1 GENERAL INTRODUCTION

1.1 Rice Facts and Figures

1.1.1 Rice Production and Utilisation

Rice (Oryza sativa L.), the staple food for more than half of the world's population, is also the source of most of the nutrients (macro and micro) for the nutritionally-at-risk groups and the economically-challenged people specially in most Asian countries where 90% of the world's rice is grown and consumed (www.irri.org). Rice is generally considered a semiaquatic annual grass plant (Oelke *et al*., 1997). About 20 species of the genus *Oryza* are recognised, but nearly all cultivated rice is *O. sativa* L. A small amount of *O. glaberrima*, a perennial species, is grown in Africa. The so-called "wild rice" (*Zizania palustris*), grown in the Great Lakes region of the United States (US) and now in California, is more closely related to oat than to rice (Juliano, 2003).

Figure 1.1. Farm-grown Rice (*Oryza sativa L*)

Domestication of rice produced the two cultivated species *Orzya sativa* and *O.*

glaberrina. The two major forms of *Orzya sativa* are thought to have diverged 2-3 million years ago to form the South Asian (*Indica*) and Chinese form (*Japonica*). The earliest archaeological evidence of domestication of rice dates back to 4000 BC when grains of rice from *O.sativa* were found in a pot in Thailand by Wilhelm G. Solheim in 1966.

Because of its long history of cultivation and selection under diverse environments, rice has acquired a broad range of water/soil regimens from deeply flooded land to dry, hilly slopes. Rice can be grown in soil direct, in partial flooding or completely submerged. Each of these methods has their own merits and demerits (Juliano, 2003).

The four major ecosystems where rice is planted are (1) irrigated, (2) rainfed lowland, (3) upland and (4) flood-prone wetland. The irrigated ecosystem has the highest yield and constitutes the major total rice area and source of rice production followed by the rainfed lowland and upland being the lowest. Most of the international rice (IR) lines developed are suitable for irrigated ecosystem (www.irri.org).

Irrigated systems have good water control and the rice is flooded throughout the growing season while upland rice is grown in conditions without surface water, relying solely on rainfall. Rice grown in rainfed lowland is dependent on rainfall, although fields are bunded or embanked to retain water (De Datta 1981).

The steps in rice cultivation include seed selection, seedbed and land preparation, transplanting, weeding, fertilizing, pest management, harvesting, threshing, drying and marketing.

There are estimated to be around 100,000 rice varieties, but only a small proportion is widely cultivated. They vary in grain weight, size and shape; degree of dormancy; longevity; and seedling vigor; and some have red to purple-black pigments (Juliano, 2003).

Regarded as the grain of life, scientists are keen on reengineering rice to add nutrients that can make the grain a more healthful source of vitamins and minerals.

Rice is a model plant system for genome analysis due to its small genome size, high synteny to other monocots, its efficient transformation system, the availability of large-scale analyses of expressed sequence tags and dense molecular genetic maps, large-insert libraries, and abundance of genetic resources (Harushima *et al*., 1998). The rice genome was the first crop genome to be successfully mapped, allowing scientists to identify and functionally characterize the genes and biochemical pathways that are responsible for agronomic performance, resistance to biotic and abiotic stress and consumer quality, among others. Traits for nutritional quality are now the next target and recently led to the development of “Golden Rice”, a genetically modified rice with higher beta-carotene content.

Figure 1.2. Greenhouse grown IR72 at the University of Nottingham

1.1.2 Rice Structure, Chemistry and Composition

The structure and gross composition of the rice grain is shown in Figure 1.3. A mature rice grain is harvested as a covered grain (rough rice or paddy), in which the caryopsis (brown or unpolished rice) is enclosed in a tough siliceous hull or husk. The caryopsis is enveloped by the husk, composed of two modified leaves (lemmae) – the palea (dorsal) and the larger lemma (ventral). The palea and lemma are hold together by two hooklike structures. Some varieties have an awn attached to the tip of the lemma.The caryopsis itself is a single-seeded fruit, wherein the pericarp is fused to the seed (composed of seed coat, nucellus, endosperm, and embryo). Pigments in coloured rices are usually in the pericarp or the seed coat which varies retention on milling across varieties (Juliano, 2003).

The aleurone layer which is the outermost layer of endosperm tissue and

completely surrounds the rice grain, differs in both morphology and function from the starchy endosperm. Varieties differ in the thickness of the aleurone layer. Coarser or bolder short-grain rices tend to have more cell layers than slender, long-grain rices. The embryo (germ) is very small and located on the ventral side at the base of the grain. It is bounded on the outside by the aleurone layer and by the fibrous cellular remains of the pericarp, seed coat and the caryopsis coat. The starchy endosperm borders the inner edge of the embryo (Juliano, 2003).

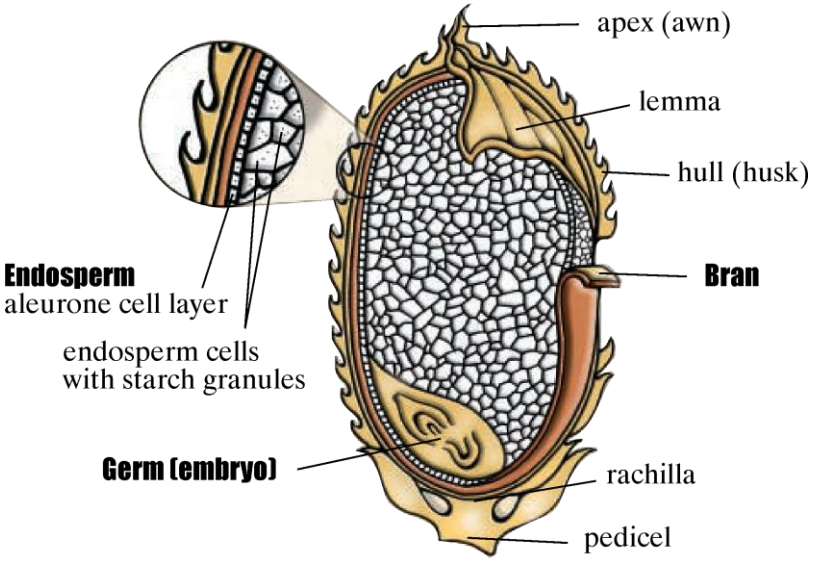


Figure 1.3. Longitudinal section of the rice grain (modified from Juliano, 2003)

According to Chen *et al*., 1999, brown rice is composed of surface bran (6-7% by weight), endosperm (~90%) and embryo (2-3%).

Carbohydrates, mainly starch, are the major constituents of the rice grain. Endosperm starch is derived mainly from photosynthesis after flowering, hence the high correlation between yield and solar radiation during panicle formation through

grain filling while grain protein comes mainly from translocation of accumulated plant nitrogen at flowering stage (Juliano, 2003).

Nutrient composition and genetic diversity in rice were reviewed by Kennedy and Burlingame (2003). Protein content in brown rice is slightly higher than in milled rice because of the higher protein level in the bran. Vitamins and minerals like thiamine, riboflavin, niacin, pantothenic acid, folate, vitamin E, Fe and Zn are also higher in rice bran. Microscopy and energy dispersive X-ray microanalysis of globoids in rice embryo and endosperm tissues showed that P, Mg and K are present in all globoids (Wada and Lott, 1997).

1.1.3 Rice Nutritional Value and Importance

Rice is mainly consumed as boiled, milled grain. The per capita (kg/person/year) consumption of rice in Southeast Asia is 144 in 2005 (FAO, 2006) such that a small increase in the nutritional value of the grain would have a significant impact.

Brown rice has one of the highest energy contents among whole cereals, similar to millet and second only to oat (USDA, 2008). In addition, it has the lowest protein content and total dietary fiber among the cereals which may be due to the removal of the hull. The fat content of brown rice is higher than that of wheat but lower than that of corn. Rice oil has the lowest linoleic acid (18:2) and linolenic acid (18:3)

content among the cereal oils. Nevertheless, rice bran oil has cholesterol-lowering effect comparable to oils from other cereals (McCaskill and Zhang, 1999).

Nutritional anaemias are widespread among rice-consuming countries. The causes are low dietary intake of Fe, B12, (McCance and Widdowson, 2004) and folic acid, low biological availability from food, blood loss caused by intestinal parasites, and unfulfilled demand associated with rapid growth (young children) and pregnancy (pregnant women).

Rice also plays a fundamental role in world food security and socio-economic development (Fresco, 2005) as it provides employment for millions of rice producers, processors and traders worldwide. As reported by FAO in 2003, rice provides 27% of the dietary energy supply and 20% of the dietary protein intake worldwide. In Asia alone, more than 2 billion people obtain 60-70% of their energy intake from rice and its derived products. Currently, rice is the most rapidly growing food source in Africa.

In 2004, the United Nations declared the International Year of Rice (IYR), where nutritional improvement is one of the key aims of the programme. Rice research, breeding and production have traditionally focused on ways to increase yield to cope with the population growth. Recently, the focus is on enhancing the nutritional quality of the grain in order to make impact on micronutrient deficiencies and promote improved human health.

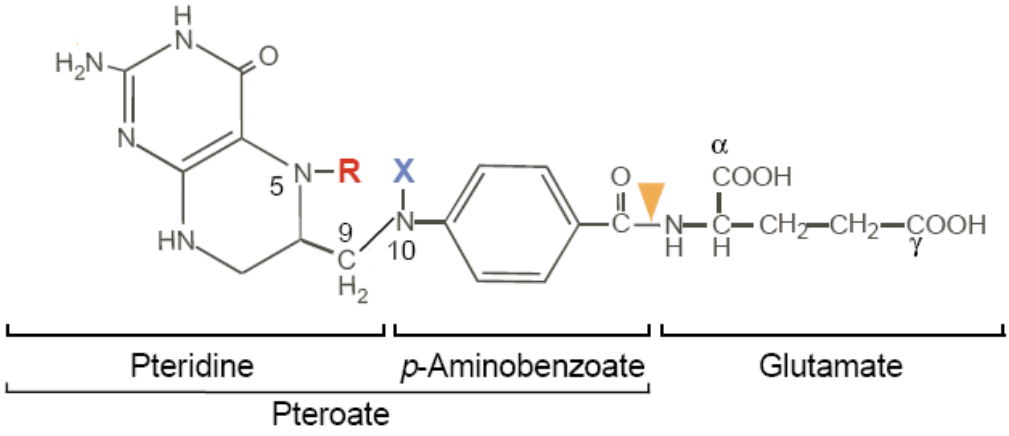
1.2 Folates and Folic acid

1.2.1 Folate Chemistry, Structure and Properties

Folic acid (pteroylmono-L-glutamic acid) and folate are water soluble forms of a B vitamin formerly known as B9 which was first identified in brewer's yeast by Lucy Wills in 1931 which led to the first extraction in 1941 and consequently resulted in the first synthesis of folic acid in 1946. Folic acid refers to the synthetic vitamin used in supplements, whereas folate is the form found in foods. The term comes from the Latin word "folium" which means leaf because folate is found mostly in leafy vegetables. Folate exists in nature in more than 100 analogues based on folic acid, the fully oxidized and stable (monoglutamate) form (Figure 1.4) of this vitamin (McIntosh and Henry, 2008). As illustrated by Rebeille *et al*. (2006), folate consists of a pteridine ring attached to a para-aminobenzoate (*p*ABA), which in turn linked to a side chain of (3 to 11) L-glutamate residues with peptide linkage (Figure 1.5). The pteridine ring could be partially reduced at the 7,8-position (H_2folate) or fully reduced (H4folate) or fully oxidized (folic acid). Folate differs in susbtituents located in the N-5 and N-10 position of the pteridine ring and can consist of hydrogen (H), methyl (CH3), formyl (CHO), formimino (NHCH), methenyl (CH^+) and methylene (CH_2). The folate derivatives are then named depending on the nature of the

substituent and reduction level of pteridine ring. For example, a tetrahydrofolate, dihydrofolate and fully oxidized folate with a –HCO substitution at N-10 position is thus called 10-formyltetrahydrofolate (10-CHO- H_4PteGlu). The L-glutamate can be conjugated in repetitive units via γ-peptide linkage of the carboxyl group. These glutamates are called e.g. diglutamate and triglutamate, depending on the number of residues. For example, 5-methyltetrahydrofolate with 3 glutamate moieties is called 5-methyltetrahydropteroyltriglutamate (5-CH_3-H_4PteGlu$_3$). The most stable form of reduced folate is 5-formyltetrahydrofolate. All folates with more than one glutamate residue are sometimes referred to as folate polyglutamates. All fully reduced folates derived from tetrahydrofolate have two chiral centres. They are the α-C in the glutamic acid moiety and C atom in position 6 of the pteridine ring. Thus, four diastereoisomers exist: [6S, αS], [6S, αR], [6R, αS] and [6R, αR]. The naturally biologically active form of the diasteroisomer is [6S, αS] for tetrahydrofolate (H_4PteGlu), 5-methylterathydrofolate (5-CH_3-H_4PteGlu), and 5-formyltetrahydrofolate (5-CHO-H_4PteGlu). For 10-CHO-tetrahydrofolate (10- CHO-H_4PteGlu), 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) and 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4PteGlu), the active form is [6R, αS] (Groehn and Moser, 1999). The rate of folate degradation depends on its form and the food matrix with respect to pH, buffer composition, catalytic trace elements and antioxidants (Gregory, 1989). Antioxidants (e.g. ascorbic acid) in sufficient amounts may prevent oxidation of H_4folate (Blakley, 1960). Folate is soluble at very low pH as cationic forms are dominant. In the presence of ionizable α-carboxyl groups at

neutral to alkaline pH levels, polyglutamyl forms are more soluble than folic acid (Figure 1.6). Short-chain polyglutamyl folates are less hydrophobic than long-chain polyglutamyl folate at low pH where α-carboxyl groups are mostly protonated (Gregory, 1989).



Folate	R	X
THF	H	H
10-Formyl-THF	H	CHO
5-Formyl -THF	CHO	H
5-Methyl-THF	CH_3	H
5-Formimino-THF	CH=NH	H
5,10-Methenyl-THF	=CH−	
5,10-Methylene-THF	$-CH_2-$	

Figure 1.4. Chemical structure of folates (modified from Rebeille et al., 2006). Folates are tripartite molecules consisting of pteridine, PABA, and glutamate moieties, with pteridines synthesized in the cytosol and PABA in the plastids.

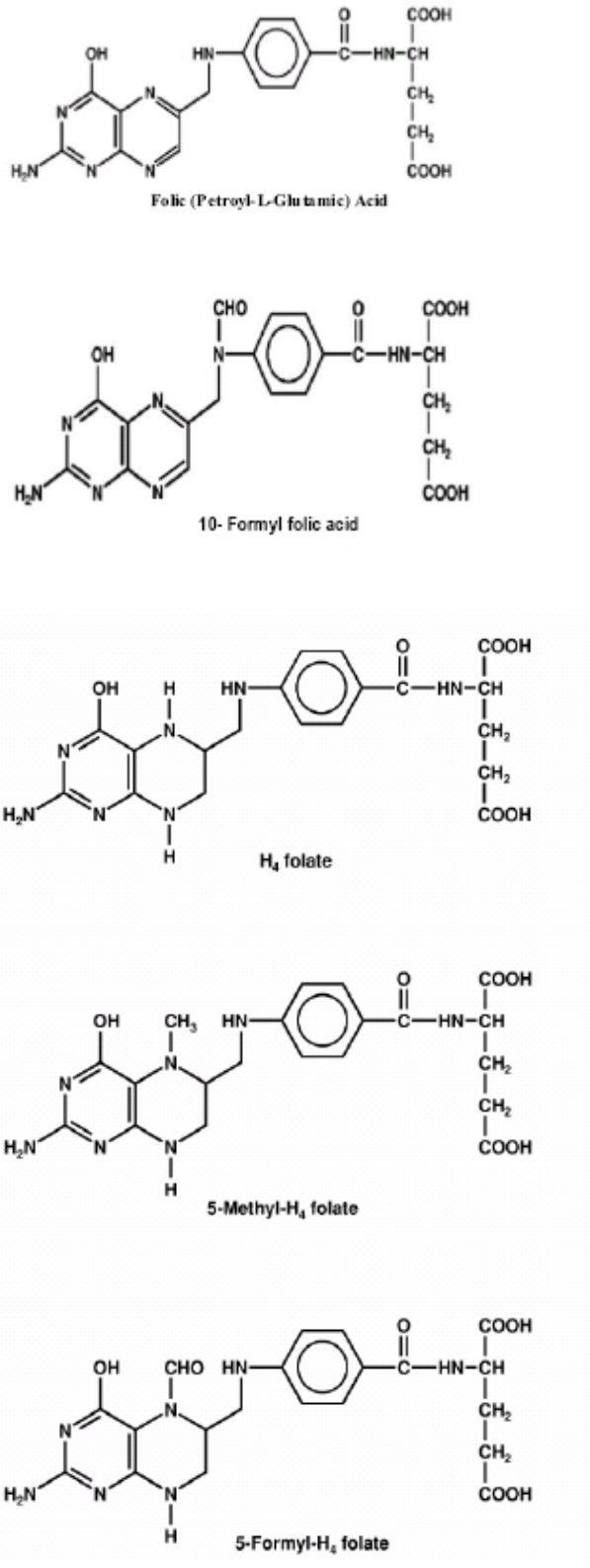


Figure 1.5. Molecular structure of folate (monoglutamates) (de Brouwer et al., 2008)

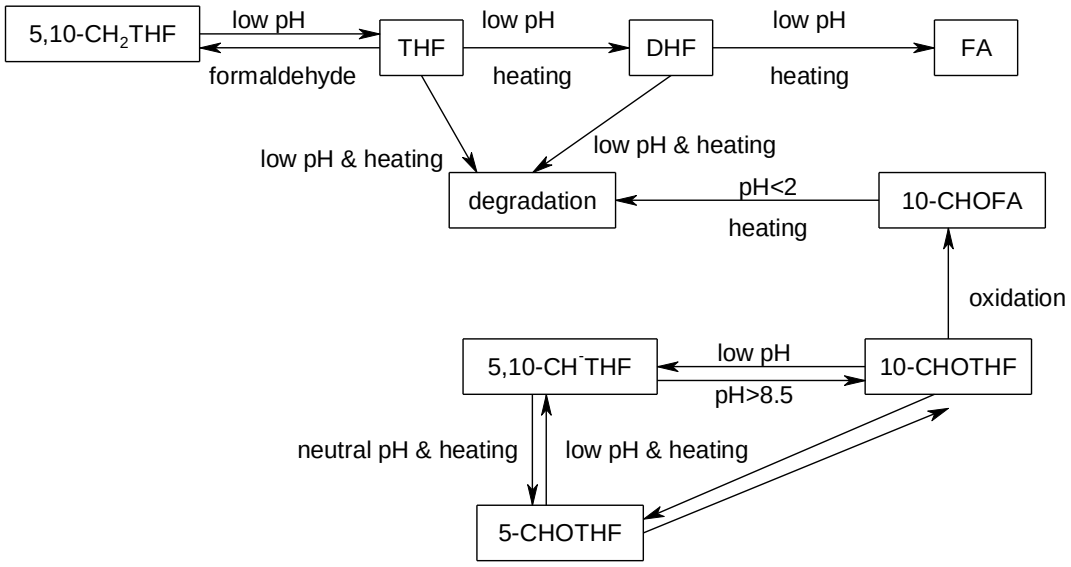


Figure 1.6. Folate interconversion at different conditions (modified from de Brouwer et al., 2007)

1.2.2 Folate in Plants and Foods

Folate synthesis and distribution in plants have been studied for a decade now (Orsomando *et al*., 2006). During plant development, folate concentration in tissue and growth stages varies depending on the physiological situation which indicates a regulated metabolism. According to Shrestha *et al*., (2000) and Mcnulty and Pentieva (2004), there is a complex partition of folate synthesis and folate-mediated reactions involved in the plant cell (Figure 1.7) : *p*ABA synthesis – where enzymes involved in the biogenesis of H_4F-Glun are distributed over the plastids, dihydropterin synthesis (cytosol) and the H_2F-Glu1 and H_4F-Glu_1 synthesis (mitochondria).

Folate is abundant in leafy green vegetables such as turnip greens or spinach, peas, beans and fruit (Catharino *et al*., 2006). Important staples like maize, wheat and rice, however, contain relatively low amounts of folates (Juliano, 2003; Tucker, 2003; Storozhenko *et al*., 2005; McIntosh *et al*., 2008). The most common form in food is poly-γ-glutamated folates which are prone to interconversion and oxidative degradation (Brouwer *et al*., 2001; Rychlik, 2004; Verlinde *et al*., 2008).

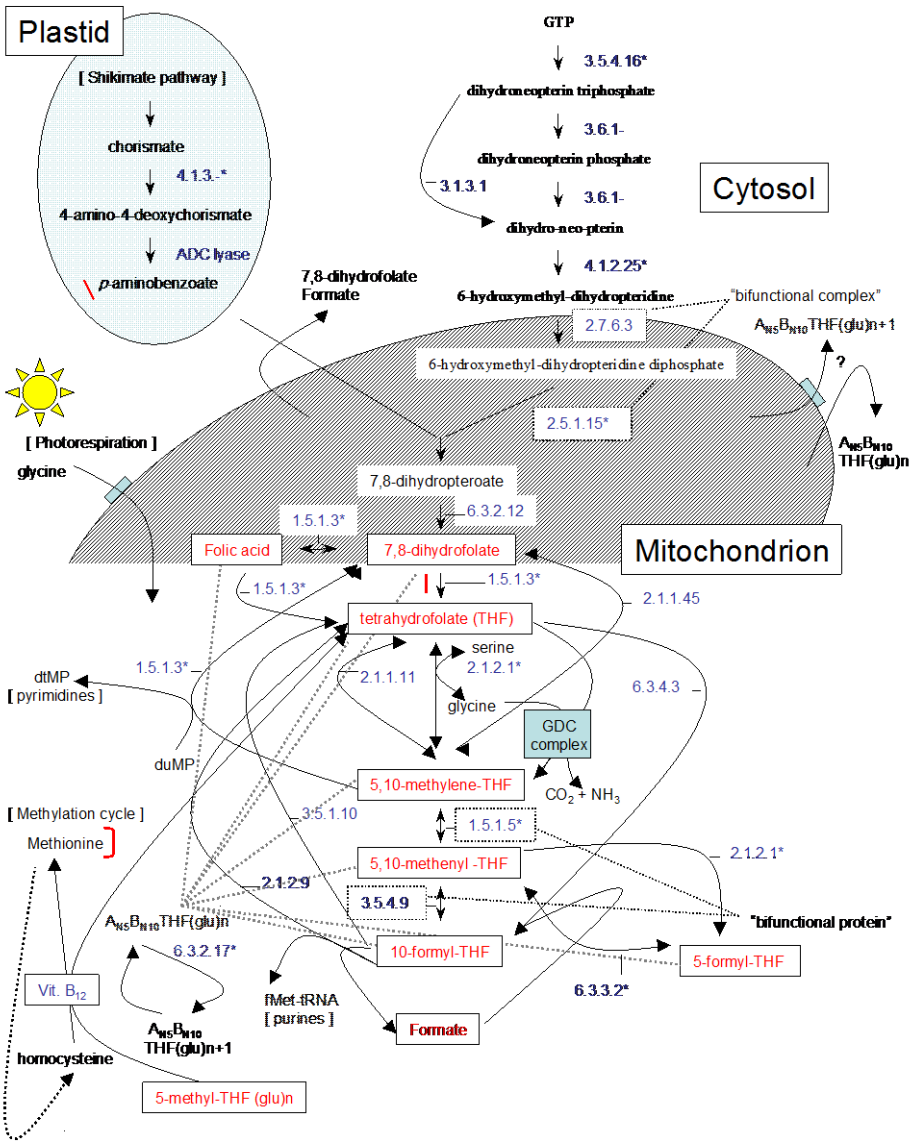


Figure 1.7. Folate biosynthetic pathway in plant, compiled using net based resources (TAIR-Arabidopsis thaliana Biochemical Pathways; BRENDA Enzyme database and KEGG Pathway database), and corrected/updated using available literature. Enzymes are represented by their EC number, with EC* indicating an enzyme for which the Arabidopsis gene has been identified. THF(glu)n represents a monoglutamated tetrahydrofolate, THF(glu)n+1 as polyglutamated form (adapted from Garratt *et al.*, 2005)

1.2.3 Folate in Cereals, Cereal Products and Rice

Folate exists in foods as various vitamers in reduced forms. Unlike many vegetables which mostly contain 5-methyltetrahydrofolate (Gregory *et al*., 2005; Vahteristo *et al*., 1997a; Konings *et al*., 2001), most cereals contain a large variety of other forms of the vitamin including methyl and formyl derivatives as well as unsubstituted tetrahydrofolate (Pfeiffer *et al*., 1997; Konings *et al*., 2001; Yazynina *et al*., 2008).

Folates in plants and cereals mostly exist in polyglutamate forms. As the microbiological assay and other analytical methods can measure only mono-, di- and to a certain extent triglutamates, the results can only be considered as rough and indicative estimates of the proportion of polyglutamyl folate when analyzed with and without conjugase (Kariluoto, 2008). Thus, to date, little information is available on the polyglutamyl distribution and the role of polyglutamate synthesis in plants and most especially in cereals.

Cereals, specifically in their whole/unpolished/brown grain form, supply much to the intake of dietary folate as they are generally consumed as staple food. A considerable variation in folate content between cereals exist and differ according to the grain species, cultivars and growing conditions (Table 1.1). Whole brown rice for example has a folate content ranging from 19 to 43 µg/100g which is

comparable to the other main cereals (wheat, rye, barley, oats). Like thiamine and other water-soluble B vitamins, it is mostly concentrated in the aleurone layer of the grain. However, folate in rice has not been investigated as intensively as folate in wheat. Data on the variation between rice cultivars are almost non-existent. Of particular concern is the fact that most rice consumption is of polished white rice which has significantly lower folate content than brown rice because most of it is removed with the outer rice layers during polishing.

Table 1.1. Total folate contents in selected cereals and cereal products according to U.S. and UK food composition databases (µg/100g edible part)

	USDA, 2005	FSA, 2002
Rye flour	60	78
Wheat flour, whole grain	44	57
Barley flour	8	-
Oats	56	60
Rice brown	20	49
Wheat bran	79	260
Wheat germ	281	-
Oat bran	52	-
Rye bread	51	24
Wheat bread	41	40
Pasta, cooked	7	4
Rice, cooked (polished)	4	10

1.2.4 Absorption, Bioavailability and Nutritional Importance

In humans, the small intestine is the major site of folate absorption (Strum, 1979; Mason *et al*., 1990). This occurs particularly in jejunum (proximal intestine) by a saturable carrier-mediated, pH and energy dependent transport mechanism as described by Strum (1979) and Mason (1990) encoded by the reduced folate carrier gene (RFC-1)(Sirotnak and Tolner, 1999). At higher concentrations absorption can also take place through a non-saturable mechanism involving passive diffusion (Gregory, 1996). The monoglutamate 5-CH_3-H_4PteGlu is the only form that goes into the blood circulation from the intestinal cell after the dietary polyglutamates are hydrolyzed by the brush-border γ-glutamyl hydrolase - also known as folylpolyglutamate carboxypeptidase or folate conjugase (Verwei *et al*., 2004). Other forms of monoglutamate and folic acid are likewise absorbed at pH 6.3 or diffused through the membrane and converted to the 5-CH_3-H_4PteGlu in the process (Veedamali and Subramanian, 2003; McKillop *et al*., 2006). After absorption, 10-20% of 5-CH_3-H_4PteGlu is stored by the liver (Figure 1.7) in the form of tetra- penta- hexa- and heptaglutamates of 5-CH_3-H_4PteGlu and 10-HCO-H_4PteGlu (Winkels *et al*., 2007). The rest of the absorbed folates are re-circulated and reabsorbed daily and as much as 100 µg is biliary excreted (McKillop *et al*., 2006; Winkels *et al*., 2007). If folic acid is consumed in amounts exceeding 5.7 x 10-7 mol (250 µg) in a single dose or as fortificant in meal, its free form can also appear in plasma (Kelly *et al*., 1997).

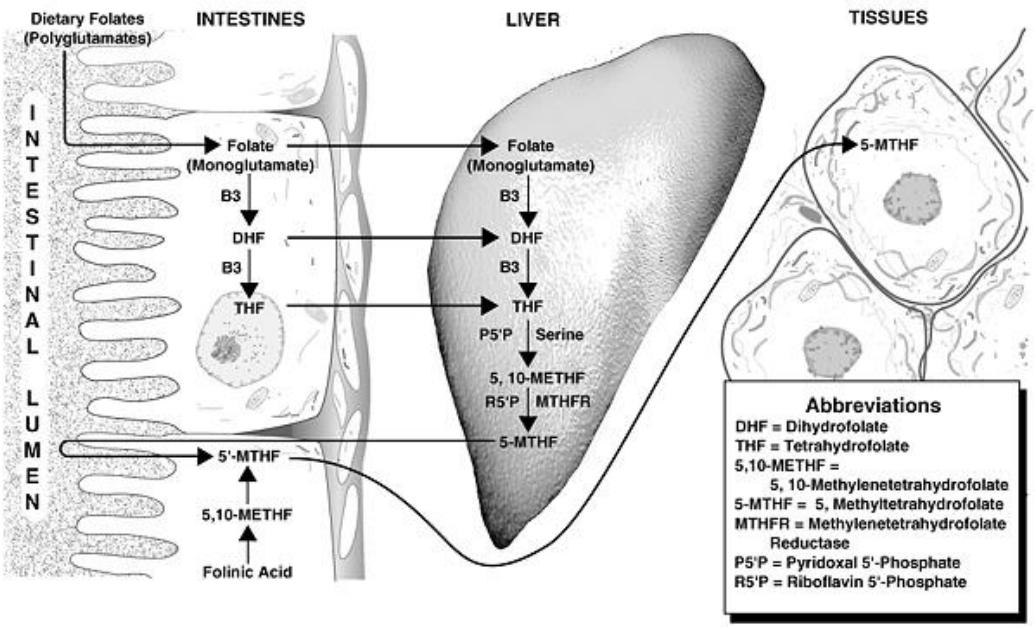


Figure 1.7. Absorption and activation of folic acid in mammals **(Miller & Kelly, 1997)**

Folate bioavailability is defined as the proportion of the ingested amount that is absorbed in the gut and that becomes available for metabolic processes (Witthöft *et al*., 2006). The reported bioavailability of monoglutamyl and polyglutamyl forms varies and depends on the release of folate from the food matrix, uptake by the brush border, deconjugation (in the case of polyglutamates), active transport and diffusion, and finally conversion to 5-CH_3-H_4folate (Al Khatib *et al*., 2006). The conservative estimate of bioavailability from a mixed human diet is about 50% (Devi *et al*., 2008).

Folate received considerable interest due to its health promoting effects first observed in 1931 in the therapeutic curing of macrocytic anemia which was first associated with the yeast and not on the unknown substance folate at that time.

Since then it was called by different names e.g. Vitamin M, Vitamin Bc, and L. casei factor as it was shown to be a growth factor for studied animals. It was in 1941 that the name folic acid was used when a research group purified an acidic substance from 4 tons of spinach leaves.

Folate reacts as a coenzyme or cofactor in various biochemical reactions in human body. Tetrahydrofolate (H_4PteGlu) is the enzymatically useful form of folate and acts as a carrier of one-carbon units. This one-carbon metabolism operates to ensure DNA synthesis for growing cells (Wagner, 1995).

Folate deficiency has been linked to numerous health problems as this vitamin is essential for a normal rate of cell division and production of red blood cells (Dayal *et al*., 2006). Rapidly growing and multiplying cells require an adequate supply of folate. Among the consequences of inadequate folate intake are: macrocytic anaemia (because of slowed DNA synthesis in new red blood cells), increased homocysteine concentration in blood resulting in hyperhomocysteinemia- a risk factor for cardiovascular disease (Boyles *et al*., 2006; Witthoft *et al*., 2006), infants born with neural tube defects, and colorectal cancer (Finglas *et al*., 2006).

Thus, it is critical to meet the recommended daily allowance (RDA) for folate (400 μg of folic acid equivalents for an adult and much higher for pregnant and lactating women and children).

1.2.5 Methods of Measurements and Analysis

Folate exists in several chemical forms in nature and differs in stability which leads to difficulties in characterizing and measuring them. In addition, folate concentrations in biological materials like plants and cereals are relatively low and the folates occur in various forms and in different stabilities adding to the challenge of identifying and quantifying them. Currently, microbiological assay (MA), radioprotein-binding assay (RPBA) and chromatography in combination with UV, fluorescence, mass spectrometry or tandem mass spectrometry are the existing methods being used to measure folates.

1.2.5.1 Microbiological assay (MA)

The only officially recognized AACC (2000) method of folic acid quantification is the microbiological assay (MA) which is so far the standard method for total folate measurement. However, it is time consuming, laborious, and lacks sufficient specificity to distinguish all different forms of natural folates. On the positive side, the possibility that the growth of the microorganism is enhanced or inhibited by non-folate compounds in the sample is very unlikely, since media is replete except for folic acid/folate. It is more likely that deconjugation is inhibited and that

reduced growth is secondary to this (AJA Wright 2009, personal communication). Still, MA is often used as reference method or standard in validating analytical methods measuring individual folate metabolites. It involves the growth measurement spectrophotometrically of *Lactobacillus rhamnosus* in a folate-deficient medium and requires the folates to be de-glutamated using conjugase enzyme to a monomer or shorter than 3 glutamate moieties of folate (Tamura *et al*., 1972) for growth. Theoretically, the growth of the microorganism (read spectrophotometrically by using a UV detector) is logarithmically proportional to total folate content in the sample. Folic acid is normally used as calibrant (Martin, 1995).

Two other methods currently being used are protein binding assays (PBA) and high performance liquid chromatography (HPLC).

1.2.5.2 Radioprotein-binding assay (RPBA)

Radioactive-labelled protein binding assay (RPBA) is an alternative method of folate analysis based on a competition between folate in sample with radiolabelled folate for binding sites on a folate-binding protein. It has been routinely used in the 1960s for vitamin B12 analysis in clinical samples. The concentration of folate in sample corresponds to the relationship between protein- bound and free species of the labeled folate. It is more convenient than MA but the application to food analysis is limited because of the problems with variations of ligand binding affinity for the

multiple folate forms present in many food stuffs (Shane *et al*., 1980; Stralsjo *et al*., 2003). Low precision between assays and laboratories, and poor agreement with the MA was also noted by Finglas *et al.* (1999) and van den Berg *et al.* (1994). On the other hand, RPBA is a good working method for the assay of folate in blood and serum where 5-CH_3-H_4PteGlu is the predominant form and so it is considered an important clinical tool to evaluate the folate status of a patient and to diagnose megaloblastic anemia (Eitenmiller and Landen, 1999). RPBA has the advantage of being less tedious, less expensive, less time consuming and less influenced by possible presence of antibiotics or substances that can promote growth of Lactobacillus casei in MA (Ball, 1998). However, as stated earlier, the lack of similar protein binding for all folate derivatives with RPBA in food analysis highlights the need for good LC methods. MA and PBA do not allow differentiation of folate forms like HPLC does.

1.2.5.3 Chromatographic methods (HPLC)

In 1903 the Russian botanist, Mikhail S. Tswett, first applied adsorption chromatography to the separation of plant pigments, using a hydrocarbon solvent and inulin powder (a carbohydrate) as stationary phase. The separation of coloured bands led to the name chromatography from the Greek words chromatos, meaning

"colour" and "graphia" meaning "writing" i.e. literally "colour writing".

The reversed-phase chromatography or HPLC is now the method of choice with the main objective to separate and quantify the different forms of folates which is important due to the different degree of bioavailability depending on the one-carbon substituents (Gregory *et al*., 1992; Hawkes and Villota, 1989). HPLC can differentiate between folate forms but requires improvement of detection limits and selectivity when analyzing complex food matrices with extremely low level of folate using this technique (Owens *et al*., 2006; Lu *et al*., 2007).

The detection is usually based on UV and fluorescence detectors, but in recent years use of mass spectrometry which is capable of measuring naturally occurring folate in mono and polyglutamates forms (Pfeiffer *et al*., 2004; Smith *et al*., 2006; Ng *et al*., 2008) has become more common.

Several HPLC techniques to separate folate derivatives in food and biological material have been established (Vahteristo *et al*., 1997, Pfeiffer *et al*., 1997, Finglas *et al*., 1998, Konings *et al*., 2001, Stokes and Webb, 1999, Ndaw *et al*., 2001, Horne, 2001, Jastrebova *et al*., 2003). However, some contradictory HPLC data were reported on contents of various folate derivatives in certain food and products, thereby stressing the importance of further method optimization with respect to identification and quantification of native folate vitamers other than 5 $-CH_3$-H_4folate (Finglas *et al*., 1999). Sample extraction, enzyme treatment, and subsequent clean-up step require optimization to allow folate analysis in various

types of samples and to be suitable for subsequent HPLC measurement.

The mass spectrometry instrument is selective and sensitivity can be enhanced for monitoring protonated analytes (Tamura, 1998; Finglas, 2000; Nilsson *et al*., 2004). Though the first application of the LC-MS method by Kiehl *et al*. in 1998 was not on folate, Garbis *et al*. (2001) measured folates in human plasma using hydrophilic interaction LC-MS/MS and Careri *et al*. (2002) reported the advances on food analysis using mass spectrometry. Kok *et al*. (2004) specifically studied folic acid and 5-CH_3-H_4PteGlu in plasma using LC-MS/MS while Zhang *et al*. (2005) focused their work on the monoglutamates in spinach. Owens *et al*. (2006) quantified folate in human red blood cells using the LC-MS/MS while Patring and Jastrebova (2007) further optimised the method for application on dietary folates by studying the effects of buffer nature and mobile phase composition on sensitivity and selectivity.

The need for a sensitive and selective method for folate quantification and characterisation of the various forms of folate in cereals and cereal products has led to many studies over the past 10 years using LC-MS and LC-MS/MS. Use of LC-MS/MS gives much higher specificity and sensitivity than LC-MS due to the possibility of performing multiple reaction monitoring (MRM) by selecting precursor ion(s) and then fragmenting it further by use of collision-induced dissociation (CID). In this way a specific spectrum of the fragmented precursor ion(s) can be obtained, and quantification be based on one of the product ions (Polettini, 2006). It was only recently when LC-MS/MS folate measurements were reported on cereals such as

rye (Karilouto, 2008) and rice (De Brouwer *et al*., 2008) and mainly made on the major monoglutamated forms of the vitamin. Garatt *et al*. (2005) made the first attempt to simultaneously profile the mono- and polyglutamated folates in plant and animal tissues using LC-MS/MS in negative ion mode. The group reported that in spinach, there were 1 to 8 glutamated folates of which the total polyglutamates equalled to 68% of the total folate pool. Moreover, monoglutamated folates were observed by the group to elute earlier than the polyglutamates. The chromatographic conditions were concluded to permit separation of mono and polyglutamated folates up to a chain of 14 conjugated glutamates within a 25 min analysis time. Using this method, an apparent high selectivity can be obtained without sample purification or adequate separations of analytes or both. However, in real samples like rice, there is still a need to optimise separation and purification since ionisation of analytes can be strongly influenced by other co-eluting matrix compounds; so called ion suppression effects or matrix effects (Careri *et al*., 2002). Matrix effects is seen as residual matrix components that alter the MS response either by ion suppression (loss of signal) or ion enhancement (gain in signal) of the targeted analytes or metabolites of interest or study (Matuszewski *et al*., 2003).

Extraction of folate from food is a critical step to the quantification using this powerful technique, as food matrix effects interfere with the analysis (McKeehen *et al*., 1996; Ruggeri *et al*., 1999; Verlinde *et al*., 2008). Another limitation of the method is its dependency on the limited number of commercially available folate

standards. To date, a limited number of methods are established to measure the polyglutamated folates.

1.3 The Biofortification Prospects

Biofortification is a relatively recent term that refers to the use of biological processes such as plant breeding and/or transgenic techniques to develop micronutrient-dense staple crops such as rice, wheat and other cereals. It is a concept that bridges agriculture, nutrition and health. This effort is seen as a more cost-effective alternative to the commodity-based nutrition interventions such as industrial fortification and pharmaceuticals supplements.

As a basis of comparison, the cost of commercial fortification is 10 US cents per person, whether the person is micronutrient-deficient or not, which is equivalent to about 20 US cents per micronutrient-deficient person reached. The current cost of iron supplementation for example is US$20 per iron-deficient person reached while the cost of biofortification would just be 1.5 US cents for each iron-deficient person reached (www.harvestplus.org). Folic acid fortification has already been implemented and considered in some European countries including the U.K. and in the U.S. but not in many developing countries where micronutrient malnutrition is a recurring problem. Folate fortification and supplementation are relatively effective in most developed countries but not in developing countries where distribution and

access are still a big challenge.

Biofortification is a more promising strategy that looks into the selective breeding of staple food crops with an increased density of micronutrients. This approach has the advantage that, once developed, the extra nutrition derived from the enriched varieties comes free with every harvest.

The crops being investigated include rice, wheat, maize, beans, cassava and sweet potato. Rice is of particular interest because it is the major food source for nearly half the world's population –most notably the half that is the poorest, most deprived and least healthy of all. Improvement in the nutritive value of rice, particularly in terms of its folate content, will thus be of significant benefit.

Recently, Storozhenko *et al.* (2007) showed 100-fold increase in rice seeds folate through over expression of the *Arabidopsis* GTPCHI and ADCS which are involved in pterin and *p*ABA production, respectively. The initial result from this study seems to be promising for further work in this area.

1.4 Aims and Objectives

To date, little is known about the natural forms and concentration of folate (especially polyglutamates) in rice. Considering the importance of this crop to supply most of the nutrients including folate in developing and developed countries, baseline data and basic information on how much and what are the derivatives present in the main form for consumption – the rice grain, are vital in designing the strategies to increase the level of the vitamin.

This research aims to optimize the analytical method of measuring folate concentration and profiling the mono- and polyglutamated forms in the rice grain using the standard microbiological assay (MA), HPLC and liquid chromatography-negative ion electrospray ionisation tandem mass spectrometry (LC-MS/MS).

The specific objectives of the research project are:

- to optimize sample pre-treatment (extraction and enzymatic treatments) for MA and LC-MS/MS quantification of folates in rice,
- to measure total folate concentration in rice grain using MA,
- to identify and quantify folate metabolites in rice using HPLC and LC-MS/MS,
- to profile/analyse range of rice materials/germplasm for genetic variation among cultivars in terms of folate content and forms, and
- to quantify folate metabolites in rice grains from wild type, knockout and transgenic (overexpressed) lines.