methanol/PO₄ extraction (Santoyo Castelazo, 2009 method);

MeOHAA – methanol/PO₄ extraction with α-amylase;

BAA (+) – aqueous/NaPO₄ extraction with soft heat treatment and α-amylase (modified Kariluoto *et al.*, 2008 method);

BAA (-) – aqueous/ NaPO₄ extraction with α-amylase (modified Brouwer *et al.*, 2008 method)

2.3.1.1 Method 1 – original MeOH/ PO₄ extraction

The original methanol/phosphate extraction method (MeOH/PO₄) without enzyme and heat treatments involved the homogenisation of 0.5 g rice samples in 3 replicates using a Retsch[®] MM301 ball mill equipment (Retsch Limited, Leeds, UK) for 1.5 min at 30.0 Hz and addition of 25 µL of the internal standards (IS) mix-methotrexate (MTX), tri-MTX and hexa-MTX (1:1:1 v/v) having a final concentration of 10 µM and 1.25 mL of ice-cold working organic-based extraction buffer (concentrated stock of 75 mM KH₂PO₄, 0.4M ascorbic acid, 0.8%(v/v) 2-mercaptoethanol, pH 6.0 in methanol (CH₃OH) (5:95)) afterwards. The solution was vortex mixed for 5 min. The sample extracts were centrifuged (15 000 g, 10 min, 4°C). The supernatant was collected and stored temporarily at -20°C for about 30 min. The residue was re-suspended with 1.5 mL organic-based extraction buffer (75 mM KH₂PO₄, 0.4 M ascorbic acid, 0.8%(v/v) 2-mercaptoethanol, pH 6.0), vortex mixed for 5 min and again centrifuged for 10 min at 15,000 x g at 4°C. The second

supernatant was collected, added to the first supernatant obtained and evaporated to dryness under nitrogen gas. After drying, it was reconstituted with 200 μ L of aqueous extraction buffer (75 mM KH_2PO_4 , 52 mM ascorbic acid, 0.1% (v/v) 2-mercaptoethanol, pH 6.0 and ultrafiltrated using 0.45 μ m Whatman[®] Vectaspin propylene microfilter (Whatman, Maidstone, UK) by centrifugation for 3 min. The sample was transferred to an amber HPLC vial with 200 μ L insert for LC-MS/MS analysis afterwards.

2.3.1.2 Method 2 - MeOHAA/ PO_4 extraction

The second method tested was a combination of MeOH/ PO_4 and α – amylase (20 μ L of 0.5 mg/mL) treatment. Three replicates of 0.5g rice grains were also used and homogenised as stated in Method 1. α – amylase was added on the first extraction with buffer and internal standards after homogenisation. Vortex mixing was modified by increasing to 15 min each time. Centrifugation was kept at 15000 x g for 10 min at 4°C. After collecting the first supernatant, the sample was re-suspended with 1.25 mL organic-based extraction buffer described in Method 1, vortex mixed for another 15 minutes and centrifuged for 10 min at 15000 x g. The second supernatant was collected and combined with the first one, mixed and dried under nitrogen gas before reconstitution with 200 μ L aqueous extraction buffer described in Method 1. Ultracentrifugation was made for 3 min using 0.45 μ m Whatman[®] Vectaspin propylene microfilter (Whatman, Maidstone, UK) before keeping the sample at -80°C prior to LC-MS/MS analysis.

2.3.1.3 Method 3 - BAA (+) modified phosphate extraction (Kariluoto *et al.*, 2008)

The third method of extraction was a modification of Kariluoto *et al.* (2008) published method on rye folate extraction. It involved the NaPO₄ (0.1 M, pH 6.1, 2% (w/v) Na ascorbate and 0.1%(v/v) 2-β-mercaptoethanol) extraction with soft heat treatment at 75°C for 5 min after addition of α-amylase and incubation at 37°C for 3 hours. Three replicates of 0.5g rice sample were used and vortex mixing was for 5 min and centrifugation at 15000 x g. The supernatant was filtered using 0.45 μm Whatman® Vectaspin propylene microfilter (Whatman, Maidstone, UK) and kept in -80°C prior to LC-MS/MS analysis.

2.3.1.4 Method 4 – BAA (-) modified aqueous/phosphate extraction (de Brouwer *et al.*, 2008)

The fourth method tried for folate extraction in rice was a modification from the NaPO₄ (50mM, with 1% ascorbic acid and 0.5% of 2-β-mercaptoethanol, pH 7.2) extraction published for monoglutamates in rice (de Brouwer *et al.*, 2008). Only α-amylase was used to treat the samples after homogenisation which followed the reported amount of 10 μL (0.5mg/mL). Incubation with α-amylase was for one hour. Boiling which is part of the original method was not done to preserve the less stable folate forms and polyglutamates. Vortex mixing was for 5 min and centrifugation for

10 min at 15000 x g. Re-suspension with 1 mL of the phosphate buffer was made after collecting the first supernatant. Vortex mixing was for another 5 min while ultracentrifugation was for another 10 min at 15000 x g. The second supernatant collected was combined with the first before ultrafiltration with 0.45 µm Whatman® Vectaspin propylene microfilter (Whatman, Maidstone, UK) for 3 min using the centrifuge. All samples were maintained at 4°C in the HPLC autosampler or at -80°C before analysis by LC-MS/MS.

2.3.3 Mono- and polyglutamated folate measurement using LC-MS/MS

2.3.3.1 Sample preparation and extraction for LC-MS/MS analysis

The method of extraction used the MeOH/PO₄ with α-amylase treatment as described in the previous section based on the recovery test results conducted.

Three replicates of 0.5 g per variety of rice were weighed and homogenized for 1.5 min at 30 Hz for 1.5 min using the Retsch® MM301 ball mill equipment. Twenty five µL of the internal standards (IS) mix- methotrexate (MTX), tri-MTX and hexa-MTX having a final concentration of 10 µM, 20 µL of 0.5 mg/mL α - amylase and 1.25 mL of ice-cold working organic-based extraction buffer (concentrated stock of 75 mM KH₂PO₄, 0.4M ascorbic acid, 0.8%(v/v) 2-mercaptoethanol, pH 6.0 in methanol (CH₃OH) (5:95)) were added to each sample replicate and the solution was vortex

mixed for 15 min. The sample extracts were centrifuged (15 000xg, 10 min, 4°C). The supernatant was collected and stored temporarily at -20°C for 25 min. The residue was re-suspended with 1.5 mL organic-based extraction buffer (75 mM KH_2PO_4 , 0.4M ascorbic acid, 0.8%(v/v) 2-mercaptoethanol, pH 6.0), vortex mixed for 15 min and again centrifuged for 10 min at 15,000 x g at 4°C. The second supernatant was collected, added to the first supernatant obtained and evaporated to dryness under nitrogen gas. After drying, it was reconstituted with 200 μL of aqueous extraction buffer (75 mM KH_2PO_4 , 52 mM ascorbic acid, 0.1% (v/v) 2-mercaptoethanol, pH 6.0 and ultrafiltrated using 0.45 μm Whatman Vectaspin propylene microfilter by centrifugation for 3 min. The sample is transferred to amber HPLC vial with 200 μL insert for analysis afterwards. All samples were maintained at 4°C in the HPLC autosampler or stored at -80°C before analysis by LC-MS/MS. All the extraction steps were done under subdued light and in amber tubes to prevent folate oxidation.

The plant QC sample used in the LC-MS/MS method was 200 mg spinach extracted using the same extraction buffers stated above but with only 0.5 mL in total volume. Initial homogenisation of the QC sample was made using mortar and pestle which was constantly cooled with liquid nitrogen. Further homogenisation was made using the Retsch[®] MM301 ball mill equipment at 30 Hz for 1 min, after the addition of 25 μL internal standards (IS) mix and of 0.5 mL of ice-cold working organic-based extraction buffer. The rest of the preparation and extraction steps were the same as described previously for rice. Three replicates of rice extracts

with known concentration (500 nM) of standard mixture of 15 folate analytes (spiked samples) shown in Table 2.1 were also prepared to verify the folate recovery from the sample matrix.

2.3.3.2 LC-MS/MS analysis

HPLC analysis was performed using Shimadzu VP series HPLC system (Milton Keynes, UK) consisting LC-10ADVP high performance pump, SIL-HT autosampler, SCL-10AVP system controller and CTO-10Avp column ovens. Luna C18 (2) 100Å analytical column (150 x 2.0 mm, 5 µm particle size) and its compatible C18 guard column were procured from Phenomenex (Macclesfield, UK) and were used throughout the experiment. Mobile phase A consisted of methanol/water (5:95, v/v) with 5mM dimethylhexylamine at pH 8.1 which was adjusted by titration with formic acid (MS grade). Mobile phase B was 5 mM dimethylhexylamine in methanol. A linear gradient from 22% B to 80% B over 20.5 min was followed by a 5 min isocratic hold at 80% B. The column was re-equilibrated for 12.5 min at 22% B afterwards. The flow rate for folate separation was 200 µL/min and the volume per injection was 20µL. The column was maintained at 35°C throughout the run.

A hybrid triple quadrupole ion trap mass spectrometer (4000 QTRAP) from Applied Biosystems (Foster City, CA, USA) was run using negative polarity. The TurbolonSpray source conditions were optimized for optimal ionization of folates as follow: gas 1 and 2 at 20 and curtain gas at 40 psi. The ion spray voltage was set at 4

kV and the turbo probe was heated at 500°C while the interface heater was on. The resolution for Q1 and Q3 was set to unit. Commercial standards were infused at 200 µL/min with a syringe pump for MRM transitions. Declustering potential (DP) and collision energies (CE) for each folate standard were optimized for compound dependent parameters using the quantitative optimization wizard of the Analyst software (version 1.4.2) installed in the PC which is connected to the equipment.

2.3.3.3 Calibration and validation

The procedure used to validate the method followed the recommendations for bioanalytical method validation (US Department of Health and Human Services, 2001), and used the following criteria to assess the analytical protocol: linearity, sensitivity, intra-and inter-day accuracy and precision, metabolite recovery and an examination of the influence of matrix effects. Due to the lack of an appropriate “blank” matrix, analytes were spiked into the rice grain to evaluate accuracy, precision, and recovery of the method and corrections were made for the endogenous levels of individual folate forms.

To quantify the endogenous levels of folate metabolites in rice grain, thirteen-point calibration curves containing the following amounts of 15 folate standards: 0.2, 0.5, 1, 3, 5, 10, 25, 50, 100, 250, 500, 1000, 1500 nM, were prepared using the sample extraction procedure, for each of the standard analytes: PteGlu, 5,10-CH₂-H₄PteGlu, 5-CH₃-H₄PteGlu, 5-CHO-H₄PteGlu, 5-CH₃-H₄PteGlu₂, PteGlu₃, 5-CH₃-H₄PteGlu₃,

PteGlu₄, 5-CH₃-H₄PteGlu₄, PteGlu₅, 5-CH₃-PteGlu₅, 5-CHO-H₄PteGlu₅, PteGlu₆, 5-CHO-H₄PteGlu₆ and PteGlu₈. The ratios of the LC-MS/MS peak areas of each analyte and internal standard were calculated and used to construct calibration lines of peak area ratio against analyte concentration using linear regression analysis. The linearity and reproducibility of calibration were assessed in 6 replicate analyses.

To generate a calibration curve, the peak area ratio (metabolite area/IS area) was plotted against increasing concentration of calibrator spiked into the biological matrix. The appropriate internal standard (IS) for each analyte was selected based on structural similarities. The three internal standards used were: methotrexate (MTX) for monoglutamyl folates, tri-MTX for folates with 2,4-conjugated glutamates, and hexa-MTX for folates with five or more conjugated glutamates. The slope, intercept and r^2 value of the calibration lines were determined.

To determine intra- and inter-day precision and accuracy, analytes were spiked into the homogenised rice grains (200 mg) at a low and mid-range concentration (100 and 500 nM, respectively). Five of 100 nM and of 500 nM standard mixture of 15 commercial folate analytes were prepared and ran as blank/neat standards. The same concentrations were prepared in 5 rice sample extracts each for the 100 nM and for the 500 nM and spiked before extraction and another set were spiked after extraction.

Precision was calculated from the relative standard deviation (%RSD) by comparison of measured levels of spiked analytes with expected concentrations (%RSD).

Recovery was calculated by comparing peak area ratios of rice grain samples spiked at the same two concentrations (100 and 500 nM) prior to extraction with those spiked after extraction.

The limit of detection (LOD) was determined using serial dilutions of the standard metabolite mix, without spiking into rice samples, and was defined as the concentration at which a signal/noise ratio of 3:1 was achieved while the limit of quantification (LOQ) was obtained from the analyte response at which a signal/noise ratio of 10:1 was achieved.

Matrix effects were assessed by comparing peak area ratios of homogenised rice grain samples spiked post-extraction with the low and mid-range metabolite concentration (minus calculated endogenous levels in the nonspiked rice samples), to commercial standards mix prepared at low and mid-range (100 and 500 nM) concentrations without spiking into the rice matrix (neat standards).

2.3.3.4 Quantification

Folate forms for which commercially-available standards were available were quantified in rice and spinach extracts using an internal standard method. Quantification of individual folate used three internal standards and extracted calibration standards for all the folate species. The amount of single vitamers was calculated from the peak areas with reference to the internal standards. Methotrexate (MTX) for monoglutamyl folates, tri-MTX (MTX-3) for folates with 2,4-conjugated glutamates, and hexa-MTX (MTX-6) for folates with five or more conjugated glutamates were the three internal standards used. Certified reference material –CRM 121 (whole meal flour) was analysed with a batch of samples and standardised spinach extracts were analysed with every batch of rice samples for quality control purposes.

2.4 Statistical analysis

Significance of differences for the means of folate analytes, total folate concentration and percent distribution of folate derivatives in different samples and treatments were analysed by one way ANOVA with Tukey's post test performed using GraphPad Prism version 4.02 for Windows, GraphPad software, San Diego California, USA, www.graphpad.com.

2.5 Results and Discussion

Table 2.4. Retention times, cone voltages, and collision voltages used to identify and quantify analytes using MRM with the LC-MS/MS method.

Analyte	Retention time (min)	Declustering potential (V)	Collision energy (eV)
PteGlu	7.33	-77	-32
5-CH ₃ -H ₄ PteGlu	8.31	-79	-32
5-CHO- H ₄ PteGlu	6.76	-79	-37.5
5-CH ₃ -H ₄ PteGlu ₂	9.57	-91	-44
PteGlu ₃	10.04	-52	-68.5
5-CH ₃ -H ₄ Pteglu ₃	10.52	-52	-68.5
PteGlu ₄	10.85	-50	-86
5-CH ₃ -H ₄ PteGlu ₄	11.21	-50	-86
PteGlu ₅	11.44	-65	-45
5-CH ₃ -H ₄ PteGlu ₅	11.63	-65	-45
5/10-CHO-H ₄ PteGlu ₅	11.39	-65	-45
PteGlu ₆	11.88	-70	-53
5/10-CHO-H ₄ PteGlu ₆	11.97	-70	-53
PteGlu ₈	12.54	-75	-67
<i>Internal standards</i>			
Methotrexate (MTX)	9.78	-80	-30
Tri-MTX	11.07	-50	-27
Hexa-MTX	12.34	-71	-44

2.5.1 Optimisation of rice folate extraction

Most of the existing extraction protocols which involve enzyme and heat treatments in order that polyglutamate folates are converted to monoglutamates may promote folate decomposition and interconversion despite the presence of antioxidants such as ascorbate. This study used and modified the most common extraction methods employed in cereals and rice to investigate the possibility of

measuring the mono- and polyglutamated folate species simultaneously in rice grain matrix. The findings of this study showed that the combination of methanol and phosphate extraction with monoenzyme (α -amylase) treatment (MeOHAA/PO₄) provided the best recovery of mono- and polyglutamated folate forms found in the rice grains (Table 2.5). Only 5-CH₃-H₄PteGlu was detected in extract of rice using the method with heat treatment (BAA(+)) as this is the only stable folate form up to 120°C as shown in previous studies (Iniesta *et al.*, 2009; Melse-Boonstra *et al.*, 2002; Indrawati *et al.*, 2004). Lower recovery for most of the folate forms except for 5/10-CHO-H₄PteGlu was also observed using the NaPO₄/aqueous extraction without heating (BAA(-)). Recovery of folates using MeOH/PO₄AA method (Method 2) gave 84.9% \pm 9.5 recovery for 5-CH₃-H₄PteGlu, 71.6% \pm 12.2 for 5/10-CHO-H₄PteGlu, 47.2% \pm 15.6 for 5-CH₃-H₄PteGlu₄, 32.3% \pm 9.1 for 5-CH₃-H₄PteGlu₅, and 37.7% \pm 14.6 for 5/10-CHO-H₄PteGlu₅. Thus, the MeOH/PO₄ was adapted in this study for the sample preparation and extraction of folates in rice for LC-MS/MS profiling of the mono-and polyglutamated forms.

Table 2.5. Recovery of folate metabolites from various extraction methods

Folate Form	%Recovery (mean \pm SD, <i>n</i> =3)			
	MeOH	MeOHAA	BAA(+)	BAA(-)
5-CH ₃ -H ₄ PteGlu	50.5 \pm 8.3	84.9 \pm 9.5	21.3 \pm 5.7	43.0 \pm 3.3
5/10-CHO-H ₄ PteGlu	47.4 \pm 17.5	71.6 \pm 12.2	nd	53.4 \pm 19.8
5-CH ₃ -H ₄ PteGlu ₄	nd	47.2 \pm 15.6	nd	17.8 \pm 6.2
5-CH ₃ -H ₄ PteGlu ₅	nd	32.3 \pm 9.1	nd	33.9 \pm 1.6
5/10-CHO-H ₄ PteGlu ₅	nd	37.7 \pm 14.6	nd	34.2 \pm 2.1

meOH – methanol/PO₄ extraction (Garratt *et al.*, 20005; Santoyo Castelazo, 2009 method);

meOHAA – methanol/PO₄ extraction with α -amylase;

BAA(+)₄ – aqueous/NaPO₄ extraction with soft heat treatment and α -amylase (modified Kariluoto *et al.*, 2008 method) and SPE purification;

BAA(-)₄ – aqueous/ NaPO₄ extraction with α -amylase (modified Brouwer *et al.*, 2008 method)

nd – not detected

2.5.2 Method validation

Accuracy and precision are just two of the important factors to be tested to validate the bioanalytical method established. Accuracy is the closeness of the mean test results obtained using the method to the true value or concentration of the analyte while precision is the closeness of individual measures per analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of sample matrix (USDHHS, 2001). It is important to assess the precision and accuracy of any bioanalytical method to check the overall performance of the chromatographic system, reproducibility of the detector response, reproducibility of sample preparation procedure, consistency of recovery of analytes from biological samples and to ensure the absence of matrix effect on the quantification (Matuszewski *et al.*, 2003).

The validation method involved the use of fifteen individual folate standards (listed in Table 2.4) and 3 internal standards (MTX, tri-MTX and hexa-MTX). The recovery for individual folates is summarized in Table 2.6. These recoveries were taken into account in quantifying the folate content in the rice samples.

Table 2.6. Individual folate recovery from the rice grain matrix spiked with 5-CH₃/CHO-H₄PteGlu₁₋₈ and PteGlu_n standards.

Analyte	Recovery (%)±RSD (%), n=5)	
	100 nM	500 nM
PteGlu	68.8 ± 7.5	69.2 ± 6.5
5,10-CH ₂ -H ₄ PteGlu	49.0 ± 8.7	57.5 ± 4.8
5-CH ₃ -H ₄ PteGlu	62.9 ± 14.8	70.6 ± 7.8
5-CHO-H ₄ PteGlu	55.4 ± 4.3	48.0 ± 7.2
5-CH ₃ -H ₄ PteGlu ₂	52.4 ± 4.3	58.6 ± 5.7
PteGlu ₃	55.2 ± 10.6	61.5 ± 9.3
5-CH ₃ -H ₄ PteGlu ₃	48.5 ± 4.4	55.4 ± 12.1
PteGlu ₄	51.3 ± 14.5	65.1 ± 7.9
5-CH ₃ -H ₄ PteGlu ₄	68.2 ± 10.1	73.2 ± 9.5
PteGlu ₅	73.2 ± 10.1	64.9 ± 10.7
5-CH ₃ -H ₄ PteGlu ₅	62.7 ± 10.4	51.8 ± 5.1
5-CHO-H ₄ PteGlu ₅	68.7 ± 14.8	64.4 ± 4.2
PteGlu ₆	40.7 ± 6.9	31.7 ± 11.6
5-CH ₃ -H ₄ PteGlu ₆	54.1 ± 7.1	64.9 ± 22.4
5-CHO-H ₄ PteGlu ₆	41.5 ± 12.0	52.9 ± 4.1
PteGlu ₇	31.8 ± 25.4	38.3 ± 25.8
PteGlu ₈	40.7 ± 2.8	31.7 ± 3.7

Matrix effect was examined by comparing the MS/MS response (peak areas) of each analyte at a given concentration (500 nM) in neat solution to the response of each analyte spiked into the rice grain extract after extraction. The post-extraction spike method quantitatively assessed the matrix effects.

Table 2.7 summarises the intra- and inter-day precision and accuracy using the 15 folate standards at two different concentrations, 100 and 500 nM per sample. The intra-run precision was within an RSD of 0.5 to 16.6% and inter-run was between 2.8 and 20.4%. The intra-day and inter-day accuracy ranged from 83.3 to 117.1% and from 81.9 to 125.2%, respectively. The values obtained for the accuracy were within the accepted value of 70% to 130% of theory at the LOQ level. The values for

recovery of folate by the methanol phosphate extraction with α -amylase treatment were generally highly reproducible at all concentrations and ranged from $31.7\% \pm 3.7$ to $73.2\% \pm 10.1$. It was noted that most of the folates and in particular, polyglutamyl forms gave a lower recovery at the lower concentrations. Identities of extracted folate species from rice was confirmed by comparing the negative ion ESI-MS spectrum of an extracted folate with that of its commercial standard. A maximum of eight glutamate residues of folates can be quantified using this method as commercial standards with higher number of conjugated glutamates are not available. The use of ion-pairing agent, dimethylhexylamine (DMHA) was needed for the retention of folate polyglutamates in negative electrospray ionisation mode (ESI) which is known to be less sensitive for the mono-glutamates (Garratt *et al.*, 2005). Hence, the method may have reduced sensitivity compared with those methods using positive ESI but this is an acceptable compromise to allow simultaneous detection of both mono- and polyglutamyl folate species.

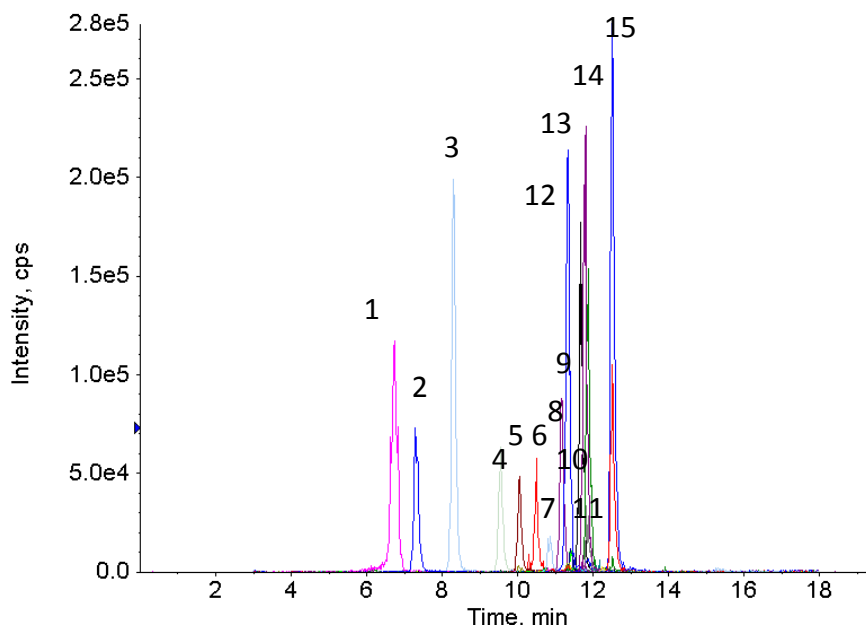


Figure 2.1. representative total ion chromatogram of 15 folate standards at 500 nM which was extracted using the MeOHAA/PO₄ method. The numbers correspond to (1)5-CHO-H₄PteGlu, (2) PteGlu, (3) 5-CH₃-H₄PteGlu, (4) 5-CH₃-H₄PteGlu₂, (5) PteGlu₃, (6) 5-CH₃-H₄PteGlu₃, (7) PteGlu₄, (8) 5-CH₃-H₄PteGlu₄, (9) PteGlu₅, (10) 5-CH₃-H₄PteGlu₅, (11) 5-CHO-H₄PteGlu₅, (12) PteGlu₆, (13) 5-CHO-H₄PteGlu₆, (14) PteGlu₇, (15) PteGlu₈

Table 2.7. Intra- and inter-day precision (n=5) and accuracy results for the determination of folates in rice matrix using LC-MS/MS.

Analyte	Intra-day precision (%RSD, n=5)		Intra-day accuracy (%) ± RSD (% , n=5)		Inter-day precision (% RSD, n=5)		Inter-day accuracy (%) ± RSD (% , n=5)	
	100 nM	500 nM	100 nM	500 nM	100 nM	500 nM	100 nM	500 nM
	PteGlu	4.7	6.5	109.4 ± 5.2	96.7 ± 8.2	10.9	16.6	88.6 ± 7.3
5,10-CH ₂ -H ₄ PteGlu	15.2	16.6	98.9 ± 25.1	104.8 ± 19.8	20.4	18.7	87.9 ± 19.8	116.6 ± 17.1
5-CH ₃ -H ₄ PteGlu	7.8	7.6	85.6 ± 8.7	100.2 ± 4.7	11.1	8.4	87.3 ± 14.8	93.3 ± 11.7
5-CHO-H ₄ PteGlu	6.3	10.8	89.9 ± 4.7	106.9 ± 11.0	14.9	7.8	101.8 ± 13.6	99.8 ± 8.0
5-CH ₃ -H ₄ PteGlu ₂	8.7	8.3	85.4 ± 6.1	90.7 ± 7.1	7.2	4.7	90.3 ± 8.8	81.9 ± 10.4
PteGlu ₃	9.4	5.5	93.9 ± 5.5	92.3 ± 6.3	3.6	2.8	97.1 ± 5.3	95.2 ± 4.7
5-CH ₃ -H ₄ PteGlu ₃	4.4	8.8	87.8 ± 9.6	87.1 ± 15.3	4.9	12.1	89.6 ± 12.9	86.3 ± 15.8
PteGlu ₄	14.4	11.8	92.7 ± 13.6	83.2 ± 18.3	7.8	7.9	92.6 ± 7.2	82.7 ± 4.5
5-CH ₃ -H ₄ PteGlu ₄	5.7	8.8	91.3 ± 7.8	84.5 ± 11.2	10.6	12.5	105.9 ± 4.0	86.8 ± 4.1
PteGlu ₅	5.6	13.1	96.9 ± 16.5	114.5 ± 7.5	10.1	10.7	86.9 ± 8.5	117.8 ± 6.4
5-CH ₃ -H ₄ PteGlu ₅	0.5	8.1	117.1 ± 5.5	112.9 ± 6.4	16.5	9.9	107.3 ± 17.1	109.6 ± 9.9
5/10-CHO-H ₄ PteGlu ₅	10.2	8.2	103.4 ± 6.5	112.6 ± 6.4	9.2	14.0	94.8 ± 16.2	89.3 ± 15.5
PteGlu ₆	7.1	7.3	110.7 ± 6.3	94.5 ± 8.6	4.7	9.5	103.4 ± 9.2	125.2 ± 5.5
5-CH ₃ -H ₄ PteGlu ₆	9.1	8.0	83.3 ± 17.0	89.8 ± 8.4	20.2	18.1	98.0 ± 13.1	96.6 ± 7.1
PteGlu ₈	6.6	9.9	103.7 ± 13.7	99.4 ± 8.5	17.3	11.6	113.0 ± 19.3	92.9 ± 7.0

The method developed and validated here is unable to detect the very unstable tetrahydrofolate (H₄PteGlu) which was probably degraded within 1 month of storage of the rice sample regardless of temperature and even in the presence of antioxidants as observed in tomato (Iniesta *et al.*, 2009) and this represents a discrepancy with results from previous studies (de Brouwer *et al.*, 2008; Witthöft *et al.*, 2006) which showed that H₄PteGlu is about 5% of the total folate in the rice grain. Due to the hydrophilic properties of folate, reversed-phase chromatography coupled to electrospray ionisation (ESI) is more suitable than atmospheric pressure chemical ionisation (APCI) (Nelson *et al.*, 2001; Stokes and Webb, 1999). Another reason for using the negative ion mode was because it provided cleaner mass spectra than positive ion mode. As the method was designed to profile both the mono- and polyglutamated folates which are over 10 species in 25 min run, sensitivity is slightly compromised and relatively low compared to other methods measuring only monoglutamates and less than 10 analytes in one run time. The limit of detection and limit of quantification for folates measurement in rice using the LC-MS/MS technique was reported by de Brouwer *et al.* (2008) to range between 0.2 and 1.2 µg/100g and between 0.6 and 4 µg/100g, respectively for six monoglutamates and four internal standards. In this study, LOD and LLOQ for 14 folates range from 0.02 to 1.2 µg/100g and from 0.13 to 4.1 µg/100g, respectively (Table 2.9). Not all LC-MS/MS methods applied on folate have been fully validated particularly with respect to % recovery and matrix effects (de Brouwer *et al.*, 2007; Nelson *et al.*, 2004) as was done in this particular study.

The evaluation of matrix effect on the results of quantitative determination of folates in rice extracts is also an important element of method validation. The matrix effect was calculated as $(B-C)/A \times 100$ with (A) being the peak area of neat standards in the extraction buffer, (B) as the peak area of the rice matrix spiked with 500 nM standards and (C) as the peak area of the endogenous folate in the rice matrix. The internal standards were added after sample preparation. Table 2.10 shows that ion suppression of the signal was somehow compensated for by use of the internal standards.

The developed and validated methodology was applied in determining folate analytes in wild type, knockout and transgenic rice materials which are presented and discussed in the next two experimental chapters.

Table 2.8. Calibration data for analysis of folates using LC-MS/MS.

Analyte	Slope ($\times 10^{-3}$)	Intercept	Correlation coefficient (r^2)	Retention time(min) (n = 5)
PteGlu	1.2	+0.0320	0.992	7.33
5,10-CH ₂ -H ₄ PteGlu	0.2	-0.0072	0.991	6.95
5-CH ₃ -H ₄ PteGlu	8.7	-0.1387	0.993	8.31
5-CHO-H ₄ PteGlu	2.3	-0.3121	0.998	6.76
5-CH ₃ -H ₄ PteGlu ₂	1.0	-0.8140	0.996	9.57
PteGlu ₃	5.8	+0.1657	0.998	10.04
5-CH ₃ -H ₄ PteGlu ₃	4.5	+0.2106	0.993	10.52
PteGlu ₄	2.0	-0.1447	0.992	10.85
5-CH ₃ -H ₄ PteGlu ₄	8.1	+0.1452	0.999	11.21
PteGlu ₅	6.3	-2.9912	0.996	11.44
5-CH ₃ -H ₄ PteGlu ₅	2.3	-1.3584	0.998	11.63
5-CHO-H ₄ PteGlu ₅	2.8	+1.3469	0.995	11.39
PteGlu ₆	2.3	+0.0263	0.994	11.88
5-CH ₃ -H ₄ PteGlu ₆	0.3	+0.0020	0.993	12.11
5-CHO-H ₄ PteGlu ₆	3.4	+0.0397	0.994	11.87
PteGlu ₇	9.3	+0.2927	0.991	12.27
PteGlu ₈	8.0	+0.8838	0.997	12.54

Table 2.9. Limit of detection (LOD) and lower limit of quantification (LLOQ) for individual folates measured using LC-MS/MS.

Analyte	LOD ($\mu\text{g}/100\text{g}$)	LLOQ ($\mu\text{g}/100\text{g}$)
PteGlu	0.25	0.39
5,10-CH ₂ -H ₄ PteGlu	0.5	0.9
5-CH ₃ -H ₄ PteGlu	0.02	0.13
5-CHO-H ₄ PteGlu	1.1	3.8
5-CH ₃ -H ₄ PteGlu ₂	0.3	0.6
PteGlu ₃	0.7	3.1
5-CH ₃ -H ₄ PteGlu ₃	0.2	1.4
PteGlu ₄	0.6	3.3
5-CH ₃ -H ₄ PteGlu ₄	0.1	1.1
5-CH ₃ -H ₄ PteGlu ₅	0.5	2.8
5/10-CHO-H ₄ PteGlu ₅	0.2	1.3
PteGlu ₆	0.4	2.4
5-CH ₃ -H ₄ PteGlu ₆	1.2	4.1
PteGlu ₈	0.9	3.7

Table 2.10. Ion suppression of individual folates. Matrix effect% = $(B-C)/A \times 100$ with (A) as the peak area of the neat standards in extraction buffer, (B) the peak area of rice matrix spiked with the 500 nM standards and (C) the peak area of the endogenous amount of folates present in the rice matrix.

Analyte	Matrix effect	
	Without Internal Standards	With Internal Standards
PteGlu	80.4	107.0
5,10-CH ₂ -H ₄ PteGlu	78.4	85.9
5-CH ₃ -H ₄ PteGlu	47.1	104.4
5-CHO-H ₄ PteGlu	28.6	76.7
5-CH ₃ -H ₄ PteGlu ₂	50.0	109.1
PteGlu ₃	43.3	100.7
5-CH ₃ -H ₄ PteGlu ₃	42.3	105.7
PteGlu ₄	38.0	93.9
5-CH ₃ -H ₄ PteGlu ₄	43.5	92.8
5-CH ₃ -H ₄ PteGlu ₅	51.6	118.9
5/10-CHO-H ₄ PteGlu ₅	25.5	86.7
PteGlu ₆	35.2	64.3
5-CH ₃ -H ₄ PteGlu ₆	27.2	68.6
PteGlu ₈	19.8	43.8

2.5.3 Application of the method to certified reference material (CRM 121) and plant (spinach) quality control (QC)

Commercially available certified reference material, whole meal flour (CRM 121) containing 50 µg/100g total folate was chosen to check for the accuracy of the method. As no blank matrix is available, we used the CRM 121 to represent the matrix of the rice samples giving an indication of how the system will be when injected with the similar sample composition. Another set of CRM 121 was spiked with known concentration of internal standards (IS mix of MTX, MTX3 and MTX6 at 100 nM) for recovery test.

Due to the limited availability of CRM 121 which was kindly shared by the group of Cornelia Witthöft (Swedish University of Agricultural Sciences, Uppsala, Sweden), spinach was also used as QC in all quantitative analytical runs. Non-spiked and spiked (with 500 nM of 13 folate standards) samples were analysed together with rice samples each time. Table 2.11 shows the average value of 37.4 ± 1.4 µg/100 g 5-CH₃-H₄PteGlu of the 41.97 µg/100 total folate in CRM 121. Other minor forms of folate found were: 5/10-CHO-H₄PteGlu (3.7 ± 0.3), 5-CH₃-H₄PteGlu₄ (0.11 ± 0.01), 5-CH₃-H₄PteGlu₅ (0.18 ± 0.03) and 5-CHO-H₄PteGlu₅ (0.58 ± 0.08). The value obtained is close to but lower than the reported total folate in CRM 121 based on MA which is 51 µg/100g (Finglas *et al.*, 1999). The LC-MS/MS result was 18% lower than the MA value as nonfolate compounds are suspected to have a similar folate activity increasing bacterial growth (Konings *et al.*, 2001; Ruggeri *et al.*, 1999). Reference

materials like CRM are good indicators of the performance of an analytical method and most of the international interlaboratory studies carry out folate analysis using in-house QC (Puwastien *et al.*, 2005) and/or reference materials.

Table 2.11. Folate species in CRM 121 as measured using LC-MS/MS. Values are mean of triplicates with error bars (standard deviation).

Analyte	Mean concentration ($\mu\text{g}/100\text{g}$) \pm SD
5-CH ₃ -H ₄ PteGlu	37.4 \pm 1.4
5/10-CHO-H ₄ PteGlu	3.7 \pm 0.3
5-CH ₃ -H ₄ PteGlu ₄	0.11 \pm 0.01
5-CH ₃ -H ₄ PteGlu ₅	0.18 \pm 0.02
5/10-CHO-H ₄ PteGlu ₅	0.58 \pm 0.1
total folate	41.9 \pm 1.83

2.6 Conclusion

A novel monoenzyme method of folate extraction from the rice grains has been developed in this work to enable the quantitative determination of mono- and polyglutamated forms of folate. Validation involved investigation of matrix effects, determination of recovery by standard addition method and repeatability tests. With this method, 17 folates were successfully separated within 20 minutes, five of which were present or detected in the rice samples. Using the advantageous capabilities of the QTRAP™, MRM was used as survey scan to trigger an EPI scan to confirm the presence and structure of each folate form in the rice extract in a single experimental run. To our knowledge, this is the first extensive study, on the polyglutamate folates in rice. This is also the first time that a method can measure simultaneously all the mono- and polyglutamated folates present in the rice matrix. The data presented here confirmed that this new method is a useful tool for the determination of folate forms in rice and can be applied in the same food or cereal matrices.