3 APPLICATION OF MICROBIOLOGICAL ASSAY AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY IN THE QUANTIFICATION OF RICE GRAIN FOLATES

Abstract

Microbiological assay (MA) and LC-MS/MS were applied in the measurement of total concentration and folate forms in unpolished rice grains of selected cultivars. Fifty one rice varieties were screened for their total folate content using MA and seven among them were profiled for their mono- and polyglutamated folates using LC- MS/MS. Total folate content in 51 different rice cultivars varied from 6.7 to 86.6 µg/100g. MA revealed large variations among rice cultivars in terms of total folate concentration while LC-MS/MS provided novel information on the polyglutamated folate forms 5-CH_3-H_4PteGlu$_4$, 5-CH_3-H_4PteGlu$_5$ and 5-CHO-PteGlu$_5$ that can be found in the grain in addition to the widely studied and reported monoglutamates (e.g. 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu). The folate content in some rice cultivars was relatively high and has potential to be further increased by targeted breeding. The folate vitamer distribution of whole grain rice was characterised by a large proportion of 5-methyltetrahydrofolate followed by formyltetrahydrofolate species.

3.1 Introduction

3.1.1 Measurements of food folates

Folates in foods exist as various metabolites differing in oxidation status and single carbon substituents with variable glutamyl residues (Gregory, 1989). Metabolites are the intermediates and products of metabolism. So far, the only officially accepted method (AOAC Official Method 992.05) of folate quantification is the microbiological assay (MA) limited to folic acid in infant formula and not specifically recommended for total folate determination in foods (DeVries *et al*., 2005). For the past decades though, the microbiological method has been applied to many food items for the publication of food composition data (Phillips *et al*., 2007) which serve as the basis for the folate fortification programs and recommendations in the U.S. and in other developed countries. After several studies reported the reliability, reproducibility and usefulness of the MA data (Tamura 1989; Rader *et al*., 1998; O'Broin and Kelleher, 1992; Pandrangi and Laborde, 2004) it became the standard (AOAC, 2000) and the basis of comparing data obtained from the more recently developed chromatographic measurements of folates in cereals.

Since folate vitamers differ in their chemical characteristics, it is important to examine their distribution in a matrix. Moreover, targeted metabolic profiling is needed to identify a selected number of metabolites in a complex cereal extract to enhance the folate content in cereals via conventional breeding or metabolic

engineering as a sustainable and complementary way of improving human nutrition.

Chromatographic methods separate and quantify the different forms of folate, in particular the monoglutamates, and recently many researchers proved the usefulness of the technique in quantifying the vitamers in biological extracts. O'Broin *et al*. (1972) were able to measure some of the naturally occurring monoglutamate derivatives of folic acid. In 1989, Gregory III developed the method in measuring dietary folates. Then Finglas *et al*., (1999) Konings (1999), and Ruggeri *et al*. (1999) measured the naturally occurring folate forms in several food items and diets. Further refinement of the method happened from 2000 to 2005 when application of the affinity and reversed-phase chromatography was made by Bagley and Selhub (2000); then the method was applied on raw and processed beetroots (Jastrebova *et al*., 2003); later on the solid-phase extraction for HPLC analysis of dietary folates was made (Nillson *et al*. 2004); and Johansson *et al*. (2005) and Patring *et al*. (2005) compared the purification techniques prior to HPLC analysis of folates and evaluated the impact of antioxidants on the stability of the different forms of extracted folates from food, respectively.

Recently, publications on individual folate measurements in the food matrices increased significantly. Most of these published works used HPLC in combination with MS and MS/MS on the monoglutamate folate forms in fruits, vegetables and

other cereals like wheat and rye. Only limited information is available on folates in rice.

3.1.2 Folates in cereals and cereal products

According to Gregory *et al*.(1984), Vahteristo *et al*. (1997) and Konings *et al*. (2001), cereals and cereal products often contain methyl and formyl derivatives as well as unsubstituted tetrahydofolate which are difficult to measure due to their low concentration and instability. To date, the role of polyglutamate synthesis is not well understood and polyglutamyl distribution in cereals is also not known. Only estimates of the proportion of polyglutamyl folate were reported by Konings *et al*. (2001) for breads (66± 27%); by Arcot *et al*. (2002) for wheat bread (44%) and Australian wheats (60%); and by Muller (1993) for grains (23.5%) and for bakery products (34.4%). Little information is available on how much and what are the folate forms in most staple crops like rice. This could be attributed to the large proportion of matrix-bound folates. Some findings showed that approximately 40% of folates in wheat and rye are bound to starch (Cerna and Kas, 1983) and on average, 41% matrix-bound folate in cereals (63% for wheat flour) and grain products (39% for white bread) (Yon and Hyun, 2003).

The use of proteolytic and amylolytic enzymes during extraction, especially for cereal based foods, are becoming more and more established (De Souza and Eitenmiller, 1990; Martin, Landen and Soliman, 1990; Pfeiffer *et al.*, 1997; DeVries, Keagy, Hudson and Rader, 2001; Tamura, Mizuno, Johnston and Jacob, 1997) since

they facilitate the release of protein- and starch-bound folates resulting in a more complete extraction of folates that may be bound to or trapped in the matrices of protein and polysaccharides (Arcot and Shrestha, 2005).

A number of folate extraction protocols have been published for various plant tissues but most of them were designed to only accurately measure the monoglutamated folate forms. The LC-MS/MS method developed in the earlier part of the study and applied here is novel, specifically in the measurement of the polyglutamated forms of this particular B vitamin. To our best knowledge, this is the first attempt to measure polyglutamyl forms of natural folates in rice.

3.2 Aims and objectives

The main aim of the study is to probe the natural variation among rice cultivars in terms of total folate content and forms which can aid further work in developing the folate-enhanced rice. Specifically, the objectives of the work are:

- To measure total folate in rice by microbiological assay,
- To measure mono-glutamated folate by HPLC-UV/FLD, and
- To measure mono- and polyglutamated folates using LC-MS/MS.

3.3 Materials and Methods

3.3.1 Rice materials

Rice cultivars obtained (Table 3.1) from the Genebank of the International Rice Research Institute (IRRI) consisting of FAO and MLS germplasm collections, Plant Science Greenhouse (April – November 2007 and 2008) grown IR72, Nipponbare, IR64 and other varieties which were harvested during the 2007 dry season at the Philippine Rice Research Institute (PhilRice) were analysed for total folate concentration using the MA based on Tamura *et al*., (1998), Rader *et al*., (1998) and Shrestha *et al*., (2000) with slight modifications as per consultation with Mr. Anthony J.A. Wright of the Institute of Food Research (IFR), Norwich. Samples of each cultivar or variety were derived from pools of 10-15 plants.

IR72, IR64, Nipponbare and Moroberekan were grown in the greenhouse using a 1:1 combination of two soils (soil mixes shown in Table 3.2) and addition of 40 g Osmocote (after 4 weeks) in 16 cm pots with a 12h light cycle at 27-29 ^{o} C (day time) and 21 ^{o} C (night time) were included in the analysis. Humidity was not controlled, but was monitored and in the region of 70% relative humidity. The plants were grown in soils for at least 4 months or until rice grains were mature enough (at least 80% of the grains were golden or yellow) for harvesting. Seeds were collected and dried in a convection oven at 40°C overnight to attain the 11-14% moisture content

of paddy or rough rice for storage. Dehusking was made using the Satake® THU-35A rice dehusker at the IRRI or PhilRice or by hand using forceps at the University of Nottingham. All samples were kept at -80°C prior to homogenization and extraction for analyses. All tests were done in three replications. The growing conditions in the greenhouse of the Plant and Crop Sciences Department, University of Nottingham were optimised and established by the author in 2006 and 2007.

Table 3.1. Rice materials screened for total folate concentration using microbiological assay

Rice variety	Description	Source country of original seed
Moroberekan	Upland rice	Guinea, West Africa
IR72	Irrigated lowland rice	Philippines
IR64	Irrigated lowland rice	Philippines
IR60	Irrigated lowland rice	Philippines
IR42	Irrigated lowland rice	Philippines
IR66	Irrigated lowland rice	Philippines
IR29	Irrigated lowland rice	Philippines
IR65	Irrigated lowland rice	Philippines
IR8	Irrigated lowland rice	Philippines
Punjab Basmati	Basmati lowland rice	India
Karnal Basmati	Basmati rice	Pakistan
Asahi	Japonica rice	Japan
Heenati	Rainfed lowland	Sri Lanka
Taichung Senyo 223	Irrigated lowland rice	Taiwan
IR40	Irrigated lowland rice	Philippines
Ishikare Shiroke	Japonica rice	Japan
Milagrosa	Upland rice	Philippines
Taichung Senyo Glutinous	Irrigated lowland rice	Taiwan
Goo Meuang	Irrigated lowland rice	Thailand
Domsiah	Rainfed lowland	Iran
Chao Khao	Rainfed lowland	Laos
Dinorado	Upland rice	Philippines
Basmati 370	Basmati rice	India
Basmati	Basmati rice	Pakistan
Pusa Basmati	Basmati rice	India
Cartuna	Upland rice	Indonesia

Table 3.1. cont…

Rice variety	Description	Source country of original seed
Nipponbare	Japonica rice	Japan
Norin	Japonica rice	Japan
Milyang 15	Japonica	Korea
Taipei 105	Japonica	Taiwan
Basmati 217	Basmati rice	India
Aichi Asahi	Japonica	Japan
China Rice - 203	Irrigated lowland rice	Myanmar
Dehradun Basmati	Basmati rice	India
Sarang Barung	Rainfed lowland	Indonesia
Dourado Agulha	Rainfed lowland	Brazil
Sadri	Rainfed lowland	Iran
Ranbir Basmati	Basmati rice	India
Karnal Local	Basmati rice	Taiwan
Palawan	Upland rice	Philippines
DV110	Upland rice	Bangladesh
Suweon 339	Irrigated lowland rice	Korea
Toride	Japonica rice	Japan
Lemo Besar	Rainfed lowland	Indonesia
ADT 36	Irrigated lowland rice	India
Balam	Upland purple	Bangladesh
IRI 353	Japonica rice	Korea
C702015	Irrigated lowland rice	Taiwan
Camor	Rainfed lowland	Indonesia
C662083	Irrigated lowland rice	Taiwan
PSBRc60	Irrigated lowland rice	Philippines

Table 3.2. Materials for growing rice in pots in the greenhouse

Growth Medium	Substrate Type	Fertilizer Content
Levington M3 (LM3) John Innes No. 2 (JI2)	peat Loam/peat/sand	N_{280}, $P_{205\ 160}$, K_{20}, 350g/m^3 K_2P0_4
Osmocote	Slow release fertilizer	N_{16}, P_{11}, K_{11}, B $_{0.02}$, Cu $_{0.047}$, Fe $_{0.40}$, Mn, Mo $_{0.02}$, Zn $_{0.015}$, Mg $_{3.0}$ (all in %)

3.3.2 Microbiological determination of total folate concentration in unpolished rice grains

The sample preparation and extraction done by the author at the Centre for Analytical Biosciences, School of Pharmacy, University of Nottingham were based on the established method of Wright *et al*. (2005) of the IFR, Norwich, UK with slight modifications.

Approximately 15 ml of boiled extraction buffer (0.1M sodium phosphate buffer pH 6.2 with 0.5% sodium ascorbate) was added to glass tubes containing 1.0 g brown rice. Using Ultra-Turrax® probe (IKA Staufen, Germany), the samples were homogenized for 1.5 min. Extracts were quantitatively transferred to 20 ml volumetric flasks, made to volume with the extraction buffer and stewed in 92°C water bath for 10 min to denature endogenous enzymes. After thorough mixing, 1.0 ml of each extract was placed into 15 ml conical tubes, capped and stored frozen at -80°C for deconjugation. At an appropriate time, the 1.0 ml samples were thawed and deconjugated for 6h at 37°C with the addition of 0.5 ml chicken pancreas (CP)

solution containing 2.5 mg CP in water (Becton-Dickinson Biosciences, code 245910), 0.5 ml α-amylase containing 625 units in water (Sigma, code A-6211; from *Aspergillus oryzae)* and further addition of 3.0 ml extraction buffer. Enzymatic activity was terminated by placing the 5.0 ml deconjugation tubes into a boiling water bath for 10 minutes. The system was rapidly cooled in ice afterwards and 2 x 1.5 mL aliquots were stored in amber eppendorf at -80°C prior to assay.

To test the capacity of CP to deconjugate folate polyglutamates, yeast sample was prepared the same way as the rice samples. In principle, there should be little if any folate available to cause growth in the assay because all yeast folate is high chain polyglutamate that does not support growth (Wright personal communication, 2007). In addition, enzyme and yeast blanks were also prepared and corrections were made for all the folate calculations to correct for the endogenous folate present in the enzyme treatments. All steps were carried out under subdued light.

The actual folate assay of most rice extracts was performed by Mr. AJA Wright at the Institute of Food Research, Norwich, UK for most of the rice samples.

Total folate levels in unpolished rice grains was determined using a cryopreserved and chloramphenicol-resistant *Lactobacillus rhamnosus* (NCIMB 10463) microbiological assay (O'Broin SO & Kelleher B, 1992) which was relying on the turbidimetric growth of this indicator microorganism. Growth of *L. rhamnosus* in test extracts was compared to growth in the presence of varying concentrations of folic acid with a detection limit of 40 ng g^{-1}. Final folate concentrations were adjusted for enzyme folate content. The activity of CP folate deconjugating capacity

in the 5.0 ml system was assessed by its ability to deconjugate folate polyglutamates in a calibration series (0.125 and 0.5 g) of extracts of dried yeast (Becton-Dickinson Biosciences, code 212740). Day to day 'precision' of the method was closely monitored by including in each analytical run high, medium and low 'in-house' Quality Controls (QCs) from aliquots of three plasma pools stored at -80°C. Additionally, 'accuracy' of the method was monitored monthly with the use of two external QCs; WHO 1st Serum Folate International Standard IS 03/178, and WHO 1st Whole Blood International Standard IS 95/528.

3.3.3 Monoglutamate folate measurement in unpolished rice grain using HPLC-UV/FLD

This experiment was conducted by the author at the Department of Food Science, Swedish University of Agricultural Sciences in Uppsala, Sweden with the folate team under the supervision of Cornelia Witthöft from 16 to 25 September 2008.

For quite some time now, the folate team in the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden have been working on the measurement of folates in foods using HPLC techniques. The dienzyme treatment made on rice samples was a method adapted from the group and which they are currently using on rice and other cereals (Witthöft and Jagerstad, 2008, personal communication) to measure folate forms. Their method is very similar to the MA sample preparation

where α-amylase and chicken pancreas or rat serum are used to release folates from the rice matrix and to deconjugate the polyglutamated folates to di- or monoglutamated forms, respectively. An additional purification step through the solid-phase extraction (SPE) follows to eliminate impurities which would interfere in the analysis. The details of the method can be found fully described in their published papers (Witthöft *et al*., 1999; Jastrebova *et al*., 2003; Johansson *et al*., 2005; Patring and Jastrebova, 2007) and steps are enumerated below:

3.3.3.1 Extraction and deconjugation of folates in rice

Unpolished rice samples were weighed and homogenized using the Retsch® ball mill (Retsch Limited, Leeds, UK) for 30 seconds at a speed of 30.0 Hz. Two replicates of around 0.5 g of each rice sample were weighed, placed in the extraction tubes and 15 mL extraction buffer containing 0.1M phosphate buffer, pH 6.1 with 2% w/v Na ascorbate and 0.1% v/v 2,3-dimercaptopropanol was added to each. Sixty μL of alpha-amylase (Sigma, code A-6211; from *Aspergillus oryzae*) was also added to each sample replicate before sealing the system with argon gas. The tubes were vortex mixed, boiled for 12 minutes for folate extraction, cooled in ice and centrifuged for 20 minutes at 13000x g under 4°C. The supernatant was collected and made up to 25 mL volume with the extraction buffer. An aliquot of four mL was transferred to another extraction tube for each sample and 160 μL of rat serum was added. Another 40 μL of alpha-amylase was added, vortex mixed and sealed again using argon gas before incubation for 2 hours in a shaking water bath at 37°C. After

incubation, the samples were boiled for five minutes to inactivate the enzymes, cooled in ice and centrifuged for another 20 minutes at 13000x g in the pre-cooled centrifuge. The supernatant (3.5 mL) was set aside for SPE extraction. Another 2 replicates of each homogenised sample were weighed in aluminum pans which were placed in the oven set at 105°C for 6 hours, placed in dessicator for 30 minutes and weighed afterwards for dry matter calculation and moisture content determination (AOAC, 1995).

3.3.3.2 Solid phase purification of folate extracts

A Visiprep SPE vacuum manifold (57030-U Supelco, USA) was set up under the fumehood with SAX columns (Isolute) which were activated with methanol (2.5 mL x 2 applications; 2 droplets per second). The columns were equilibrated with milliQ water (2.5 mL x 2 applications; 2 droplets per second) afterwards prior to the application of the 3.5 mL (1.75 mL each time) sample at a rate of one droplet per second. The columns were then washed with 2.5 x 2 mL water at a rate of one droplet per second and void volume was eluted with 0.5 mL elution buffer (0.1M Na acetate, pH 4.5 with 10% v/v NaCl, 1% w/v Na ascorbate and 0.1% v/v 2,3-dimercaptopropanol). The folates were then eluted with 4 mL (2x2 applications) elution buffer at one droplet per second rate into the pre-weighed Sarstedt® tubes. The columns were run dry until all the purified extracts were collected. The tubes were weighed again and 100 μL of each collected extracts was placed and sealed with argon gas in HPLC vials for analysis.

3.3.3.3 HPLC measurement of purified folate extracts

An Agilent 1100 HPLC system with a gradient quarternary pump, thermostated autosampler (8ºC), thermostated column compartment (23°C), fluorescence detector (excitation/emission of 290/360 nm for reduced folates and 360/460 nm for 10-HCO-folic acid) and a multi-wavelength detector (280, 290,300 nm) was used for folate measurement. Mobile phases consisted of 30 mM Phosphate buffer at pH 2.3 and acetonitrile. Folate forms were separated in Zorbax C8 or Aquasil C18 column at flow rate of 0.4 mL/min using an injection volume of 20 μL. The linear gradient condition started with 6% acetonitrile for 5 min, increased to 25% within 16 min and kept constant for 2 min before returning to initial values within 1 min. The column was re-equilibrated at 6% acetonitrile for 8 min prior to the next run. Tetrahydrofolate (H_4PteGlu), 5-CH_3-H_4PteGlu and 10-HCO-folic acid were identified in fluorescence detector by their retention times. External calibration was made (n=7) by plot area peak against standard concentrations.

3.3.4 Mono- and polyglutamated folate profiling of rice using LC-MS/MS

The LC-MS/MS method developed earlier in this study was based on the published method of Garratt *et al*. in 2005 and which further optimised by Santoyo Castelazo

(2009). Sample preparation and extraction for LC-MS/MS analysis was as described in the previous chapter. HPLC and MS/MS settings and conditions used in the final method validation in the previous study were also employed in this study. The structural identities of folates extracted from rice grains were confirmed by comparison with commercial standards and with an in-house folate spectral library using HPLC retention time, diagnostic precursor and product ions, and full-scan mass spectra by EPI. Full details of the LC-MS/MS method are given in chapter 2.

3.4 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 using Tukey's as the post test performed in GraphPad Prism version 4.02 for Windows, GraphPad software, San Diego, California USA, www.graphpad.com.

3.5 Results and Discussion

3.5.1 Total folates in rice varieties as measured by microbiological assay with dienzyme treatment

Fifty one rice cultivars screened for their total folate concentration are shown in Table 3.1. The varieties used in this study represented various sub-species and ecosystems with original seeds sourced from different countries (Table 3.3) through the effort of the International Rice Research Institute.

The assay revealed natural variation in the total folate content in rice. As shown in Table 3.3, most of the Elite Indica, irrigated lowland and Basmati cultivars contain less than 50 µg/100g total folate in unpolished grains. Much lower folate levels were observed with Japonica as most of them contain less than 30 µg/100g total folate. The upland purple rice from Bangladesh, Balam, showed the highest total folate concentration of 86.6 µg/100g among the 51 cultivars analysed. This is followed by Moroberekan, an upland rice from Guinea (West Africa) with 63.6 µg/100g total folate content (Figure 3.1). These two varieties had been used as a bridge variety for crossing Indica and Japonica rice cultivars due to its wide compatibility (S'5 gene located in chromosome 6) which produces hybrids with normal pollen and spikelet fertility when crossed with both Indica and Japonica subspecies (Harushima *et al*., 1998; Cyranoski, 2003; Lafitte *et al*., 2006). Wide

compatibility varieties (WCVs) are a special class of rice (*Oryza sativa L.*) germplasm that produces hybrids with normal pollen and spikelet fertility when crossed with both Indica and Japonica subspecies. The wide compatibility gene $S5^n$ has been used extensively in intersubspecific hybrid breeding programs. The S5 locus to a 2.2-cM genomic region between RM253 and R2349 on chromosome 6 was previously mapped, using a population of 356 F_1 plants derived from the three-way cross (www.knowledgebank.irri.org)

Wide-compatibility (WC) is one of the most important traits in rice, which can overcome the fertility barrier in the Indica/Japonica hybrids, and hence makes it possible to utilize the higher yield potential of inter-subspecific hybrids. The S^n_5 gene located on chromosome 6 has been previously reported to be responsible for the wide-compatibility in rice (Harushima *et al*., 1998; Ikehashi and Araki, 1998).

A gene for wide-compatibility (or affinity), which overcomes the partial sterility frequently encountered in the progeny of Indica-Japonica crosses (Ikehashi and Araki, 1988), can be used for exploiting heterosis of distant crosses in hybrid rice breeding and in biofortification work.

A Sri Lankan indica rice cultivar Heenati which is resistant to all four biotypes of the brown planthopper (BPH) (www.knowledgebank.irri.org) also showed a relatively high total folate concentration of 46.8 µg/100g followed by the traditional irrigated

lowland rice Goo Meuang from Thailand which contain 35.6 µg/100g total folate in the brown rice grain (Figure 3.1).

Like any other cereals, most of the rice cultivars studied were relatively low in folate if compared to other main cereals like rye and wheat (Arcot *et al*., 2002; Karilouto *et al*., 2004; Gujska and Majewska, 2005). Most of the rice materials we studied (26 out of 51) contain less than 20 µg/100g total folate (Figure 3.2). The mean total folate content of the 51 rice cultivars was 24.9 µg/100g. The calculated mode for total folate concentration of all the cultivars studied was 15.8 µg/100g while the median was 22.1 µg/100g. As a general observation, traditional and wild types tend to have higher total folate concentrations than did common cultivated types.

For six months after harvest and dehusking, total folate of unpolished IR72 rice seeds were monitored under cold (CT =-28°C) and ambient (RT = 21-29°C) storage. A significant decrease in total folate (39%) was observed for the ambient stored IR72 after 3 months which was very similar to the observation of Iniesta *et al*. (2009) in bottled stored tomato juice which exhibited 24.6 to 39.1% total folate losses after a month. Total folate loss after six months of rice storage at ambient temperature reached 32% (Figure 3.3). However, no significant change was observed in the total folate concentration of the cold stored IR72 after 6 months. This is similar to the observation of Stralsjo *et al* (2002) on strawberries stored at room temperature for 9 days at 4°C. The group observed a relatively high stability

of folate in berries at cold storage and lower as expected at room temperature even though strawberries are rich in ascorbic acid that can stabilise folates. Other reports (Kalt *et al*., 1999) minimal losses in other fruits high in organic acids and phenolic compounds after 8 days of storage compared to green leafy spinach which showed losses up to 90% within 3 days after harvest (Diplock *et al*., 1998).

Some genotypes seem to load more folate into seeds than other genotypes which is the same trend observed with other micronutrients, in particular, minerals like iron and zinc (Fresco, 2005; McKeehen *et al*., 1996). The large variation for different rice varieties in this study might also result from the fact that although all of them were grown at the same location, they were grown in different seasons and years. The data collected suggest that there is sufficient genetic variability to significantly increase folate concentrations in rice grains and some of these genotypes can be potentially used as parents in breeding high folate rice either by conventional or transgenic approach.

To our best knowledge, the values obtained in this study are the largest collection of folate content of the edible part of the cereal and can be used to accurately target folate biofortification in rice.

Table 3.3. Microbiologically determined folate contents of different rice varieties (mean ± SD; n=3)

Category	Variety	Source Country/Origin	Total Folate (µg/100g)
Elite Indica and Irrigated lowland	IR72	IRRI/Philippines	41.6± 2.6
	IR64	IRRI/Philippines	30.3± 1.3
	IR60	IRRI/Philippines	17.5± 2.1
	IR42	IRRI/Philippines	6.7± 0.4
	IR66	IRRI/Philippines	14.4± 2.0
	IR29	IRRI/Philippines	15.8± 0.7
	IR65	IRRI/Philippines	9.3± 2.3
	IR8	IRRI/Philippines	18.2± 0.8
	IR40	IRRI/Philippines	13.1± 2.3
	PSBRc60	IRRI/Philippines	14.4± 1.2
	Goo Meuang	Thailand	35.6± 5.3
	Taichung Senyo 223	Taiwan	15.6± 0.1
	Suweon 339	Korea	12.1± 1.5
	ADT 36	India	40.3± 3.3
	C702015	Taiwan	30.7± 3.5
	C662083	Taiwan	41.0± 1.5
	China rice-203	Myanmar	24.9± 1.4
Basmati	Punjab Basmati	India	30.5± 1.7
	Karnal Basmati	Pakistan	8.9± 2.2
	Basmati 370	India	7.7± 1.0
	Basmati	Pakistan	18.3±0.7
	Pusa Basmati	India	35.7±2.0
	Basmati 217	India	23.2± 1.4
	Ranbir Basmati	India	16.0±4.0
	Dehradun Basmati	India	44.1±4.5
	Karnal Local	Taiwan	15.8± 0.2

Table 3.3. cont…

Category	Variety	Source Country/Origin	Total Folate (µg/100g)
Japonica	Asahi	Japan	9.6± 3.7
	Ishikare Shiroke	Japan	14.9± 0.3
	Nipponbare	Japan	24.5± 6.5
	Norin	Japan	18.0± 3.6
	Milyang 15	Korea	19± 1.1
	Taipei 101	Taiwan	16.9± 0.9
	Aichi Asahi	Japan	22.8± 6.2
	Toride	Japan	24.8± 4.7
	IRI 353	Korea	28.8± 3.4
Upland	Moroberekan	Guinea, West Africa	63.6± 1.7
	Milagrosa	Philippines	17.9± 1.29
	Dinorado	Philippines	29.3± 4.0
	Cartuna	Indonesia	22.1± 2.1
	Balam	Bangladesh	86.6± 14.4
	Palawan	Philippines	24.1± 1.6
	DV110	Bangladesh	38.5± 4.2
Rainfed lowland	Heenati	Sri Lanka	46.8± 4.7
	Domsiah	Iran	26.1± 0.6
	Chao Khao	Laos	12.7± 4.1
	Sarang Barung	Indonesia	17.8± 2.5
	Dourado Agulha	Brazil	12.6± 1.2
	Sadri	Iran	11.4± 1.9
	Lemo Besar	Indonesia	26.5± 3.5
	Camor	Indonesia	30.6± 5.5

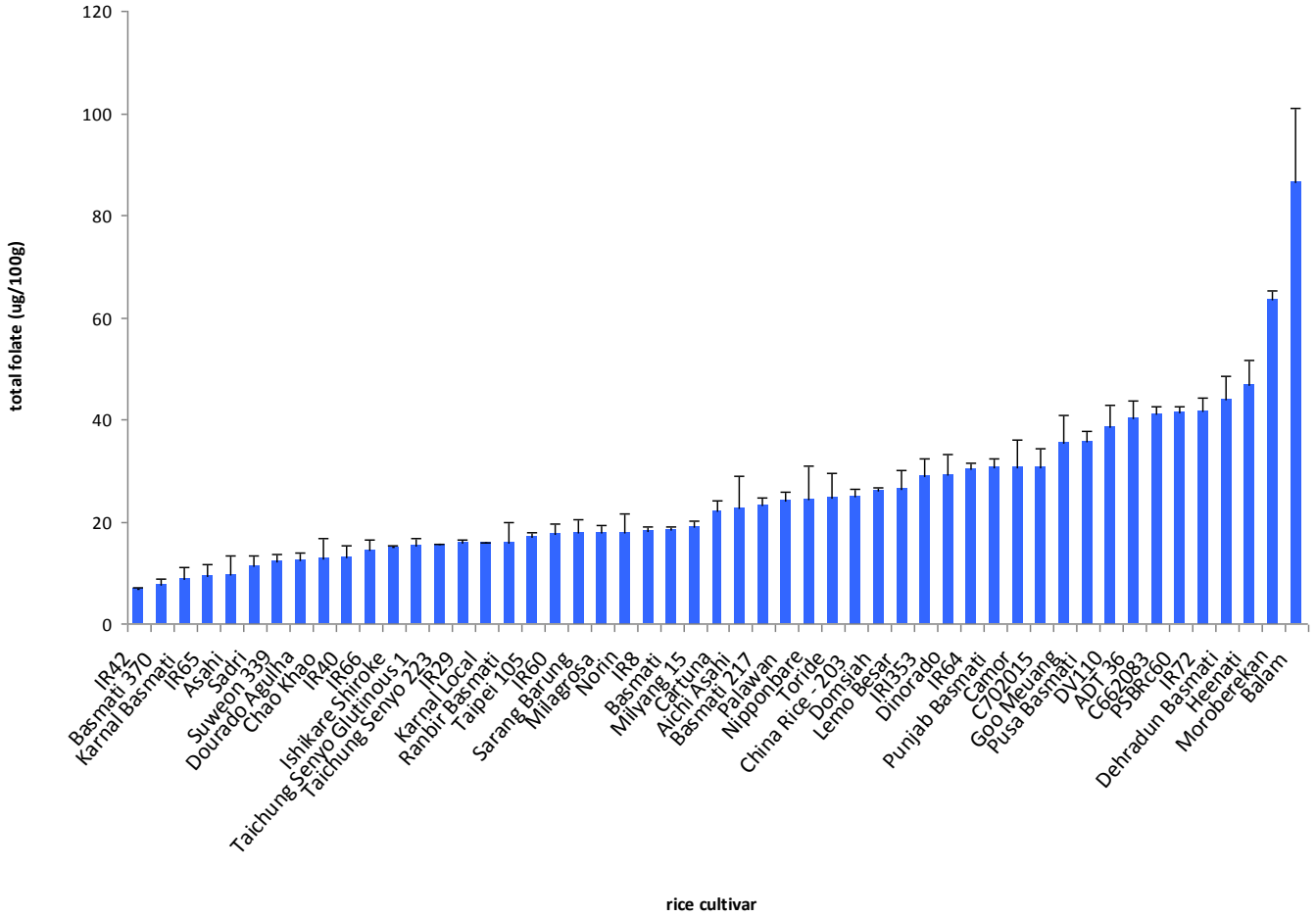

Figure 3.1. Total folate content (µg/100g) in 51 rice cultivars. Values are mean of triplicates with error bars (standard deviation).

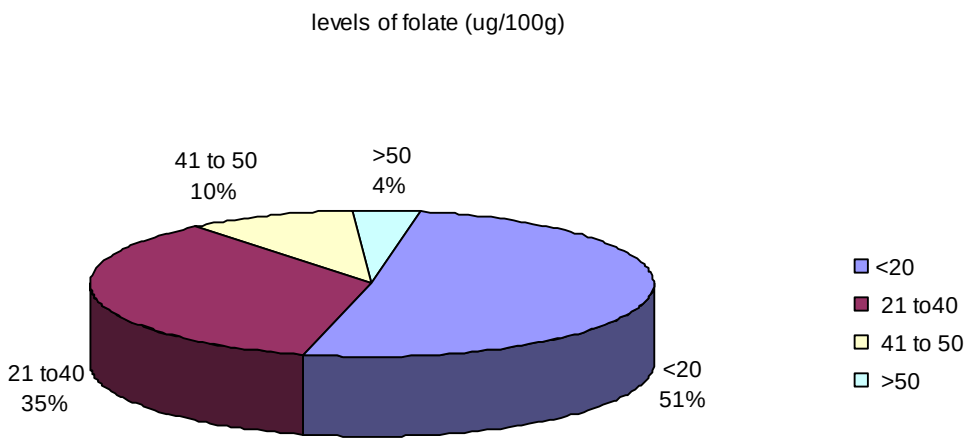

Figure 3.2. Different levels of total folate (µg/100g) in various rice cultivars screened using the microbiological assay

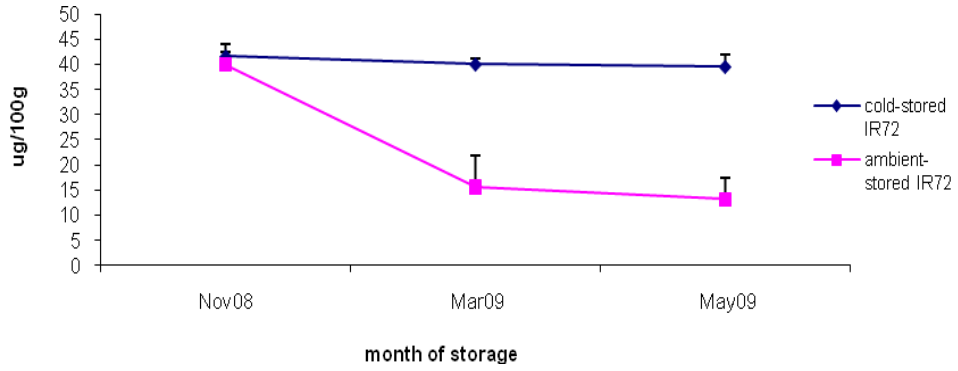

Figure 3.3. Changes in total folate content of unpolished IR72 grain stored in ambient (21-28°C) and cold condition (-28°C). Values are mean of triplicates with error bars (standard deviation)

3.5.2 Determination of monoglutamate folate species in unpolished rice grains using HPLC – UV/FLD

Analysis of a naturally high (Moroberekan) and two relatively low (IR72 and IR64) folate containing rice varieties were made using the method of Witthöft *et al*. (2006) at the Swedish University of Agricultural Sciences, Uppsala, Sweden. The summary of the results of the analysis made is shown in Table 3.4. Consistent with the results obtained from the MA, Moroberekan showed the highest total folate content (58.9 µg/100g) followed by IR72 (42.2 µg/100g) and then by IR64 (27.2 µg/100g) showing the lowest values (Table 3.4). 5-CH_3-H_4PteGlu is the most abundant form found in the grain. 10-CHO-PGA was detected in all the rice grain samples but H_4PteGlu which is one of the very unstable metabolites of the vitamin was only detected in Moroberekan at a very low concentration of 5.0 ± 0.2 µg/100g (Figure 3.4). Another reported mono-glutamated folate form in rice was 5,10-CH^+-H_4PteGlu (deBrouwer *et al*., 2008) at pH 3 which was not detected using the pH 6.1 phosphate extraction buffer in this particular study.

The recovery for individual folates is summarised in Table 3.5. The mean recovery for 5-CH_3-H_4PteGlu was 87.8% and 91.2% at two spiking levels, whereas the recoveries for 10-CHO-PGA was considerably higher, 99.2 – 100%, which may be

explained by higher stability of this folate form. These recoveries were taken into account in quantifying the folate content in the rice grain samples reported in Table 3.4. The recovery test was only made with IR72 rice grains due to the limited availability of Moroberekan seeds. Using the dienzyme (α-amylase and rat serum) extraction, only two forms of folate (5-CH_3-H_4PteGlu and 10-CHO-PGA) were recovered from IR72 grains. These results were similar to the previous work made on rice flour (Yazynina *et al*., 2008) where 5-CH_3-H_4PteGlu was also the major folate form (5.0 ± 0.1 of 6.3 ± 0.2 µg/100g total folate) and H_4PteGlu was just 1.4 ± 0.1 µg/100g. As this group was a very known team to establish an HPLC method for folates measurement in cereal matrices, this study was conducted in collaboration with them for the author to gain the knowledge and skills in developing the method for LC-MS/MS measurement of folates in rice later on.

Table 3.4. Folate contents in unpolished rice (µg/100g), determined by dienzyme method (mean ± SD; *n*=3) in HPLC-UV/FLD

Rice Variety	5-CH_3-H_4PteGlu	H_4PteGlu	10-CHO-PGA	Total Folate
Moroberekan	51.5 ± 7.2	5.0 ± 0.2	2.5 ± 0.8	58.9
IR72	39.0 ± 1.5	ND	3.2 ± 0.3	42.2
IR64	24.4 ± 3.8	ND	2.8 ± 0.7	27.2

ND – not detected

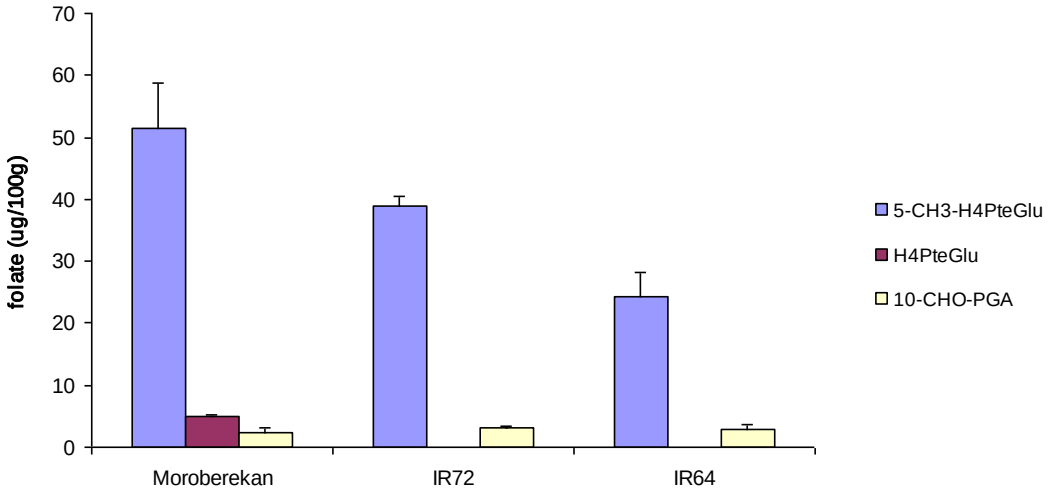

Figure 3.4. Folate forms detected in unpolished Moroberekan, IR72 and IR64 rice grains using HPLC-UV/FLD. Values are mean of 3 replicates with error bars (standard deviation)

Table 3.5. Recovery of dienzyme method for folate determination in unpolished rice grain using HPLC-UV/FLD

Folate Form	Amount in sample (µg/100g)	Added (µg/100g)	Found (µg/100g)	Recovery % Mean ± SD (n=3)
5-CH_3-PteGlu	33.6	45.38	73.47	87.82± 6
		44.64	74.33	91.24± 2
10-CHO-PGA	4.59	2.43	7.02	100.00± 5
		2.39	7.0	99.18± 7

3.5.3 LC-MS/MS measurement of folate forms in the rice grain

Folate profiling of some low, medium and high folate (total concentration based on MA screening results) rice cultivars revealed that 5-CH_3H_4PteGlu is the most abundant form of folate in the rice grain. Although the same mono- and polyglutamated folate forms were detected in most rice samples, they vary in amount between rice cultivars with relatively low, medium and high total folate concentration. A sample of a typical LC-MS/MS total chromatogram of rice grain folates is shown in Figure 3.5.

The major polyglutamated forms of folate detected and identified in unpolished rice grains were 5-CH_3-H_4PteGlu$_4$, 5-CH_3-H_4PteGlu$_5$ and 5-CHO-H_4PteGlu$_5$ (Figures 3.7 to 3.10). Structural confirmation of these folate species were made by matching their mass spectra with their corresponding mass spectral library. Santoyo Castelazo (2009) improved this feature from the method published by Garratt *et al*.,2005 . It was observed that the mass accuracy was in some cases ± 0.2 or ± 0.1 mass units away from the correct mass but this is within the normal range of mass accuracy for LC-MS/MS. Figure 3.6 clearly shows that the major mono- and polyglutamated folates in rice are in methylated forms and the formylated forms are considerably in minor quantities which concur with other studies on tomato (Iniesta *et al*., 2009) which shown to contain mostly 5-CH_3-H_4PteGlu and 10% H_4PteGlu; leeks with 29.5%

5-CH_3-H_4PteGlu and 33.5% polyglutamated form of 5-CH_3-H_4PteGlu, cauliflower with 8.5% 5-CH_3-H_4PteGlu and 48.7% polyglutamated form of 5-CH_3-H_4PteGlu, and green beans (Melse-Boonstra *et al*., 2002) containing 27.8% 5-CH_3-H_4PteGlu and 26.5% of polyglutamated 5-CH_3-H_4PteGlu; rice flour with 1.4 µg/100g H_4PteGlu and 5.0 µg/100g 5-CH_3-H_4PteGlu, and breads with 5.1 µg/100g H_4PteGlu and 31.9 µg/100g 5-CH_3-H_4PteGlu (Yazynina *et al*., 2008); and wheat (Piironen *et al*., 2008) had 6-9% H_4PteGlu, 11-23% 5-CH_3-H_4PteGlu, 8-12% 10-CHO-H_2PteGlu, 16-23% 10-CHO-PGA, 34-47% 5-CHO- H_4PteGlu and 5-10% PteGlu.

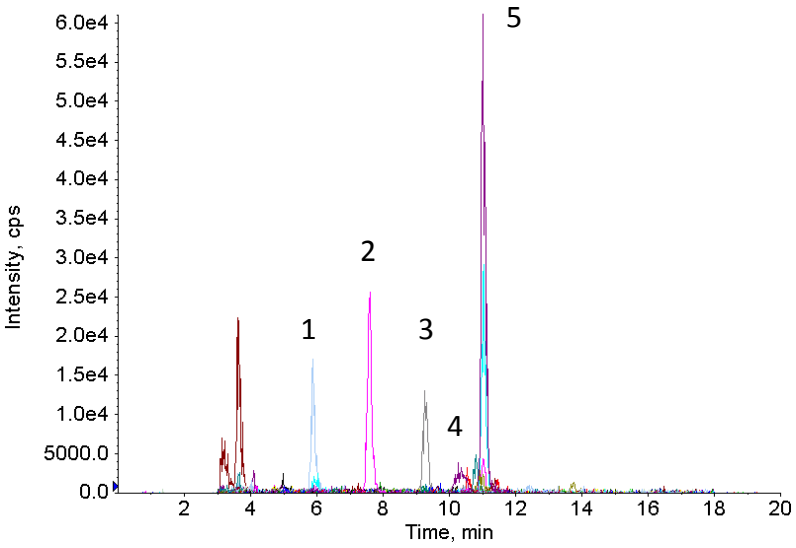

Figure 3.5.LC-MS/MS chromatogram of folates detected in unpolished rice grain. The numbers correspond to (1) 5/10-CHO-H_4PteGlu, (2) 5-CH_3-H_4PteGlu, (3) 5-CH_3-H_4PteGlu$_4$, (4) 5-CH_3-H_4PteGlu$_5$, and (5) 5/10-CHO-H_4PteGlu$_5$.

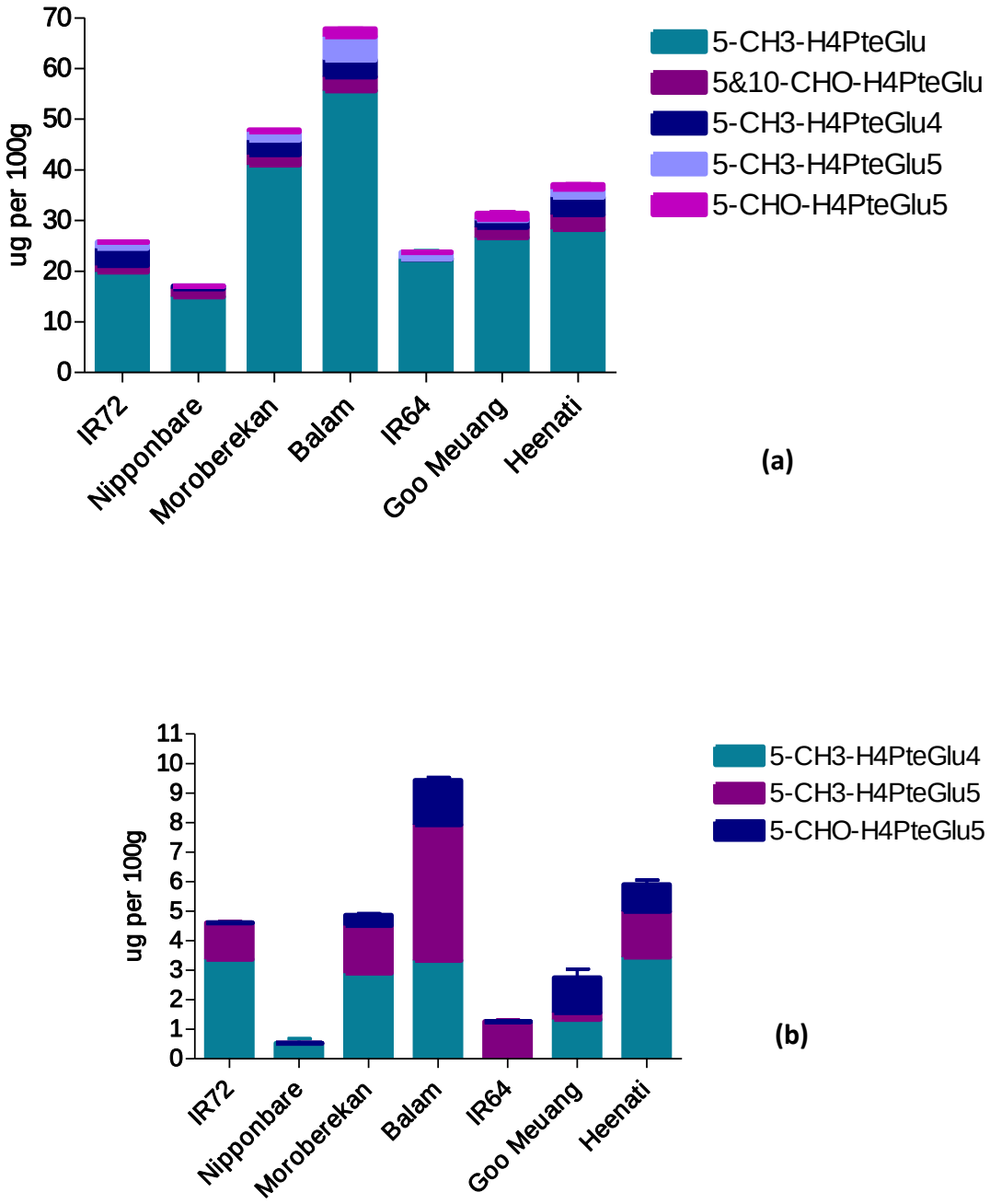

Figure 3.6. Folate profile of rice varieties with low, medium, and high total folate concentration (a). Polyglutamate folates distribution in the same set of rice (b). Values are mean of triplicates with error bars (standard deviation)

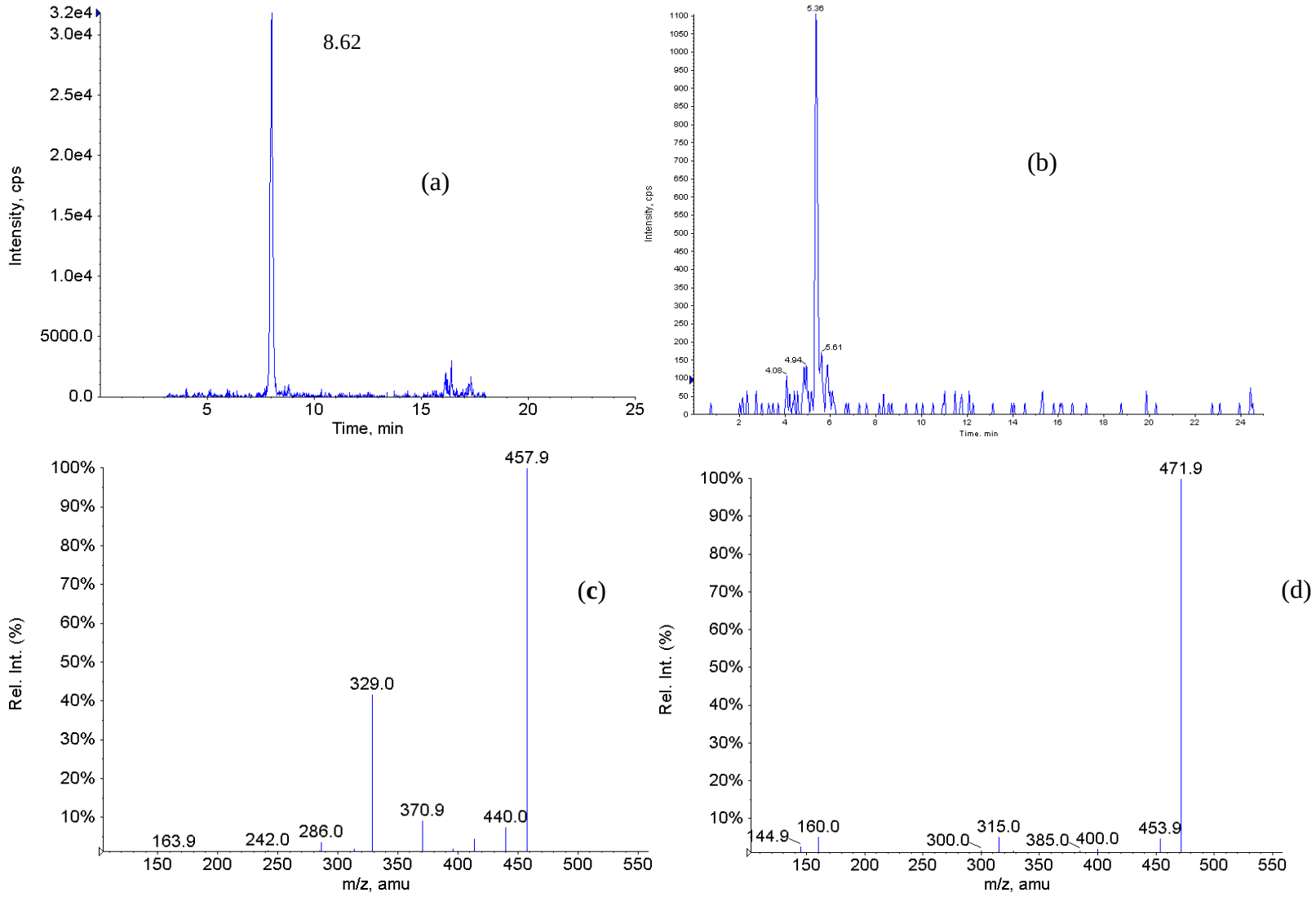

Figure 3.7. Chromatograms of 5-CH3-H4PteGlu (a) and of 5-CHO-H4PteGlu (b) in rice grain and their corresponding EPI spectra (c, d)

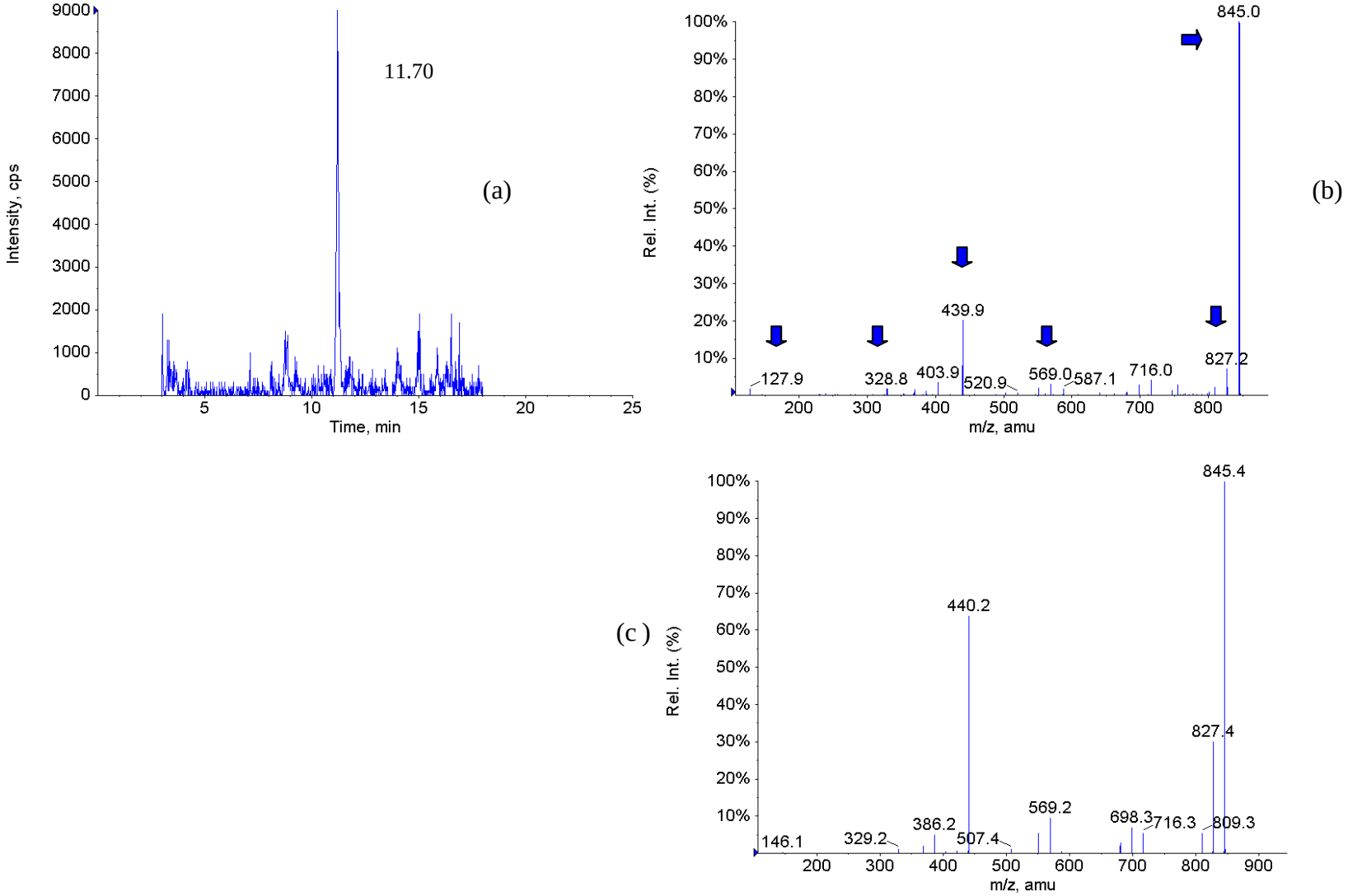

Figure 3.8. Chromatogram (a) and EPI spectra of 5-CH_3-H_4PteGlu$_4$ in rice grain (b) and verified with the library spectra (c)

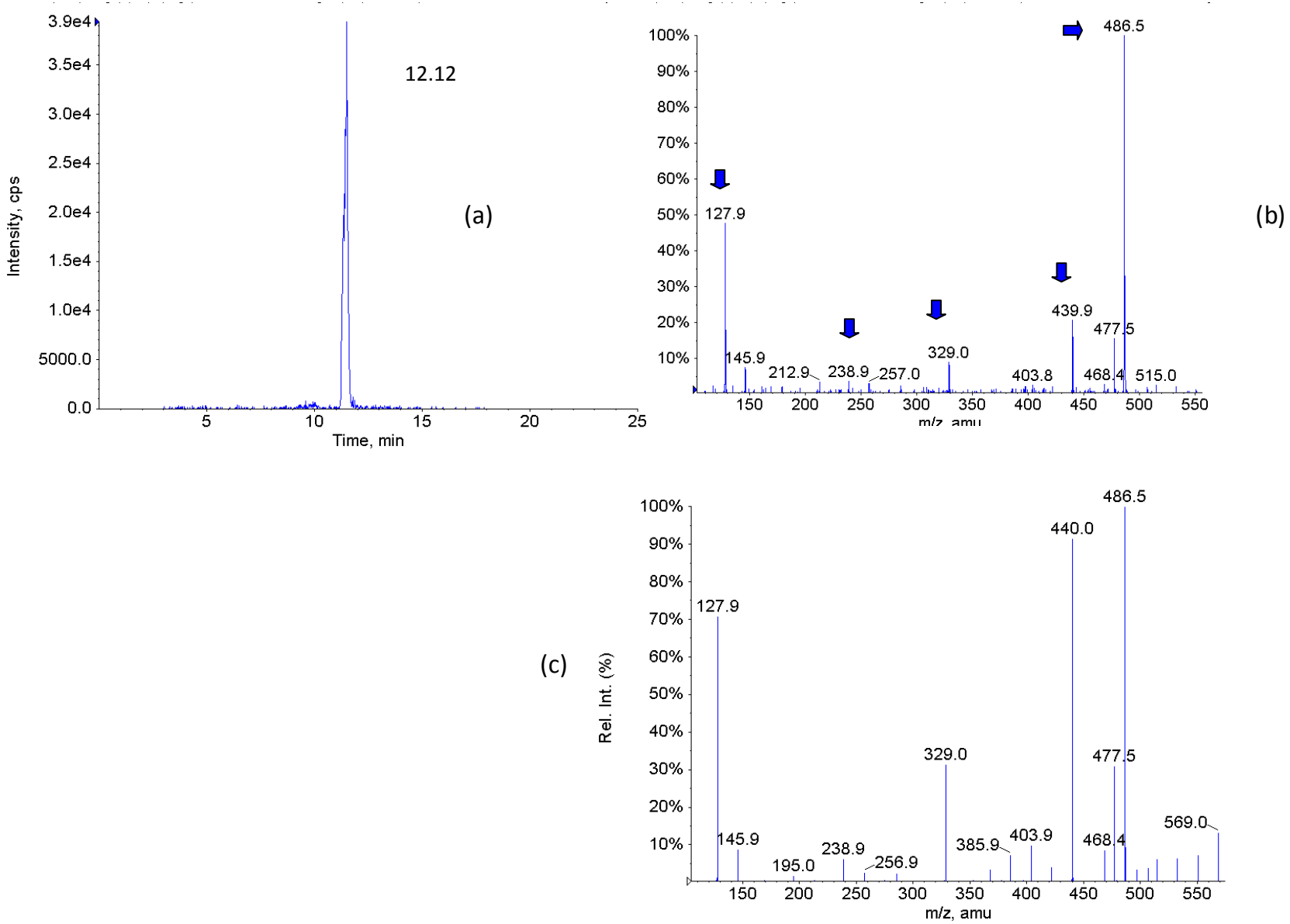

Figure 3.9. Chromatogram (a) and EPI spectra of 5-CH_3-H_4PteGlu$_5$ in rice grain (b) and verified with the library spectra(c)

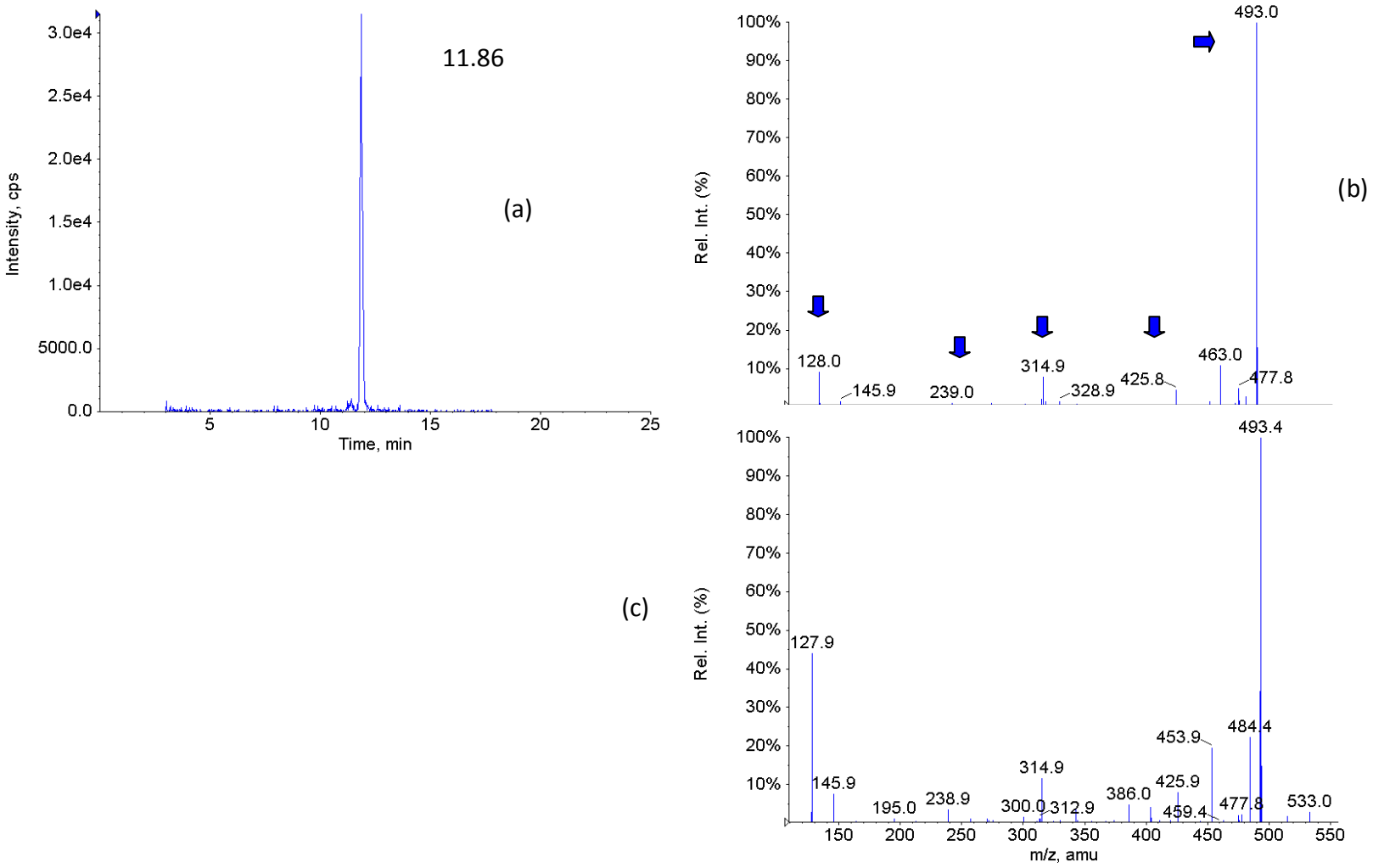

Figure 3.10. Chromatogram (a) and EPI spectra of 5-CHO-H_4PteGlu$_5$ in rice grain (b) and verified with library spectra (c)

3.5.3.1 Folate vitamer distribution

5-CH_3-H_4folate was the most abundant folate vitamer in unpolished rice grain (Figure 3.11). This is in good agreement with the findings of De Brouwer *et al*. (2008); Pfeiffer *et al*. (1997) and Konings *et al*. (2001) showing that methyl folates accounted for approximately ≥50% of total folate in rice (Figure 3.11) and the high proportions of methyl and formyl folates in cereals like rye, wheat and rice. This pattern is different from that of most vegetables like spinach and broccoli, where the predominant vitamer is 5-CH_3-H_4PteGlu and other vitamers typically account for 0 to 20% of the total folate pool (Vahteristo *et al*., 1997; Konings *et al*., 2001).

In this study, minor amounts of three polyglutamated folates (5-CH_3-H_4PteGlu$_4$, 5-CH_3-H_4PteGlu$_5$ and 5-CHO-PteGlu$_5$) were detected in the rice grains (Figure 3.6b). The attempt by Garratt *et al*. (2005) to measure polyglutamylated folates in biological materials showed that total polyglutamates in spinach as a percentage of the total folate pool equalled 68%. Limited reports are available for individual polyglutamate forms in rice although de Brouwer *et al*. (2008) estimated the amount of polyglutamates to be 40% in the rice grain in terms of total folate pool. In pea leaves, tetra- and penta-glutamated folate forms were reported to be predominant (Imeson *et al*., 1990) while di-glutamated folate was known to be most abundant in carrot root (Cossins and Chen, 1997).

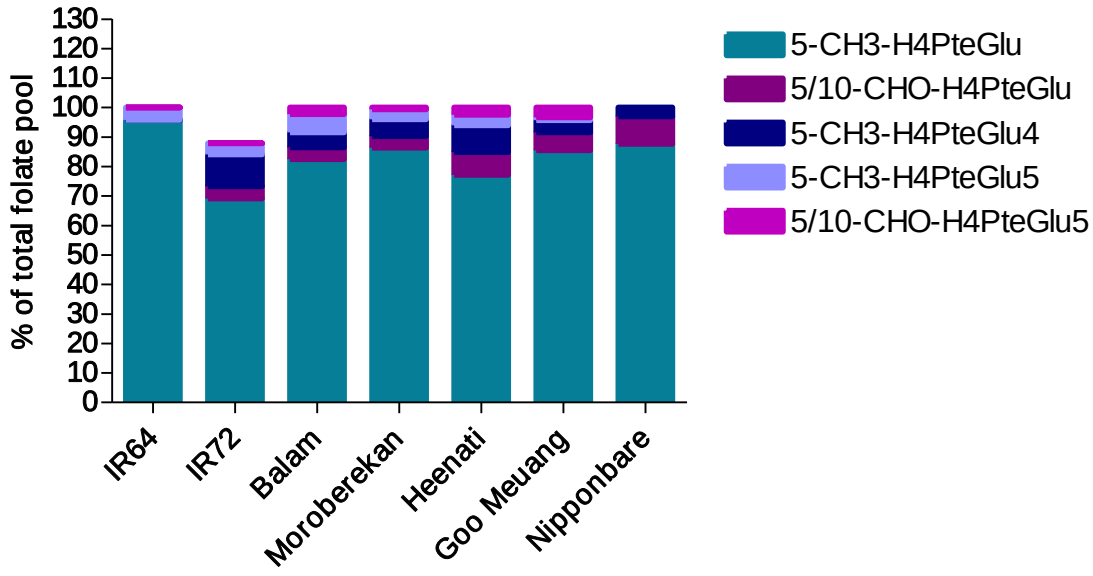

Figure 3.11. Percent distribution of different folate species in rice

3.5.4 Comparison between the MA, HPLC-UV/FLD and LC-MS/MS results

To date, there are only few works comparing HPLC and microbiological results for folates (Pfeiffer *et al*., 1997; Finglas *et al*., 1999; Lawrance, 1996). Data comparison (Table 3.6) of total folate in rice grain using different methods showed that the LC-MS/MS results are >20% lower than the MA results but almost similar with the results obtained from the HPLC-UV/FLD following the method of Witthöft *et al*., 2006.

Though the folate contents in rice determined by LC-MS/MS are lower than the values obtained from the microbiologically determined folate contents, they correlated well as also shown in the study made by Pfeiffer et al. (1997). The same pattern was observed with the HPLC-UV/FLD results for three varieties analysed in Uppsala where the obtained values were lower than the microbiological results. Konings et al.,(2001) and Ruggeri et al., (1999) also observed the same pattern when the HPLC analysed foods and diet resulted in 24-52% lower values than the MA values which suggested that nonfolate compounds influence the bacterial response by stimulating their growth or with similar folate activity, resulting in higher folate contents with the microbiological assay. It may also be that HPLC methods underestimate total folate by approximately 1/3. This and other reasons for the discrepancy are evidently complex and require further investigation.

Table 3.6. Some data comparison of total folate in brown rice grain (results obtained from three methods of analysis, µg/100g)

Rice sample	MA	SLU HPLC-UV/FLD	LC-MS/MS
IR72	41.6	32.2	32.2
IR64	30.3	27.2	29.4
Moroberekan	63.6	58.9	50.0
Nipponbare	24.5	na	16.6
Balam	86.6	na	67.9
Goo Meuang	35.6	na	31.1
Heenati	46.8	na	36.4

na – not analysed

3.6 Conclusion

In this study, rice materials were screened and profiled for their folate content in the grains using MA and LC-MS/MS. Total folate in 51 cultivars ranged from 6.7 to 86.6 µg/100g. Natural variation in terms of total folate concentration and forms exist in rice. The folate forms found in rice grains were 5-CH_3-H_4PteGlu, 5- and 10-CHO-H_4PteGlu, 5-CH_3-H_4PteGlu$_4$, 5-CH_3-H_4PteGlu$_5$ and 5-CHO-PteGlu$_5$ of which 5-CH_3-H_4PteGlu is the most predominant form followed by CHO-H_4PteGlu. Quantification of individual folate species in the rice grain was possible without prior sample purification of extracts. The method developed for the rice matrix enabled the measurement of the major mono- and polyglutamated folates in rice. The knowledge about the form and distribution of individual folate in rice grain is of practical significance for the development of new rice varieties with more folate in the grains. This information will be useful for selecting the best rice cultivars for breeding. By choosing rice cultivars loading more folate in the grain as a parent or donor of the genes responsible in the process of grain folate accumulation, folate-rich rice can be developed.