Biological availability and efficacy of folates from bioengineered rice

Filip Kiekens

Master in Industrial Engineering – Chemistry
Master in Environmental Management and Sanitation

Promoters: Prof. Dr. Christophe P. Stove
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Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

2016

Ghent University
Faculty of Pharmaceutical Sciences
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Samenvatting - Summary

Biologische beschikbaarheid en impact van folaten aanwezig in genetisch gewijzigde rijst

Het basisidee achter de ontwikkeling van een genetisch gewijzigde rijstvariant met een sterk verhoogde folaatconcentratie is te vinden in de beperkte mogelijkheid om voldoende folaten op te nemen uit het dieet in afgelegen agrarische regio's. Omwille van de relatie tussen een tekort aan folaten en het voorkomen van neuraalbuisdefecten, die in ernst variëren van een lichte rugpijn tot een niet levensvatbare foetus, is een folaatrijk dieet aangewezen. Eén mogelijkheid om de folaatstatus te verhogen is door synthetisch foliumzuur te verwerken in alledaagse voedingswaren zoals ontbijtgranen en brood. Dergelijke geïndustrialiseerde technieken zijn echter niet beschikbaar in afgelegen regio's. Folaatrijst kan echter lokaal verbouwd worden en kan in deze regio's als een belangrijke bron van folaten in de voeding fungeren.

Echter, aangezien folaatrijst een genetisch gewijzigde rijstvariant is, is het noodzakelijk om wetenschappelijk onderbouwd bewijs voor te leggen dat deze rijstvariant inderdaad een waardevolle bron van folaten is. Dit aangezien enkele genetisch gewijzigde voedingsmiddelen recent bekritiseerd werden omwille van hun beperkte bijdrage tot de totale vitamine- of mineraalinname.

Er werd voor een dierenproef gekozen omwille van wettelijke verplichtingen en omdat dit een strikte controle van de studieparameters mogelijk maakt. Gezien de complexiteit van het folaatmetabolisme en de verdeling in het lichaam, werd voor een langdurige studie gekozen. Tijdens deze studie werden het folaatgehalte in het bloed, andere folaatgerelateerde klinische parameters en de algemene gezondheid van de dieren periodiek bekeken. Om de resultaten te kunnen evalueren werden de dieren opgesplitst in verschillende groepen die ofwel geen folaten, een beetje folaten uit 'normale' rijst, een grotere hoeveelheid foliumzuur uit gefortifieerde rijst of natuurlijke folaten uit folaatrijst of een grote hoeveelheid foliumzuur kregen.

Om het biologisch effect van de verschillende diëten op het folaatgehalte in het bloed zichtbaar te maken, werd een analysemethode ontwikkeld om de hoeveelheid folaten te bepalen in 2 bloedfracties, namelijk in plasma en rode bloedcellen. Aangezien de term folaten enkele chemisch verschillende moleculen omvat die in zeer lage concentraties voorkomen, werd gebruik gemaakt van vloeistofchromatografie gekoppeld aan massaspectrometrie. Deze methode is in staat om de verschillende folaten van elkaar te onderscheiden en de concentratie individueel te bepalen.

Tevens werd een specifieke analysemethode ontwikkeld voor de bepaling van individuele folaten in knaagdiervoeders om de mogelijke invloed van folaten aanwezig in het gebruikte knaagdiervoeder uit te sluiten.

Bovendien werd de invloed van het folaatgehalte in de bloedbaan op de werking van het immuunsysteem onderzocht. Dit gebeurde door het uitvoeren van een studie naar de invloed van het gehalte van individuele folaten op de deling van witte bloedcellen.

Biological availability and efficacy of folates from bioengineered rice

The basic concept of folate rice is to develop a means for rural areas to combat folate deficiency. Folate deficiency is of concern given the relation between folate status and birth defects, ranging in severity from mild back ache to stillbirth, due to a malformation of the neural tube. While western populations benefit from diverse and nutrient rich diets, even these populations are at risk for folate deficiency. One method to alleviate the health burden associated with folate deficiency is to add synthetic folic acid to commonly consumed food items such as breakfast cereals or bread. However, remote, mostly rural communities, do not have free access to folic acid fortification. As such, a rice variety with a high natural folate content that could be grown locally may serve to achieve an adequate folate status.

However, since folate rice is a genetically engineered rice variety, public and governmental acceptance depends on scientific evidence that the folates contained within these rice grains are released into the bloodstream when consumed. This is because some genetically engineered food items have been criticized as a result of their limited influence on nutrient status. It is therefore of paramount importance to evaluate the impact of folate rice consumption on folate status.

Given the need for strict control of nutrient intake and regulatory constraints, a rodent feeding trial was devised to evaluate the efficacy of folate rice as a dietary folate source. Due to the complexity of folate metabolism and body distribution, a long term study was performed including regular evaluation of folate status, folate related clinical parameters and general health. These results were compared between groups receiving no folate, a small amount of folate present in ‘normal’ rice, a larger amount of folate in either folate rice or folic acid fortified rice or ample folate as an optimal scenario.

To quantify the biological outcome of folate rice consumption, an analytical method was developed to measure the amount of folates in 2 separate blood fractions, i.e. plasma and red blood cells. Given the low concentrations in which folates are present, a sensitive method using liquid chromatography coupled to tandem mass spectrometry was used. Since the term folate signifies several different molecules with different chemical and biological properties, the developed method required the measurement of individual folate species.

Also, to exclude the influence of folates which might be present in the rodent diets used during the rodent trial, a separate method was developed to determine the folate concentration in the rodent diets used.

Due to the necessity of folates for DNA-synthesis in white blood cells, the influence of folate concentration and speciation on the functioning of the immune system was investigated as well.

Structure

List of abbreviations

AA	L-ascorbic acid
ADCS	aminodeoxychorismate synthase
amu	atomic mass unit
CVD	cardiovascular disease
5,10CH^+THF	5,10-methenyltetrahydrofolate
5,10CH_2THF	5,10-methylenetetrahydrofolate
DBS	dried blood spot(s)
(d)FBS	(dialysed) foetal bovine serum
DFE	dietary folate equivalents
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
FA	folic acid
FAR	folic acid fortified rice group
FBP	folate binding protein
FOLH1	folate hydrolase 1
10FoFA	10-formylfolic acid
5FoTHF	5-formyltetrahydrofolate
10FoTHF	10-formyltetrahydrofolate
FPGS	folylpolyglutamyl synthase
GAR	folate rice group
GC	gas chromatography
GCPII	γ-carboxypeptidase II
GGH	γ-glutamylhydrolase
GTPCHI	guanosine triphosphate cyclohydrolase 1
HILIC	hydrophilic interaction liquid chromatography
hmTHF	4-α-hydroxy-5-methyltetrahydrofolate
HRP	horseradish peroxidase

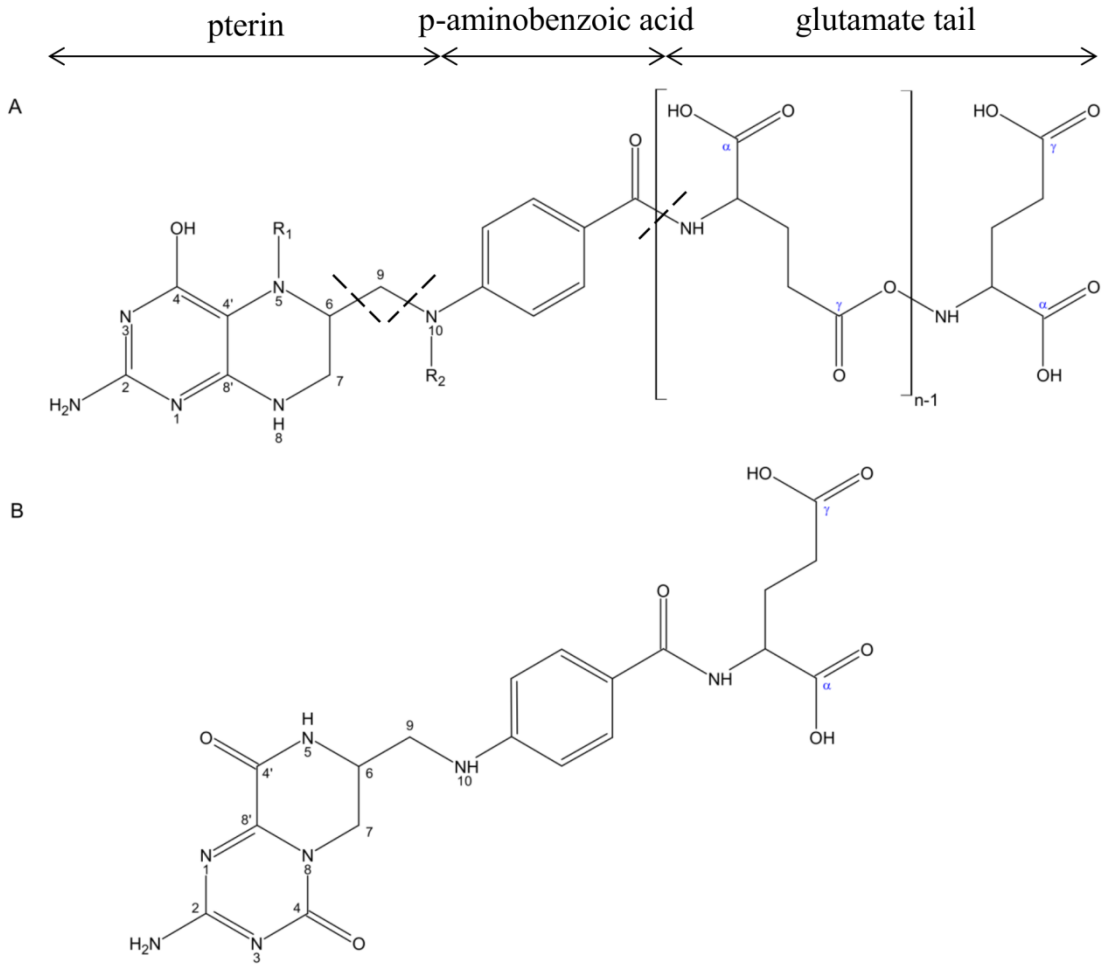
IL-2	interleukin 2
IS	internal standard
LC	liquid chromatography
LLOQ	lower limit of quantitation
LOD	limit of detection
MA	microbiologic assay
MeFox	pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate
5MeTHF	5-methyltetrahydrofolate
MS(/MS)	(tandem) mass spectrometry
MTHFR	methylenetetrahydrofolate reductase
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
NEG	negative control group
NTD	neural tube defect
pABA	para-aminobenzoic acid
PBA	protein binding assay
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCFT	proton-coupled folate transporter
PHA	phytohaemagglutinin
POS	positive control group
RBC(s)	red blood cell(s)
RFC	reduced folate carrier
SNP	single-nucleotide polymorphism
SPE	solid-phase extraction
THF	tetrahydrofolate
UHPLC	ultra high performance/pressure liquid chromatography
ULOQ	upper limit of quantitation
WHO	World Health Organisation
WTR	wild type rice group

Chapter I: General introduction

Table of contents

I.1. What are folates?

Within the water soluble vitamins, the group of folates, also known as vitamin B9, is essential for the maintenance of adequate methylation potential in an individual and is involved in the synthesis of purines and pyrimidines.(Shane 2010)



folate (A)	abbreviation	5 – 6	7 – 8	R_1	R_2
tetrahydrofolate	THF	–	–	-H	-H
dihydrofolate	DHF	=	–	-H	-H
folic acid (pteroyl glutamate)	FA	=	=	-H	-H
5-methyltetrahydrofolate	5MeTHF	–	–	$-CH_3$	-H
5-formiminotetrahydrofolate	5FiTHF	–	–	-CHNH	-H
5-formyltetrahydrofolate	5FoTHF	–	–	-CHO	-H
10-formyltetrahydrofolate	10FoTHF	–	–	-H	-CHO
10-formylfolic acid	10FoFA	=	=	-H	-CHO
5,10-methylenetetrahydrofolate	$5,10CH_2THF$	–	–	$-CH_2-$	
5,10-methenyltetrahydrofolate	$5,10CH^+THF$	–	–	$-CH^+-$	

Figure I.1: Chemical structure and typical MS/MS fragmentation (indicated by broken lines) of a number of relevant folates (A) and chemical structure of MeFox, the metabolically inactive degradation product of 5MeTHF (B)

Folates consist of three distinct parts: a pterin moiety with a varying oxidation state, a para-aminobenzoic acid (pABA) molecule and a tail of repeating glutamic acid molecules bound at the γ-position. Based on the latter, mono-, oligo- and polyglutamates can be differentiated. As shown in Figure I.1, different functional groups may be bound at either or both the 5 and 10 position.(De Brouwer *et al*. 2007) Folates can be present in 3 oxidation states, i.e. a fully oxidized form, known as folic acid (FA), a fully reduced form, known as tetrahydrofolate (THF), and an intermediate form, known as dihydrofolate (DHF). Stability of folates depends on both the oxidation state and the substitution with a methyl- or formyl group at the 5 and/or 10-position. Folate polyglutamylation is an important factor for both intracellular folate retention and enzyme binding.(Osborne *et al*. 1993) FA is the fully oxidized synthetic form, commonly used in food fortification. It is, however, 5-methyltetrahydrofolate (5MeTHF) that is the predominant species in both natural food items and in clinical samples.

I.2. Folates: biological accessibility and availability

While plants and microorganisms are able to form folates *de novo* from pterin, p-aminobenzoic acid and glutamate, most vertebrates, including humans, lack the enzymes necessary to perform folate synthesis. As a result, folates are essential vitamins and food items rich in folates should be an integral part of the human diet to assure an adequate folate intake.(Klipstein and Samloff 1966; Hoffbrand *et al*. 1971)

Prior to the release in the systemic circulation, the absorption of folates from food items requires a multistep process which involves several enzyme systems to transport the molecules through membranes and change the oxidative state and substitution.(Shane 2010)

While the term bioavailability may be used to describe different processes, in this thesis, the term bioavailability is used to indicate the fraction of a compound which is released in the gastro-intestinal (GI) tract, transferred to the systemic circulation and takes part in a metabolism. The term bioaccessibility only includes the release of the compound prior to absorption by the bowel epithelium. Biological efficacy describes the ability of a compound to induce a certain effect.

2.1. Release of folates from food items

Food items are chewed, mixed with saliva and swallowed, releasing a food bolus into the esophagus. Saliva contains an amylase and lipase activity and therefore marks the start of the digestion of the food matrix into smaller components. Upon passage through the stomach proteases and hydrochloric acid are released to turn the food bolus into a semifluid substance called chyme which is released into the small intestine.(Pedersen *et al*. 2002)

In the small intestine folate polyglutamates present in the food item are cut to folate monoglutamates by the glutamate carboxypeptidase II-enzyme (GCPII), a γ-glutamyl hydrolase enzyme (GGH), present in the brush border of the bowel mucosa. This is important since only folate monoglutamates can be transported across the membrane by folate carrier proteins.(Chandler *et al*. 1986)

Several factors influence the accessibility of folates from dietary food sources. The effect of fats, acids and fibers on the absorption of folates in the bowel has been investigated, as has the structure of the food item. In general, acidic food items such as orange juice seem to reduce the activity of the GCPII-enzyme, thereby reducing the bioaccessibility of folate polyglutamates. Also, folates can remain trapped within the plant cells.(Ball 2005; Gregory 3rd 2001)

2.2. Absorption in the small intestine

The reduced folate carrier (RFC) and the proton coupled folate transporter (PCFT) are known folate carriers.(Chandler *et al*. 1986) The latter of both transporters is most active at an acidic pH and is ubiquitously present in the small intestine. It has a similar affinity for both reduced folates and FA (binding affinity 0.5 to 0.8 µM at pH 5.5).(Shane 2010) The RFC-protein is largely inactive at acidic pH (< 6.5). As such, it is unlikely to be a major transporter due to the low pH, i.e. ±6, in the small intestine.(Shane 2010)

Oxidized folates, such as folic acid, are reduced to DHF and further to THF by the DHF reductase (DHFR) enzyme in the epithelial cells of the small intestine. These reduced folates can then be methylated to 5MeTHF and either released into the portal circulation (i.e. the circulation system between the intestines and the liver) or stored as 5-methyltetrahydro-pteroylpolyglutamates. These can be deconjugated by the GGH enzyme and are ultimately released into the portal circulation.(Shane 2010; Visentin *et al*. 2014) This process is schematically depicted in Figure I.2.

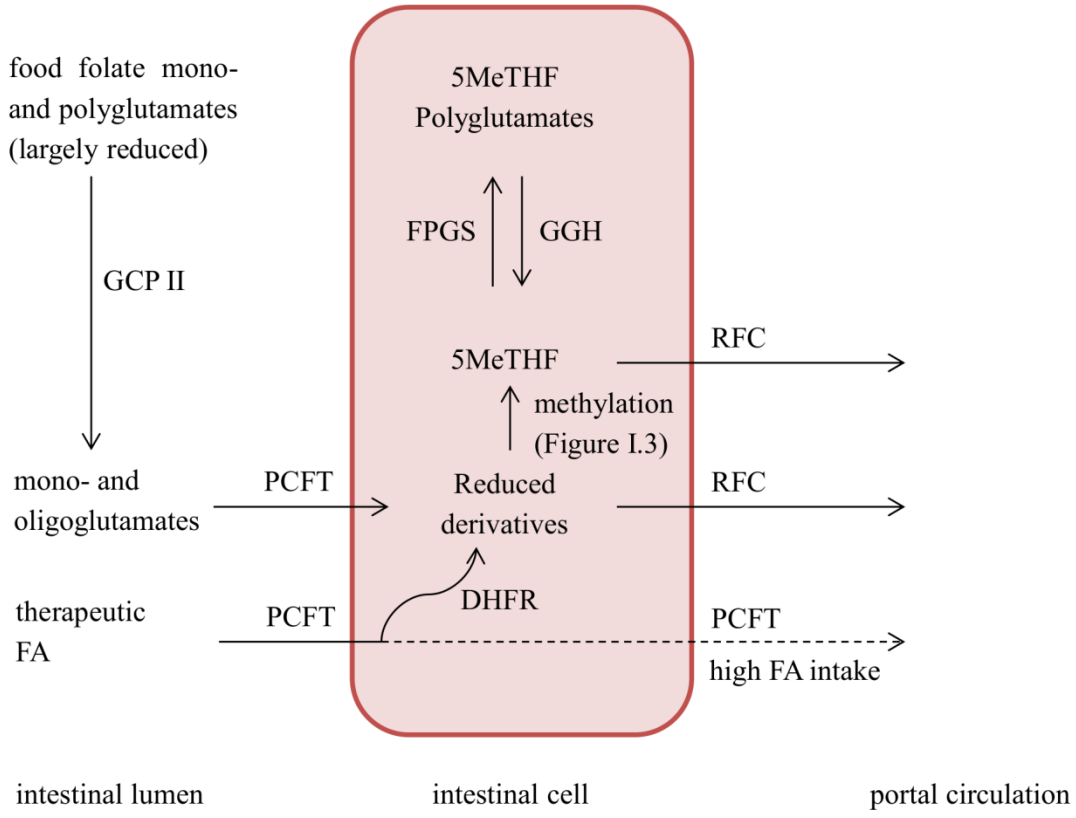


Figure I.2: Schematic representation of the intestinal absorption of folates (Shane 2010; Visentin *et al*. 2014) DHFR: dihydrofolate reductase; FPGS: folylpolyglutamate synthase; GCP II: γ-carboxypeptidase II; GGH: γ-glutamylhydrolase; PCFT: proton-coupled folate transporter; RFC: reduced folate carrier.

2.3. Folate absorption in the large intestine

Apart from dietary intake, folates produced by the microflora in the bowel provide a minimal source of folate which is absorbed into the circulatory system. This can account for up to 5% of the average folate requirement for a healthy adult.(Aufreiter *et al*. 2009) As such, it is clear that the bowel microflora is not capable of maintaining an adequate folate status. However, under normal conditions, the folate intake can never be zero.

2.4. Portal circulation and the liver

Folates absorbed through the lumen of the small intestine are transported to the liver where they are either transported to the systemic circulation or undergo what is called an enterohepatic recirculation. Here, the folates are released into the bile and are excreted through the gall bladder back into the small intestine. These folates are then reabsorbed by the lumen carrier enzymes and return to the portal circulation. This cycle repeats itself until the concentration of folates near the carrier proteins is too low for effective transport across the membrane to occur.(Visentin *et al*. 2014)

2.5. Systemic circulation and storage pools

When folates are released into the systemic circulation by the liver, a portion is captured by folate carrier proteins freely circulating in the plasma fraction of the blood. Most specific is the folate binding protein (FBP). Other carriers are less specific, such as serum albumin. However, as this protein forms a significantly larger fraction of the total amount of protein present, it is capable of retaining far greater amounts of folate. The retention of folates by carrier proteins is of great importance to prevent the renal excretion of this vitamin.(Shane 2010)

Unlike in the small intestine, the RFC is responsible for the uptake of folates from plasma into the cytoplasm of the cells. It has, however, a significantly lower affinity (Kt= 3 µM) for the reduced folates than for FA (Kt= 200 µM).(Matherly and Goldman 2003)

While folates take part in key metabolic processes, body storage of folates is limited. Clifford *et al*. investigated the distribution of folates in rats fed various levels of FA. In this study, the liver was found to contain the largest folate storage pool, with 26% of total body folate in case of prolonged (25 days) zero folate intake, to 44% of total body folate in case of ample folate intake. This indicates that the liver is capable of releasing folates in case of periodic low folate intake. The kidneys (5-6%), testes (2-1%), lungs (0.2-0.4%), spleen (0.1-0.3%) and heart (0.1-0.2%) contain smaller fractions of the total body folate pool. Red blood cells (RBCs) also serve as a folate storage pool, with 8 (limited folate intake) to 3% (ample folate intake) of total body folate. While the absolute concentration in the RBCs rose, it was not possible to match the speed of the rise in the liver, likely due to the average lifespan of the RBCs of approximately 90 days. Distributing folate across the body, plasma also serves as a readily available storage pool, containing 0.03 to 0.3% of the total body folate. The GI tract can be seen as a folate storage pool since the bowel microflora contains approximately 3% of the total body folate.(Clifford *et al*. 1990)

I.3. Folates: key players in human health

3.1. Intracellular metabolism

In human cells, folates take part in two different metabolic cycles, as depicted in Figure I.3. In the methylation cycle, the methyl group of 5MeTHF is transferred via vitamin B12 (cobalamin) to homocysteine, resulting in the formation of THF and methionine. Methionine is then activated by addition of an adenosyl group to S-adenosylmethionine. The latter is involved in many different metabolic processes, including methylation of lipids and DNA and the formation of DOPA and myelin, the insulating material of neural tissue.(Blom and Smulders 2011) In these processes, S-adenosylhomocysteine is formed, which can either be converted to pyruvate or accumulate in tissue. Given the role of vitamin B12 as a cofactor for the methionine synthase enzyme, deficiency for this vitamin will result in an accumulation of 5MeTHF and homocysteine, the impairment of methylation processes and eventually neural damage.

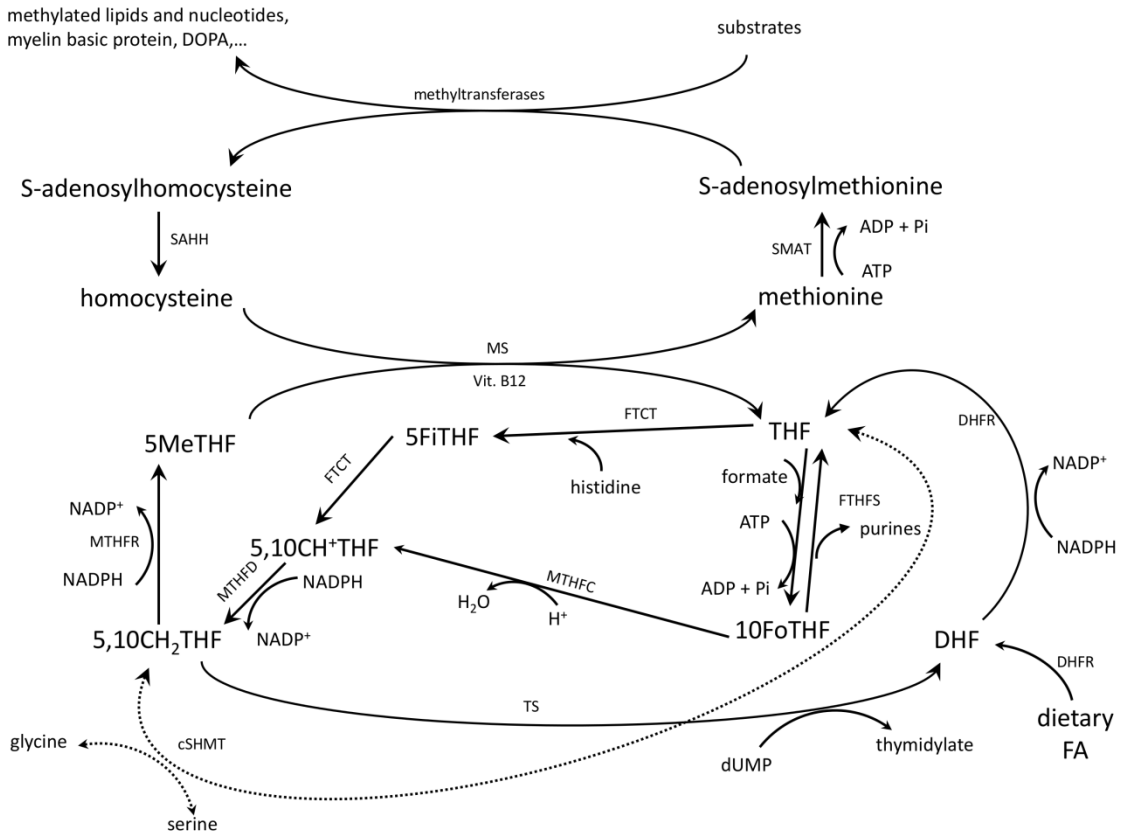


Figure I.3: Cytosolic folate metabolism; 5,10CH$^+$THF, 5,10-methenylTHF; 5,10CH$_2$THF, 5,10methyleneTHF; cSHMT, cytoplasmic serine hydroxymethyltransferase; DHFR, dihydrofolate reductase; FA, folic acid; 5FiTHF, 5-formiminoTHF; 10FoFA, 10-formylFA; 5FoTHF, 5-formylTHF; 10FoTHF, 10-formylTHF; FTCT, glycine formiminotransferase/ formimidoyltetrahydrofolate cyclodeaminase and glutamate formiminotransferase/ formimidoyltetrahydrofolate cyclodeaminase; FTHFS, 10-formyltetrahydrofolate synthetase; hmTHF, 4α-hydroxy-5-methylTHF; MeFox, pyrazino-s-triazine derivative of hmTHF; 5MeTHF, 5-methylTHF; MS, vitamin B12 dependent methionine synthase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methenyltetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SAHH, S-adenosyl-L-homocysteine hydrolase; SMAT, methionine adenosyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase. (based on: Scott 1999; Stover 2010)

Disruption of the methylation cycle as a result of a vitamin B12 and/or folate deficiency is clinically measured as an elevated plasma homocysteine concentration, known as hyperhomocysteinemia. This is associated with an increased risk for cardiovascular diseases (CVD).(Ueland *et al*. 2000; Blom and Smulders 2011) Supplementation with folates is the method of choice to treat hyperhomocysteinemia. However, folate supplementation may promote cell proliferation and inflammation, which are crucial processes in atherosclerotic plaque formation and would therefore lessen the effects of a decrease in homocysteine concentrations.(Blom and Smulders 2011) Hyperhomocysteinemia has also been associated with an increased risk for neural diseases including dementia and Alzheimer's disease.(Reynolds 2006) Best established is undoubtedly the profound influence of the folate status on the prevalence of neural tube defects (NTDs). During early pregnancy, a low folate status can lead to failure of the neural tube to close in the developing fetus, resulting - depending on the severity- in disability or death.(Iyer and Tomar 2009)

Secondly, 5,10-methylenetetrahydrofolate (5,10CH_2THF) and 10-formyltetrahydrofolate (10FoTHF) take part in the *de novo* synthesis of pyrimidines and purines, respectively. Especially the methylation of uracil to thymidine is strongly influenced by the folate status. Folate deficiency has for instance been implicated in uracil incorporation in DNA, leading to chromosomal damage.(Blount *et al*. 1997) Severe folate deficiency also inhibits cell replication because it results in an inability to synthesize new DNA. This leads to the presence of enlarged RBCs in the bloodstream, called megaloblastic anemia. The presence of these enlarged cells is also used as an indicator for vitamin B12 deficiency. This deficiency leads to a halted folate cycle with accumulation of 5MeTHF. The use of FA as a folate supplement is problematic in this respect, since the presence of FA in the bloodstream leads to a bypass of the methionine synthase enzyme. Although this allows DNA replication to be maintained, alleviating megaloblastic anemia, the formation of methionine is still blocked, potentially leading to neural damage. (Molloy 2010)

In cells, folates accumulate as polyglutamate derivatives, which are active in cellular metabolism.(Osborne *et al*. 1993) The folylpolyglutamate synthase (FPGS) activity is reported to be the highest in liver, while activity appears to be negligible in muscular tissue and mature blood cells.(Moran and Colman 1984) However, in RBCs folates are nearly completely present in the polyglutamate form.(van Haandel *et al*. 2012; Kiekens *et al*. 2015a) Although methods do exist for distinguishing individual folate polyglutamate species (Wang *et al*. 2010), the lack of pure reference standards limits the use of these methods for true quantitative folate determination in clinical matrices.

3.2. Folate status

Folate deficiency has received interest from a medical point-of-view since Minot and Murphy found, in 1923, that consumption of large amounts of liver cured pernicious anemia and since Lucy Wills' discovery, in 1931, of a factor in yeast that corrected macrocytic anemia during pregnancy. In the 1930's and 40's, this factor, now known to be folate, was isolated from spinach, receiving its name from the Latin word for leaf: folium.(Hoffbrand and Weir 2001)

Since then, folate deficiency and related health issues have been intensely studied. While several anemias and neural tube defects can be directly linked to an inadequate folate status, direct proof of the role of folate in cardiovascular diseases, neural disorders and cancer remains elusive.(Stover 2004)

In 2012, the World Health Organization (WHO) released an updated guideline on the use of folate concentrations in serum (or plasma) and red blood cells (RBCs) to assess the folate status in different populations. Such an assessment is useful to monitor trends in folate status and to evaluate the impact of public health interventions.(WHO 2012) A quantitative value for an adequate folate status is not easily determined since this depends on the metabolic indicator used to define the cut-off value. Since the 1970's the prevalence of macrocytic anemia, a hematological indicator of folate deficiency, was used to categorize patients with various blood folate concentrations. While patients with serum or plasma folate concentrations below 6.8, between 6.8 and 13.4, between 13.5 and 45.3, or above 45.3 nM were respectively categorized as deficient, possibly deficient, normal and elevated, only a cut-off value of 226.5 nM in RBCs was used to indicate folate deficiency.(WHO 1972; WHO 1975) In 2005, the elevation of plasma homocysteine concentrations was chosen as a marker for folate deficiency, based on National Health and Nutrition Examination Survey (NHANES) III data. This population survey investigated the effect of dietary intake on folate status, related clinical parameters and health outcomes. The cut-off levels indicating folate deficiency (i.e. the folate concentrations below which homocysteine concentrations started to rise) were set at 10 and 340 nM for serum (plasma) and RBCs, respectively.(Selhub *et al*. 2008; de Benoist 2008; Pfeiffer *et al*. 2012) As folate requirements are increased during pregnancy and lactation, a 2015 WHO guideline for women of reproductive age recommends in this population a RBC folate concentration above 906 nM to achieve the greatest reduction of NTD risk.(WHO 2015) In Belgium, a daily folate intake of 200 to 300 µg/day is recommended for adults. For women that want to get pregnant, a daily intake of 400 µg/day is advised to limit the risk for NTDs (Table I.1).(Hoge Gezondheidsraad 2015)

Age	**Sex**	**DFE [µg/day]**
0-6 months	male/female	50
7-12 months	male/female	50
1-3 years	male/female	100
4-6 years	male/female	130
7-10 years	male/female	150
11-14 years	male/female	180
15-18 years	male/female	200
adults (19-70 years)	male/female	200-300
> 70 years	male/female	200
pregnancy	female	400
breastfeeding	female	300

Table I.1: recommended daily folate intake in Belgium.(Hoge Gezondheidsraad 2015) Dietary folate equivalents (DFE) = µg folate present in food + (1,7 x µg folic acid supplements)

I.4. Folate fortification

Based on a review of folate status in populations worldwide by McLean *et al*., it is clear that in some countries folate deficiency is a serious health risk. However, the situation is not uniform over different social groups within a single country, as can be seen in Figure I.4. In the United Kingdom for instance, folate deficiency is nearly absent in the adult population while more than 10% of the elderly appear to be folate deficient. This may be a result of differences in food or supplement consumption.(McLean *et al*. 2008) It is therefore difficult for a governing body to introduce one size fits all guidelines to achieve an adequate folate status in the entire population.

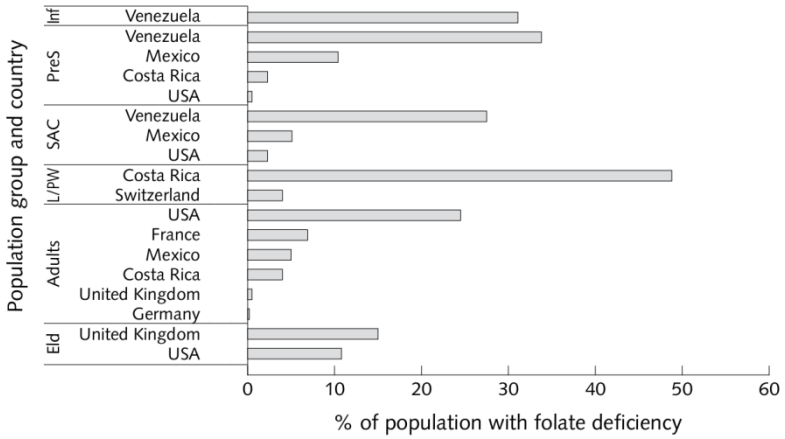


Figure I.4: Prevalence of folate deficiency; figure obtained from McLean *et al.*, 2008 Food and Nutrition Bulletin, 29,2: S38-S51; Inf: infant; PreS: preschool children; SAC: school aged children; L/PW: lactating or pregnant women; Eld: elderly persons.

4.1. Traditional fortification strategies

Some countries have instituted mandatory fortification programs to add synthetic folic acid to commonly consumed food items such as wheat flour. The countries implementing folic acid fortification in June 2010 can be found in Figure I.5. For instance, the United States started a fortification program in 1998 by adding 140 µg of FA per 100 g of enriched cereal grain product, which has been estimated to provide 100 – 200 µg of FA per day to women of childbearing age.(Crider *et al*. 2011)

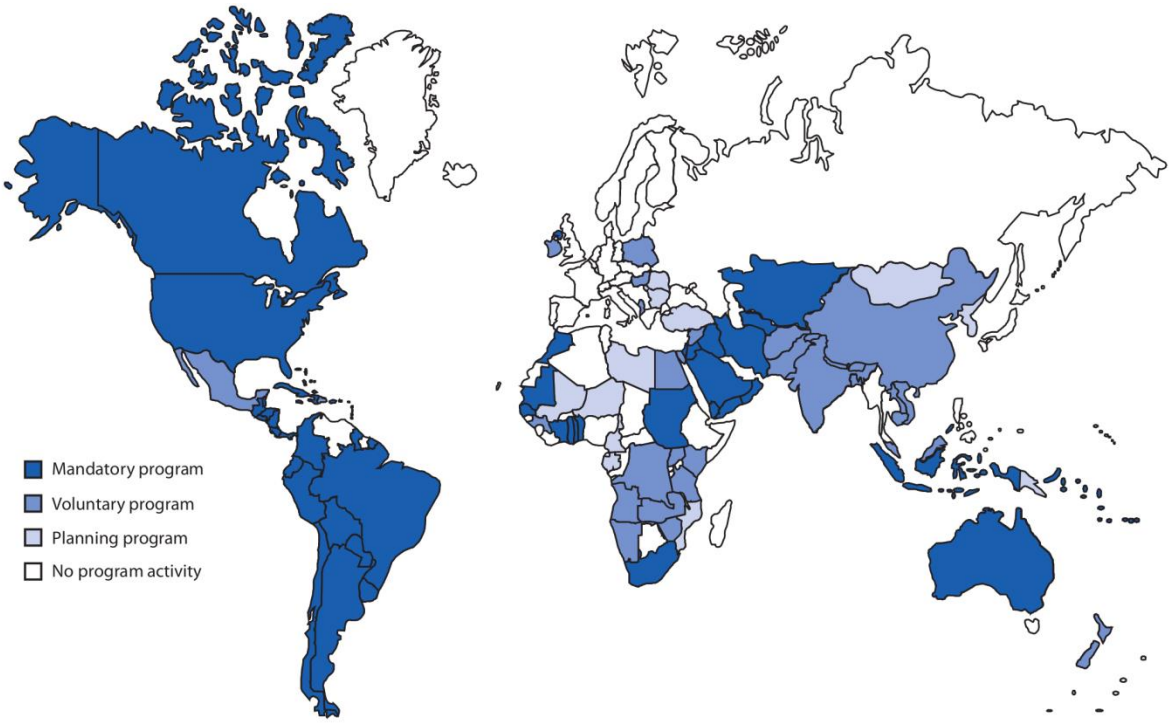


Figure I.5: Fortification programs; figure obtained from Centers for Disease Control and Prevention 2010.

Although EUROCAT, a European network of population-based registries for the epidemiologic surveillance of congenital anomalies, advised the introduction of mandatory folate fortification in 2009 (de Walle and Abramsky 2009), the European Food Safety Authority (EFSA) is more reluctant. While the working group that EFSA instigated acknowledges the beneficial relation between folate intake and a reduction of NTD risk, it questions the efficacy of the advised 400 µg/day dietary supplementation for women who may become pregnant and the effect of FA supplementation on the prevalence of cancer.(EFSA 2009)

While folates are essential vitamins, an excessive intake of FA, which is the synthetic folate species commonly used as a supplement, leads to the unnatural presence of this folate species in the bloodstream. This has been associated with an increased risk of colorectal cancer and it may delay the detection of a vitamin B12 deficiency, leading to neural damage.(Crider *et al*. 2011) It is in this respect that the EFSA concluded that not enough data are available concerning the long term effects of FA supplementation to justify the implementation of a mandatory fortification of food items.(EFSA 2009) However, given the globalized market, some products are available in European shops which are fortified with FA. These items, such as breakfast cereals, essentially provide an additional source of FA without the knowledge of the consumer.

The introduction of FA fortification of cereal grain products in the USA in 1998 succeeded in increasing the folate status at a population level. This intervention doubled the plasma folate concentrations and increased the red blood cell folate concentrations by more than 50%. While these results clearly show that FA fortification is able to increase the folate status of a population, the desired folate levels, leading to a significant impact on the prevalence of

neural tube defects, were not reached.(Dietrich *et al*. 2005) However, when evaluating the effectiveness of FA fortification in the USA, the prevalence of neural tube defects decreased by 20 to 30%. This vastly exceeded the 2 to 10% decline expected to result from fortification at the 140 µg/100g level. A possible reason for this discrepancy is the higher than expected intake due to a large consumption of products containing fortified wheat flour.(Grosse *et al*. 2005)

4.2. Biofortification

An alternative strategy for folate fortification is the introduction of 'natural' folates in common food items. These mostly reduced folates resemble natural food items rich in folates and are unlikely to negatively impact colorectal cancer and do not bypass the methylation cycle. Therefore they do not influence the detection of vitamin B12 deficiency by the appearance of megaloblasts (immature large RBCs) in the bloodstream.(Crider *et al*. 2011)

While careful selection and consumption of food items rich in folates can increase individual folate intake, it requires a change to the daily diet. As such, focus has shifted towards alteration of recipes and bioprocessing including fermentation. Wine, beer, bread and yoghurt are examples of food items in which the folate content can be increased by using folate rich cereal strains or fermentation by yeast and lactic acid producing bacteria. Although this can double or quadruple the folate content within these food items (Jägerstad *et al*. 2005), the absolute increase in folate content remains rather limited.

Another alternative is biofortification of food items, which aims at increasing the nutrient content by modifying the expression of metabolic enzymes. This can be performed either by increasing the expression level of naturally present metabolic enzymes or by introducing exogenous enzymes which are, for instance, not feedback regulated. This approach aims at eliminating bottlenecks in folate synthesis and ultimately, at increasing the folate concentration within the edible part of the plant. This technique has now been applied to increase folate concentrations in tomato fruit (Díaz de la Garza *et al*. 2007), wheat and rice seeds.(Storozhenko *et al*. 2007, Blancquaert *et al*. 2015, Piironen *et al.* 2008)

Food fortification by genetic engineering is not limited to folate. To alleviate β-carotene deficiencies, a rice variety was developed, by introducing phytoene synthase and lycopene β-cyclase genes from *Narcissus pseudonarcissus* and later from maize, which contains up to 37 µg/g of β-carotene.(Beyer *et al.* 2002 and Paine *et al.* 2004) This rice variety, also referred to as golden rice, is undoubtedly the best known example of a vitamin-fortified crop, and has already been shown to be an effective source of β-carotene in a human study.(Tang *et al.* 2009)

4.3. Folate rice, a recipe for success?

Already well over a decade, since 2004, a consortium within Ghent University, composed of the Laboratory of Functional Plant Biology, the Laboratory of Toxicology and the Department of Agricultural Economics, focuses on a better understanding of the folate pathway and the improvement of the folate content in crops. The role of the different partners lies in i) the generation of transgenic crops and their molecular and functional characterization, ii) the set-

up and deployment of validated analytical strategies to steer and evaluate the success of the genetic modifications, as well as to monitor the biological effectiveness *in vivo*, and iii) the evaluation of economic aspects, amongst which the market potential and cost effectiveness.

Being the most consumed staple crop worldwide, rice was an obvious candidate for biofortification. The metabolically engineered rice or folate rice was created in the Nipponbare variety. Several genetic interventions have been evaluated during the development of this rice variety. Arabidopsis GTP cyclohydrolase I (*GTPCHI*) and aminodeoxychorismate synthase (*ADCS*) genes from *Arabidopsis thaliana* were introduced into the rice genome as part of a co-expression strategy. The enzymes encoded by these genes are responsible for an increased formation of the deoxyneopterin and p-aminobenzoic acid building blocks, respectively (see Figure I.1), which, upon combination, yield a vast increase in folates, especially 5MeTHF.(Storozhenko *et al.* 2007 and Blancquaert *et al*. 2014)

The success of this concerted effort can be deduced from the generation of rice lines with a >100-fold increased folate levels in seeds (Storozhenko et al., 2007) and from the scientific output, with currently 4 publications in *Nature Biotechnology*, in addition to many other publications in high-ranking (top 5-10%) peer-reviewed journals. One of the prime aims of this consortium is to offer a feasible alternative for folate supplementation, by developing folate rice as a valuable source of dietary folate, to decrease the prevalence of neural tube defects in severely affected regions.

I.5. Analytical determination of folates

The analytical determination of a folate concentration in a matrix largely depends on the objective of the method (i.e. measurement of total folate concentration *versus* the concentration measurement of individual folate species) and the properties of the matrix. Also the appropriate procedure for method validation is largely matrix dependent and no folate specific procedures are available. Given the substantial difference between food and clinical matrices, the determination of folates in these distinct matrices is discussed separately below. For both, the classical guidelines for bioanalytical method validation, as issued by EMA and/or FDA, can be followed.(European Medicines Agency 2011, U.S. Department of Health and Human Services 2015) More particularly, these guidelines state that the following should be included in a bioanalytical method validation: evaluation of selectivity, carry-over, limit of detection (LOD) and lower limit of quantification (LLOQ), linearity, precision (including incurred sample reanalysis), accuracy, matrix effect, recovery and stability.

5.1. Plants and foodstuffs

While most data on folate concentrations in food databases originate from microbiologic assays (MA) which only allow to determine the total folate content, hyphenated liquid chromatography – (tandem) mass spectrometry (LC-MS(/MS)) based methods have been developed to determine individual folate species. To determine the success of fortification of a food item with FA, methods using ultraviolet detection are used most.(Strandler *et al*. 2015)

Although the same analytical techniques are used to determine folates in various food items, the sample preparation needed varies widely between different food matrices. For starchy matrices like rice, a classical trienzyme treatment is performed, including i) α-amylase to hydrolyze starch into sugars and thereby facilitate the extraction of the folates from the matrix; ii) a protease to reduce or eliminate the protein binding of folates and iii) a folate conjugase to cut the folate polyglutamates to monoglutamates which can be assayed.(Tamura *et al*. 1997) This classical trienzyme treatment is adapted and optimized for each individual matrix. For the determination of folates in spinach for instance, the use of α-amylase is not strictly necessary since green leafy vegetables contain little starch.

Since the start of the folate biofortification project at Ghent University, the measurement of folates in food items has been important to steer the genetic modifications. Starting with a method in which the folates were oxidized and cleaved into pABA to determine the total folate content using LC coupled to fluorescence detection, experience was gained within the lab and the research group in the field of food folate determination. (Zhang *et al.* 2003) Later, the introduction of MS detection increased method sensitivity and allowed to determine individual folate species. At this time, the trienzyme treatment was developed and used for folate determination in rice.(De Brouwer *et al*. 2008) The introduction of ultra-high pressure liquid chromatography (UHPLC) led to a further decrease of the minimal quantifiable concentrations and allowed a higher sample throughput. (De Brouwer *et al*. 2010)

Lately, research into the determination of food folates has been oriented towards folate profiling in potatoes, in the context of an effort to increase the folate content also in this common staple crop (Van Daele *et al*. 2014) and a better understanding of the biofortification process by developing ways to quantitatively determine pterins, one of the folate building blocks.(Van Daele *et al*. 2016) Additionally, a method was developed for folate determination in rodent diets (Kiekens *et al*. 2015b; described in Chapter IV) as part of the bioavailability study described in Chapter VI.(Kiekens *et al*. 2015c) Also within the context of the determination of folates in food matrices, we developed a method for the determination of folate polyglutamates, as described in Chapter VIII.

5.2. Clinical matrices

Over the past decade, the quantitation of clinical folate status using a MA or a protein binding assay (PBA) has been evaluated based on emerging LC-MS/MS assays. These chromatographic assays provide an orthogonal determination strategy, focusing on exact chemical determination rather than relying on biological activity or on recognition by a folate binding protein. While the MA currently remains the gold standard method for the determination of biologically active folates, the PBA is susceptible to differences between the folate species present in the sample and the species, mostly FA, used for assay calibration.(Pfeiffer *et al*. 2004)

The accuracy and precision obtained using LC-MS/MS and the possibility to quantify individual folate species has led to an increased interest to implement this technology for clinical folate research. The implementation of this technique in a routine clinical laboratory will depend on the automation of the sample preparation steps and the availability of LC-

MS/MS equipment. PBAs are currently used most to determine the amount of folates in patient samples, primarily owing to the ease of these assays and their automatability. Comparability of analytical results with other labs is not a major concern for the clinician since the use of reference intervals specific for the assay will suffice to distinguish folate deficient patients from those with an adequate folate status. For scientific research purposes, however, method comparability is necessary to compare findings with those obtained by other research groups. As such, for research purposes, as well as for any other scenario where insight into the distinct folate species is required, LC-MS/MS is the method of choice.

Whole blood or RBC folate analysis is not yet regularly performed for the determination of long term folate status over the lifetime of the RBCs, i.e. approximately 3 months. As this measurement is not influenced by recent folate intake, contrary to plasma or serum folate determination, a fasting period is not strictly necessary.

Since plasma and RBC folate concentrations provide complementary information, respectively for acute and chronic folate deficiency, we opted to develop a method that could analyze both, using a very comparable sample treatment procedure to limit the use of resources, i.e. time, equipment and reagents. If routine clinical analysis using an LC-MS/MS assay is considered, such an approach should make a cost effective procedure achievable.

Given the importance of folate determination in plasma and RBCs for the bioavailability trial discussed in Chapter VI and the novelty of this method within the research group, this subject is discussed further in Chapter III.

I.6. References

Aufreiter, S., Gregory, J.F.III, Pfeiffer, C.M., Fazili, Z., Kim, Y.-I., Marcon, N., Kamalaporn, P., Pencharz, P.B. and O'Connor, D.L. 2009. "Folate Is Absorbed across the Colon of Adults: Evidence from Cecal Infusion of (13)C-Labeled [6S]-5-Formyltetrahydrofolic Acid." *Am J Clin Nutr* 90 (1): 116–23.

Ball, G.F.M. 2005. *Vitamins In Foods: Analysis, Bioavailability, and Stability*. Vol. 1. CRC Press.

Beyer, P., Al-Babili, S., Xudong, Y., Lucca, P., Schaub, P., Welsch, R. and Potrykus, I. 2002. "Golden Rice: Introducing the β-Carotene Biosynthesis Pathway into Rice Endosperm by Genetic Engineering to Defeat Vitamin A Deficiency." J Nutr (132 (3): 506S-10S.

Blancquaert, D., De Steur, H., Gellynck, X., Van Der Straeten, D. 2014. "Present and future of folate biofortification of crop plants." *J Exp Bot* 65 (4): 895–906.

Blancquaert, D., Van Daele, J., Strobbe, S., Kiekens, F., Storozhenko, S., De Steur, H., Gellynck, X., Lambert, W.E., Stove, C.P. and Van Der Straeten, D. 2015. "Improving Folate (vitamin B9) Stability in Biofortified Rice through Metabolic Engineering." *Nat Biotechnol* 33 (10): 1076–8.

Blom, H.J. and Smulders, Y. 2011. "Overview of Homocysteine and Folate Metabolism. With Special References to Cardiovascular Disease and Neural Tube Defects." *J Inherit Metab Dis* 34 (1): 75–81.

Blount, B.C., Mack, M.M., Wehr, C.M., MacGregor, J.T., Hiatt, R.A., Wang, G., Wickramasinghe, S.N., Everson, R.B. and Ames, B.N. 1997. "Folate Deficiency Causes Uracil Misincorporation into Human DNA and Chromosome Breakage: Implications for Cancer and Neuronal Damage." *Proc Natl Acad Sci USA* 94 (7): 3290–5.

Centers for Disease Control and Prevention 2010. 'CDC Grand Rounds: Additional Opportunities to Prevent Neural Tube Defects with Folic Acid Fortification' *Morbidity and Mortality Weekly Report* 59 (31): 980-4.

Chandler, C.J., Wang, T.T., and Halsted, C.H. 1986. "Pteroylpolyglutamate Hydrolase from Human Jejunal Brush Borders. Purification and Characterization." *J Biol Chem* 261 (2): 928–33.

Clifford, A.J., Heid, M.K., Muller, H.G. and Bills, N.D. 1990. "Tissue Distribution and Prediction of Total Body Folate of Rats." *J Nutr* 120 (12): 1633–9.

Crider, K.S, Bailey, L.B. and Berry, R.J. 2011. "Folic Acid Food Fortification-Its History, Effect, Concerns, and Future Directions." *Nutrients* 3 (3): 370–84.

de Benoist, B. 2008. "Conclusions of a WHO Technical Consultation on Folate and Vitamin B12 Deficiencies." *Food Nutr Bull* 29 (2 Suppl): S238–44.

De Brouwer, V., Zhang, G.F., Storozhenko, S., Van Der Straeten, D. and Lambert, W.E. 2007. "pH Stability of Individual Folates during Critical Sample Preparation Steps in Prevision of the Analysis of Plant Folates." *Phytochem Anal* 18 (6): 496–508.

De Brouwer, V., Storozhenko, S., Van De Steene, J., Wille, S., Van Der Straeten, D. and Lambert, W.E. 2008. "Optimisation and validation of a liquid chromatography-tandem mass spectrometry method for folates in rice." *J Chrom A* 1215 (1-2): 125-32.

De Brouwer, V., Storozhenko, S., Stove, C., Van Daele, J., Van Der Straeten, D. and Lambert, W.E. 2010. "Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the sensitive determination of folates in rice." *J Chrom B* 878 (3-4): 509-13.

de Walle, H. and Abramsky L.. 2009. "Prevention of Neural Tube Defects with Periconceptional Folic Acid Supplementation in Europe." http://www.eurocat-network.eu/content/Special-Report-NTD-3rdEd-2009.pdf (consulted: 01/30/2016).

Díaz de la Garza, R.I., Gregory, J.F.III and Hanson, A.D. 2007. "Folate Biofortification of Tomato Fruit." *Proc Natl Acad Sci USA* 104 (10): 4218–22.

Dietrich, M., Brown, C.J.P. and Block, G. 2005. "The Effect of Folate Fortification of Cereal-Grain Products on Blood Folate Status, Dietary Folate Intake, and Dietary Folate Sources among Adult Non-Supplement Users in the United States." *J Am Coll Nutr* 24 (4): 266–74.

EFSA. 2009. "ESCO Report Prepared by the EFSA Sc Ientific Cooperation Working Group on Analysis of Risks and Benefits of Fortification of Food with Folic Acid." http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3e.pdf (Consulted: 01/30/2016).

European Medicines Agency. 2011. "Guideline on Bioanalytical Method Validation." Edited by Committee for Medicinal Products for Human Use (CHMP).

Gregory J.F.III 2001. "Case Study: Folate Bioavailability." *J Nutr* 131 (4 Suppl): 1376S – 82S.

Grosse, S.D., Waitzman, N.J., Romano, P.S. and Mulinare, J. 2005. "Reevaluating the Benefits of Folic Acid Fortification in the United States: Economic Analysis, Regulation, and Public Health." *Am J Public Health* 95 (11): 1917–22.

Hoffbrand, A.V., Tabaqchali, S., Booth, C.C. and Mollin, D.L. 1971. "Small Intestinal Bacterial Flora and Folate Status in Gastrointestinal Disease." *Gut* 12 (1): 27–33.

Hoffbrand, A.V., and Weir, D.G. 2001. "The History of Folic Acid." *Br J Haematol* 113 (3): 579–89.

Hoge Gezondheidsraad. 2015. "Voedingsaanbevelingen Voor België - Partim i: Vitaminen en Sporenelementen" http://www.health.belgium.be/sites/default/files/uploads/fields/fpshealth_theme_file/hgr_9164_9174_advies_voedingsaanbevelingen.pdf (Consulted: 05/15/2016)

Iyer, R. and Tomar, S.K. "Folate: A Functional Food Constituent." *J Food Sci* 74 (9): R114–22.

Jagerstad, M., Piironen, V., Walker, C., Ros, G., Carnovale, E., Holasova, M. and Nau, H. 2005. "Increasing Natural Food Folates through Bioprocessing and Biotechnology." *Trends Food Sci Technol* 16 (6-7): 298–306.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015a. "A Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Selective Analysis of Free and Total Folate in Plasma and Red Blood Cells." *J Chromatogr A* 1398: 20–28.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015b. "Determination of five folate monoglutamates in rodent diets." *J Agric Food Chem* 63 (45): 10089–95.

Kiekens, F., Blancquaert, D., Devisscher, L., Van Daele, J., Stove, V., Delanghe, J., Lambert, W.E. and Stove, C.P. 2015c. "Folates from metabolically engineered rice: a long-term study in rats." *Mol Nutr Food Res* 59 (3): 490–500.

Klipstein, F A, and Samloff, I.M.. 1966. "Folate Synthesis by Intestinal Bacteria." *Am J Clin Nutr* 19 (4): 237–46.

Matherly, L.H, and Goldman, D.I. 2003. "Membrane Transport of Folates." *Vitamins and Hormones* 66 (January): 403–56.

McLean, E., de Benoist, B. and Allen, L.H. 2008. "Review of the Magnitude of Folate and Vitamin B12 Deficiencies Worldwide." *Food Nutr Bull* 29 (2 Suppl): S38–51.

Molloy, A.M. 2010. "Folate-Vitamin B12 Interrelationships Links to Disease Risk" In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 381–408. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Moran, R.G. and Colman, P.D. 1984. "Measurement of Folylpolyglutamate Synthetase in Mammalian Tissues." *Anal Biochem* 140 (2): 326–42.

Osborne, C.B., Lowe, K.E. and Shane, B. 1993. "Regulation of Folate and One-Carbon Metabolism in Mammalian Cells. I. Folate Metabolism in Chinese Hamster Ovary Cells Expressing Escherichia Coli or Human Folylpoly-Gamma-Glutamate Synthetase Activity." *J Biol Chem* 268 (29): 21657–64.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. 2005. "Improving the nutritional value of Golden Rice through increased pro-vitamin A content." Nat Biotechnol 23: 482-87.

Pedersen, A.M., Bardow, A., Beier Jensen, S. and Nauntofte, B. 2002. "Saliva and Gastrointestinal Functions of Taste, Mastication, Swallowing and Digestion." *Oral Dis* 8 (3): 117–29.

Pfeiffer, C.M., Fazili, Z., McCoy, L., Zhang, M. and Gunter, E.W. 2004. "Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay." *Clin Chem* 50 (2): 423–32.

Pfeiffer, C.M., Hughes, J.P., Lacher, D.A., Bailey, R.L., Berry, R.J., Zhang, M., Yetley, E.A., Rader, J.I., Sempos, C.T. and Johnson, C.L. 2012. "Estimation of Trends in Serum and RBC Folate in the U.S. Population from Pre- to Postfortification Using Assay-Adjusted Data from the NHANES 1988-2010." *J Nutr* 142 (5): 886–93.

Piironen, V., Edelmann, M., Kariluoto, S., Bedo, Z. 2008. "Folate in wheat genotypes in the HEALTHGRAIN diversity screen." *J Agric Food Chem* 56: 9726–31.

Reynolds, E. 2006. "Vitamin B12, Folic Acid, and the Nervous System." *Lancet Neurol* 5 (11): 949–60.

Scott, J.M. 1999. "Folate and Vitamin B12." *P Nutr Soc* 58 (02): 441–8.

Selhub, J., Jacques, P.F., Dallal, G., Choumenkovitch, S. and Rogers, G. 2008. "The Use of Blood Concentrations of Vitamins and Their Respective Functional Indicators to Define Folate and Vitamin B12 Status." *Food Nutr Bull* 29 (2 Suppl): S67–73.

Shane, B. 2010. "Folate Chemistry and Metabolism." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 1–24. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Storozhenko, S., De Brouwer,V., Volckaert, M., Navarrete, O., Blancquaert, D., Zhang, G.F., Lambert, W.E. and Van Der Straeten, D. 2007. "Folate Fortification of Rice by Metabolic Engineering." *Nat Biotechnol* 25 (11): 1277–9.

Stover, P.J. 2004. "Physiology of Folate and Vitamin B12 in Health and Disease." *Nutr Rev* 62 (suppl 1): S3–12.

Stover, P.J. 2010. "Folate Biochemical Pathways and Their Regulation." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 49–74. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Strandler, H.S., Patring, J., Jagerstad, M. and Jastrebova, J. 2015. "Challenges in the Determination of Unsubstituted Food Folates: Impact of Stabilities and Conversions on Analytical Results." *J Agric Food Chem* 63: 2367−77.

Tamura, T., Mizuno, Y., Johnston, K.E. and Jacob, R.A. 1997. "Food Folate Assay with Protease, α-Amylase, and Folate Conjugase Treatments." J Agric Food Chem 45:135-9.

Tang, G., Qin, J., Dolnikowski, G.G., Russell, R.M. and Grusak, M.A. 2009. "Golden Rice is an effective source of vitamin A." *Am J Clin Nutr* 89 (6): 1776-83.

Ueland, P.M., Refsum, H., Beresford, S.A. and Vollset. S.E. 2000. "The Controversy over Homocysteine and Cardiovascular Risk." *Am J Clin Nutr* 72 (2): 324–32.

U.S. Department of Health and Human Services. 2015. "Analytical Procedures and Methods Validation for Drugs and Biologics."

Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W., Stove, C. 2014. "Folate profiling in potato (Solanum tuberosum) tubers by ultrahigh-performance liquid chromatography-tandem mass spectrometry." *J Agric Food Chem* 62 (14): 3092-100.

Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W., Stove, C. 2016. "Degradation and Interconversion of Plant Pteridines during Sample Preparation and Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry." *Food Chem* 194: 1189-98.

van Haandel, L., Becker, M.L., Williams, T.D., Stobaugh, J.F. and Leeder, J.S. 2012. "Comprehensive Quantitative Measurement of Folate Polyglutamates in Human Erythrocytes by Ion Pairing Ultra-Performance Liquid Chromatography/tandem Mass Spectrometry." *Rapid Commun Mass Spectrom* 26 (14): 1617–30.

Visentin, M., Diop-Bove, N., Zhao, R. and Goldman, I.D. 2014. "The Intestinal Absorption of Folates." *Annu Rev Physiol* 76: 251–74.

Wang, C., Riedl, K.M., and Schwartz, S.J. 2010. "A Liquid Chromatography-Tandem Mass Spectrometric Method for Quantitative Determination of Native 5-Methyltetrahydrofolate and Its Polyglutamyl Derivatives in Raw Vegetables." *J Chromatogr B* 878 (29): 2949–58.

WHO. 1972. "Nutritional Anemias. Report of WHO Group of Experts." *World Health Organ Tech Rep Ser* 503: 1–29.

WHO. 1975. "Control of Nutritional Anemia with Special Reference to Iron Deficiency. Report of an IAEA/USAIS/WHO Joint Meeting." *World Health Organ Tech Rep Ser* 580: 5–71.

WHO. 2012. "Serum and Red Blood Cell Folate Concentrations for Assessing Folate Status in Populations." World Health Organization. http://apps.who.int/iris/bitstream/10665/75584/1/WHO_NMH_NHD_EPG_12.1_eng.pdf (consulted: 01/30/2016)

WHO. 2015. "Guideline: Optimal Serum and Red Blood Cell Folate Concentration in Women of Reproductive Age for Prevention of Neural Tube Defects." World Health Organization. http://apps.who.int/iris/bitstream/10665/161988/1/9789241549042_eng.pdf?ua=1 (consulted: 01/30/2016)

Zhang, G.-F., Maudens, K.E., Storozhenko, S., Mortier, K.A., Van Der Straeten, D., Lambert, W. 2003. "Determination of Total Folate in Plant Material by Chemical Conversion into para-Aminobenzoic Acid Followed by High Performance Liquid Chromatography Combined with On-Line Postcolumn Derivatization and Fluorescence Detection." *J Agric Food Chem* 51 (27): 7872-78.

Chapter II: Objectives

Main objective

Already well over a decade, a consortium within Ghent University, composed of the Laboratory of Functional Plant Biology, the Laboratory of Toxicology and the Department of Agricultural Economics, focuses on a better understanding of the folate pathway and the improvement of the folate content in crops. The role of the different partners lies in i) the generation of transgenic crops and their molecular and functional characterization, ii) the set-up and deployment of validated analytical strategies to steer and evaluate the success of the genetic modifications, as well as to monitor the biological effectiveness *in vivo*, and iii) the evaluation of economic aspects, amongst which the market potential and cost effectiveness. The success of this concerted effort can e.g. be deduced from the generation of rice lines with a >100-fold increased folate levels in seeds (Storozhenko et al., 2007).

One of the prime aims of the consortium is to offer a feasible alternative for folate supplementation, by developing folate rice as a valuable source of dietary folate, to decrease the prevalence of neural tube defects in severely affected regions. However, although the ability to increase folate concentrations in rice grains represents a major achievement, this does not necessarily imply that this rice variety is a good source of dietary folate. To investigate whether the folates in folate rice are bio-accessible and bio-effective, an *in vivo* trial was necessary. As a means to quantify the effect of folate supplementation by providing folate rice, a method for the quantification of folates in plasma and red blood cells was necessary. Also, to prevent that folates present in rodent diet could influence the obtained results, a method had to be developed to determine the folate content in these diets.

Hence, within this thesis, we developed and validated the analytical tools that were required to evaluate the biological effect of folate rice consumption. This was done within the context of the available knowledge concerning folate determination in food matrices using liquid-chromatography with ultraviolet and fluorescence detection in spinach (Zhang *et al.* 2003) and liquid chromatography with mass spectrometric detection in rice (De Brouwer *et al.* 2010), *Arabidopsis thaliana* and green leafy vegetables and alongside the development of methods for the LC-MS/MS-based determination of folates in potato (Van Daele *et al.* 2014) and pterins (Van Daele *et al.* 2016) in plant matrices.

Outline and detailed objectives

In **Chapter I**, which gave a general background on folates, their relevance for human health, fortification and the relevance of folate determination, it was already pointed out that the analytical determination of a folate concentration can be looked at from different viewpoints. While in some instances knowledge about the (order of magnitude of a) total folate concentration might suffice, in other instances quantitative measurement of individual folate species is required. In the work described in this thesis, quantitative determination of individual folate species was required for both rodent feed (to be used in a rodent feeding trial), as for plasma and red blood cells (sampled following the rodent feeding trial). In the context of the latter, **Chapter III** points out the distinct points of attention that need to be taken into account when embarking upon folate determination in a clinical context, with special attention to LC-MS/MS.

Consumption of wild type rice only results in a very limited folate intake. Therefore, in the rodent feeding trial we performed, the determination of folates in rodent diets required limits of detection and quantitation that were as low as possible to assure that only rice was the source of folate in the diet. A wide applicability of this method was also necessary since different ingredients may require different sample treatments, e.g. a protease treatment to disrupt the protein binding of folates. Using this method, the details of which can be found in **Chapter IV**, a truly folate free diet was selected for use during the rodent feeding trial.

Since folate concentrations in plasma and red blood cells are key to study the outcome during the bioavailability trial, the development of a method for folate quantification in plasma and red blood cells, described in **Chapter V**, was required. While the LC-MS/MS settings were available from the determination of folates in food matrices, a new sample treatment procedure had to be developed. Given that both plasma (or serum) and red blood cell folates provide complementary information on folate status, the development of a method to measure the folate concentration in both matrices allows to obtain the maximal amount of information from a rodent feeding trial. A factor complicating the development of such a method was the limited amount of whole blood that can be obtained from periodic sampling of rats. Therefore, a method was required that allowed to determine both plasma and red blood cell folates from only one 300 µl aliquot of whole blood instead of two separate samples commonly used in literature. Ease of use was also a priority during method development since large amounts of samples would need to be processed periodically during the feeding trial. To treat plasma and RBC samples simultaneously, a sample treatment procedure including similar steps for both plasma and red blood cell folate analysis was required. Also, the implementation of a 96-well solid-phase extraction format was to be evaluated to increase sample throughput.

The evaluation of the biological availability and efficacy of folate rice consumption was performed by means of a rodent feeding study, described in **Chapter VI**. In contrast to traditional studies, only recording the pharmacokinetics of a bolus folate intake, a long-term

study was to devised to evaluate the efficacy of folate rice consumption on both short and long term. However, this required that an appropriate feeding strategy was set up and a feasible manner for sample collection was found. Using the rodent feeding trial, we aimed at answering the simple question "is folate rice able to maintain an adequate folate status when it is part of the diet?" To have a benchmark for the folate levels measured, we compared the results obtained from rats that were fed folate rice with those obtained from rats fed wild type or folic acid fortified rice. Also a positive and negative control group, receiving respectively ample and no folate in their diet, were included. Over a period of three months, we not only determined the folate levels in plasma and RBCs, but also the homocysteine levels (a metabolic marker linked to folate status), as well as other parameters.

As we could observe in our rodent trial, folates are essential for maintaining a healthy status, by their effects on many physiological processes. One of the systems whose function is influenced by folate status is the immune system. Although this was evaluated to a limited extent by others (Courtemanche *et al*. 2004), the question remained whether a low folate status leads to low intracellular folate concentrations and whether or not the folate species used during *in vitro* incubation influences the intracellular folate concentrations and the proliferation rate of the cells. As we had the analytical tools at hand, we decided to investigate this relationship, and added either folic acid or 5-methyltetrahydrofolate to *in vitro* cultures of peripheral blood mononuclear cells, at different concentration levels. To determine the intracellular folate concentration, an adapted method for folate determination in lymphocyte lysates and the development of procedures for cell incubation and the selective isolation of different cell types were required. How these experiments were performed can be found in **Chapter VII**.

For biofortified rice, information on the degree of folate polyglutamylation was available via the determination of folate monoglutamates and total folates (monoglutamates + polyglutamates). However, the measurement of individual folate polyglutamate species was to be investigated since this would provide further information on the folate polyglutamate chain length in rice samples with varying gene expression. Since not all folate polyglutamate standards were commercially available, the *in house* synthesis of the necessary reference materials was required to achieve a method able to quantify all relevant polyglutamate species. The analytical method and procedure for the preparation of the reference standards can be found in **Chapter VIII**.

In **Chapter IX**, the broader international context, relevance and future perspectives are described. A comparison between folate rice and traditional folate fortification strategies and the prevalence of folate deficiency can be found in this chapter.

A summary of the project described in this thesis, as well as the conclusions, can be found in **Chapter X**.

References

Courtemanche, C., Elson-Schwab, I., Mashiyama, S.T., Kerry, N. and Ames, B.N. 2004. "Folate Deficiency Inhibits the Proliferation of Primary Human CD8+ T Lymphocytes in Vitro." *J Immunol* 173 (5): 3186–92.

De Brouwer, V., Storozhenko, S., Stove, C., Van Daele, J., Van Der Straeten, D., Lambert, W. 2010. "Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the sensitive determination of folates in rice." *J Chrom B* 878 (3-4): 509-13.

Storozhenko, S., De Brouwer,V., Volckaert, M., Navarrete, O., Blancquaert, D., Zhang, G.F., Lambert, W.E. and Van Der Straeten, D. 2007. "Folate Fortification of Rice by Metabolic Engineering." *Nat Biotechnol* 25 (11): 1277–79.

Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W., Stove, C. 2014. "Folate profiling in potato (Solanum tuberosum) tubers by ultrahigh-performance liquid chromatography-tandem mass spectrometry." *J Agric Food Chem* 62 (14): 3092-100.

Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W., Stove, C. 2016. "Degradation and Interconversion of Plant Pteridines during Sample Preparation and Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry." *Food Chem* 194: 1189-98.

Zhang, G.-F., Maudens, K.E., Storozhenko, S., Mortier, K.A., Van Der Straeten, D., Lambert, W. 2003. "Determination of Total Folate in Plant Material by Chemical Conversion into para-Aminobenzoic Acid Followed by High Performance Liquid Chromatography Combined with On-Line Postcolumn Derivatization and Fluorescence Detection." *J Agric Food Chem* 51 (27): 7872-78.

Chapter III: Determination of clinical folate status

Based on:

Kiekens, F., Blancquaert, D., Van Daele, J., De Steur, H., Gellynck, X., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2016. “Determination of folate status: practical considerations”

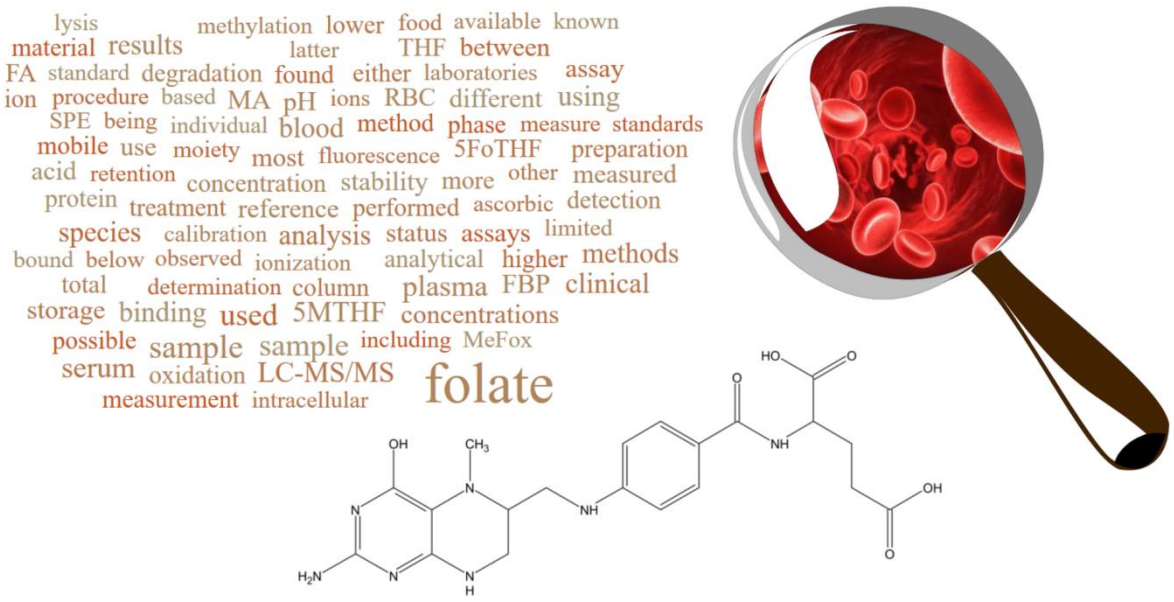
Table of contents

III.1. Abstract

Public interest for the influence of nutrient status on human health has increased over the past decade. Adequate nutrient intake serves a preventive function in relation to human health. By implementing fortification programs, an adjustment to adequate nutrient levels has been attempted in several countries. One such fortification strategy consists of the addition of folic acid to cereal grain products. However, a folic acid supplementation strategy on an industrial scale is not always possible, especially for remote communities.

The limited dietary diversity in remote regions increases the risk for folate deficiency, increasing the need for an effective supplementation strategy. As such, the development of a rice variety with a substantially increased folate content could, when cultured locally, provide a source of additional folate intake in these regions. This rice species, known as folate rice, should be capable of significantly decreasing the burden of folate related illnesses on these communities. However, given that folate rice is genetically modified and contains higher than normal folate concentrations, the efficacy of folate rice as a source of dietary folate was to be verified.

Evaluation of further developments in folate research depends on the availability of analytical methods capable of determining the different folate species in both food matrices and clinical samples. Substantial improvement in method comparability is key to evaluate the effect of different folate fortification strategies. Also, the use of short term studies to evaluate folate bioavailability proves problematic given the differences in absorption of different folate species. As such, the measurement of red blood cell folate concentrations can provide additional information on folate status over greater lengths of time. This approach could be beneficial for population based studies since this measurement is less influenced by recent folate intake.



III.2. Clinical folate analysis

Since the introduction of liquid chromatography tandem-mass spectrometry (LC-MS/MS) in clinical laboratories, folate analysis has shifted from microbiological (MA) or protein binding assays (PBA) to chromatographic methods. Although several chromatographic methods have been developed, validated and published, interlaboratory variability limits the comparability of the results. In this chapter we provide an overview of the strategies for sample treatment and analysis and how these may influence the final analytical result. Analyte stability in function of pH and temperature, measures taken to counter oxidation and the use of anticoagulants will all be covered. Also the use of certified reference materials is discussed.

2.1. Introduction

The multitude of sample treatment procedures preceding LC-MS/MS hinders comparability of clinical and scientific data on one hand and of international reference ranges on the other. This has proven to be the Achilles' heel of current folate determinations in clinical samples. In this chapter, we focus on the use of LC-MS/MS for folate quantitation in blood, plasma and serum, by providing a non-exhaustive overview of methods improving the existing analytical procedures since the year 2000. We will focus especially on strategies and trends that have emerged during the last 5 to 10 years to address the issues concerning folate quantitation in these matrices.

2.2. Reference range

Based on the homocysteine response, the cut-off levels indicating folate deficiency (i.e. the folate concentrations below which homocysteine concentrations started to rise) were set at 10 and 340 nM for serum (plasma) and RBCs, respectively. Since these reference concentrations encompass different folate species, the quantification of these different species requires the utmost sensitivity for the less abundant folate species. Under normal conditions, total folate concentrations are unlikely to exceed 100 and 1000 nM for plasma and RBCs respectively (see also Chapter V and VI).(Selhub *et al*. 2008; de Benoist 2008; Pfeiffer *et al*. 2010)

2.3. Genetic variation vs. folate status

Given the complexity of the folate pathway and the numerous enzymes involved, it is not surprising that several genetic polymorphisms in the human genome have been identified that play a role in the folate biosynthesis pathway. The most studied is a single nucleotide polymorphism (SNP) in the methylene tetrahydrofolate reductase gene at nucleotide 677 (*MTHFR 677 C>T*), changing an alanine to a valine, which results in a thermolabile and less efficient enzyme.(Huang *et al*. 2008) This SNP is very rare in the African population but approximately 10% of Caucasians and 20% of Asians are homozygous for this functional polymorphism.(Franco *et al*. 1998) While intracellular folates in *677C* homozygotes and in heterozygotes mostly consist of 5MeTHF, *677T* homozygotes have a much lower fraction of 5MeTHF and are at higher risk for low folate status, leading to hyperhomocysteinemia and related CVDs, and posing a higher risk for NTDs.

Additionally, other polymorphisms have been described that may influence folate status. As described by DeVos *et al.*, SNPs can occur in nearly all metabolically relevant enzymes such as FPGS and GGH enzymes governing polyglutamylation, MTHFR, as well as methionine synthetase (5MeTHF-homocysteine methyltransferase, MTR). Also the folate carriers PCFT and RFC, as well as the folate hydrolase 1 (FOLH1) present in the brush border, are subject to polymorphic variation.(DeVos *et al*. 2008) However, not all polymorphisms have a significant influence on the intracellular folate profile and/or concentration. Ideally, a method for the analysis of folates in plasma or serum and RBCs should be able to discern differences in concentrations of all relevant folate species, including the degree of polyglutamylation.

2.4. The measurement of folates in clinical matrices

The relevant folate species are 5MeTHF, THF, DHF, 5,10CH_2THF and 10FoTHF.(Bailey and Gregory 3rd 1999) However, as a result of folate instability and interconversions, not all of these can be quantified directly. Moreover, oxidation of 5MeTHF leads to the formation of 4'-hydroxy-5-methyltetrahydrofolate (hmTHF), which structurally rearranges to a pyrazino-s-triazine derivative known as MeFox. This folate species is metabolically inactive. However, since it may be formed during sample treatment or storage and may interfere with determination of other folates, it is of interest to measure this compound as well when aiming at an accurate folate profiling of a sample.(Pfeiffer *et al*. 2015)

Plasma or serum folate measurement is widely applied in clinical laboratories and, to this day, provides clinicians with an indication of the folate status. However, this method relies on an adequate (overnight) fasting period to assure that steady-state folate concentrations are measured, since only these correlate with folate stores elsewhere in the body. Absorption following ingestion of chemically pure 5MeTHF is rapid, with peak plasma concentrations readily occurring after one hour. Food 5MeTHF is absorbed more slowly, with peak plasma concentrations after only 2 hours. Following consumption of oxidized folate, i.e. FA, peak plasma concentrations are reached after only 3 hours because a reduction by DHFR is required prior to release in the portal circulation.(Konings *et al*. 2002)

2.4.1. Sample preparation

Samples for clinical folate analysis are typically obtained following venepuncture. Serum samples are more commonly used for folate analysis than plasma samples. Ethylenediaminetetraacetic acid (EDTA) is the most used anticoagulant to generate plasma as well as for whole blood folate analysis. An alternative sampling strategy, limiting the burden on the patient, is the collection of dried blood spots (DBS), obtained following a finger or heel prick.(Velghe *et al*. 2016) Already in 1999, O'Broin and Gunter reported on the use of a MA for screening the folate status in neonates in DBS.(O'Broin and Gunter 1999) Recently, Kopp and Rychlik published a stable isotope dilution assay using LC-MS/MS to determine 5MeTHF in dried blood and serum spots.(Kopp and Rychlik 2015) Hereby, aliquots of blood, plasma or serum were spotted on paper pretreated with L-ascorbic acid (AA). The folates were extracted from 3 mm punches by sonication in a buffer containing 2-(N-morpholino)-ethanesulfonic acid, Triton X-100 and dithiothreitol (DTT). To measure total folate, whole blood spot extracts were treated with rat serum and chicken pancreas GGH. Plasma folates could be determined from whole blood spots by first performing heat denaturation to abolish

endogenous GGH activity. These authors found that 5MeTHF could be determined at LOD's of 1.5, 2.2 and 9.1 nM for serum, plasma and whole blood, respectively, and that spotted samples were stable for 11 days when kept desiccated at -20°C. During storage and extraction, the presence of labeled internal standards compensated for the degradation of the folates present. Pretreating the paper with AA did not provide adequate stabilization of THF, which consequently could not be quantified.(Kopp and Rychlik 2015) For longer storage, lower storage temperatures (-80°C) are required, as shown by Zimmerman *et al*.(Zimmerman *et al*. 2013)

2.4.2. Anticoagulant vs sample stability

Using a variety of analytical methods, Hannisdal *et al*. investigated the influence of anticoagulants on folate stability during storage at room temperature. Strikingly, these authors found a significant impact of EDTA-treatment on the stability of 5MeTHF. During the first hours of storage a significant oxidation of this folate to MeFox was observed. Given its biological inactivity, this compound is not measured using a MA. However, the total folate content (including MeFox), either measured via LC-MS/MS or gas chromatography mass spectrometry (GC-MS) analysis of pABA-glutamate(s), is not influenced by this oxidation reaction.(Hannisdal *et al*. 2009a) In a study investigating the stability of fat- and water-soluble vitamins during long term storage at -20°C, a similar pattern was observed for EDTA-plasma samples analyzed by a competitive binding assay. Based on the data obtained, storage was considered acceptable up to 6 months, provided that samples were kept at -20°C.(Ocké *et al*. 1995) Serum samples as well as citrate or heparin treated plasma samples showed less oxidation and should therefore be preferred when long term storage is foreseen.(Hannisdal *et al*. 2009a) However, depending on the anticoagulant used, measured plasma folate concentrations may differ to a limited extent.(Fazili and Pfeiffer 2004) Also the folate concentration in erythrocytes was found to be influenced by the anticoagulant. O'Broin *et al*. found substantial losses of folate activity (assessed by MA) in the presence of EDTA during storage at room temperature, which were observed to a lesser extent in heparinized samples. While heparinization may improve sample stability upon storage, fresh heparinized samples readily had a slightly lower (3.61%) folate activity as compared to fresh EDTA-treated samples.(O'Broin *et al*. 1980)

2.4.3. Stability during sample preparation

Erythrocyte folate analysis is commonly performed by 10-fold dilution of whole blood with a 1% AA solution in deionized water (pH=±3). The use of an antioxidant during cell lysis ensures immediate protection against oxidative damage of intracellular folates. Also when an alternative lysis procedure is performed, for instance using a selective ammonia based buffer (Kiekens *et al*. 2015; Chapter V), the addition of AA is required to protect the intracellular folates against degradation. However, the utilized procedure differs between labs and is mentioned as a possible source of variability between methods due to incomplete lysis. This was shown in a comparative study, in which lysis with a 1% AA solution without neutralization provided complete and reproducible lysis.(O'Broin and Kelleher 2001)

Addition of a thiol such as DTT or 2-mercaptoethanol is required to capture the formaldehyde released by degradation of AA at elevated temperature. This is relevant since free formaldehyde causes the methylation of THF to 5,10-methenyltetrahydrofolate ($5,10CH^{+}THF$) and, as such, influences the measured folate distribution.(De Brouwer *et al*. 2007)

Though performed in a food matrix, Patring *et al*. investigated the effect of different reducing agents on the stability of folates during sample preparation. The addition of 2-mercaptoethanol, DTT, 2,3-dimercapto-1-propanol and 2-thiobarbituric acid in combination with sodium ascorbate was evaluated for different experimental conditions. The choice of antioxidant had an effect on the measurement of THF following various sample treatment procedures including heat treatment, freeze-thawing and frozen storage. While 2,3-dimercapto-1-propanol was most effective, these authors suggested to evaluate the stability of all folates for each specific sample treatment procedure, a suggestion we concur.(Patring *et al*. 2005)

Whole blood or RBC samples are commonly incubated at 37 °C for extended periods of time to deconjugate the folate polyglutamates present, enabling determination of total folate content. Since folates are relatively sensitive to elevated temperatures and oxidative stress, their stabilization during this step is critical. De Brouwer *et al*. investigated the influence of pH on folate recovery following 2 hours of incubation in a phosphate buffer with a pH ranging between 4 and 8.(De Brouwer *et al*. 2007) While most folates proved stable at the different pH levels, some degradation was observed for THF at acidic pH. Both DHF and $5,10CH_2THF$ are extremely sensitive to incubation at a pH lower than 8. Though Strandler *et al*. suggest the possible occurrence of $5,10CH_2THF$ in living cells, this compound -being the active intermediary for enzymatic methylation of uridylate to thymidylate- has yet to be observed in clinical samples due to its limited stability.(Strandler *et al*. 2015) This property implies that $5,10CH_2THF$, as well as DHF, cannot be measured in RBC samples using the currently available methods.(De Brouwer *et al*. 2007; Strandler *et al*. 2015) Likewise, 10FoTHF, a possible intermediary during the purine biosynthesis, cannot be detected when acidic conditions (e.g. a mobile phase with a pH<8) are employed, since it converts to $5,10CH^{+}THF$.(De Brouwer *et al*. 2007)

Akhtar *et al*. studied the influence of light on the stability of FA in aqueous solutions and found that a high pH, exceeding 10.0, was optimal to minimize degradation.(Akhtar *et al*. 1999) However, since most sample treatment steps are performed at or below neutral pH, sample treatment is best performed under subdued light.

2.4.4. Enzyme treatment

Theoretically, no enzyme treatment is necessary for the analysis of folates in serum or plasma samples: while intracellular folate retention depends on polyglutamylation, only mono-glutamates have been observed in plasma.(Shane 2010) This is owing to the presence of GGH in plasma, an exopeptidase that deconjugates any polyglutamate that would be released in plasma following cell lysis.(Galivan *et al*. 2000)

Large quantities of proteins capable of binding folates, most importantly FBP, are found in clinical samples, rendering folates unavailable for analysis. Total folate measurement requires release of these bound folates via protease treatment or via gentle acidification, since both the specific (FBP) and non-specific binding capacity is negligible below pH 4.(Kamen and Caston 1986) In folate binding assays, strong alkali in combination with reducing agents like DTT are used to denature all endogenous folate binding proteins.(Beggs *et al*. 1995)

2.4.5. Microbiological assay

The oldest, though still used, method for clinical folate analysis consists of the incubation of diluted serum with the bacterium *Lactobacillus rhamnosus* (formerly known as *Lactobacillus casei*). The growth rate of this bacterium, measured as optical density, can be correlated to the folate concentration in the cellular environment.(Hoffbrand *et al*. 1966; O'Broin *et al*. 1973) This method remains the benchmark for clinical folate analysis given its clear correlation with a biological outcome. Though originally developed to measure folates in serum samples (O'Broin *et al*. 1973), this method is also applied to whole blood lysates as a measure of RBC folates. In 2011 Pfeiffer *et al*. published the results of a sample exchange study between 3 laboratories performing the MA.(Pfeiffer *et al*. 2011) The results were found to be significantly influenced by the bacterial strain used during the assay (chloramphenicol resistant ATCC 27773 vs. wild-type ATCC 7469) and even more by the folate species used to construct the calibration curve. FA, which is relatively stable and therefore relatively easy to manipulate, is predominantly used to calibrate the assay. However, bacterial growth is ±25% lower, and therefore analytical results ±25% higher, compared to calibration with 5MeTHF. Although calibration with 5MeTHF, the main folate species in clinical samples, likely resembles actual samples more closely, its limited stability complicates calibration. Last, it should be remarked that the presence of antibiotics -inhibiting bacterial growth- will negatively influence the results.(Shane 2011)

2.4.6. Protein binding assay

While the MA requires overnight to 2 days of incubation, the protein binding assay does not. This significantly speeds up the procedure, allowing compatibility with high-throughput systems readily available in clinical labs. Additionally, in contrast to the MA this assay is not influenced by the presence of antibiotics in the sample. The assay was originally performed as a competitive binding radiolabel assay using ^{3}H-FA.(Archibald *et al.* 1972) More recent assays use ruthenium labeled FBP (Elecsys® Folate III) or FBP coated paramagnetic microparticles (Abbott Architect Folate) in a competition reaction to measure folates in the sample. Following incubation of the sample with the respective FBP preparation, biotinylated FA and magnetic microparticle-bound streptavidin (Elecsys® Folate III) or a FA – acridinium conjugate are added. The readout is based on chemiluminescence, the light emission being inversely proportional to the folate concentration in the sample. As such, high signal intensities are measured at low concentrations, improving sensitivity. While both are automated assays, a similar principle - with either colorimetric or chemiluminescent readout- can be applied off-line (e.g. in a 96-well format). In these assays, endogenous folates and a FA – horseradish peroxidase (HRP) conjugate compete for binding to immobilized FBP (e.g. VAST CLIA Microwells® and VAST ELISA Microwells® Folate Test System from

Monobind Inc. and AccuDiag™ Folate from Cortez Diagnostics).(Roche Diagnostics International 2014; Abbott Ireland 2010; Monobind Inc. 2015a; Monobind Inc. 2015b; Diagnostic Automation/ Cortez Diagnostics Inc. 2014)

Following a comparison of 5 binding assays to the reference radiolabel assay, Owen and Roberts found that although comparable results were obtained for serum folate, results of RBC samples strongly varied between different methods. These authors suggested that the obtained results strongly depend on the lysis procedure, utilizing AA solutions with differing concentration or detergents such as saponin or Triton X-100.(Owen and Roberts 2003) Moreover, as protein binding assays rely on the selective binding of folates to FBP that is added in the assay, an optimized sample pretreatment step is necessary to release folates from endogenous FBP. Finally, it should also be noted that FBP has a different affinity towards distinct folate species, which may lead to a bias.(Dakshinamurti 1994)

2.4.7. LC-MS/MS analysis

Multiple chromatographic methods have been developed for the measurement of 5MeTHF and other folate species. The availability of LC-MS(/MS) equipment has fueled the development of higher-order assays to measure folates in both serum or plasma and RBCs. Below, we focus on the most recent developments for clinical folate analysis. For an in-depth overview of other methods, we refer to Pfeiffer *et al*.(Pfeiffer *et al*., 2010)

GC-MS based methods rely on the cleavage of the 9-10 bond between the pterin and the pABA-Glu moiety, followed by derivatization.(Santhoshkumar *et al*. 1995) Though these methods allow for a total folate measurement, they do not allow for individual folate speciation and rely on a quantitative (i.e. complete and selective) folate cleavage.

While most LC-MS/MS methods attempt to quantify the different folate species as they occur at the time of phlebotomy, some methods explicitly interconvert some folate species. By acidifying the final sample, both Wang *et al*. and van Haandel *et al*. determined 10FoTHF and 5FoTHF as 5,10CH$^+$THF.(Wang *et al*. 2010; van Haandel *et al*. 2012) While at a pH below 10, almost instantaneous conversion of 10FoTHF to 5,10CH$^+$THF will occur, it is possible to determine 5FoTHF and 5,10CH$^+$THF separately (cfr. Fazili and Pfeiffer 2004, Smith *et al*. 2006, De Brouwer *et al*. 2007, Kirsch *et al*. 2010, Fazili *et al*. 2013a, and Kiekens *et al*. 2015 (Chapter V)).

2.4.7.1. Sample Cleanup

To limit the presence of matrix compounds and assure method selectivity, sample cleanup is performed prior to LC-MS/MS analysis. This can range from simple protein precipitation to combined affinity and solid phase extraction (SPE). As with any sample cleanup procedure, simplicity and robustness need to be balanced against the sensitivity and throughput that will eventually be required.

While extensive sample treatment is often preferred to obtain purified extracts, some methods simply use protein precipitation with an organic solvent, like methanol or acetonitrile, followed by evaporation to dryness and reconstitution in mobile phase.(Garbis *et al*. 2001)

For serum or plasma, this approach seems feasible since these are relatively clean matrices. For RBCs, however, either SPE alone or SPE combined with affinity extraction is needed.

Given its selectivity, FBP is ideally suited to extract folates from complex matrices. While FBP is now commercially available in purified form, it can also be obtained from bovine milk. For affinity extraction procedures, FBP is bound to agarose beads and stored refrigerated in the presence of sodium azide as a preservative.(Hart *et al*. 2002; Nelson *et al*. 2004) Sample preparation is performed in a column configuration, with elution under acidic conditions since FBP shows little folate retention at a pH below 4. The fact that FBP does not have equal affinity for all folate species necessitates a substantial excess of binding sites to quantitatively retain all folates.(Dakshinamurti 1994) The number of samples which can be purified using one column is limited due to degradation of and irreversible binding to the protein. As such, there is a gradual decrease of binding capacity, which has to be controlled to avoid erroneous sample handling.(Nygren-Babol and Jägerstad 2012)

Recently developed methods have shown the value of SPE. Especially for the analysis of numerous samples, SPE is preferred over FBP columns. For low-throughputs, manual column-format SPE can be used, while for higher throughputs, the different SPE steps can be automated and/or 96-well formats can be used.(Fazili *et al*. 2013a) Both reversed phase and ion exchange sorbents can be used to separate folates from matrix constituents, the former being used more frequently. Phenyl and octadecyl sorbents are most frequently used and elution is often performed using a small percentage of organic solvent, either methanol or acetonitrile, acidified to correspond to the mobile phase. Samples can be concentrated prior to injection by evaporation or by using a vacuum system, followed by reconstitution in the initial mobile phase or in another appropriate solvent.(Garbis *et al*. 2001; Hart *et al*. 2002; Pfeiffer *et al*. 2004; Nelson *et al*. 2004; Smith *et al*. 2006; Owens *et al*. 2007; Thorpe *et al*. 2007; Hannisdal *et al*. 2009b; Kirsch *et al*. 2010; Monch *et al*. 2010; Kirsch *et al*. 2012; Fazili and Pfeiffer 2013b; Kiekens *et al*. 2015 (Chapter V))

2.4.7.2. Chromatographic separation

Both standard HPLC and UHPLC have been applied for the separation of folate species in clinical samples. Using traditional reversed phase columns, typically C_8 or C_{18}, folate species can relatively easily be separated. MeFox, despite being an oxidation product of 5MeTHF, is also worth measuring as it allows insight into possible oxidative stress the sample was subject to during e.g. sample storage or treatment. However, care should be taken to differentiate MeFox from its isobar 5FoTHF, which requires optimization of chromatography.(Fazili and Pfeiffer 2013b; Hannisdal *et al*. 2009a) Alternatively, when using MS/MS-based detection, it is possible to distinguish both folate species by selecting certain product ions (see 2.4.7.3), although maximal sensitivity may not be achieved this way.(Fazili and Pfeiffer 2013b)

Separation is mostly achieved by applying gradient elution with acidic mobile phases containing either acetic or formic acid in ultrapure water and methanol, acetonitrile or a combination of both. The use of salt buffers is not common and mainly reserved for HILIC chromatography.(Garbis *et al*. 2001) In 2006, Patring *et al*. investigated the elution pattern of folates when varying the stationary phase and the mobile phase constituents. The ionization of

the α- (pKa= 3.1 - 3.5) and γ- (pKa= 4.6 - 4.8) carboxyl groups of the glutamate part of the folate molecule was found to influence the retention behavior. A pH<3 is needed to completely suppress the ionization of both carboxyl groups. At higher pH (>3), the retention of the folates decreases to a varying extent depending on the column chemistry. This also negatively impacts peak symmetry and peak width. This effect is also noticed when the amount of organic solvent at the time of injection is increased. High retention of folates was achieved on columns with either polar endcapping or with a high carbon content. When volatile modifiers are used, i.e. either acetic or formic acid, retention is generally increased while peak shape deteriorates, though this effect was most pronounced using polar endcapped columns.(Patring *et al*. 2006) As evidenced by column usage of the methods shown in Table III.1, it is hard to predict which column will work best, based on column characteristics.

2.4.7.3. Detection methodology

Both ultraviolet, fluorescence (Vahteristo *et al*. 1996; Ndaw *et al*. 2001) and electrochemical (Bagley and Selhub 2000) detection have been applied for folate analysis in food items. Reduced folates show strong fluorescence (290-295 nm→ 356 nm), making this type of detection highly efficient and specific for the detection of THF, 5MeTHF and 5FoTHF in food matrices and for quality control purposes. Typical methods using fluorescence detection for 5MeTHF in food items have an LOD of 20 to 40 fmol on column. (Vahteristo *et al*. 1996; Ndaw *et al*. 2001) Oxidized folates do not fluoresce but all folates can be detected using a UV detector (±280 nm), albeit at the expense of sensitivity, which drops with a factor 50 to 100 compared to fluorescence detection.(Ndaw *et al*. 2001) With detection limits down to 0.2 to 0.4 fmol on column, recent LC-MS methods for the determination of folates in plasma or serum are 50 to 100 times more sensitive than fluorescence detection. As such, the use of fluorescence detection for clinical folate analysis would be limited to the quantification of RBC or whole blood folates due to the higher folate concentration in these samples. UV and electrochemical detection are not able to match the sensitivity of fluorescence detection and cannot be used for clinical samples as they would require vast sample volumes.(Bagley and Selhub 2000)

For clinical analyses, MS (and more particularly MS/MS) is most used as detection technique hyphenated with liquid chromatography. Folates, containing both alkaline (pterin moiety) and acidic (glutamate moiety) sites, can be positively and negatively charged in the pH range of mobile phases containing volatile modifiers such as acetic and formic acid. As such, MS systems can be operated both in positive and negative mode. Electrospray ionization (ESI) is most commonly used as an interface system to vaporize the mobile phase. To achieve high ionization efficiencies, a modifier (i.e. formic or acetic acid) is commonly used and is either included in the mobile phase (cfr. 2.4.7.2) or infused post column.(Owens *et al*. 2007) In general, folates show predominant single ionization in either negative or positive mode, with the latter being used most. However, double ionization can occur, though more in positive than in negative mode. As such, singly charged ions are typically used as Q1 ions (MW+1 or MW-1) during MS/MS-analysis. Generally, 2 fragment ions are monitored, as this allows to add the ion ratio as an extra identification criterion. In positive mode, the most abundant fragment ion, used as quantifier, generally consists of the ionized remainder (pterin-pABA$^{+\bullet}$),

following neutral loss of the glutamic acid• moiety.(Smith *et al*. 2006; Patring and Jastrebova 2007) 5,10CH^+THF differs from the other folates in that the glutamate moiety is not split off due to the withdrawal of electrons from the nitrogen atom at the 10 position and the resulting influence on ion stabilization.(Smith *et al*. 2006) During fragmentation, the formyl groups bound to the nitrogen at the 5 or 10 position have a tendency to split off. This phenomenon, the expulsion of CO, does not influence the charge of the remaining ion and has little influence on the signal intensity of the ion which is monitored.(Freisleben *et al*. 2003) The second fragment ion is typically formed via cleavage of the bond between position 6 and 9 or 9 and 10, yielding ions of 166 (representing the reduced pterin cation), 176 or 194 Da (the latter two resulting from loss of both Glu and pABA). For 5,10CH^+THF, the second fragment ion that is typically followed is formed after the loss of glutamate-C=O.(De Brouwer *et al*. 2008) The quantitative LC-MS/MS determination of 5FoTHF and MeFox is complicated by their identical molecular mass of 474 amu and the fact that both are predominantly fragmented to a fragment ion of 327 amu. Both compounds also have a tendency to coelute. Though baseline resolution is achievable, the less abundant ions of 284 and 299 amu, specific for MeFox and 5FoTHF, respectively, can be used as quantifiers.(Fazili and Pfeiffer 2013b)

2.4.8. Method comparability

Method comparability has remained a substantial issue hindering folate analysis because of the influence of several procedural aspects such as the use of calibrators, strains of micro-organisms and antioxidants. To this day, the MA, despite its limitations, is still considered the gold standard for folate measurement. As such, the performance of alternative methods such as PBA and LC-MS/MS assays is measured against this MA.(Yetley *et al*. 2011) For serum or plasma, the total folate results obtained using the MA (calibrated with 5MeTHF) and LC-MS/MS analysis were found to be in good agreement, although slightly higher concentrations were found with LC-MS/MS. Results obtained using a PBA (calibrated with FA) were much (i.e. close to 30%) lower than those of either the MA or LC-MS/MS assay. This is likely due to a lower recovery of certain folate species, amongst which 5MeTHF, in the PBA.(Fazili *et al*. 2007) As such, results depend on whether 5MeTHF or FA is used as a calibrator. (Fazili *et al*. 2007) For RBC folates, a good agreement was found between results obtained by MA and those obtained by LC-MS/MS, although here total folates determined by LC-MS/MS were 10% lower than those scored by MA. Again, a substantial difference was seen between the results obtained by MA and PBA, the results in the latter being 45% lower. Importantly, this difference was genotype dependent, with as an underlying cause a differential recovery of different folate species.(Fazili *et al*. 2008) As such, the MA was maintained as reference method for whole blood or RBC folate measurement.(Yetley *et al*. 2011)

plasma/serum						
method	year	sample source	Folate species monitored	sample preparation	LOD-range [nM]	start volume [µl] ($^{+}$whole blood)
Garbis *et al.*	2001	plasma	5MeTHF, THF, FA, 5FoTHF	PP-EV	0.04 - 0.43	2000
Hart *et al.*	2002	plasma	5MeTHF	PP-AE	0.2	5000
Rychlik *et al.*	2003	plasma	5MeTHF	PP-SPE	3.5	1000
Kok *et al.*	2004	EDTA plasma	5MeTHF, FA	PP-AE-SPE-EV	0.012, 0.05	2000
Pfeiffer *et al.*	2004	serum	5MeTHF, 5FoTHF, FA	SPE	0.05-0.13	275
Nelson *et al.*	2004	serum and citrate plasma	5MeTHF	PP-AE or AE-SPE-EV	0.07 (affinity) or 0.87 (SPE)	500
Huang *et al.*	2008	EDTA plasma	5MeTHF, 5,10CH^{+}THF, THF, FA	SPE-EV	1.5	300
Liu *et al.*	2009	plasma	6S-5MeTHF, 6S-5FoTHF, 6R-5FoTHF	PP-EV-SPE	54.41, 105.7*	200
Hannisdal *et al.*	2009b	serum	5MeTHF, FA, 5FoTHF, hmTHF (now known to be MeFox)	PP-EV	0.07 - 0.52	60
Mönch *et al.*	2010	EDTA plasma	5MeTHF, THF, FA, 5FoTHF, 10FoFA	SPE	0.05 - 5	400
Kirsch *et al.*	2010	serum	5MeTHF, 5,10CH^{+}THF, THF, FA, 5FoTHF	SPE-EV	0.09 - 0.9	250
van Haandel *et al.*	2012	EDTA plasma	5MeTHF, 5,10CH^{+}THF, THF, FA	PP	0.1 - 0.5	50
Fazili *et al.*	2013a	serum	5MeTHF, 5,10CH^{+}THF, THF, FA, 5FoTHF, MeFox	SPE	0.06 - 0.31	150
Kiekens *et al.*	2015	EDTA plasma	5MeTHF, 5,10CH^{+}THF, THF, FA, 5FoTHF, 10FoFA	PP-SPE	0.33-0.5	300^{+}
Zheng *et al.*	2015	plasma	5MeTHF, FA	PP-EV	11.0, 0.56	500
RBC						
method	year	sample source	folate species monitored	sample preparation	LOD-range [nM]	start volume [µl]
Fazili and Pfeiffer	2004	EDTA whole blood	5MeTHF, 5,10CH^{+}THF, THF, FA, 5FoTHF, 10FoFA	SPE	0.05 - 2.5	100
Smith *et al.*	2006	EDTA whole blood	5MeTHF, 5,10CH^{+}THF, 5,10CH$_2$THF, DHF, THF, FA, 5FoTHF, 10FoTHF	PP-AE-SPE-EV	0.4 - 20*	100
Huang *et al.*	2008	EDTA whole blood	5MeTHF, 5,10CH^{+}THF, THF, FA	SPE-EV	1.5	500
Mönch *et al.*	2010	lyphilized RBCs	5MeTHF, THF, FA, 5FoTHF, 10FoFA	SPE	4 - 54	100
Kirsch *et al.*	2012	EDTA whole blood	5MeTHF, THF, FA, 5FoTHF, 10FoFA	SPE-EV	0.12 - 0.40	200
van Haandel *et al.*	2012	EDTA whole blood	5MeTHF, 5,10CH^{+}THF, THF, FA	PP	0.2 - 1.0	50
Kiekens *et al.*	2015	EDTA whole blood	5MeTHF, 5,10CH^{+}THF, THF, FA, 5FoTHF, 10FoFA	PP-SPE	0.33-0.5	300

Table III.1: Overview of recent LC-MS methods for folate measurement. *LLOQ as no LOD was reported; AE, affinity extraction using folate binding protein; EV, evaporation to dryness; LLOQ, lower limit of quantification; LOD, limit of detection; PP, protein precipitation; SPE, solid phase extraction.

2.4.8.1. Assay calibration

One of the major issues complicating folate analysis is the comparability of analytical results between labs throughout the world. Therefore, correct calibration of an assay is of paramount importance to obtain correct results.

Perhaps the most important part of an assay is its calibration using pure reference standards. These can be obtained from various sources, such as Merck Eprova (Schaffenhausen, Switzerland), Schircks Laboratories (Jona, Switzerland) or Sigma-Aldrich (Saint-Louis, MO, USA). As mentioned above, several authors have indicated that the calibrator choice can strongly influence analytical results.(Pfeiffer *et al*. 2004; Fazili *et al*. 2007) More specifically, owing to a difference in recovery for individual folate species, the protein-binding assay is not able to match the results of both an LC-MS/MS assay or MA. In LC-MS/MS, the actual panel of folates that is measured will determine the eventual total folate concentration, as the latter is derived from the sum of the individual folate species. On the other hand, the composition of the panel of folates that can be reliably quantified, depends on the availability of pure reference standards.

While the use of MS allows to differentiate individual folate species, the technique does suffer from matrix-associated effects, including differences between individual samples influencing the measured signal. For this reason, isotopically labeled internal standards are commonly used for LC-MS/MS folate analyses, then named stable isotope dilution assays, to compensate for the influence of the matrix on recovery and ionization efficiency. For this purpose, isotopologues of the parent compounds are used in which either carbon or hydrogen atoms are replaced with ^{13}C or ^{2}H atoms. A mass difference of 4 to 5 atomic mass units (amu) ensures that the analyte and the IS can be monitored separately, given the presence of natural isotopologues in samples (up to +2 amu).(Freisleben *et al*. 2002) While deuterated ISs are easier to manufacture, deuterium ions from the IS and hydrogen atoms from the aqueous solvent can interchange, which impacts the isotopic purity of the IS and may influence the analytical result. Also, given their location at the outside edge of the molecule, deuteration can change the charge density of the molecule, thereby slightly influencing its retention time.(Stokvis *et al*. 2005) For this reason, ^{13}C-labeled ISs are preferred over deuterium labeled ISs. Unfortunately, not every folate form has its own labeled IS commercially available. In that case, the nearest folate species in terms of stability and elution time is used as an IS. However, it should be evaluated on a case-by-case basis which IS is most appropriate for a specific folate.

The limited stability of folates also requires careful storage of prepared calibrators and, if these are gravimetrically prepared, likewise careful storage of the powder form. Reduced folates are susceptible to oxidative degradation while some, like 5FoTHF, are very hygroscopic. Therefore, it is advised to verify the purity of the standard material as part of the preparation of stock solutions. This can be performed spectrophotometrically based on the molar extinction measured by Blakley.(Blakley 1969) In addition, it is worth bearing in mind that AA, commonly used to stabilize folates in solutions, absorbs light at the same wavelengths as folates, making the spectrophotometric verification of folate concentrations in the presence of this antioxidant impossible.(Karayannis *et al.* 1977)

2.4.8.2. Possible certified reference materials and the values measured

Key to the evaluation of method performance is the use of reference materials with a known folate content. These are commercially available samples that allow the comparison of results obtained in different laboratories. Ideally, the concentration obtained should match, although for sensitive compounds, such as folates, this may be a tall order.

To accurately assess the performance of an entire method, a certified reference material (CRM) should resemble a real sample as close as possible. The spiking of folates to samples can be problematic due to differences in protein binding and may lead to divergent extraction behavior. It is therefore preferred to mix different blood pools to obtain a certain folate concentration. However, this is not always possible due to the limited and unpredictable presence of non-5MeTHF-folates.

Two WHO international standards are available for folates through The National Institute for Biological Standards and Control (NIBSC), one consisting of lyophilized serum (03/178) and the other of a lyophilized whole blood lysate (95/528). The NIBSC serum reference material has been assigned reference values for total folate, as well as for 5MeTHF, 5FoTHF and FA. The consensus concentrations were reached following the analysis of the standard material by 24 laboratories in 7 different countries, published by Thorpe *et al*. in 2007.(Thorpe *et al*. 2007) These included 19 protein binding assays, 3 MA and 2 LC-MS/MS assays. Though significant variability was observed between the results obtained using the different assays, this study showed the potential of such a reference material. When the reference standard 03/178 for folates in serum was used to correct for the systematic error between the laboratories, significant gains in comparability were obtained.(Thorpe *et al*. 2007) Also NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) has issued a standard for folates in serum, the standard reference material (SRM) 1955.(Satterfield *et al*. 2006) To replace the SRM 1955 when stocks were depleted, a new standard reference material was prepared, i.e. the SRM 3949. This is a set of three serum samples, devised to contain, apart from 5MeTHF and FA, the minor folate species THF, 5FoTHF, MeFox and 5,10CH$^+$THF. Given the limited availability of analytical results, consensus concentrations for the different folate species using a variety of methods have not been reached yet.(Camara *et al*. 2015)

For whole blood lysate (95/528), a consensus value was assigned following a study in which 13 laboratories participated for a total of 34 assay results. Unfortunately, only MA and PBAs were used.(Thorpe *et al*. 2004) However, recently we published an LC-MS/MS method for the analysis of folates in RBC samples which includes data obtained using this reference material.(Kiekens *et al*. 2015; Chapter V) Table III.2 lists the consensus concentrations for the reference materials (serum and whole blood) for the different analytical techniques, where available.

			MA	PBA	LC-MS/MS			
	matrix	level	total folate [nM]	total folate [nM]	FA [nM]	5MeTHF [nM]	5FoTHF [nM]	sum [nM]
NIST SRM 1955	serum	I	5.6±1.2	4.5±0.4	0.49±0.17	4.26±0.25	1.3±0.4	6.0±0.4
		II	14±3	10±1	1.05±0.16	9.73±0.24	2.3±0.8	13±1
		III	44±11	25±3	1.07±0.24	37.1±1.4	3.6±1.3	41±2
NIBSC 03/178	serum		12.6±3.3	8.75-19.2[a]	0.74±0.47	9.75±1.1	1.59±0.13	12.1±3
NIBSC 95/528	whole blood		9.45-19.33[b]	6.75-16.68[b]	n.a.	n.a.	n.a.	n.a.

Table III.2: Consensus concentrations for NIST and NIBSC reference materials. Values obtained from Thorpe *et al.* a: for serum (Thorpe *et al.* 2007) and b: for whole blood (Thorpe *et al.* 2004); MA: microbiologic assay; PBA: protein-binding assay; n.a.: not available.

III.3. Conclusion

Despite the knowledge obtained following decades of research investigating the dietary sources and metabolism of folate, the analytical determination of these essential vitamins in clinical samples remains difficult. Due to the relative instability of folates in solution, care must be given during sample preparation to avoid conversions between folate species and their degradation. While there is increasing evidence that sample preparation factors may strongly impact analytical results, it remains difficult to find an optimal sample preparation procedure that allows the accurate measurement of folates in the original sample.

Given the problematic storage stability of EDTA-anticoagulated samples, heparin or citrate may be preferred over EDTA to limit the degradation and interconversion during sample storage. While whole blood lysis using a diluted AA solution is commonly performed, small variations in the procedure can significantly influence the analytical outcome. The use of a thiol in combination with AA has proven to be required for the stabilization of THF in food samples. This approach may prove beneficial to stabilize this folate species in clinical samples as well. Higher order methods consisting of SPE followed by LC-MS/MS analysis are being implemented in some clinical labs to allow for a detailed evaluation of the folate status of an individual. However, given the problematic analytical calibration, a harmonization of folate calibrator preparation is recommended to compare results obtained using different procedures. In this respect, the use of certified reference materials, analyzed using a variety of methods, including MA, PBA and, importantly, also LC-MS/MS assays, would prove beneficial to compare analytical results.

III.4. References

Abbott Ireland. 2010. "Architect System - Folate." http://www.ilexmedical.com/files/PDF/Folate_ARC.pdf. (consulted: 02/21/2016)

Akhtar, M., Jamil, M., Ataullah, K., and Iqbal, A. 1999. "Photodegradation of Folic Acid in Aqueous Solution." *J Pharmaceut Biomed* 19 (3–4): 269–75.

Archibald, E.L., Mincey, E.K., and Morrison, R.T. 1972. "Estimation of Serum Folate Levels by Competitive Protein Binding Assay." *Clin Biochem* 5 (4): 232–41.

Bagley, P.J. and Selhub, J. 2000. "Analysis of Folate Form Distribution by Affinity Followed by Reversed- Phase Chromatography with Electrical Detection." *Clin Chem* 46 (3): 404–11.

Bailey, L.B. and Gregory, J.F.III 1999. "Folate Metabolism and Requirements." *J Nutr* 129 (4): 779–82.

Beggs, M.J., Sohn, L.J., Herrmann, R.J., Hsu, S., Hawksworth, D.J. and Pinkus, M.S. 1995. "Folate Immunoassay Utilizing Folate Binding Protein in a Multiclonal Antibody Format." Google Patents. http://www.google.com/patents/US5434087. (consulted: 03/11/2016)

Blakley, R.L. 1969. *The Biochemistry of Folic Acid and Related Pteridines*. North-Holland Publishing Company.

Camara, J.E., Pritchett, J.S., Daniels, Y.C., Phinney, K.W., Sander, L.C., and Lowenthal, M.S. 2015. "Value Assignment of Candidate Standard Reference Material® 3949 Folate Vitamers in Frozen Human Serum by Isotope-Dilution LC-MS/MS." Edited by The Association for Mass Spectrometry: Applications to the Clinical Lab. *MSACL 2015 US*. San Diego, CA.

Dakshinamurti, K. 1994. *Vitamin Receptors: Vitamins as Ligands in Cell Communication - Metabolic Indicators*. Cambridge University Press, Cambridge, UK.

de Benoist, B. 2008. "Conclusions of a WHO Technical Consultation on Folate and Vitamin B12 Deficiencies." *Food Nutr Bull* 29 (2 Suppl): S238–44.

De Brouwer, V., Zhang, G.F., Storozhenko, S., Van Der Straeten, D. and Lambert, W.E. 2007. "pH Stability of Individual Folates during Critical Sample Preparation Steps in Prevision of the Analysis of Plant Folates." *Phytochem Anal* 18 (6): 496–508.

De Brouwer, V., Storozhenko, S., Van De Steene, J.C., Wille, S.M.R, Stove, C.P., Van Der Straeten, D. and Lambert, W.E. 2008. "Optimisation and Validation of a Liquid Chromatography–tandem Mass Spectrometry Method for Folates in Rice." *J Chromatogr A* 1215 (1–2): 125–32.

DeVos, L., Chanson, A., Liu, Z., Ciappio, E.D., Parnell, L.D., Mason, J.B., Tucker, K.L. and Crott, J.W. 2008. "Associations between Single Nucleotide Polymorphisms in Folate Uptake and Metabolizing Genes with Blood Folate, Homocysteine, and DNA Uracil Concentrations." *Am J Clin Nutr* 88 (4): 1149–58.

Diagnostic Automation/ Cortez Diagnostics Inc. 2014. "AccuDiagTM Folate ELISA Kit." http://www.rapidtest.com/pdf/Folate_3127-15%2808-21-2014%29.pdf. (consulted: 02/21/2016)

Fazili, Z. and Pfeiffer, C.M. 2004. "Measurement of Folates in Serum and Conventionally Prepared Whole Blood Lysates: Application of an Automated 96-Well Plate Isotope-Dilution Tandem Mass Spectrometry Method." *Clin Chem* 50 (12): 2378–81.

Fazili, Z. and Pfeiffer, C.M. 2013b. "Accounting for an Isobaric Interference Allows Correct Determination of Folate Vitamers in Serum by Isotope Dilution-Liquid Chromatography-Tandem MS." *J Nutr* 143 (1): 108–13.

Fazili, Z., Pfeiffer, C.M. and Zhang, M. 2007. "Comparison of Serum Folate Species Analyzed by LC-MS/MS with Total Folate Measured by Microbiologic Assay and Bio-Rad Radioassay." *Clin Chem* 53 (4): 781–4.

Fazili, Z., Pfeiffer, C.M., Zhang, M., Jain, R.B. and Koontz, D. 2008. "Influence of 5,10-Methylenetetrahydrofolate Reductase Polymorphism on Whole-Blood Folate Concentrations Measured by LC-MS/MS, Microbiologic Assay, and Bio-Rad Radioassay." *Clin Chem* 54 (1): 197–201.

Fazili, Z., Whitehead, R.D.Jr., Paladugula, N., and Pfeiffer, C.M. 2013a. "A High-Throughput LC-MS/MS Method Suitable for Population Biomonitoring Measures Five Serum Folate Vitamers and One Oxidation Product." *Anal Bioanal Chem* 405 (13): 4549–60.

Franco, R.F., Araujo, A.G., Guerreiro, J.F., Elion, J. and Zago, M.A. 1998. "Analysis of the 677 C--> T Mutation of the Methylenetetrahydrofolate Reductase Gene in Different Ethnic Groups." *Thromb Haemostasis* 79 (1): 119–21.

Freisleben, A., Schieberle, P. and Rychlik, M. 2002. "Syntheses of Labeled Vitamers of Folic Acid to Be Used as Internal Standards in Stable Isotope Dilution Assays." *J Agric Food Chem* 50 (17): 4760–8.

Freisleben A., Schieberle P. and Rychlik M. 2003. "Specific and Sensitive Quantification of Folate Vitamers in Foods by Stable Isotope Dilution Assays Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry." *Anal Bioanal Chem* 376 (2): 149–56.

Galivan, J., Ryan, T.J., Chave, K., Rhee, M., Yao, R. and Yin, D. 2000. "Glutamyl Hydrolase: Pharmacological Role and Enzymatic Characterization." *Pharmacol Therapeut* 85 (3): 207–15.

Garbis, S.D., Melse-Boonstra, A., West, C.E. and van Breemen, R.B. 2001. "Determination of Folates in Human Plasma Using Hydrophilic Interaction Chromatography-Tandem Mass Spectrometry." *Anal Chem* 73 (22): 5358–64.

Hannisdal, R., Ueland, P.M., Sven Jens Eussen, S.J., An Svardal, A. and Hustad, S. 2009. "Analytical Recovery of Folate Degradation Products Formed in Human Serum and Plasma at Room Temperature." *J Nutr* 139 (7): 1415–8.

Hannisdal, R., Ueland, P.M. and Svardal, A.. 2009. "Liquid Chromatography-Tandem Mass Spectrometry Analysis of Folate and Folate Catabolites in Human Serum." *Clin Chem* 55 (6): 1147–54.

Hart, D.J., Finglas, P.M., Wolfe, C.A., Mellon, F., Wright, A.J.A. and Southon, S. 2002. "Determination of 5-Methyltetrahydrofolate (13C-Labeled and Unlabeled) in Human Plasma and Urine by Combined Liquid Chromatography Mass Spectrometry." *Anal Biochem* 305 (2): 206–13.

Hoffbrand, A.V., Newcombe, F.A. and Mollin, D.L. 1966. "Method of Assay of Red Cell Folate Activity and the Value of the Assay as a Test for Folate Deficiency." *J Clin Pathol* 19 (1): 17–28.

Huang, Y., Khartulyari, S., Morales, M.E., Stanislawska-Sachadyn, A., Von Feldt, J.M., Whitehead, A.S. and Blair, I.A . 2008. "Quantification of Key Red Blood Cell Folates from Subjects with Defined MTHFR 677C>T Genotypes Using Stable Isotope Dilution Liquid Chromatography/mass Spectrometry." *Rapid Commun Mass Spectrom* 22 (16): 2403–12.

Kamen, B.A. and Caston J.D. 1986. "Properties of a Folate Binding Protein (FBP) Isolated from Porcine Kidney." *Biochem Pharmacol* 35 (14): 2323–9.

Karayannis, M.I., Samios, D.N. and Gousetis, C.H.P. 1977. "A Study of the Molar Absorptivity of Ascorbic Acid at Different Wavelengths and pH Values." *Anal Chim Acta* 93: 275–9.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015. "A Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Selective Analysis of Free and Total Folate in Plasma and Red Blood Cells." *J Chromatogr A* 1398: 20–8.

Kirsch, S.H, Knapp, J.P., Herrmann, W. and Obeid, R. 2010. "Quantification of Key Folate Forms in Serum Using Stable-Isotope Dilution Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry." *J Chromatogr B* 878 (1): 68–75.

Kirsch, S.H., Herrmann, W., Geisel, J. and Obeid, R. 2012. "Assay of Whole Blood (6S)-5-CH3-H4folate Using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry." *Anal Bioanal Chem* 404 (3): 895–902.

Kok, R.M., Smith, D.E., Dainty, J.R., Van Den Akker, J.T., Finglas, P.M., Smulders, Y.M., Jakobs, C., De Meer, K. 2004. "5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism." *Anal Biochem* 326 (2):129-38.

Konings, E.J., Troost, F.J., Castenmiller, J.J., Roomans, H.H., Van Den Brandt, P.A. and Saris, W.H. 2002. "Intestinal Absorption of Different Types of Folate in Healthy Subjects with an Ileostomy." *Br J Nutr* 88 (3): 235–42.

Kopp, M. and Rychlik, M. 2015. "Quantitation of 5-Methyltetrahydrofolic Acid in Dried Blood Spots and Dried Plasma Spots by Stable Isotope Dilution Assays." *PloS One* 10 (11): e0143639.

Liu, K., Dai, X., Zhong, D., Deng, P., Ma, J., Chen, X. 2009. "Simultaneous determination of 6R-leucovorin, 6S-leucovorin and 5-methyltetrahydrofolate in human plasma using solid phase extraction and chiral liquid chromatography-tandem mass spectrometry." *J Chrom B* 877(10):902-10

Mönch, S., Netzel, M. Netzel, G. and Rychlik, M. 2010. "Quantitation of Folates and Their Catabolites in Blood Plasma, Erythrocytes, and Urine by Stable Isotope Dilution Assays." *Anal Biochem* 398 (2): 150–60.

Monobind Inc. 2015a. "VAST CLIA Microwells Folate/Vitamin B12." https://system.netsuite.com/core/media/media.nl?id=579735&c=445858&h=6278ee8823cc8e196510&_xt=.pdf&ck=UfNFrcwCAs0Uon0M&vid=UfNFrcwCAtEUoirZ&cktime=131788&addrcountry=US. (consulted: 02/21/2016)

Monobind Inc. 2015b. "VAST ELISA Microwells Folate/Vitamin B12." https://system.netsuite.com/core/media/media.nl?id=443113&c=445858&h=e31908ec17f31fa16bf8&_xt=.pdf&ck=UfNFrcwCAs0Uon0M&vid=UfNFrcwCAtEUoirZ&cktime=131788&addrcountry=US. (consulted: 02/21/2016)

Ndaw, S., Bergaentzle, M.,Aoude-Werner, D.,Lahely, S. and Hasselmann, C. 2001. "Determination of Folates in Foods by High-Performance Liquid Chromatography with Fluorescence Detection after Precolumn Conversion to 5-Methyltetrahydrofolates." *J Chromatogr A* 928 (1): 77–90.

Nelson, B.C., Pfeiffer, C.M., Margolis, S.A. and Nelson, C.P. 2004. "Solid-Phase Extraction-Electrospray Ionization Mass Spectrometry for the Quantification of Folate in Human Plasma or Serum." Anal Biochem 325 (1): 41–51.

Nygren-Babol, L., Jägerstad, M. 2012. "Folate-binding protein in milk: a review of biochemistry, physiology, and analytical methods." *Crit Rev Food Sci Nutr* 52 (5):410-25.

O'Broin, J.D., Scott, J.M. and Temperley, I.J. 1973. "A Comparison of Serum Folate Estimations Using Two Different Methods." *J Clin Pathol* 26 (1): 80–1.

O'Broin, J.D., Temperley, I.J. and Scott, J.M. 1980. "Erythrocyte, Plasma, and Serum Folate: Specimen Stability before Microbiological Assay." *Clin Chem* 26 (3): 522–4.

O'Broin, S.D. and Gunter, E.W. 1999. "Screening of Folate Status with Use of Dried Blood Spots on Filter Paper." *Am J Clin Nutr* 70 (3): 359–67.

O'Broin, S., and Kelleher, B. 2001. "Optimization of Erythrocyte Folate Extraction." *Clin Chem* 47 (12): 2181–2.

Ocké, M.C., Schrijver,J., Obermann-De Boer, G.L., Bloemberg, B.P.M., Haenen, G.R.M.M. and Kromhout, D. 1995. "Stability of Blood (pro) Vitamins during Four Years of Storage At− 20 C: Consequences for Epidemiologic Research." *J Clin Epidemiol* 48 (8): 1077–85.

Owen, W.E. and Roberts, W.L. 2003. "Comparison of Five Automated Serum and Whole Blood Folate Assays." *Am J Clin Pathol* 120 (1): 121–6.

Owens, J.E., Holstege, D.M., and Clifford, A.J. 2007. "High-Throughput Method for the Quantitation of Total Folate in Whole Blood Using LC-MS/MS." *J Agric Food Chem* 55 (9): 3292–7.

Patring, J.D.M., Johansson, M.S., Yazynina, E. and Jastrebova, J.A. 2005. "Evaluation of Impact of Different Antioxidants on Stability of Dietary Folates during Food Sample Preparation and Storage of Extracts prior to Analysis." *Anal Chim Acta* 553 (1): 36–42.

Patring, J.D., Lanina, S.A. and Jastrebova, J.A. 2006. "Applicability of Alkyl-Bonded Ultra-Pure Silica Stationary Phases for Gradient Reversed-Phase HPLC of Folates with Conventional and Volatile Buffers under Highly Aqueous Conditions." *J Sep Sci* 29 (6): 889–904.

Patring, J.D. and Jastrebova, J.A. 2007. "Application of Liquid Chromatography-Electrospray Ionisation Mass Spectrometry for Determination of Dietary Folates: Effects of Buffer Nature and Mobile Phase Composition on Sensitivity and Selectivity." *J Chromatogr A* 1143 (1-2): 72–82.

Pfeiffer, C.M, Fazili, Z., McCoy, L., Zhang, M. and Gunter, E.W. 2004. "Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay." *Clin Chem* 50 (2): 423–32.

Pfeiffer, C.M., Fazili, Z., and Zhang, M. 2010. "Folate Analytical Methodology." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 517–574. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Pfeiffer, C.M., Zhang, M., Lacher, D.A., Molloy, A.M., Tamura, T., Yetley, E.A., Picciano, M.F. and Johnson, C.L. 2011. "Comparison of Serum and Red Blood Cell Folate Microbiologic Assays for National Population Surveys." *J Nutr* 141 (7): 1402–9.

Pfeiffer, C.M., Sternberg, M.R., Fazili, Z., Lacher, D.A., Zhang, M., Johnson, C.L., Hamner, H.C. *et al*. 2015. "Folate Status and Concentrations of Serum Folate Forms in the US Population: National Health and Nutrition Examination Survey 2011–2." *Br J Nutr* 113 (12): 1965–77.

Roche Diagnostics International Ltd. 2014. "Elecsys® Folate III." http://www.roche-diagnostics.ch/content/dam/corporate/roche-dia_ch/documents/broschueren/professional_diagnostics/serumarbeitsplatz/immunologie/anamie/EN_Anemia_Factsheet_Folate.pdf. (consulted: 02/21/2016)

Rychlik, M., Netzel, M., Pfannebecker, I., Frank, T., Bitsch, I. 2003. "Application of stable isotope dilution assays based on liquid chromatography-tandem mass spectrometry for the assessment of folate bioavailability." *J Chromatogr B* 792 (2):167-76.

Santhoshkumar, C.R., Deutsch, J.C., Hassell, K.L., Kolhouse, N.M. and Kolhouse, J.F. 1995. "Quantitation of Red Blood Cell Folates by Stable Isotope Dilution Gas Chromatography-Mass Spectrometry Utilizing a Folate Internal Standard." *Anal Biochem* 225 (1): 1–9.

Satterfield, M.B., Sniegoski, L.T., Sharpless, K.E., Welch, M.J., Hornikova, A., Zhang, N.-F. Pfeiffer, C.M., Fazili, Z., Zhang, M. and Nelson, B.C. 2006. "Development of a New Standard Reference Material: SRM 1955 (homocysteine and Folate in Human Serum)." *Anal Bioanal Chem* 385 (3): 612–22.

Selhub, J., Jacques, P.F., Dallal, G., Choumenkovitch, S. and Rogers, G. 2008. "The Use of Blood Concentrations of Vitamins and Their Respective Functional Indicators to Define Folate and Vitamin B12 Status." *Food Nutr Bull* 29 (2 Suppl): S67–73.

Shane, B. 2010. "Folate Chemistry and Metabolism." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 1–24. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Shane, B. 2011. "Folate Status Assessment History: Implications for Measurement of Biomarkers in NHANES." *Am J Clin Nutr* 94 (1): 337S – 342S.

Smith, D. E., Kok, R.M., Teerlink, T., Jakobs, C. and Smulders, Y.M. 2006. "Quantitative Determination of Erythrocyte Folate Vitamer Distribution by Liquid Chromatography-Tandem Mass Spectrometry." *Clin Chem Lab Med* 44 (4): 450–9.

Stokvis, E., Rosing, H. and Beijnen, J.H. 2005. "Stable Isotopically Labeled Internal Standards in Quantitative Bioanalysis Using Liquid Chromatography/mass Spectrometry: Necessity or Not?" *Rapid Commun Mass Spectrom* 19 (3): 401–7.

Strandler, H.S., Patring, J., Jagerstad, M., and Jastrebova, J. 2015. "Challenges in the Determination of Unsubstituted Food Folates: Impact of Stabilities and Conversions on Analytical Results." *J Agric Food Chem* 63 (9): 2367–77.

Thorpe, S.J., Sands, D., Heath, A.B. Hamilton, M.S., Blackmore, S. and Barrowcliffe, T. 2004. "An International Standard for Whole Blood Folate: Evaluation of a Lyophilised Haemolysate in an International Collaborative Study." *Clin Chem Lab Med* 42 (5): 533–9.

Thorpe, S.J., Heath, A., Blackmore, S., Lee, A. Hamilton, M., O'broin, S., Nelson, B.C. and Pfeiffer, C.M. 2007. "International Standard for Serum Vitamin B(12) and Serum Folate: International Collaborative Study to Evaluate a Batch of Lyophilised Serum for B(12) and Folate Content." Clin Chem Lab Med 45 (3): 380–6.

Vahteristo, L.T., Ollilainen, V., Koivistoinen, P.E. and Varo, P. 1996. "Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography." *J Agric Food Chem* 44 (2). 477–82.

van Haandel, L., Becker, M.L., Williams, T.D., Stobaugh, J.F. and Leeder, J.S. 2012. "Comprehensive Quantitative Measurement of Folate Polyglutamates in Human Erythrocytes by Ion Pairing Ultra-Performance Liquid Chromatography/tandem Mass Spectrometry." *Rapid Commun Mass Spectrom* 26 (14): 1617–30.

Velghe, S., Capiau, S. and Stove, C.P. 2016. "Opening the Toolbox of Alternative Sampling Strategies in Clinical Routine: A Key-Role for (LC-)MS/MS." *Trends Anal Chem* (accepted for publication) doi:10.1016/j.trac.2016.01.030

Wang, C., Riedl, K.M., and Schwartz, S.J. 2010. "A Liquid Chromatography-Tandem Mass Spectrometric Method for Quantitative Determination of Native 5-Methyltetrahydrofolate and Its Polyglutamyl Derivatives in Raw Vegetables." *J Chromatogr B* 878 (29): 2949–58.

Yetley, E.A., Pfeiffer, C.M., Phinney, K.W., Fazili, Z., Lacher, D.A. Bailey, R.L., Blackmore, S., *et al*. 2011. "Biomarkers of Folate Status in NHANES: A Roundtable Summary." *Am J Clin Nutr* 94 (1): 303S – 312S.

Zheng, X.H., Jiang, L.Y., Zhao, L.T., Zhang, Q.Y., Ding, L. 2015. "Simultaneous quantitation of folic acid and 5-methyltetrahydrofolic acid in human plasma by HPLC–MS/MS and its application to a pharmacokinetic study" *J Pharm Anal* 5 (4):269-75.

Zimmerman, R.K., Slater, M.E., Langer, E.K., Ross, J.A. and Spector, L.G. 2013. "Long-Term Stability of Folate in Dried Blood Spots Stored in Several Conditions." *J Pediatr* 163 (2): 596–97.

Chapter IV: Folate determination in rodent diets

Based on:

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Author contributions

FK, CPS and WEL designed the research; FK conducted the research; DB and DVS provided essential materials; FK analyzed the data and conducted statistical analysis; JVD reviewed the data; FK and CPS wrote the paper, which was revised by DVDS, WEL, JVD, and DB; FK and CPS are responsible for the final content.

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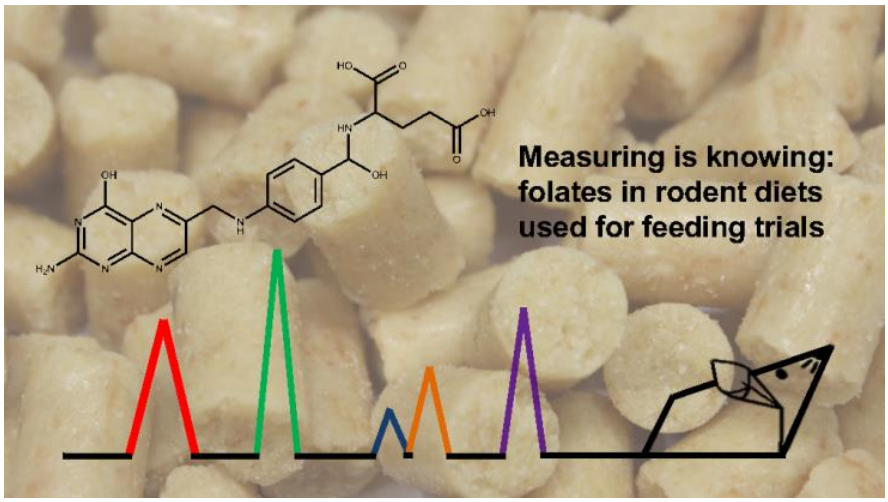
IV.1. Abstract

A method for the quantitative determination of folates in rodent diets is very important for correct interpretation of folate intake during feeding trials, given the possible discrepancy between the actual folate concentration in the diet and that mentioned on the product sheet. This discrepancy may be accounted for by e.g. inaccurate folic acid (FA) supplementation and/or the presence of endogenous reduced and substituted folates. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) is the method of choice to differentiate and quantify the individual folate species present. We developed a method, validated based on Food and Drug Administration (FDA) guidelines, that allows the measurement of added and endogenous folates by quantitative determination of 5 folate monoglutamates with linear ranges from 8 µg to 2 mg/kg feed. This information, combined with feed intake data, allows insight into the actual folate intake in animal feeding studies. The relevance of this method was illustrated by the analysis of several feed samples of varying composition, by the investigation of the effect of casein incorporation and by evaluating the variability of the folate content between pellets and production batches.



IV.2. Introduction

Deficiencies of folates have been related to several health disorders such as neural tube defects (NTDs) (Zeisel 2009), neural damage (Crider *et al*. 2012) and an increased cancer risk.(Laird and Jaenisch 1996) Animal studies investigating the relation between folate intake and health disorders, e.g. studies in which mice receive a folate depleted diet to investigate the prevalence of NTDs (Heid *et al*. 1992), typically use an amino acid based diet, supplemented with various amounts of FA. However, due to a variety of factors, such as a limited chemical stability, the presence of endogenous folates in feed ingredients and analytical inaccuracies , the actual folate concentration may differ from the concentration mentioned on the product sheet. Also, folate concentrations may vary as a result of differences in production and storage conditions or feed formulation. Therefore, it is key to quantify the exact amount of folates ingested by the animals as determined in the diet at time of consumption.

Rodent diets used for animal studies can generally be subdivided into three categories. Firstly, unrefined diets are composed of primary ingredients obtained from natural sources. Second, and most used, are purified diets in which the ingredients are purified or consist of refined forms of natural sugars, proteins and oils and purified micronutrients. A third class of diets is composed of chemically pure ingredients such as peptides and reagent grade micronutrients.(Reeves 1997) Though for open diets the exact formulation is known, i.e. disclosed by the manufacturer, this is not the case for a closed formula diet. However, its basic components remain the same and will include some form of proteins, peptides, starches, sugars, fibers, oils and fats. These ingredients are ground when not in powdered form, homogenized and pressed into pellets.

Though FA is generally added to the rodent diet as a source of folate, the addition of natural food constituents such as plant starches and animal or plant based protein can result in the presence of endogenous folates.(Reeves *et al*. 1993) The presence of these non-FA folates may impede a correct interpretation of short term feeding trials due to a difference in absorption characteristics, as observed by Castorena-Torres *et al*.(Castorena-Torres *et al*. 2014) We recently demonstrated that during long term studies, it is essential to precisely determine the folate content to accurately interpret the clinical data resulting from a specific folate intake.(Kiekens *et al*. 2015; Chapter VI) Another factor possibly hindering correct interpretation of data from a feeding trial is the labile nature of folates. Due to oxidative and thermal degradation during production and transport, the folate content as specified by the manufacturer may deviate substantially from that actually consumed by the animals.(Scott *et al*. 2000)

Several methods have been developed for the analysis of other compounds in rodent diets, for instance for quantification of estrogenic isoflavones (Degen *et al*. 2002) or the vitamins A and E.(Rushing *et al*. 1991) To our knowledge, we are the first to report a method that not only allows the total folate content to be measured, but also enables folate speciation in rodent feed. However, publications describing analytical methods for the analysis of folates in food items are plentiful. One such method for food analysis, i.e. the analysis of folates in potato (Van Daele *et al*. 2014), features a tri-enzyme treatment followed by ultrafiltration and ultra-high pressure/performance LC-MS/MS (UPLC®-MS/MS) analysis. Though a tri-enzyme treatment is indispensable (Tamura *et al*. 1997), ultrafiltration is not adequate for feed analysis due to the presence of loosely bound minerals, triglycerides and proteins within the feed matrix. This can be solved by incorporation of a solid phase extraction (SPE) step to purify the raw samples, as was also used for specific food analyses.(Vahteristo *et al*. 1996; Doherty and Beecher 2003) Moreover, an optimized method should have a wide linear range to allow quantification of both high concentrations of FA, when added to the diet, and the endogenous folates, which are present at far lower concentrations.

IV.3. Objectives

When performing a bioavailability study, all sources of dietary folate must be taken into account to evaluate the observed results. Since rodent diets often incorporate natural ingredients such as casein, starches and cellulose, it is conceivable that the folate concentration in a diet is not zero even without added folate. To investigate the presence of folates in commercial rodent diets, an analytical method was developed.

IV.4. Materials and methods

4.1. Reagents and materials

Tetrahydrofolate (THF), 5-methyltetrahydrofolate (5MeTHF), 5,10-methenyltetrahydrofolate (5,10CH$^+$THF), 10-formylfolic acid (10FoFA), 5-formyltetrahydrofolate (5FoTHF) and FA were purchased from Schircks Laboratories (Jona, Switzerland). ^{13}C-labeled internal standards (ISs), with a labeling yield higher than 98%, were obtained from Merck Eprova (Glattbrugg, Switzerland). $^{13}C_5$-FA was used as internal standard for FA, 10FoFA and 5FoTHF, while $^{13}C_5$-THF, $^{13}C_5$-5MeTHF and $^{13}C_5$-5,10CH$^+$THF were used as ISs for their respective isotopologues. All calibrators and IS solutions were prepared in a final concentration of 100 µg/ml in a 50 mM sodium phosphate buffer, pH 7.4, containing 1% L-ascorbic acid (AA) and 0.5% DL-dithiothreitol (DTT) / methanol (MeOH) (50/50 v/v%). Deionized water (H_2O-MQ) was produced in house by means of a Synergy UV water-purification system from Millipore (Billerica, MA, USA). Acetonitrile (ACN) and MeOH of LC-MS quality were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, phosphoric acid and ammonium hydroxide were obtained from Merck & Cie. (Darmstadt, Germany). Formic acid, trisodium phosphate, AA and DTT, α-amylase (Type Ia,

700-1400 U/mg protein; E.C. 3.2.1.1) and protease type XIV (>3.5 U/mg; E.C. 3.4.24.31) were purchased from Sigma Aldrich (Diegem, Belgium). The protease powder was dissolved at a concentration of 5 mg/ml in deionized water. As a source of γ-glutamylhydrolase enzyme (GGH; E.C. 3.4.19.9), non-sterile, non-hemolyzed rat serum was obtained from Harlan Laboratories (Horst, The Netherlands), while lyophilized chicken pancreas powder was obtained from Pel-Freez Biologicals (Rogers, AR, USA). Both the protease solution and the rat serum were stirred on ice for 1 hour with 100 mg/ml of activated charcoal (Sigma-Aldrich) to remove endogenous folates.(De Brouwer *et al*. 2008) Following removal of the activated charcoal by centrifugation at 4500 g for 15 minutes at 4 °C, the solutions were filtered over a 0.45 µm syringe filter (CA-S 30/0.45, Whatman, GE Healthcare, Little Chalfont, UK), divided into aliquots (1.5 ml) and frozen at -20 °C. The chicken pancreas solution was prepared by dissolving 400 mg powder in 25 ml of a 0.2 M phosphate buffer (pH 4.6) to which a suspension of 0.5 g activated charcoal and 0.05 g dextran in 5 ml of H_2O-MQ were added. After a 30 minute incubation, centrifugation and filtration were performed as described for protease and rat serum, after which the pH was adjusted to 6.1 and the solution was aliquoted and frozen at -20 °C.(Pedersen 1988) SPE was performed using Bond Elut SAX tubes containing 500 mg sorbent in 3 ml tubes, purchased from Agilent (Palo Alto, CA, USA).

Five different feed samples were used in this study. TD.06691 is a closed formula chemically pure diet in which casein is replaced by single peptides to remove all endogenous folates. The TD.95247 FA deficient diet is a purified diet in which the casein has been extracted with ethanol while TD.94045 is an AIN-93G diet in which standard casein is used as a protein source, with FA added in a concentration of 2 mg/kg. The 7012 diet is an unrefined diet using primary ingredients without casein and 7 mg/kg added FA. To evaluate the influence of casein on the folate speciation in a rodent diet both standard casein and ethanol-extracted casein were tested. All these samples were obtained from Teklad Diets (Harlan Laboratories, Madison, WI, USA). As a standard unrefined diet using primary ingredients and casein, a rat & mice maintenance diet (Carfil Quality, Oud-Turnhout, Belgium) was used.

4.2. Calibrator and QC-matrix

The blank amino acid defined diet (TD.06691) was used to prepare both the calibrators and QC-samples. This diet also contains 1% succinylsulfathiazole which is used in many rodent feeding trials to inhibit the *de novo* synthesis of folates by intestinal bacteria. The concentration of calibrators, quality controls and validation samples can be found in Table IV.1.

4.3. Method optimization

To evaluate the optimal pH during deconjugation with rat serum (5.3 to 7.4), equal amounts of 5MeTHF di-, tri-, tetra- and hexaglutamate (Schircks Laboratories) were spiked to blank rodent feed at a total concentration of 500 µg 5MeTHF/kg while the pH was altered using an

8% phosphoric acid solution. The addition of stripped chicken pancreas extract, as a secondary source of GGH, or oxalic acid, to capture zinc ions which may inhibit deconjugation by rat serum deconjugase (Horne *et al*. 1981), was also evaluated. Finally, the amount and\or incubation time of α-amylase (10, 20 or 50 µl; 15 or 30 min), protease (100 or 200 µl; 30, 60 or 120 min) and rat serum (100, 150 or 200 µl; 120 min) were evaluated by duplicate analysis.

sample	THF [µg/kg]	FA [µg/kg]	5MeTHF [µg/kg]	5,10CH$^+$THF [µg/kg]	10FoFA [µg/kg]	5FoTHF [µg/kg]
zero	0	0	0	0	0	0
calibrator 1	7.980	7.840	8.041	8.161	7.840	8.197
calibrator 2	23.94	23.52	24.12	24.48	23.52	24.59
calibrator 3	59.85	58.80	60.31	61.21	58.80	61.48
calibrator 4	147.6	145.0	148.8	151.0	145.0	151.6
calibrator 5	498.8	490.0	502.6	510.0	490.0	512.3
calibrator 6	997.5	980.0	1005	1020	980.0	1025
calibrator 7	1995	1960	2010	2040	1960	2049
QC1	39.90	39.20	40.20	40.80	39.20	40.99
QC2	718.2	705.6	723.7	734.5	705.6	737.7
LLOQ	7.980	7.840	8.041	8.161	7.840	8.197
3*LLOQ	23.94	23.52	24.12	24.48	23.52	24.59
MID	199.5	196.0	201.0	204.0	196.0	204.9
0.75*ULOQ	1496	1470	1508	1530	1470	1537

Table IV.1: Concentration of calibrators, quality controls (QCs) and validation samples. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

4.4. Sample preparation

All materials used were light protected and all manipulations were performed under subdued light from a yellow 15 W bulb. A homogenous aliquot of 200 mg rodent diet was weighed in a 15 ml polypropylene centrifuge tube. To this, 5 ml of a phosphate solution (50 mM sodium phosphate, 1% L-AA, 0.5% DTT, pH 7.4) including internal standards (ISs) at 250 µg/kg was added. After thorough mixing, the tubes were placed in a boiling water bath for 10 minutes followed by chilling on ice to room temperature.

4.5. Tri-enzyme treatment

When at room temperature, 25 µl of the α-amylase solution was added to the suspension of rodent diet and phosphate solution, which was then incubated for 15 minutes at room temperature. A further incubation at 37 °C for 30 minutes was performed after addition of 100 µl of protease solution. After incubation, the sample was placed in a boiling water bath for 10 minutes, chilled on ice to room temperature and centrifuged for 15 minutes at 4500 g and 4 °C. While minimally disturbing the remainder of the rodent feed on the bottom and the fatty layer on top, 3.5 ml of supernatant was transferred to a new light protected centrifuge tube.

The pH was adjusted to 6±0.15 by adding phosphoric acid. The sample was incubated for 2 hours at 37°C after the addition of 200 µl of stripped rat serum. After deconjugation, the pH was adjusted to 7.4, using 5 N NaOH, after which the samples were again placed in a boiling water bath for 10 minutes and subsequently chilled on ice. Prior to centrifugation for 15 minutes at 4500 g and 4 °C, 3.25 ml of H_2O-MQ was added to reduce the ionic strength.

4.6. Sample cleanup

SPE was performed using Bond Elut SAX tubes containing 500 mg sorbent per 3 ml tube. The sorbent was activated with 3 ml of hexane followed by 3 ml of MeOH and conditioned with 4 x 2.5 ml of 1.22 mM sodium phosphate (pH 7.4). Next, 5.5 ml of the supernatant obtained after tri-enzyme treatment (cfr. 4.5) was loaded onto the sorbent. To remove interferences, the sorbent was washed twice with 1.5 ml of 25 mM sodium phosphate, 0.5% L-AA, 0.25% DTT and 10% MeOH. The folates were eluted in two steps of 1 ml 0.52M ammonium acetate, 1% AA, 0.5% DTT and 5% MeOH (pH 7.4).

4.7. LC-MS/MS analysis

Samples were analyzed using a Waters Acquity UPLC® system coupled to an ABSciex API 4000™ triple quadrupole mass spectrometer equipped with a TurboIonSpray® probe. The autosampler was equipped with a 10 µl sample loop and samples were kept at 4 °C prior to injection. After injection the needle was cleaned with 600 µl of 10/90 v/v% H_2O-MQ/ACN followed by 1200 µl of 90/10 v/v% H_2O-MQ/ACN. Chromatographic separation was achieved using a mobile phase composed of H_2O-MQ (A) and ACN (B), each containing 0.1% of formic acid, with a flow rate of 0.6 ml/min on a Waters HSS T3-column (150 mm x 2.1 mm; 1.8 µm, equipped with Vanguard pre-column) held at 60 °C. The composition of the mobile phase starts with 100% solvent A and was kept constant for 1 minute. Thereafter the amount of solvent B was increased linearly to 10% in 2 minutes and to 12% in 1 minute after which the column was cleaned for 1 minute with 95% solvent B and equilibrated to initial solvent composition for 3 minutes. This amounts to a total time of approximately 8.5 minutes between injections. Mass spectrometric detection was performed in scheduled multiple-reaction monitoring mode. Electrospray ionization was used to ionize the analytes with the ion spray voltage set at 2500 V and a temperature of 600 °C. Nitrogen was used as curtain gas, gas 1 and gas 2, with respective pressure settings at 25, 75 and 90 psig, and as collision gas at setting 11. The interface heater was switched on. In Figure IV.1 chromatograms of a blank diet, a blank diet spiked at LLOQ level and a standard rodent diet are shown. The signal obtained for 5MeTHF in the blank diet originates from incomplete removal of this folate species from stripped rat serum and is lower than 20% of the LLOQ. The transitions monitored per compound were the same as described before (Van Daele *et al*. 2014), specific transitions and settings can be found in Table IV.2.

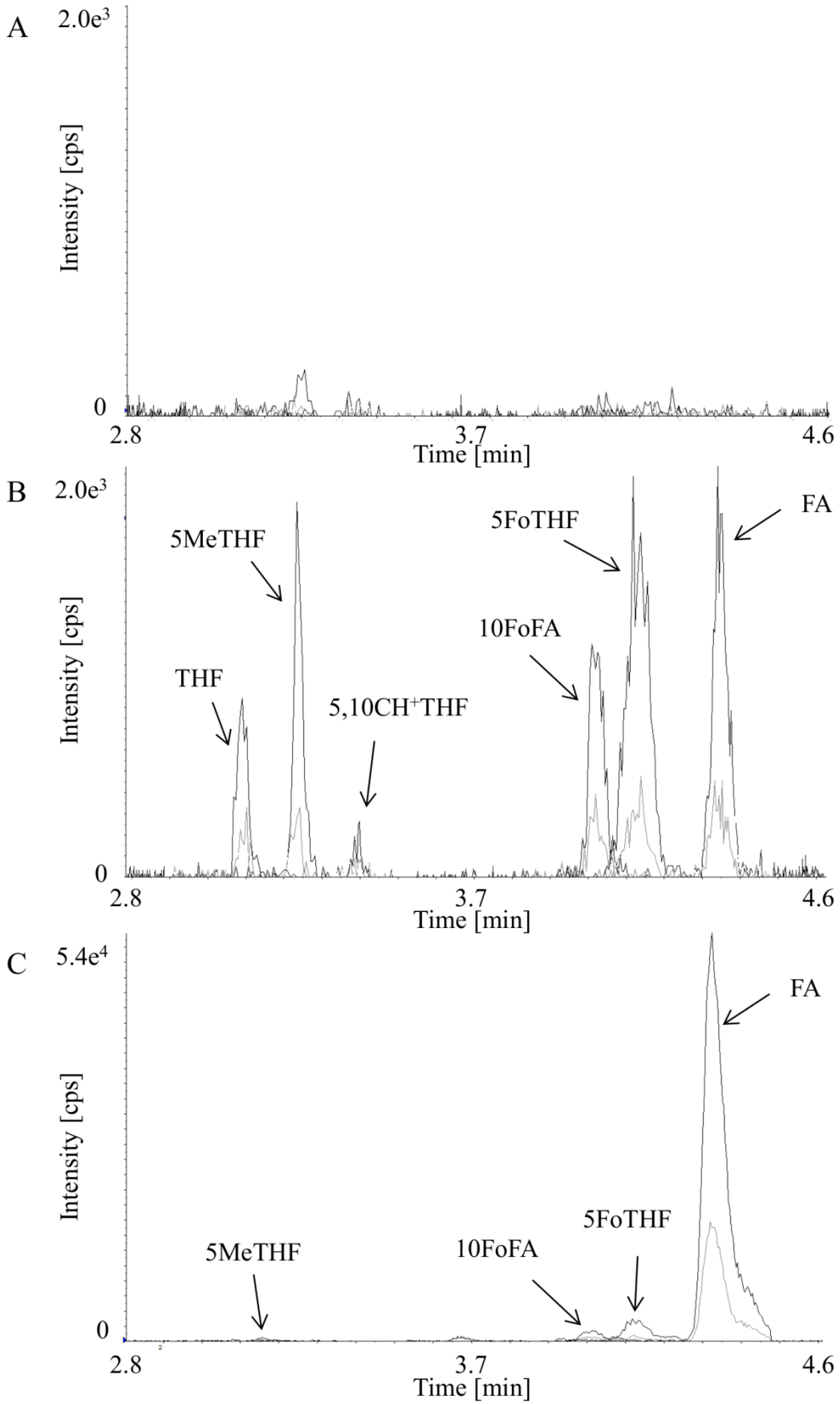


Figure IV.1: UHPLC-MS/MS chromatogram (A) of a "blank" feed sample, (B) a blank feed sample spiked at approximately 8 µg/kg, (C) a sample of a standard rodent diet (Carfil). Please note the difference in scale of the y-axis in panel C compared to panels A and B.

compound	Q1 ion [Da]	Q3 ion [Da]	DP [V]	EP [V]	CE [V]	CXP [V]
THF	446.2	**299.3**	59	4	26	13
		166.3		10	54	12
FA	442.1	**295.3**	54	9	24	22
		176.2		9	60	16
5MeTHF	460.4	**313.2**	91	10	27	8
		194.2		8	45	14
5,10CH$^+$THF	456.2	**412.1**	115	9	44	13
		282.1		8	63	16
10FoFA	470.1	**295.3**	76	2	32	16
		176.2		4	66	10
5FoTHF	474.2	**327.1**	46	6	27	18
		299.3		6	43	8
$^{13}C_5$-THF	451.1	299.3	64	6	27	16
$^{13}C_5$-FA	447.2	295.3	65	3	27	26
$^{13}C_5$-5MeTHF	465.3	313.2	66	6	27	13
$^{13}C_5$-5,10CH$^+$THF	461.0	416.2	79	8	45	9

Table IV.2: Mass spectrometric settings compound related MS parameters using an ABSciex API4000™ instrument. For 10FoFA and 5FoTHF the internal standard of FA is used. The transition used as quantifier is indicated in bold. DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential; V: volt.

4.8. Validation

The method was validated based on the FDA guidelines for bioanalytical method validation. Selectivity, carry-over, precision, accuracy, linearity, matrix effect, recovery and stability were evaluated.(U.S. Department of Health and Human Services 2001)

4.8.1. Selectivity and carry-over

Method selectivity was tested using blank rodent diet (TD.06691) obtained from Harlan. This is a purified diet without casein, which should therefore contain no endogenous folates. Cross-interferences were evaluated by adding 6 folates and 4 labelled ISs individually to this diet in a concentration of 2 mg/kg. For the analytes, the acceptance criterion for the selectivity assessment was set at 20% of the peak area corresponding to the LLOQ level, while for the labelled ISs only 5% was considered acceptable. Carry-over was evaluated by injecting blank samples after calibrators with identical concentrations and acceptance criteria as for the evaluation of selectivity.

4.8.2. Linearity, accuracy and precision

The actual LLOQ was verified as the concentration which could be measured with a %bias and relative standard deviation (%RSD) below 20%. Duplicate analysis of 1 zero sample and 7 calibrators was performed on 4 non-consecutive days to evaluate linearity. Homoscedasticity was evaluated by plotting the residuals versus the nominal values. The sum of the residual error was used to evaluate the need for a weighted or transformed model (1/x, $1/x^2$, 1/y, $1/y^2$, $\sqrt{x}$, ln(x), log(x)). For the selected model to be acceptable, back-calculated mean concentrations should be within 15% of the nominal value (20% at LLOQ-level). Accuracy and precision were evaluated using 4 spiked samples (LLOQ - 0.75*ULOQ, Table IV.1), prepared and analyzed in duplicate on 4 non-consecutive days. A single-factor ANOVA was used to calculate intra- and interbatch variability (%RSD). Accuracy (%bias) was calculated as the measured value divided by the nominal value. Acceptance criteria for %bias and %RSD are 15%, except at LLOQ, where they are 20%. Also, the dilution integrity of samples exceeding the ULOQ was verified by fivefold dilution of blank matrix spiked at a concentration of 8 mg/kg with sample buffer prior to solid phase extraction (n=2).

4.8.3. Recovery and matrix effects

Matrix effect and recovery were evaluated at 3 concentration levels (3*LLOQ – 0.75*ULOQ, Table IV.1) in blank matrix material TD.06691 according to Matuszewski *et al*.(Matuszewski *et al*. 2003) The analytes were spiked in duplicate either before (A) or after (B) the extraction and SPE procedure. Also, analytes were spiked in SPE elution buffer to obtain a sample free of matrix (C). Absolute recovery was calculated as the percent ratio of peak areas A over B, while absolute matrix effect was calculated as the percent ratio of the peak areas B over C. Relative recovery and matrix effect are expressed as %RSD of the absolute recovery and matrix effect values and should not exceed 15%.

4.8.4. Stability

To investigate the stability of folates in rodent diets, six folate monoglutamates were spiked to blank matrix at a 100 and 1 000 µg/kg level. Triplicate analysis of samples stored at 4 or -20 °C was performed weekly over a total period of 4 weeks. Autosampler stability, at 4 °C, was assessed by reinjection of samples stored for 24 hours in the autosampler while concentrations were calculated based on fresh calibrators. Also, the effect of up to three freeze-thaw cycles was evaluated by freezing and thawing the samples in cycles of 1 hour. Stability is acceptable if the concentration measured does not deviate more than 15% from that measured initially.

4.9. Application

The accuracy of the method was evaluated via the measurement of a certified reference material, namely: BCR 121 Wholemeal Flour issued by the Institute for Reference Materials and Measurements of the European Commission.(Unit for Reference Materials EC-JRC-IRMM 2007) Also, the folate content was evaluated in five different rodent diets with varying composition and expected folate content. To evaluate the reproducibility of the folate concentration between different feed batches and between different pellets within one batch, pellets of two different production batches were obtained of which 4 individual pellets were analyzed in duplicate per batch. Also, casein and ethanol extracted casein (Fonterra, Auckland, New Zealand), used as a protein source in many diets, were analyzed to evaluate the efficacy of the ethanol extraction. To correlate the results between feed analysis and casein treatment, casein samples consisted of only 40 mg instead of 200 mg for rodent diets since the rodent diets used in this study contain on average 20% protein.

IV.5. Results and discussion

5.1. Method optimization

Due to the application of LC-MS/MS analysis, the results are not influenced by preservatives and anti-microbial agents which may be present in rodent feed. This is a major improvement over microbiologic assays commonly used for food and feed analysis. Sample preparation encompasses classical tri-enzyme treatment with an amylase, protease and folate conjugase treatment to degrade starches and proteins and to hydrolyze folate polyglutamates, respectively. However, since the feed matrix may have an effect on the activity of the enzyme, the amount of enzyme and length of incubation were optimized.

5.2. Optimization of polyglutamate deconjugation

Though GGH is reported to have a pH optimum between 6.2 and 7.2 (Horne *et al*. 1981), deconjugation was found to be optimal at a pH of approximately 6.00 with an optimal range between 5.85 and 6.15 (see Figure IV.2). No compounds exerting an inhibitory effect could be inferred based on literature.(Galivan *et al*. 2000) Neither the addition of oxalate nor chicken pancreas extract improved polyglutamate deconjugation and was therefore not

included for further method optimization. The optimized enzyme amounts and incubation times were as described in section 4.5.

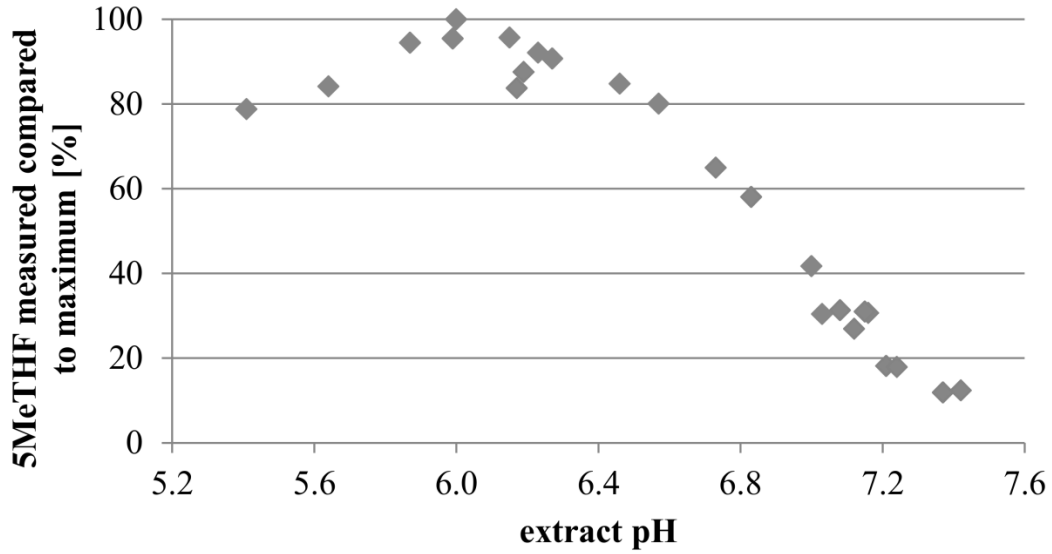


Figure IV.2: Influence of pH on the deconjugation efficiency using rat serum γ-glutamylhydrolase following spiking the di-, tri-, tetra- and hexaglutamate form of 5-methyltetrahydrofolate (5MeTHF) to a total concentration of 500 µg/kg.

5.2.1. Separation of oils and fats

The addition of water-immiscible organic solvents, such as hexane, ethyl acetate and 1-octanol, followed by vortexing and centrifugation, was evaluated to separate fats and oils from the feed extract prior to tri-enzyme treatment. Although this indeed led to a better separation after centrifugation, the layer formed at the interface stuck to the pipette tip and fragments were sucked into the aqueous extract upon transfer. Hence, this step did not improve the analytical results and was therefore omitted.

5.3. Solid phase extraction

Early during method development, reversed phase (C_{18} and Phenyl) and ion-exchange solid-phase extraction were compared. While equivalent folate recovery was obtained, the strong-anion exchange (SAX) packing gave less background noise and was therefore preferred.

5.4. Method validation

5.4.1. Selectivity and carry-over

No interferences or cross-talk was observed for any of the folates analyzed. However, 5,10CH$^+$THF was partly oxidized to 5FoTHF during initial experiments with a boiling step at pH 6.0. Therefore, it is necessary to adjust the pH to 7.4 prior to performing the boiling step after conjugase treatment. This prevents the oxidation of 5,10CH$^+$THF at elevated temperature, which was also observed by De Brouwer *et al*. and Jagerstad and Jastrebova.(De Brouwer *et al*. 2007; Jagerstad and Jastrebova 2013) Carry-over measured in a blank sample injected after the highest calibrator did not exceed 2.8% of the LLOQ, which is acceptable according to the FDA guideline.(U.S. Department of Health and Human Services 2001)

5.4.2. Linearity, accuracy and precision

Based on the evaluation of the sum residual error and homoscedasticity, 1/x was chosen as weighting factor for all folates. This transformation also led to the best intra- and interbatch precision and accuracy. The values for both precision (%RSD) and accuracy (%bias) can be found in Table IV.3 (raw data in Table IV.4) and did not exceed the 15 or 20% acceptance criteria. Also, the back-calculated concentrations of the calibrators fell within the acceptance criteria. The LLOQ and ULOQ for the different folates are 8 µg/kg and 2 mg/kg, respectively (Table IV.1).

folate	conc.	intrabatch precision [%RSD]	interbatch precision [%RSD]	accuracy [%bias]
THF	LLOQ	14.1	14.1	-0.8
	3*LLOQ	9.8	9.8	-1.9
	MID	3.9	3.9	-1.4
	0.75*ULOQ	4.1	4.1	-0.8
FA	LLOQ	7.4	8.0	-4.5
	3*LLOQ	2.4	4.6	0.8
	MID	2.5	2.5	1.0
	0.75*ULOQ	1.4	4.2	-2.9
5MeTHF	LLOQ	10.1	10.1	3.0
	3*LLOQ	6.5	10.7	-2.0
	MID	3.5	6.2	3.4
	0.75*ULOQ	4.3	4.3	2.3
5,10CH$^+$THF	LLOQ	16.1	18.7	2.2
	3*LLOQ	5.1	13.8	-3.5
	MID	10.5	10.5	8.1
	0.75*ULOQ	3.7	3.7	3.2
10FoFA	LLOQ	7.2	12.3	-5.0
	3*LLOQ	9.4	9.4	-3.0
	MID	5.2	5.2	-4.8
	0.75*ULOQ	3.5	6.6	-5.1
5FoTHF	LLOQ	9.0	9.0	5.5
	3*LLOQ	9.9	9.9	3.3
	MID	3.4	3.4	3.0
	0.75*ULOQ	1.7	3.0	0.2

Table IV.3: Accuracy and precision data at 4 concentration levels analyzed in twofold on 4 non-consecutive days. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

When the folate concentration measured exceeds the ULOQ, fivefold dilution with extraction buffer without internal standards prior to solid phase extraction allows to extend the measurable range up to 10 mg/kg feed. Duplicate analysis of a diluted sample originally spiked at a concentration of 8 mg/kg gave acceptable results, with a difference less than 10.5% compared with the nominal concentration. As such, it is possible to measure individual

folate species in a concentration range of 8 µg/kg to 10 mg/kg. This encompasses the normal range present in rodent feed and allows quantification of natural folates present in feed ingredients and of synthetic folate, generally FA, added to the diet.

folate [µg/kg]	level	replicate	nominal value	day			
				1	2	3	4
THF	LLOQ	a	7.980	7.740	7.496	8.597	8.861
		b	7.980	7.833	9.103	6.334	7.352
	3*LLOQ	a	23.94	24.90	23.56	25.17	26.35
		b	23.94	23.26	21.59	20.52	22.57
	MID	a	199.5	185.5	197.2	209.7	198.7
		b	199.5	199.5	197.0	195.1	191.5
	0.75*ULOQ	a	1496	1490	1524	1466	1511
		b	1496	1555	1390	1505	1433
FA	LLOQ	a	7.840	6.712	7.551	8.428	8.180
		b	7.840	7.367	7.775	7.006	7.970
	3*LLOQ	a	23.52	23.23	24.31	24.68	23.67
		b	23.52	24.40	24.50	25.48	22.88
	MID	a	196.0	202.7	204.5	200.6	201.7
		b	196.0	190.2	203.5	207.2	201.0
	0.75*ULOQ	a	1470	1432	1425	1503	1432
		b	1470	1403	1433	1526	1474
5MeTHF	LLOQ	a	8.041	7.650	7.565	8.545	7.094
		b	8.041	7.632	6.950	8.763	8.365
	3*LLOQ	a	24.12	27.30	26.51	25.14	25.14
		b	24.12	23.46	21.52	22.11	25.99
	MID	a	201.0	216.7	209.5	210.1	194.5
		b	201.0	221.7	194.3	200.7	203.1
	0.75*ULOQ	a	1508	1499	1585	1577	1519
		b	1508	1494	1463	1486	1627
5,10CH$^+$THF	LLOQ	a	8.161	5.070	7.642	8.067	9.196
		b	8.161	8.562	8.777	8.237	9.722
	3*LLOQ	a	24.48	19.90	26.41	21.98	22.74
		b	24.48	22.53	25.74	21.02	24.44
	MID	a	204.0	209.2	217.2	179.8	226.2
		b	204.0	234.6	222.9	233.0	202.1
	0.75*ULOQ	a	1530	1604	1549	1601	1511
		b	1530	1453	1504	1586	1545
10FoFA	LLOQ	a	7.840	7.605	6.999	9.181	7.568
		b	7.840	6.985	7.396	7.961	6.969
	3*LLOQ	a	23.52	22.50	23.57	22.71	25.19
		b	23.52	24.58	25.60	21.89	19.82
	MID	a	196.0	188.2	186.8	188.8	175.0
		b	196.0	205.2	186.9	192.4	196.7
	0.75*ULOQ	a	1470	1503	1390	1368	1437
		b	1470	1520	1461	1370	1315
5FoTHF	LLOQ	a	8.197	9.012	7.791	8.556	8.992
		b	8.197	7.622	8.628	9.062	7.685
	3*LLOQ	a	24.59	27.14	23.13	22.15	27.45
		b	24.59	24.96	27.61	22.67	22.70
	MID	a	204.9	210.2	200.2	213.3	194.7
		b	204.9	212.9	202.0	200.3	209.6
	0.75*ULOQ	a	1537	1532	1479	1471	1519
		b	1537	1550	1517	1461	1461

Table IV.4: Raw concentration data for the assessment of accuracy and precision. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

5.4.3. Recovery and matrix effects

Given the use of carbon-13 labelled ISs, the IS-corrected matrix effect remained limited (within 15%) for all folates at all three concentration levels. The relative matrix effect ranged from 0.2 to 11.3%. Given the limited recovery of 5,10CH$^+$THF without compensation by the IS (±4.7%), it is not possible to reliably obtain a quantitative result for this compound. For all other folates, non-IS corrected absolute recovery exceeded 30% while IS-corrected absolute recovery exceeded 88.0% and was reproducible as relative recovery only exceeded the acceptance criterion of 15% for 5MeTHF at the MID concentration level (15.9%). Detailed data can be found in Table IV.5 (raw peak area data: Table IV.6).

	conc.	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
absolute ME without IS	3*LLOQ	57.3	89.8	72.9	61.4	96.8	88.3
	MID	55.0	84.3	68.2	54.7	88.5	89.3
	0.75*ULOQ	54.3	86.5	60.8	50.2	85.6	82.5
absolute ME with IS	3*LLOQ	91.9	101.7	100.2	93.6	109.7	99.9
	MID	96.8	98.3	96.5	99.0	103.2	104.1
	0.75*ULOQ	101.5	106.9	88.8	93.3	105.8	101.9
relative ME without IS	3*LLOQ	1.7	1.7	3.4	1.3	7.4	8.2
	MID	6.7	4.3	3.9	3.0	0.7	1.1
	0.75*ULOQ	2.1	1.5	4.3	0.2	0.3	0.9
relative ME with IS	3*LLOQ	9.3	2.5	2.1	11.3	7.7	8.8
	MID	7.7	4.4	9.5	7.1	0.2	2.0
	0.75*ULOQ	2.4	4.5	4.5	4.1	6.7	5.0
absolute RE without IS	3*LLOQ	46.8	37.7	45.2	4.9	33.1	40.0
	MID	36.6	36.7	38.2	4.5	31.3	39.5
	0.75*ULOQ	44.2	37.7	48.2	4.7	35.6	49.6
absolute RE with IS	3*LLOQ	112.6	107.1	111.8	114.5	94.5	112.3
	MID	98.5	102.8	104.6	105.8	88.0	110.3
	0.75*ULOQ	98.0	97.5	102.8	118.6	92.2	128.5
relative RE without IS	3*LLOQ	11.9	1.2	6.0	0.0	8.2	5.4
	MID	8.6	2.0	9.8	0.2	3.2	6.9
	0.75*ULOQ	3.1	2.5	3.7	0.2	1.1	0.1
relative RE with IS	3*LLOQ	6.6	9.2	12.2	4.1	14.3	1.5
	MID	9.0	0.8	15.9	13.2	5.0	14.1
	0.75*ULOQ	0.1	0.6	7.4	18.8	9.5	9.5

Table IV.5: Results following the analysis of matrix effects and recovery. The maximal achievable recovery of 53.5% was not taken into account in the values of the absolute recovery without IS. LLOQ: lower limit of quantitation; ME, matrix effect; MID: intermediate concentration; RE, recovery; ULOQ: upper limit of quantitation.

Analyte								
type	level	replicate	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
spiked at the start	3*LLOQ	a	1.72E+03	4.58E+03	3.31E+03	6.08E+02	2.70E+03	5.22E+03
		b	2.32E+03	5.05E+03	4.36E+03	5.59E+02	3.53E+03	5.79E+03
	MID	a	1.15E+04	3.52E+04	2.41E+04	4.17E+03	2.25E+04	4.08E+04
		b	1.38E+04	3.96E+04	3.23E+04	4.02E+03	2.51E+04	5.14E+04
	0.75*ULOQ	a	1.23E+05	2.93E+05	2.51E+05	3.05E+04	1.90E+05	4.22E+05
		b	1.10E+05	2.72E+05	2.34E+05	2.73E+04	1.93E+05	4.46E+05
spiked at the end	3*LLOQ	a	4.48E+03	1.24E+04	8.09E+03	1.24E+04	9.86E+03	1.45E+04
		b	4.20E+03	1.31E+04	8.81E+03	1.14E+04	9.08E+03	1.32E+04
	MID	a	3.76E+04	9.99E+04	7.70E+04	9.65E+04	7.73E+04	1.18E+05
		b	3.23E+04	1.04E+05	7.16E+04	8.77E+04	7.48E+04	1.16E+05
	0.75*ULOQ	a	2.65E+05	7.42E+05	4.94E+05	6.26E+05	5.46E+05	8.52E+05
		b	2.62E+05	7.56E+05	5.14E+05	5.94E+05	5.31E+05	8.98E+05
without matrix	3*LLOQ	a	7.99E+03	1.40E+04	1.15E+04	2.05E+04	9.66E+03	1.54E+04
		b	7.18E+03	1.44E+04	1.17E+04	1.83E+04	9.92E+03	1.60E+04
	MID	a	6.30E+04	1.23E+05	1.08E+05	1.70E+05	8.78E+04	1.31E+05
		b	6.43E+04	1.19E+05	1.09E+05	1.67E+05	8.40E+04	1.31E+05
	0.75*ULOQ	a	5.02E+05	8.68E+05	8.55E+05	1.25E+06	6.36E+05	1.04E+06
		b	4.70E+05	8.63E+05	8.05E+05	1.18E+06	6.22E+05	1.08E+06
Internal Standard								
type	level	replicate	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
spiked at the start	3*LLOQ	a	3.21E+04	1.50E+04	5.95E+04	4.97E+03	1.50E+04	1.50E+04
		b	4.59E+04	1.85E+04	8.49E+04	4.31E+03	1.85E+04	1.85E+04
	MID	a	2.91E+04	1.51E+04	5.53E+04	4.28E+03	1.51E+04	1.51E+04
		b	3.38E+04	1.61E+04	6.39E+04	3.74E+03	1.61E+04	1.61E+04
	0.75*ULOQ	a	2.57E+04	1.09E+04	5.42E+04	2.72E+03	1.09E+04	1.09E+04
		b	2.36E+04	9.99E+03	4.51E+04	2.02E+03	9.99E+03	9.99E+03
spiked at the end	3*LLOQ	a	9.80E+04	4.62E+04	1.75E+05	1.13E+05	4.62E+04	4.62E+04
		b	8.97E+04	4.83E+04	1.77E+05	1.03E+05	4.83E+04	4.83E+04
	MID	a	8.76E+04	4.38E+04	1.65E+05	9.55E+04	4.38E+04	4.38E+04
		b	8.30E+04	4.37E+04	1.64E+05	9.39E+04	4.37E+04	4.37E+04
	0.75*ULOQ	a	5.42E+04	2.68E+04	1.04E+05	5.88E+04	2.68E+04	2.68E+04
		b	5.51E+04	2.72E+04	1.07E+05	5.79E+04	2.72E+04	2.72E+04
without matrix	3*LLOQ	a	1.49E+05	5.21E+04	2.45E+05	1.60E+05	5.21E+04	5.21E+04
		b	1.51E+05	5.49E+04	2.39E+05	1.68E+05	5.49E+04	5.49E+04
	MID	a	1.50E+05	5.13E+04	2.40E+05	1.75E+05	5.13E+04	5.13E+04
		b	1.51E+05	5.07E+04	2.25E+05	1.68E+05	5.07E+04	5.07E+04
	0.75*ULOQ	a	1.06E+05	3.45E+04	1.54E+05	1.13E+05	3.45E+04	3.45E+04
		b	9.87E+04	3.22E+04	1.54E+05	1.04E+05	3.22E+04	3.22E+04

Table IV.6: Raw peak area data for the assessment of matrix effect and recovery. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

5.4.4. Stability

Both storage conditions proposed by the manufacturer were tested, i.e. 4 and -20 °C. As can be seen in Table IV.7, samples stored at 4 °C were not adequately stable since especially THF was susceptible to degradation with on average 17% loss already after one week. Up to one week of storage at 4 °C was acceptable for the other folates. Stability at -20 °C was acceptable up to 2 weeks of storage according to the 15% acceptance criterion except for FA at the high concentration level which was 20% higher than at the start.

		weeks at 4°C					weeks at -20°C				
folate	conc.	0	1	2	3	4	0	1	2	3	4
THF	low	100	84.9	54.8	32.2	25.3	100	108	98.8	83.6	50.4
	high	100	81.5	78.1	52.1	33.6	100	92.8	96.6	55.3	43.9
FA	low	100	104	89.2	60.8	47.5	100	115	99.0	88.7	49.6
	high	100	100	100	77.2	46.8	100	108	120	75.2	53.9
5MeTHF	low	100	98.4	84.6	65.4	48.5	100	98.5	106	72.7	45.9
	high	100	97.8	108	82.6	50.4	100	90.9	114	91.5	47.5
5,10CH$^+$THF	low	100	112	82.6	79.8	57.0	100	85.1	98.4	77.1	55.5
	high	100	84.2	94.8	82.7	50.1	100	82.7	94.1	78.6	48.3
10FoFA	low	100	112	79.8	55.3	66.3	100	96.9	89.8	67.2	70.6
	high	100	95.8	79.4	73.4	69.9	100	90.3	87.0	70.3	59.6
5FoTHF	low	100	123	82.7	49.9	66.2	100	97.2	90.5	60.9	68.2
	high	100	96.2	80.7	66.8	71.8	100	97.1	94.1	67.1	63.6

Table IV.7: Results obtained during the evaluation of long term stability. Results are depicted as % recovery relative to the concentration measured at the 0 time point. Low concentration=0.1 mg/kg; high concentration=1.0 mg/kg.

The evaluation of the stability of samples in the autosampler of the UPLC® instrument revealed no problems during storage up to 24 hours (cfr. Table IV.8). Only for THF did freeze-thaw cycles affect the measured folate concentration. After 3 cycles, the degradation of THF exceeded the 15% acceptance criterion (cfr. Table IV.9).

deviation [%]	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
low concentration	-1.51	1.95	0.24	-9.39	-8.46	-10.7
high concentration	-4.95	0.36	-4.40	-1.77	-12.7	-9.63

Table IV.8: Autosampler stability expressed as the percentage difference between samples analyzed immediately after sample treatment and following 24 hours in the autosampler (n=3; average values are shown). Quantitation of the latter was done using a freshly prepared calibration curve.

% bias	# cycles	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
low concentration	1	7.2	-0.8	-0.3	-4.0	-0.8	3.5
	2	8.1	-1.6	-1.5	5.1	6.3	6.9
	3	-28.4	-1.9	-0.3	3.4	-1.0	3.3
high concentration	1	2.9	5.0	3.3	-5.8	3.7	5.1
	2	-4.4	-1.6	-3.5	-9.4	-0.2	0.8
	3	-18.8	-3.6	-3.1	-9.2	-16.9	-16.0

Table IV.9: Results obtained for 1 to 3 freeze-thaw cycles. Percent bias is expressed relative to the average value (n=3) obtained without any freeze-thaw cycle.

5.5. Application

The analysis of five feed varieties (in duplicate) clearly illustrated the relevance of a method for the analysis of folates in rodent diets. Both the TD.06691 and the TD.95247 diets are reported to be FA deficient. However, though in the former no folates could be quantified, the incorporation of purified casein in the latter leads to a total folate content of 45.6 µg/kg, with FA and 10FoFA present in quantifiable amounts (28.6 and 17.0 µg/kg respectively). According to the specification sheet, 7 mg of folate/kg is present in the 7012 diet (as added FA). This was confirmed by a measured total folate content of 6.91 mg total folate/kg. However, only 82.9% of this consists of FA (5.73 mg/kg), while also 5FoTHF (6.7%; 0.465 mg/kg), 5MeTHF (5.8%; 0.402 mg/kg), 10FoFA (4.0%; 0.279 mg/kg) and THF (0.6%; 41.5 µg/kg) are present. Though the product sheet of the TD.94045 diet reports the addition of 2 mg/kg FA, only 1.33 mg/kg FA (95.9% of the total amount of folates present) was measured, which is 31% lower than reported by the manufacturer. However, also trace amounts of 5FoTHF (2.2%; 31.0 µg/kg) and 10FoFA (1.4%; 20.0 µg/kg) were present in this diet, leading to a total folate content of 1.38 mg total folate/kg. Like the TD.94045 diet, 2.00 mg FA/kg is added to the rodent maintenance diet obtained from Carfil, according to the product sheet. However, a total folate concentration of 5.63 mg/kg was measured of which 4.98 mg/kg was FA (86.9%) while also 5FoTHF (7.1%; 0.404 mg/kg), 10FoFA (5.3%; 0.303 mg/kg) and 5MeTHF (0.8%; 43.9 µg/kg) were present. These results, including total folate concentration and speciation are represented graphically in Figure IV.3A. The variability of folate concentrations between different production batches was evaluated using the diet obtained from Carfil. For two batches, we performed duplicate analysis of four pellets (Figure IV.3B).

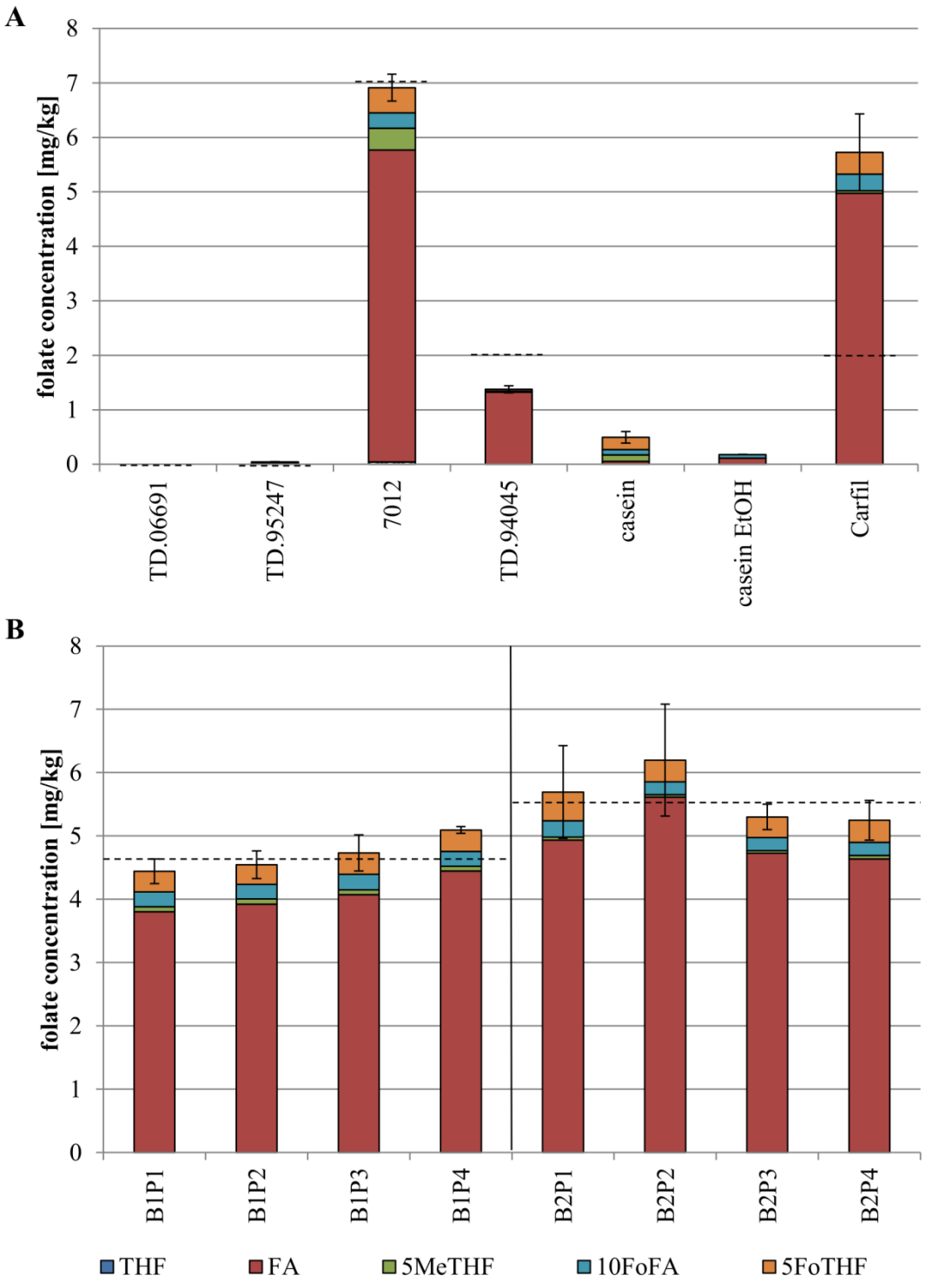


Figure IV.3: Measured folate species in various rodent diets, analyzed in duplo, including standard deviation on total folate content and a dotted line indicating the FA concentration mentioned by the manufacturer (A) and evaluation of homogeneity of the folate concentration of 2 production batches and 4 different pellets per batch indicated as B(#batch)P(#pellet). The average total folate concentration was indicated for each batch by a dotted line. Significant differences were observed for FA, 5MeTHF and total folate (B).

Though no appreciative difference was observed between pellets of each production batch, there was a significant difference between these batches for FA (p=0.002), 5MeTHF (p=0.000) and total folate (p=0.002), analyzed using an independent t-test at the 95% confidence level. An 18.9% difference in FA concentration was measured between both batches (4.98 and 4.04 mg/kg) while the 5MeTHF concentration differed by 41.2% (45.7 and 79.5 µg/kg, respectively), as can be seen in Figure IV.3B. As such, the use of pellets from different production batches during a long term feeding trial may exert an influence because of variable folate concentrations, especially since the concentrations of the distinct folates do not differ equally. These results again illustrate the importance of an analytical method capable of quantifying individual folate species.

When analyzing casein without the rest of the ingredients present, a total folate concentration of 494 µg/kg casein was found. Here, mainly 5FoTHF (45.5%; 225 µg/kg), 5MeTHF (24.3%; 120 µg/kg), 10FoFA (19.5%; 101 µg/kg) and FA (10.8%; 53.2 µg/kg) were present. While commonly performed to remove endogenous compounds, amongst which folates, ethanol extraction only succeeds partly in removing folates from casein, with a measured remaining total folate concentration of 179 µg/kg. While 5FoTHF was the major folate form present in untreated casein, only FA (63.4%; 114 µg/kg) and 10FoFA (36.6%; 65.5 µg/kg) could be quantified in ethanol extracted casein. As such, ethanol extraction is demonstrated to be insufficient to completely remove all endogenous folates.

The BCR 121 certified reference material consists of a ground wholemeal flour with a certified total folate concentration of 500 µg/kg with an uncertainty of 70 µg/kg. Though wholemeal flour differs from rodent feed in terms of fat, protein and starch content and consistency, it was used to evaluate the accuracy of the method. Following duplicate analysis, we measured a total folate content of 443 µg/kg with a standard deviation of 4 µg/kg, which corresponds to the certified range. Also, individual folate concentrations (59, 25, 48, 111 and 199 µg/kg for THF, FA, 5MeTHF, 10FoFa and 5FoTHF respectively) were comparable to those found in literature.(Doherty and Beecher 2003; Pawlosky *et al*. 2003; Van Daele *et al*. 2014)

IV.6. Conclusion

It is clear that for the correct interpretation of results obtained during animal feeding trials, it is very important to determine the actual feed intake and the folate concentration at the time of feeding. This conclusion is supported by our analysis of various feed samples, in which we observed substantial differences between the specified folate content and the actual folate content and the presence, up to 17%, of reduced and substituted folates.

IV.7. References

Castorena-Torres, F., Ramos-Parra, P.A., Hernandez-Mendez, R.V., Vargas-Garcia, A., Garcia-Rivas, G. and de la Garza, R.I. 2014. "Natural Folates from Biofortified Tomato and Synthetic 5-Methyl-Tetrahydrofolate Display Equivalent Bioavailability in a Murine Model." *Plant Foods Hum Nutr* 69 (1): 57–64.

Crider, K.S., Yang, T.P., Berry, R.J. and Bailey, L.B. 2012. "Folate and DNA Methylation: A Review of Molecular Mechanisms and the Evidence for Folate's Role." *Adv Nutr* 3 (1): 21–38.

De Brouwer, V., Zhang, G.F., Storozhenko, S., Van Der Straeten, D. and Lambert, W.E. 2007. "pH Stability of Individual Folates during Critical Sample Preparation Steps in Prevision of the Analysis of Plant Folates." *Phytochem Anal* 18 (6): 496–508.

De Brouwer, V., Storozhenko, S., Van De Steene, J.C., Wille, S.M.R., Stove, C.P., Van Der Straeten, D. and Lambert, W.E. 2008. "Optimisation and Validation of a Liquid Chromatography–tandem Mass Spectrometry Method for Folates in Rice." *J Chromatogr A* 1215 (1–2): 125–32.

Degen, G.H., Janning, P., Diel, P. and Bolt, H.M. 2002. "Estrogenic Isoflavones in Rodent Diets." *Toxicol Lett* 128 (1–3): 145–57.

Doherty, R.F., and Beecher, G.R. 2003. "A Method for the Analysis of Natural and Synthetic Folate in Foods." *J Agric Food Chem* 51 (2): 354–61.

Galivan, J., Ryan, T.J., Chave, K., Rhee, M., Yao, R. and Yin, D. 2000. "Glutamyl Hydrolase: Pharmacological Role and Enzymatic Characterization." *Pharmacol Therapeut* 85 (3): 207–15.

Heid, M.K., Bills, N.B., Hinrichs, S.H. and Clifford, A.J. 1992. "Folate Deficiency Alone Does Not Produce Neural Tube Defects in Mice." *J Nutr* 122 (4): 888–94.

Horne, D.W., Krumdieck, C.L. and Wagner, C. 1981. "Properties of Folic Acid Gamma-Glutamyl Hydrolase (conjugase) in Rat Bile and Plasma." *J Nutr* 111 (3): 442–9.

Jagerstad, M. and Jastrebova, J. 2013. "Occurrence, Stability, and Determination of Formyl Folates in Foods." *J Agric Food Chem* 61 (41): 9758–68.

Kiekens, F., Blancquaert, D., Devisscher, L., Van Daele, J., Stove, V.V., Delanghe, J.R., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015. "Folates from Metabolically Engineered Rice: A Long-Term Study in Rats." *Mol Nutr Food Res* 59 (3): 490–500.

Laird, P.W., and Jaenisch, R. 1996. "The Role of DNA Methylation in Cancer Genetic and Epigenetics." *Annu Rev Genet* 30: 441–64.

Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. 2003. "Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS." *Anal Chem* 75 (13): 3019–30.

Pawlosky, R.J., Flanagan, V.P. and Doherty, R.F. 2003. "A Mass Spectrometric Validated High-Performance Liquid Chromatography Procedure for the Determination of Folates in Foods." *J Agric Food Chem* 51 (13): 3726–30.

Pedersen, J.C. 1988. "Comparison of γ-Glutamyl Hydrolase (conjugase; EC 3. 4. 22. 12) and Amylase Treatment Procedures in the Microbiological Assay for Food Folates." *Br J Nutr* 59 (2): 261–71.

Reeves, P.G. 1997. "Components of the AIN-93 Diets as Improvements in the AIN-76A Diet." *J Nutr* 127 (5 Suppl): 838S –41S.

Reeves, P.G., Nielsen, F.H. and Fahey G.C.Jr. 1993. "AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet." *J Nutr* 123 (11): 1939–51.

Rushing, L.G., Cooper, W.M. and Thompson, H.C. 1991. "Simultaneous Analysis of Vitamins A and E in Rodent Feed by High-Pressure Liquid Chromatography." *J Agric Food Chem* 39 (2): 296–9.

Scott, J., Rébeillé, F. and Fletcher, J. 2000. "Folic Acid and Folates: The Feasibility for Nutritional Enhancement in Plant Foods." *J Sci Food Agric* 80 (7): 795–824.

Tamura, T., Mizuno, Y., Johnston, K.E. and Jacob, R.A. 1997. "Food Folate Assay with Protease, α-Amylase, and Folate Conjugase Treatments." *J Agric Food Chem* 45 (1): 135–9.

U.S. Department of Health and Human Services. 2001. "Guidance for Industry, Bioanalytical Method Validation." http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf (Consulted: 05/14/2016)

Unit for Reference Materials EC-JRC-IRMM. 2007. "Certified Reference Material BCR 121." https://ec.europa.eu/jrc/sites/default/files/rm/BCR-121_cert.pdf (consulted: 01/30/2016).

Vahteristo, L.T., Ollilainen, V., Koivistoinen, P.E. and Varo, P. 1996. "Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography." *J Agric Food Chem* 44 (2): 477–82.

Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2014. "Folate Profiling in Potato (Solanum Tuberosum) Tubers by Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry." *J Agric Food Chem* 62 (14): 3092–100.

Zeisel, S.H. 2009. "Importance of Methyl Donors during Reproduction." *Am J Clin Nutr* 89 (2): 673S – 7S.

Chapter V: Folate determination in plasma and red blood cells

Based on:

Kiekens F., Van Daele J., Blancquaert D., Van Der Straeten D., Lambert W.E., Stove C.P. 2015. "A validated ultra-high-performance liquid chromatography-tandem mass spectrometry method for the selective analysis of free and total folate in plasma and red blood cells" *J Chromatogr A*, 1398, 20-8

Author contributions

FK, CPS and WEL designed the research; FK conducted the research; FK analyzed the data and conducted statistical analysis; JVD reviewed the data; FK and CPS wrote the paper, which was revised by DVDS, WEL, JVD, and DB; FK and CPS are responsible for the final content.

Table of contents

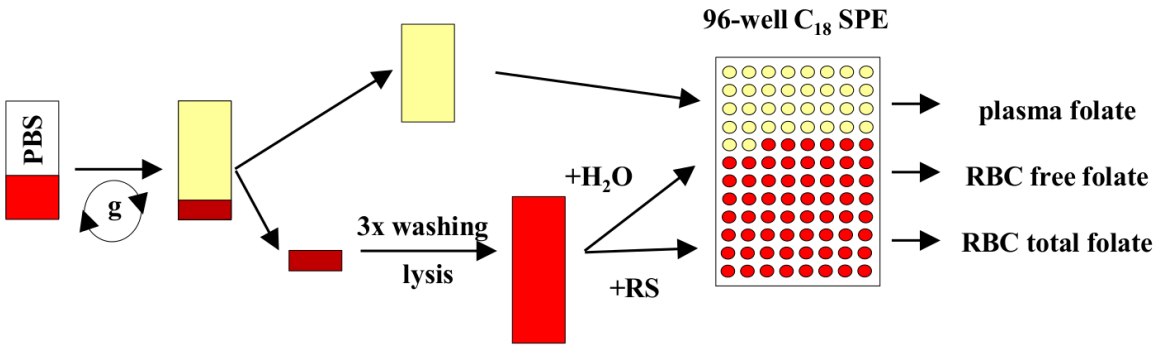
V.1. Abstract

A stable isotope dilution LC–MS/MS method is the method of choice for the selective quantitative determination of several folate species in clinical samples. By implementing an integrated approach to determine both the plasma and red blood cell (RBC) folate status, the use of consumables and time remains limited.

Starting from a single 300 µl whole blood sample, the folate status in plasma and RBCs can be determined after separating plasma and RBCs and sequential washing of the latter with isotonic buffer, followed by reproducible lysis using an ammonium-based buffer. Acidification combines both liberation of protein bound folates and protein precipitation. Sample cleanup is performed using a 96-well reversed-phase solid phase extraction procedure, similar for both plasma and RBC samples. Analyses are performed by UHPLC–MS/MS.

Method validation was successfully performed based on EMA-guidelines and encompassed selectivity, carry-over, linearity, accuracy, precision, recovery, matrix effect and stability. Plasma and RBC folates could be quantified in the range of 1–150 nM and 5–1500 nM, respectively.

This method allows for the determination of 6 folate monoglutamates in both plasma and RBCs. It can be used to determine short and long term folate status in both normal and severely deficient subjects in a single analytical sequence.



V.2. Introduction

Folates, a group of essential water-soluble B-vitamins (B9), are key cofactors in both the methylation cycle and DNA-synthesis. Folate deficiency has been implicated in several health disorders, with a proven link between periconceptional folate deficiency and the prevalence of neural tube defects.(Zeisel 2009) Other health issues such as increased cancer risk due to a disruption of DNA methylation and misincorporation of uracil may be related to folate deficiency.(Laird and Jaenisch 1996) Also, an impaired or halted methylation cycle causes neural damage through the reduced myelination of the nervous system (Crider *et al*. 2012) and leads to increased plasma homocysteine concentrations.(Shane 2010; Kiekens *et al*. 2015 (Chapter VI)) Following several decades of research it is evident that knowledge of nutrient status, including folate concentrations, is of paramount importance for the evaluation of individual health.

Clinical folate status is traditionally determined in serum by a competitive folate binding protein (FBP) assay or a microbial assay. However, more recently developed liquid chromatographic tandem mass spectrometric (LC–MS/MS) methods allow to discriminate between different metabolically active folate species for which the microbial and ligand binding assays are not equally specific.(Owen and Roberts 2003; Yetley *et al*. 2011; Pfeiffer *et al*. 2011)

For LC–MS/MS analysis of folates in plasma or serum, several methods have been published, differing in certain steps. Nelson *et al*. showed that affinity extraction with FBP led to lower detection limits, compared to reversed-phase solid phase extraction (SPE).(Nelson *et al*. 2004) However, Hannisdal *et al*. omitted the purification step and simply used protein precipitation and evaporation as sample cleanup technique, yielding similar results.(Hannisdal *et al*. 2009a) Also, Garbis *et al*. showed that hydrophilic interaction liquid chromatography can be used to separate four different folate species.(Garbis *et al*. 2001) Using an automated phenyl-based SPE system, a sensitive and high-throughput method for the measurement of serum folates was devised by Fazili *et al*.(Fazili *et al*. 2013)

Lysis of whole blood samples for folate analysis is commonly performed using an L-ascorbic acid (AA) solution as a lysing agent.(Fazili and Pfeiffer 2004; Smith *et al*. 2006; Kirsch *et al*. 2012) However, this strategy was criticized due to differences in the assayable folate concentrations, attributable to differences in solution strength, which may lead to incomplete lysis, non-linear dilution curves and variable lysis times.(Wright *et al*. 2000) The addition of AA to whole blood lysates causes deoxygenation of hemoglobin due to the acidification of the sample. Since folates are trapped within the structure of oxyhemoglobin, deoxygenation is required for deconjugation.(Wright *et al*. 1998) Alternatively, lyophilized RBCs have also been used as a starting material to which an extraction buffer is added.(Monch *et al*. 2010)

Since most LC–MS/MS procedures only measure folate monoglutamates, it is necessary to incubate the lysate at 37 °C for 2–12 h to allow human γ-glutamylhydrolase (GGH), present in plasma, to deconjugate the folate polyglutamates present in the sample. Mostly heat inactivation is used to stop this reaction.(Smith *et al*. 2006) Samples can be purified using affinity extraction with FBP, followed by SPE to concentrate the sample.(Smith et al. 2006) However, most used is ion-exchange or reversed-phase SPE without affinity extraction.(Fazili *et al*. 2005; Monch *et al*. 2010) Mixed-mode sorbents (i.e. reversed phase + ion-exchange interaction) can be used, though this necessitates an evaporation-dissolution step to remove the organic solvent from the sample.(Kirsch *et al*. 2012)

For plasma and RBCs it is difficult to obtain a blank matrix. A surrogate matrix, consisting of lyophilized egg white and sunflower oil in an isotonic sodium chloride solution, is sometimes used.(Monch *et al*. 2010) Another approach is to serially dilute samples with water in order to estimate the detection limits of the method.(Fazili *et al*. 2005) In both approaches, the obtained results may not be a true reflection of actual method performance. A better approach, as applied by e.g. Kirsch *et al*., may be to use a matrix treated with activated charcoal to assess method performance, although it should be noted that this matrix also differs in many aspects from non-treated matrix since other compounds are also removed from the matrix.(Kirsch *et al*. 2012)

To selectively quantify plasma, RBC free and RBC total folate, we developed a new LC–MS/MS method, with the focus on the sample preparation steps. This method was set up as such, that most of the treatment steps, consumables and solvents were shared for both plasma and RBC analyses, thereby optimizing the workflow and augmenting sample throughput. Also, reproducible RBC lysis, an obstacle troubling previously published methods, was achieved using a lysis buffer specific for RBCs, while addition of dithiothreitol (DTT) assures the release of folates from hemoglobin. To assess method performance, a full validation was performed, based upon European Medicines Agency (EMA) guidelines, including selectivity, carry-over, linearity, accuracy, precision, recovery, matrix effects and stability in whole blood, prepared extracts and while being in the autosampler.(European Medicines Agency 2011) Additionally, certified reference materials for both plasma and RBCs were analyzed to assure interlaboratory accuracy.

V.3. Objectives

To determine the folate status (e.g. following consumption of folate rice), a method was needed to measure folates in both plasma (short term status) and RBC (long term status). However, since in a rodent study only a small volume of whole blood can be obtained following (periodic) sampling, the method should be able to determine both plasma and RBC folates from a limited volume of whole blood.

V.4. Materials and methods

4.1. Reagents and materials

The six folate monoglutamates studied are: tetrahydrofolate (THF), 5-methyltetrahydrofolate (5MeTHF), 5,10-methenyltetrahydrofolate (5,10CH^+THF), 10-formylfolic acid (10FoFA), 5-formyltetrahydrofolate (5FoTHF) and folic acid (FA). The folate reference standards were obtained from Schircks Laboratories (Jona, Switzerland), while the labeled internal standards (ISs), with a labelling yield higher than 98%, were obtained from Merck Eprova (Glattbrugg, Switzerland). At the pH values reached during sample preparation and analysis, the metabolically active 10-formyltetrahydrofolate (10FoTHF) is detected as 5,10CH^+THF. Also, 5,10-methylenetetrahydrofolate (5,10CH_2THF) is unstable at acidic pH and is measured as THF.(De Brouwer *et al*. 2007; Jagerstad and Jastrebova 2013) All calibrators and $^{13}C_5$ labelled IS solutions were prepared at a final concentration of 200 µM in a 50 mM sodium phosphate buffer, pH 7.5, containing 1% AA and 0.5% DTT/methanol (50/50 v/v%). $^{13}C_5$-FA was used as IS for FA, 5FoTHF and 10FoFA, whereas $^{13}C_5$-THF, $^{13}C_5$-5MeTHF and $^{13}C_5$-5,10CH^+THF were used as IS for their respective isotopologues. Stripped rat serum was obtained starting from non-sterile, non-hemolyzed rat serum, obtained from Harlan (Horst, The Netherlands), which was stirred on ice for 1 h with 100 mg/ml of activated charcoal (Sigma–Aldrich, Schnelldorf, Germany) to remove endogenous folates. Following removal of the activated charcoal by centrifugation at 4500 × g for 15 min at 4 °C, the solution was filtered over a 0.45 µm syringe filter (GD/X CA 25/0.45, Whatman, GE Healthcare, Little Chalfont, UK), divided into aliquots (1.5 ml) and frozen at −80 °C. Acetonitrile (ACN) and methanol of LC–MS quality were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, trisodium phosphate, AA and DTT were obtained from Sigma–Aldrich. SPE was performed in a 96-well format with Bond Elut C_{18} 100 mg sorbent, purchased from Agilent (Palo Alto, CA, USA). Deionized water (H_2O-MQ) was produced in house by means of a Synergy UV water-purification system from Millipore (Billerica, MA, USA).

4.2. Preparation of stripped calibrator and QC-matrix

EDTA-anticoagulated blood samples were obtained from healthy volunteers, 4 male and 2 female, aged 25–37. To remove endogenous folates, plasma and RBC extracts were treated with activated charcoal as described for rat serum in Section 4.1.

4.3. Sample preparation

Folate analysis was performed starting from 300 µl EDTA-anticoagulated venous blood. Plasma extracts were prepared by adding 500 µl of phosphate buffered saline (PBS) to the whole blood sample. After centrifugation at 1000 × g for 5 min at 4 °C, 500 µl of supernatant was withdrawn. To this aliquot 50 µl of a 1% AA, 0.5% DTT solution (containing all ISs in a final concentration of 3.0 nM) was added to stabilize the folates. Following 15 min of incubation at room temperature, the samples were either analyzed immediately or frozen at

−80 °C. To the remainder of the samples, containing the RBCs, 1 ml of PBS at room temperature was added, followed by resuspension, centrifugation at 1000 × g for 5 min at 4 °C and removal of 1 ml supernatant. This washing procedure was repeated two more times. After the final removal of 1 ml supernatant, 800 µl of lysis buffer (168 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA) was added.(Chernyshev *et al*. 2008) Selective RBC lysis occurred during a 10-min incubation at room temperature, after which cell residues were spun down by centrifugation at 2500 × g for 10 min at 4 °C. One milliliter of supernatant was withdrawn and 100 µl of IS/AA/DTT solution was added. After incubation for 15 min at room temperature, these samples were analyzed further or frozen at −80 °C. A flowchart describing the sample preparation procedure is shown in Figure V.1.

4.4. Enzyme treatment and protein precipitation

To measure the total folate content in RBCs, 100 µl of stripped rat serum was added to 500 µl of RBC lysate and incubated for 2 h at 37 °C. This stripped rat serum serves as an exogenous source of GGH to deconjugate the folate polyglutamates present in RBC samples. The reaction was halted by addition of 300 µl of 10 vol% acetic acid in water. This acidification of the sample also liberates protein-bound folates and causes protein precipitation.(Kamen and Caston 1986) While no enzyme treatment is necessary to measure free monoglutamates in plasma and RBCs, these samples were also acidified by adding 275 µl or 300 µl of 10% acetic acid, respectively. The acidified samples were incubated for 20 min at 4 °C prior to centrifugation at 11,000 × g for 15 min at 4 °C.

4.5. Sample clean-up

SPE was performed using a Bond Elut 96-well plate containing 100 mg C_{18} sorbent per well. The sorbent was activated with 2 × 600 µl of methanol and conditioned with 2 × 600 µl of 1.67/30.30/68.03 v/v/v% CH_3COOH/PBS/H_2O-MQ including 303 mg AA and 30,3 mg DTT per 100 ml. Next, plasma and RBC samples were diluted with an equal volume of water of which 1 ml for plasma extracts and 800 µl for RBC extracts was loaded onto the sorbent. The sorbent was then washed with 2 × 600 µl of conditioning buffer prior to elution with 450 µl of elution buffer (7.5/92.5 v/v% ACN/50 mM trisodium phosphate with 1% AA and 0.1% DTT; pH = 7.3). The SPE procedure is also summarized in Figure V.1.

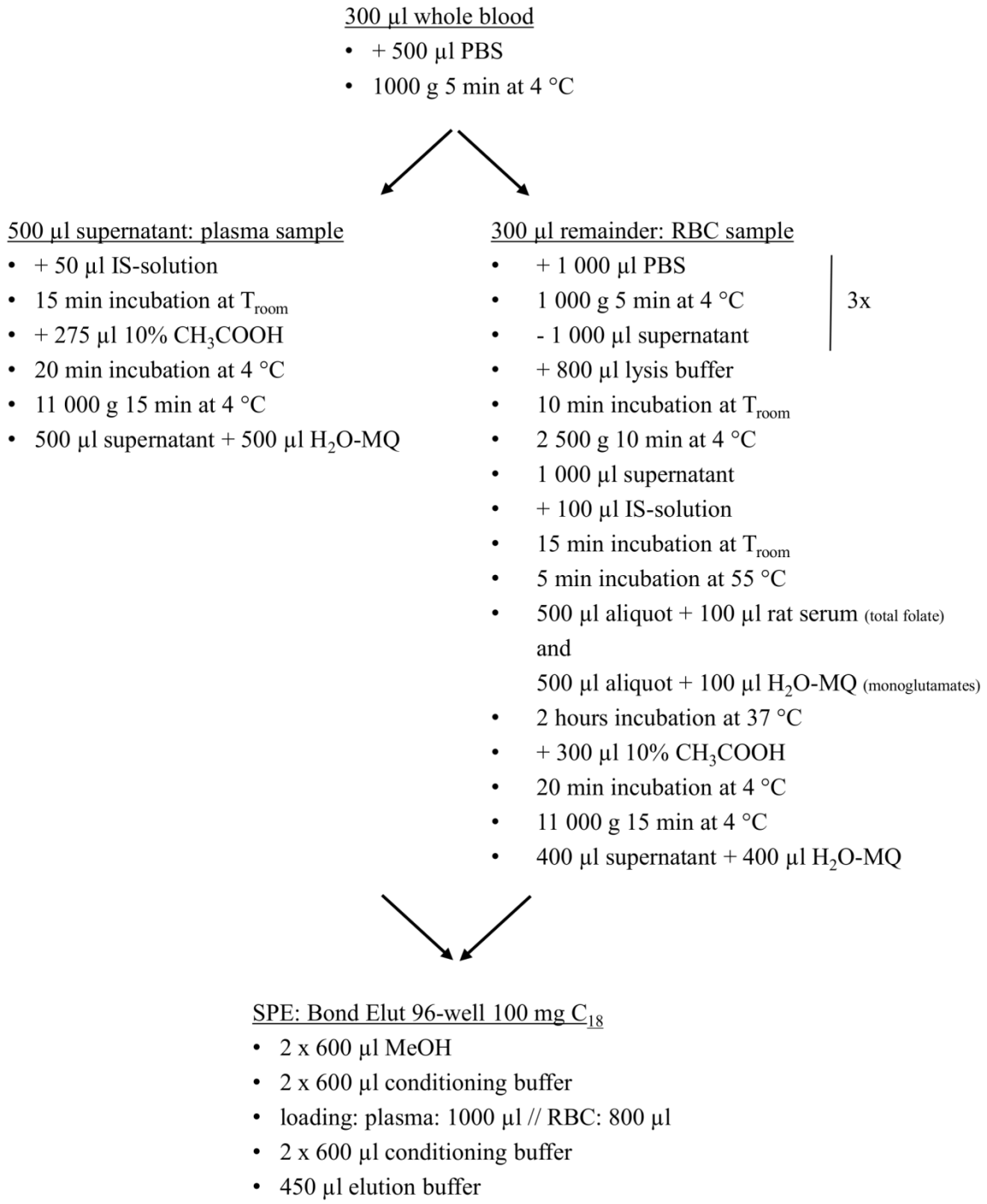


Figure V.1: Flowchart describing the sample preparation procedure for the analysis of plasma and RBC folate; IS-solution: internal standard solution containing L-ascorbic acid and DL-dithiothreitol

4.6. LC-MS/MS analysis

Samples were analyzed using a Waters Acquity UPLC® system coupled to an ABSciex API 4000™ triple quadrupole mass spectrometer equipped with a TurboIonSpray® probe. The autosampler was equipped with a sample loop of 10 µl and samples were kept at 4 °C prior to injection. After injection the needle was cleaned with 600 µl of 10/90 v/v% H_2O-MQ/ACN followed by 800 µl of 90/10 v/v% H_2O-MQ/ACN. Chromatographic separation was achieved using a mobile phase composed of H_2O-MQ (A) and ACN (B), each containing 0.1% of formic acid, with a flow rate of 0.6 ml/min on a Waters HSS T3-column (150 mm × 2.1 mm; 1.8 µm) held at 60 °C. The composition of the mobile phase starts with 100% solvent A and is kept constant for 1.6 min. Thereafter the amount of solvent B is increased to 10% in 1.2 min and to 16% in another 0.7 min after which the column is cleaned for 1.5 min with 95% solvent B and equilibrated to initial solvent composition for 3 min. This amounts to a total time of approximately 9 min between injections. Mass spectrometric detection was performed in scheduled multiple-reaction monitoring mode. Electrospray ionization was used to ionize the analytes with the ion spray voltage set at 2500 V and a temperature of 600 °C. Nitrogen was used as curtain gas, gas 1 and gas 2, with respective pressure settings at 25, 75 and 90 psig, and as collision gas at setting 11. The interface heater was switched on. The transitions monitored per compound were the same as described before (De Brouwer *et al*. 2008) and can be found together with the settings in Table V.1.

Compound	Retention time [min]	Molecular ion [Da]	Fragment ion [Da]	DP [V]	EP [V]	CE [V]	CXP [V]
THF	3.36	446.2	**299.3**	59	4	26	13
	3.36		166.3	59	10	54	12
FA	3.97	442.1	**295.3**	54	9	24	22
	3.97		176.2	54	9	60	16
5MeTHF	3.45	460.3	**313.2**	52	5	25	20
	3.45		194.2	62	2	54	14
5,10CH$^+$THF	3.57	456.2	**412.1**	115	9	44	13
	3.57		282.1	113	8	63	16
10FoFA	3.84	470.1	**295.3**	76	2	32	16
	3.84		176.2	70	4	66	10
5FoTHF	3.89	474.2	**299.3**	54	12	46	8
	3.89		166.3	52	12	52	14
$^{13}C_5$-THF	3.36	451.1	299.3	64	6	27	16
$^{13}C_5$-FA	3.97	447.2	295.3	65	3	27	26
$^{13}C_5$-5MeTHF	3.45	465.3	313.2	66	6	27	13
$^{13}C_5$-5,10CH$^+$THF	3.57	461.0	416.2	79	8	45	9

Table V.1: Compound related MS parameters as used for the instrument programming. For 10FoFA and 5FoTHF the internal standard of FA is used. DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential; V: volt.

4.7. Validation

The developed method was validated based on the EMA guidelines for bioanalytical method validation.(European Medicines Agency 2011) Selectivity, carry-over, limit of detection (LOD) and lower limit of quantification (LLOQ), linearity, precision (including incurred sample reanalysis), accuracy, matrix effect, recovery and stability were evaluated.

4.7.1. Selectivity and carry-over

Selectivity was evaluated by analyzing plasma and RBC lysates from six individual donors (4 male and 2 female), both with and without treatment with activated charcoal. Cross-interferences were evaluated by adding the 6 folates and 4 labelled ISs individually to stripped plasma or RBC extract in a concentration of 100 nM for plasma samples (150 nM for 5FoTHF) and 300 (THF and 5,10CH$^+$THF), 500 (5MeTHF, FA and 10FoFA) or 750 nM (5FoTHF) for RBC extracts. For the analytes, the acceptance criterion for the selectivity assessment was set at 20% of the peak area corresponding to the LLOQ level, while for the labelled ISs only 5% was considered acceptable. Carry-over was evaluated by injecting blank samples after calibrators with identical concentrations and acceptance criteria as for the evaluation of selectivity

4.7.2. Linearity, accuracy and precision

The actual LLOQ was verified as the concentration which could be measured with a %bias and relative standard deviation (RSD) below 20%. The LOD was calculated as one third of the LLOQ. Duplicate calibration curves of 7 calibrators and 1 zero sample for plasma or 9 calibrators (8 for THF and 5,10CH$^+$THF) and 1 zero sample for RBC were constructed on 4 non-consecutive days to evaluate linearity (Table V.2). Homoscedasticity was evaluated by plotting the residuals versus the nominal values. The sum of the residual error was used to evaluate the need for a weighted or transformed model ($1/x$, $1/x^2$, $1/y$, $1/y^2$, $\sqrt{x}$, $\ln(x)$, $\log(x)$). For the selected model to be acceptable, back-calculated mean concentrations should be within 15% of the nominal value (20% at LLOQ-level). Accuracy and precision were evaluated using 4 QCs (LLOQ to 0.75* upper limit of quantification (ULOQ), Table V.2), prepared and analyzed on 4 non-consecutive days. A single-factor ANOVA was used to calculate inter- and intrabatch variability (%RSD). Accuracy (%bias) was calculated as the measured value divided by the nominal value. Acceptance criteria for %bias and %RSD are 15%, except at LLOQ level, where they are 20%.(Wille *et al*. 2011) An RBC sample of a healthy volunteer was analyzed in duplicate on 3 non-consecutive days to evaluate the precision of a sample requiring deconjugation of the folate polyglutamates. Also, the dilution integrity of RBC extracts exceeding the ULOQ was verified by two- or fourfold dilution of RBC matrix spiked at a concentration of 600–3000 nM with SPE elution buffer prior to UHPLC–MS/MS analysis (n = 4).

A: plasma level	THF [nM]	FA [nM]	5MeTHF [nM]	5,10CH$^+$THF [nM]	10FoFA [nM]	5FoTHF [nM]
0	0	0	0	0	0	0
1	0.9990	1.001	1.001	1.000	1.000	1.500
2	1.998	2.002	2.003	2.000	2.000	3.000
3	3.995	4.003	4.005	4.001	4.000	6.000
4	9.990	10.01	10.01	10.00	10.00	15.00
5	19.98	20.02	20.03	20.00	20.00	30.00
6	39.95	40.03	40.05	40.01	40.00	60.00
7	99.89	100.1	100.1	100.0	100.0	150.0
QC1	4.994	5.004	5.007	5.001	5.001	7.500
QC2	24.97	25.02	25.03	25.01	25.00	37.50
LLOQ	0.9990	1.001	1.001	1.000	1.000	1.500
3*LLOQ	2.997	3.002	3.004	3.001	3.000	4.500
MID	14.98	15.01	15.02	15.00	15.00	22.50
0.75* ULOQ	74.91	75.06	75.10	75.02	75.01	112.4

B: RBC level	THF [nM]	FA [nM]	5MeTHF [nM]	5,10CH$^+$THF [nM]	10FoFA [nM]	5FoTHF [nM]
0	0	0	0	0	0	0
1	5.010	5.008	5.000	5.005	5.001	7.520
2	10.02	10.02	10.00	10.01	10.00	15.04
3	25.00	25.04	25.00	25.02	25.01	37.60
4	50.10	50.08	50.00	50.05	50.01	75.20
5	70.10	100.2	100.0	70.06	100.0	150.4
6	100.2	200.3	200.0	100.1	200.0	300.8
7	200.0	300.5	300.0	200.2	300.1	451.2
8	301.0	500.8	500.0	300.3	500.1	752.0
9		1002	1000		1000	1504
QC1	30.10	35.06	35.00	30.03	35.01	52.64
QC2	200.0	400.6	400.0	200.2	400.1	601.6
LLOQ	5.010	5.008	5.000	5.005	5.001	7.520
3*LLOQ	15.03	15.02	15.00	15.01	15.00	22.56
MID	80.20	250.4	250.0	80.07	250.1	376.0
0.75* ULOQ	220.0	741.2	740.0	220.2	740.2	1113

Table V.2: Calibration, quality control and validation concentrations. MID, intermediate concentration; LLOQ, lower limit of quantification; QC, quality control sample; ULOQ, upper limit of quantification.

4.7.3. Recovery and matrix effects

Matrix effect and recovery were evaluated at 3 concentration levels (3*LLOQ to 0.75*ULOQ, Table V.2) in matrix obtained from 6 individual sources according to Matuszewski *et al.*(Matuszewski *et al*. 2003) At the 3*LLOQ concentration level, charcoal treated matrix was used. To prepare the MID and 0.75*ULOQ concentration levels, the matrix was not treated with charcoal. The analytes were spiked either before (A) or after (B) the extraction and SPE procedure. Also, analytes were spiked in SPE elution buffer to obtain a sample free of matrix (C). Absolute recovery was calculated as the percent ratio of peak areas A over B, while absolute matrix effect was calculated as the percent ratio of the peak areas B over C. Relative recovery and matrix effect are expressed as %RSD of the absolute recovery and matrix effect values and should not exceed 15%.

4.7.4. Stability

The stability of folates in whole blood samples was verified by storing whole blood at 4 °C for 0, 1, 2, 7 and 10 days, while protected from light, before performing the entire sample preparation procedure in triplicate. Autosampler stability was assessed by reinjection of samples stored for 24 h in the autosampler at 4 °C, while concentrations were calculated based on fresh calibrators. Long term stability of plasma and RBC lysates at −80 °C was evaluated by analysis of samples in duplo after 0, 1, 2 and 4 weeks including freshly prepared calibration curves. Also, the effect of up to three freeze-thaw cycles was evaluated by freezing and thawing the samples (n=2) in cycles of 1 h. Stability is acceptable if the concentration measured does not deviate more than 15% from that measured initially.

4.8. Application

The accuracy of the method was evaluated via the measurement of two certified reference materials, namely: Vitamin B12 and Serum Folate (03/178) and Folate in Whole Blood Haemolysate (95/528), both WHO International Standards issued by the National Institute for Biological Standards and Control (NIBSC). The reference material for serum has previously been measured by e.g. Fazili *et al*.(Fazili *et al*. 2013)

V.5. Results and discussion

5.1. Method optimization

5.1.1. Sample treatment

The method starts from 300 µl of whole blood. Following removal of the plasma sample, the RBC pellet is washed to remove plasma residues. To evaluate the washing procedure of RBCs we monitored the removal of GGH activity by measuring the concentration of 5MeTHF monoglutamate in RBC lysates after 2 h of incubation at 37 °C without addition of rat serum GGH. Whole blood lysate, sole plasma removal and 1 to 4 washing steps were evaluated. The amount of 5MeTHF monoglutamates remained stable after at least 2 washing steps, indicating

that human GGH was sufficiently removed. One additional washing step was included to ensure reproducible removal of plasma proteins, plasma folates and other constituents. Since plasma GGH is removed prior to RBC lysis, the presence of different folate polyglutamates may be visualized using the chromatographic method developed by Wang *et al.*(Wang *et al.* 2010)

Given the need for complete and reproducible RBC lysis, several solvent compositions described in literature were evaluated. These were either based on the selective absorption of ammonia by RBCs (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA at pH 7.4), hyper- (1% AA) or hypotonicity (deionized water) or susceptibility to ethanol or saponin.(Alter *et al.* 1979; Chi and Wu 1991; Philpott *et al.* 2001; Vuorte *et al.* 2001; Arias *et al.* 2010) The addition of 1% AA, ethanol or saponin resulted in incomplete lysis and would require at least one freeze-thaw cycle for full lysis. In contrast, the ammonia lysis buffer and deionized water led to full lysis of RBCs. The former was selected because it has been reported to selectively lyse RBCs, leaving other cells unaffected.(Alter *et al.* 1979) However, this buffer showed limited shelf life, with prolonged storage resulting in increased lysis times. Therefore, it was used within fourteen days after preparation and was stored at 4 °C.

5.1.2. Folate deconjugation

Since folates trapped within the quaternary structure of oxyhemoglobin are not accessible to GGH, deoxygenation of hemoglobin is necessary to measure the total amount of folates in the sample. While other methods rely on the Bohr effect (decrease of pH) to decrease oxygen binding affinity (Wright *et al.* 2000), in our method DTT was added to RBC extracts, which gently deoxygenates hemoglobin to produce a mixture of deoxy-, met- and sulfhemoglobin, leading to a release of folates.(Herzfeld *et al.* 1990)

To deconjugate the folate polyglutamates present in RBCs, the samples are incubated with stripped rat serum, which serves as a reliable source of the necessary GGH enzyme. Addition of 100 µl stripped rat serum to 500 µl of RBC lysate, containing AA, DTT and ISs, and incubation for 2 h at 37 °C proved optimal to assure full deconjugation. However, the incubation of RBC extracts at 37 °C led to a minor conversion of THF to 5,10CH^+THF. From literature it could be concluded that this reaction, which is catalyzed by a formate-activating enzyme (or formyltetrahydrofolate synthetase) present in RBCs, can be prevented by denaturing the enzyme at 55 °C for 5 min or by addition of NaCl to a concentration of 50 mM.(Bertino *et al.* 1962) Since the latter inhibited the deconjugation of folate polyglutamates, the heat treatment was preferred.

5.1.3. Protein precipitation

A significant part of folates is bound to FBP. These folates can be released by acidification to a pH below 5.5, which strongly diminishes the binding capacity of FBP.(Kamen and Caston 1986) This offers an advantage over previously published methods which may suffer from poor reproducibility due to incomplete lysis and uncontrolled protein binding.(Wright *et al.*

2000) Thus, 10% acetic acid was added as this combines protein precipitation and the liberation of protein-bound folates. Moreover, this renders an additional treatment with protease unnecessary.

5.1.4. Solid phase extraction

Since both reversed-phase and ion-exchange SPE (Nelson *et al*. 2004; Fazili *et al*. 2005; Monch *et al*. 2010) have been used for the determination of folates in plasma and RBC extracts, the retention behavior of 6 folate monoglutamates on various sorbent types was evaluated. Strong-anion exchange (Agilent Bond-Elut SAX) showed inferior retention of 5,10CH$^+$THF, but gave less background noise. However, since 5,10CH$^+$THF was regularly detected in plasma and RBC samples, the superior recovery obtained using the reversed-phase sorbents was of greater importance. Comparable results were found with phenyl and C_{18} (both Agilent Bond-Elut) sorbents. The C_{18} sorbent was selected for further optimization.

5.2. Method validation

5.2.1. Selectivity and carry-over

Method selectivity was evaluated using spiked stripped blood samples of 6 apparently healthy volunteers. Apart from a limited conversion of THF to 5,10CH$^+$THF in RBC extracts, the MS signals for none of the folates exceeded 20% of the LLOQ of the others. A 5 min treatment at 55 °C restricted the conversion of THF to 5,10CH$^+$THF to on average 3.1% of the amount of THF present in the RBC sample. When the THF concentration exceeds 33 nM, an apparent 5,10CH$^+$THF concentration will be observed which is higher than 20% of the LLOQ. In non-stripped samples, no interfering peaks were observed that would hamper folate analysis, based on an evaluation of peak shape and baseline resolution. No unacceptable carry-over was observed in either plasma or RBCs for any of the folates analyzed (Table V.3).

Carry-over [% of LLOQ]	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
plasma	0	7.7	8.6	4.7	13.4	1.7
RBC	0	0	5.6	9.3	8.0	0.8

Table V.3: Carry-over calculated as the percentage of the peak area obtained from the chromatogram of a blank sample after injection of a high calibrator compared to the peak area at the LLOQ (n=2).

5.2.2. Linearity, accuracy and precision

The data were heteroscedastic; a linear unweighted model was preferred for all folates in both matrices, since weighting provided little benefit based on the evaluation of the sum relative error. Also, the back-calculated concentrations of the calibrators fell within the acceptance criteria using this linear model. The LLOQ and ULOQ range from 1 to 1.5 and 100 to 150 nM, respectively, for plasma and from 5 to 7.5 and 300 to 1500 nM, respectively, for RBCs (Table V.2).

In plasma the interbatch variation for 5FoTHF at the LLOQ level slightly exceeded the 20% acceptance criterion (21.3%), which may be improved using the commercially available $^{13}C_5$-

5FoTHF as IS. All other accuracy and precision values fell within 15% bias resp. RSD (or 20% at the LLOQ level) (Table V.4).

A: plasma	concentration level	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
intrabatch precision [% RSD]	LLOQ	13.3	10.0	4.9	7.7	10.9	15.9
	3*LLOQ	7.8	9.3	6.7	7.2	3.4	7.6
	MID	5.1	6.6	2.4	8.4	9.7	4.5
	0.75*ULOQ	5.5	4.8	3.1	8.4	4.4	8.3
interbatch precision [% RSD]	LLOQ	15.5	19.5	7.0	9.0	20.0	21.3
	3*LLOQ	7.8	9.5	7.9	7.2	15.2	13.2
	MID	6.8	6.6	4.1	8.4	9.7	6.7
	0.75*ULOQ	5.5	4.8	3.8	8.4	7.8	8.3
accuracy [% bias]	LLOQ	0.2	-9.7	6.7	-0.1	-9.1	-5.3
	3*LLOQ	-1.8	1.8	2.6	2.5	5.5	2.9
	MID	1.0	-1.4	-1.9	-1.3	5.7	3.5
	0.75*ULOQ	-0.9	-1.4	0.1	2.2	-1.3	3.1
B: RBC	concentration level	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
intrabatch precision [% RSD]	LLOQ	19.1	4.8	6.3	10.1	7.1	8.0
	3*LLOQ	13.9	8.5	4.4	7.5	7.8	10.7
	MID	8.9	2.1	1.2	6.4	5.1	6.5
	0.75*ULOQ	11.3	1.7	1.7	7.9	4.0	5.7
interbatch precision [% RSD]	LLOQ	19.1	10.9	10.4	13.8	9.3	10.9
	3*LLOQ	13.9	8.5	4.4	7.5	7.8	14.0
	MID	8.9	3.2	2.0	7.0	5.1	6.5
	0.75*ULOQ	11.9	2.1	0.8	7.9	5.8	5.7
accuracy [% bias]	LLOQ	16.9	-5.4	-8.3	-3.5	-4.5	14.1
	3*LLOQ	-6.7	-5.7	-3.4	0.3	2.4	2.7
	MID	-3.3	-1.2	-0.7	-7.3	-1.3	-2.5
	0.75*ULOQ	5.1	-0.2	0.4	5.9	-2.4	2.7

Table V.4: Accuracy and precision data (n=4x2) for plasma (A) and RBCs (B). MID, intermediate concentration; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

To assess the reproducibility of measurements in a true RBC extract, in which mainly folate polyglutamates are present, incurred samples were analyzed in duplicate during three different analytical runs. In these samples only 5MeTHF and 5,10CH$^+$THF could be quantified, with a respective intrabatch precision (%RSD) of 7.5 and 9.6% and a respective interbatch precision (%RSD) of 7.7 and 9.6%, at a respective average concentration of 124 and 22.8 nM. A two- or four-fold dilution of samples exceeding the ULOQ with SPE elution buffer proved possible, with overall bias and RSD of 5MeTHF, THF and 5FoTHF below 15%. For all other folates (5,10CH$^+$THF and 10FoFA) twofold dilution is possible (Table V.5).

dilution factor		THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
1/4	average [nM]	2376	2118	2099	745.1	2308	2947
	target [nM]	2398	2003	2000	600.5	2000	3008
	accuracy [%]	-0.900	5.73	4.96	24.1	15.4	2.01
	precision [%]	11.0	9.58	11.3	10.1	4.73	6.02
1/2	average [nM]	2713	2061	2130	685.2	2236	2903
	target [nM]	2398	2003	2000	600.5	2000	3008
	accuracy [%]	13.14	2.90	6.50	14.1	11.8	-3.48
	precision [%]	11.85	4.32	2.02	8.07	3.43	6.35

Table V.5: Results obtained during the assessment of the dilution integrity for RBC folates (n=3).

To investigate whether the treatment of the matrix with activated charcoal, used to prepare calibrators, affected the calibration results, two calibration curves were compared. One was prepared in stripped matrix, the other in untreated matrix. As can be seen in Table V.6, the slope of both the plasma and the RBC calibration curves deviated less than 7.3 and 6.8% between stripped and untreated matrix. Also, cross-calculated concentrations fell within the acceptance criteria, except for RBC folates at LLOQ level, which was likely due to the high background folate concentrations in untreated RBC samples. As such, the use of stripped matrix does not influence the analytical results.

	plasma			RBC		
folate	slope treated matrix	slope untreated matrix	difference [%]	slope treated matrix	slope untreated matrix	difference [%]
THF	0.0167	0.0155	-7.3	0.0069	0.0066	-5.0
FA	0.0878	0.0871	-0.8	0.0374	0.0362	-3.1
5MeTHF	0.0166	0.0173	4.4	0.0073	0.0076	4.8
5,10CH$^+$THF	0.0188	0.0181	-3.7	0.0081	0.0086	6.8
10FoFA	0.0867	0.0905	4.4	0.0399	0.0408	2.0
5FoTHF	0.0114	0.0122	7.0	0.0081	0.0077	-4.9

Table V.6: Effect of treatment of the matrix with activated charcoal on the slope of the calibration lines for both plasma and RBCs.

5.2.3. Recovery and matrix effects

Matrix effects and recoveries for both plasma and RBCs are shown in Table V.7, both with and without compensation by the ISs. Overall, IS-compensated recoveries lay between 91.2 and 107.5% for plasma and 84.8 and 117.2% for RBCs, while IS-compensated matrix effects – with a single exception for 5FoTHF – did not exceed 20%. Both 10FoFA and 5FoTHF showed slightly different recoveries and matrix effects than $^{13}C_5$-FA, which is used as an IS for these compounds. Though some values for IS-compensated relative recovery and matrix effect exceeded the 15% acceptance criterion, none were larger than 20%. The relative recovery and matrix effect values without IS-compensation demonstrate the necessity of using labelled ISs to compensate for the variability between samples.

			THF		FA		5MeTHF		5,10CH$^+$THF		10FoFA		5FoTHF	
		Conc. level	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
plasma	absolute matrix effect [%]	3*LLOQ	92.9	104.0	99.7	106.6	83.6	99.7	94.4	104.8	90.1	101.3	103.6	108.7
		MID	88.0	102.8	87.7	103.7	78.2	105.2	82.7	96.5	87.1	86.8	90.6	109.0
		0.75*ULOQ	84.8	106.2	87.3	108.7	85.6	103.9	82.5	109.0	88.3	105.8	88.6	111.2
	relative matrix effect [%RSD]	3*LLOQ	6.6	2.1	3.3	3.2	6.0	5.2	10.4	6.4	8.4	4.7	8.4	9.0
		MID	9.9	3.6	9.6	12.5	9.8	6.1	9.3	4.8	12.3	16.3	8.4	11.9
		0.75*ULOQ	7.6	6.3	9.3	9.6	8.6	3.0	8.6	2.3	7.2	9.9	7.5	9.8
	absolute recovery [%]	3*LLOQ	47.4	91.2	48.4	91.2	51.4	97.4	54.8	94.6	53.0	98.4	51.1	94.3
		MID	57.1	97.8	60.5	103.9	58.1	98.4	61.8	101.3	61.8	107.5	57.7	97.9
		0.75*ULOQ	54.5	99.4	55.3	98.0	55.8	106.2	58.0	100.8	57.5	102.7	57.0	101.1
	relative recovery [%RSD]	3*LLOQ	16.1	9.8	19.9	10.4	14.4	6.4	16.9	15.3	12.8	16.9	14.8	13.5
		MID	15.2	5.5	11.0	10.8	17.2	15.9	12.4	9.0	14.4	12.1	8.7	11.0
		0.75*ULOQ	6.4	7.6	9.0	8.3	8.0	10.7	9.1	4.2	6.9	8.9	6.6	9.7
RBC	absolute matrix effect [%]	3*LLOQ	154.4	110.0	91.5	102.6	100.9	119.9	143.2	112.3	87.6	101.9	108.3	126.4
		MID	84.5	85.3	101.0	90.6	84.1	90.4	88.9	83.7	96.1	86.2	114.7	102.9
		0.75*ULOQ	77.0	89.1	101.3	96.0	84.3	103.7	74.9	91.2	102.0	96.6	111.3	105.4
	relative matrix effect [%RSD]	3*LLOQ	3.8	6.6	7.1	9.9	34.7	19.7	7.4	6.0	2.7	8.8	4.2	5.7
		MID	9.5	8.8	7.2	3.0	9.9	7.6	9.1	7.3	5.5	4.3	5.0	2.9
		0.75*ULOQ	5.8	3.2	7.0	2.3	7.0	1.9	2.8	3.8	8.3	2.4	7.7	1.5
	absolute recovery [%]	3*LLOQ	11.9	95.8	22.5	102.5	*19.0	*102.6	30.7	106.0	29.5	110.7	27.9	105.9
		MID	14.1	94.7	25.6	97.4	33.8	91.1	43.2	105.8	30.2	115.1	29.9	113.6
		0.75*ULOQ	14.0	107.0	23.3	100.4	24.4	84.8	43.2	115.9	27.0	115.6	27.4	117.2
	relative recovery [%RSD]	3*LLOQ	34.6	11.0	30.8	3.2	*15.6	*15.4	30.9	13.1	22.2	13.3	27.0	11.6
		MID	17.9	7.5	6.9	5.8	22.3	17.8	11.9	14.6	6.5	5.9	7.7	7.0
		0.75*ULOQ	14.1	14.5	21.4	7.1	38.1	11.3	20.2	11.7	25.3	10.6	23.3	4.0

Table V.7: Results following the analysis of matrix effects and recovery. The maximal achievable recovery (plasma 64.5%; RBC: 44.4%) was not taken into account in the values of the absolute recovery. * Although not a statistical outlier, 1 extremely deviating sample was omitted. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

5.2.4. Stability

Folates present in the plasma fraction of whole blood proved stable for 24 h, while the folates present in RBCs proved stable up to 10 days (Figure V.2). As observed by Hannisdal *et al.*, the stability of plasma folates can be improved through the use of heparin or citrate instead of EDTA as anticoagulant.(Hannisdal *et al*. 2009b) Therefore, if sample stability is an issue, it is unlikely that the use of heparin as an alternative anticoagulant would negatively influence method performance.

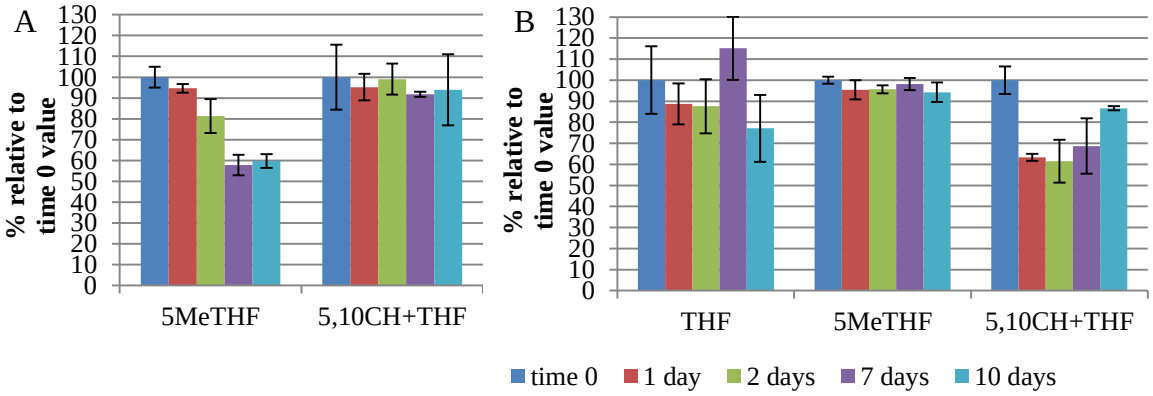


Figure V.2: Stability of folates in whole blood stored at 4°C in both plasma (A) and red blood cell fractions (B). Averages are shown, together with standard deviations (n=3).

Storage of plasma extracts at −80 °C was possible up to 4 weeks (Figure V.3 A-C). RBC folate levels in extracted samples showed a steady decrease upon storage at −80 °C, where after storage for 4 weeks losses regularly exceeded 20% (Figure V.3 D-F). Processed samples were stable for at least 24 h in the refrigerated autosampler at 4 °C (Table V.8). Although samples with a low folate concentration were more susceptible to the effects of freeze-thaw cycles, up to three freeze–thaw cycles did not have an unacceptable effect on the folate levels measured in both plasma and RBC samples at higher concentration levels (Table V.9). In general, freeze-thaw cycles should be avoided.

A: Plasma [% bias]	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
LLOQ	-15.91	-3.90	10.02	8.96	10.26	5.87
3*LLOQ	9.31	-3.67	3.31	7.40	-5.05	-0.86
MID	-6.29	-1.58	0.49	1.38	-6.25	-8.91
0.75*ULOQ	13.02	0.37	-0.53	-10.25	3.77	-6.18
B: RBC [% bias]	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
LLOQ	4.43	-6.12	-14.29	13.47	0.41	-13.84
3*LLOQ	16.13	2.36	-7.26	1.91	2.08	4.84
MID	0.17	0.61	-1.28	2.23	-0.08	3.24
0.75*ULOQ	4.85	3.05	2.77	-0.78	1.42	2.92
RBC extract	14.93	-0.55	-6.04	-1.29	<LLOQ	<LLOQ

Table V.8: Autosampler stability expressed as the percentage difference between samples analyzed immediately after sample treatment and following 24 hours in the autosampler (averages are shown, n=3). Quantitation of the latter was done using a freshly prepared calibration curve. The RBC extract was obtained from a healthy volunteer. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

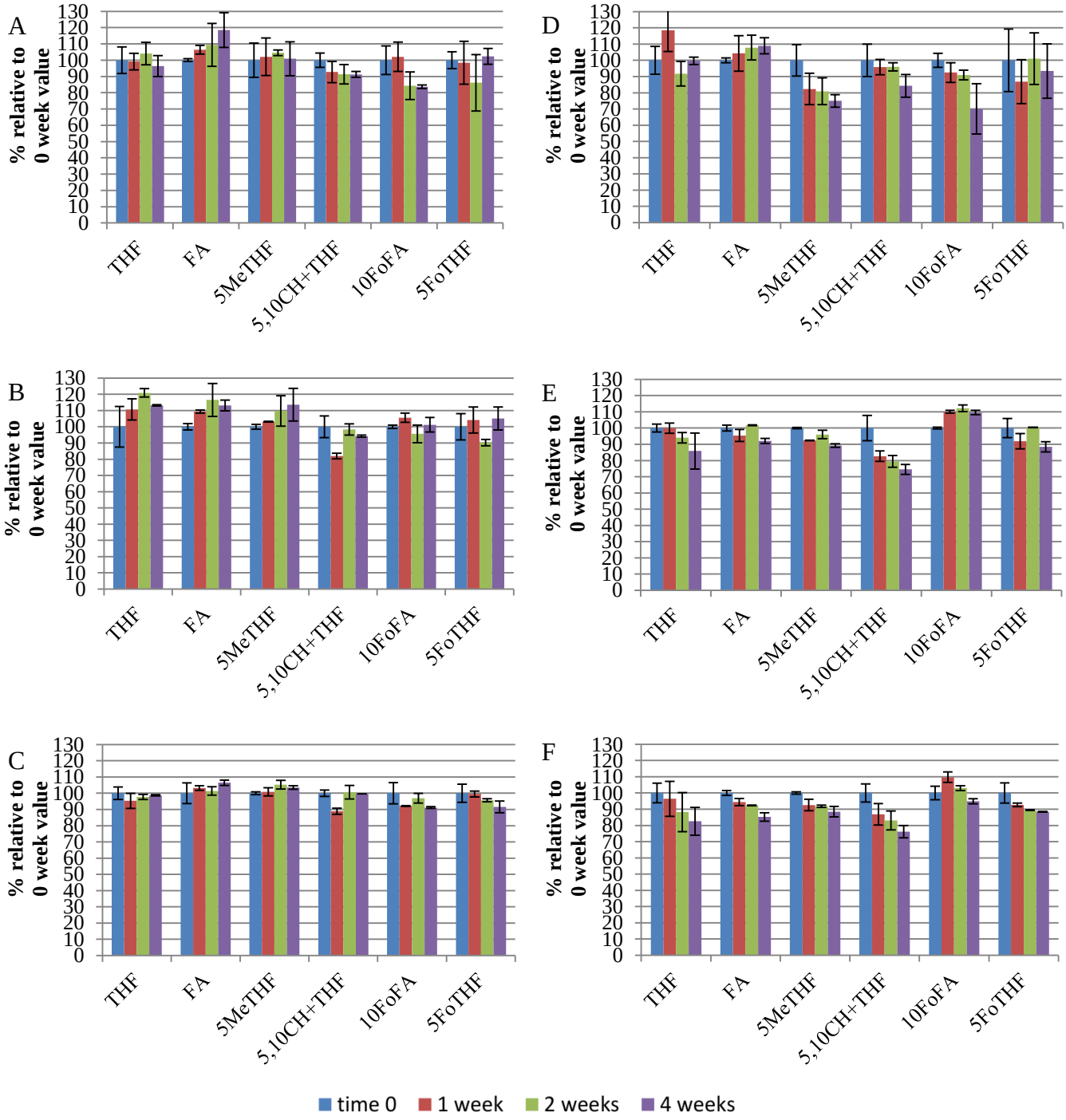


Figure V.3: Stability of folates in plasma or RBC extracts spiked at 3*LLOQ (A,D), MID (B,E) and 0.75*ULOQ (C,F), stored at -80°C until analysis. Averages are shown, together with standard deviations (n=2). LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

A: plasma [%bias]	# cycles	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
3*LLOQ	0	0	0	0	0	0	0
3*LLOQ	1	-10.2	-0.02	-9.05	-3.01	6.61	37.4
3*LLOQ	2	-11.5	2.24	-18.4	-12.5	30.1	63.1
3*LLOQ	3	-9.32	-0.48	-12.6	1.31	22.1	-6.40
MID	0	0	0	0	0	0	0
MID	1	0.62	-9.41	-1.55	10.6	2.50	-5.50
MID	2	0.22	-11.1	0.29	-0.25	-9.08	-5.94
MID	3	-6.98	-7.13	-2.44	-3.09	-0.39	5.35
0.75*ULOQ	0	0	0	0	0	0	0
0.75*ULOQ	1	4.04	2.33	1.05	-0.07	10.4	7.66
0.75*ULOQ	2	4.99	1.53	-0.54	-1.12	9.51	10.5
0.75*ULOQ	3	4.09	-5.78	2.13	-0.53	3.86	2.46
B: RBC [%bias]	# cycles	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
3*LLOQ	0	0	0	0	0	0	0
3*LLOQ	1	-6.15	7.45	3.65	-9.60	-3.29	-14.3
3*LLOQ	2	30.4	-6.21	14.0	3.81	16.20	12.8
3*LLOQ	3	28.1	6.64	35.1	13.2	7.83	24.3
MID	0	0	0	0	0	0	0
MID	1	-16.2	-1.31	-0.11	-4.54	0.26	1.01
MID	2	-2.91	-1.66	0.17	1.77	11.14	6.82
MID	3	0.05	-2.84	0.64	-0.82	9.27	-3.81
0.75*ULOQ	0	0	0	0	0	0	0
0.75*ULOQ	1	-3.77	3.17	3.73	-3.52	-0.04	0.31
0.75*ULOQ	2	-3.58	7.30	5.96	-4.87	9.89	11.1
0.75*ULOQ	3	4.26	-0.60	2.18	-2.02	1.62	0.14
RBC extract	0	<LLOQ	0	0	0	0	<LLOQ
RBC extract	1	<LLOQ	2.78	-4.28	-4.30	10.70	<LLOQ
RBC extract	2	<LLOQ	2.23	2.30	-17.2	1.37	<LLOQ
RBC extract	3	<LLOQ	-1.41	0.05	-16.2	-7.54	<LLOQ

Table V.9: Stability results obtained for 1 to 3 freeze-thaw cycles. Percent bias is expressed relative to the average value (n=2) obtained without any freeze-thaw cycle. The RBC extract was obtained from a healthy volunteer. LLOQ: lower limit of quantitation; MID: intermediate concentration; ULOQ: upper limit of quantitation.

5.3. Application

In the plasma certified reference material (NIBSC 03/178), analyzed in sevenfold, only 5MeTHF could be detected and quantified in a concentration of 11.1 nM (SD: 0.233 nM). While this concentration is slightly higher than that reported by the NIBSC and by Fazili *et al*. (Fazili *et al*. 2013) (resp. 13.8 and 7.8%), the total folate concentration measured is slightly lower than that reported by the NIBSC (8.3%). Also, Fazili *et al*. (Fazili *et al*. 2013) measured higher total folate (15.9%), reporting on the presence of FA and MeFox (an oxidation product of 5MeTHF) as well (Table V.10). There is no real discrepancy between our results and those of Fazili *et al*. First, although the FA concentration reported by these authors is low, lying between the LOD and LLOQ of our method, we did not detect FA. We cannot exclude that slight differences in FA formation during sample processing may account for this. Second, MeFox was not included in our method given the lack of commercially available reference standards. Also, MeFox was not detected in samples using the mass transitions described for this compound.

For RBCs, peak areas are back-calculated to the folate concentration in the reconstituted reference material. THF, 5MeTHF and 5,10CH$^+$THF could be quantified in the reference material. Also FA and 10FoFA were present, with peak areas between the LOD and LLOQ, while no 5FoTHF was detected. The sum of the individual folates only exceeds the total folate measurement reference value set by the NIBSC by 6.0%. All values can be found in Table V.10.

		THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	*5FoTHF	*MeFox	sum
plasma	Average (n=7) [nM]	<LOD	<LOD	11.1	<LOD	<LOD	<LOD	-	11.1
	SD [nM]	-	-	0.233	-	-	-	-	0.233
	CV [%]	-	-	2.09	-	-	-	-	2.10
	value NIBSC [nM]	-	0.74	9.75	-	-	1.59	-	12.1
	deviation [%]	-	-	13.8	-	-	-	-	8.26
	Fazili et al. [nM]	-	0.66	10.3	-	-	-	2.24	13.2
	deviation [%]	-	-	7.77	-	-	-	-	15.9
RBC	Average (n=2) [nM]	0.820	<LLOQ	28.8	0.400	<LLOQ	<LOD	-	30.0
	SD [nM]	0.029	-	0.387	0.017	-	-	-	0.433
	CV [%]	3.54	-	1.34	4.25	-	-	-	1.44
	value NIBSC [nM]	-	-	-	-	-	-	-	28.3
	deviation [%]	-	-	-	-	-	-	-	6.01

Table V.10: Results obtained from the analysis of the NIBSC reference materials (Vitamin B12 and Serum Folate - 03/178 and Folate in Whole Blood Hemolysate - 95/528) and comparison with reference values. *Since not all LC-MS/MS methods for 5FoTHF and MeFox achieve a complete separation of these compounds, it is possible that the 5FoTHF measured in the reference material is actually MeFox.(Fazili *et al.* 2013) CV: coefficient of variation; LOD: limit of detection; LLOQ: lower limit of quantitation; SD: standard deviation.

The method was tested on blood samples obtained from 6 healthy volunteers (4 male and 2 female) after overnight fasting. Results are depicted in Figures V.4 and V.5. The total plasma folate concentration ranged from 6.30 to 16.0 nM. The main folate form was 5MeTHF, minor folate forms exceeding the LLOQ were 10FoFA, THF and FA.

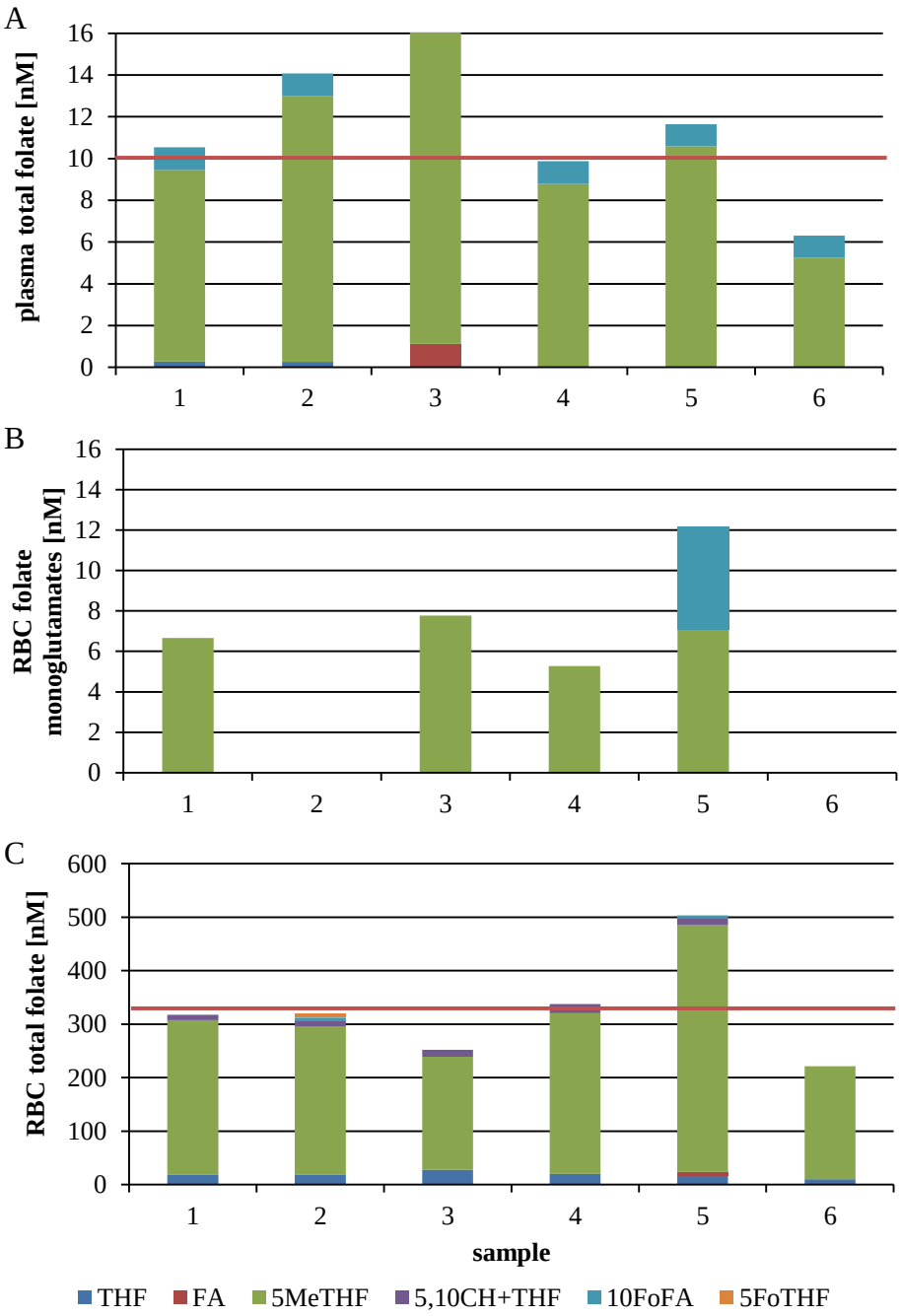


Figure V.4: Results following the analysis of folates in plasma and red blood cells (RBCs) in samples obtained from 6 healthy volunteers. Values lower than the lower limit of quantitation are not depicted. Threshold for folate deficiency (plasma: 10 nM and RBC total folate: 340 nM; see I.3.2) is indicated by a red line.

In only four out of six subjects, RBC folate monoglutamates exceeded the LLOQ, 5MeTHF being the major folate species and THF being quantifiable in only 1 sample. Total folate concentrations in RBCs ranged from 222 to 504 nM. Similarly to plasma, 5MeTHF is the major folate species in RBCs, while THF and 5,10CH^{+}THF are present in minor amounts. FA, 10FoFA and 5FoTHF could also be quantified in some samples. Based on the total folate concentration and the concentration of folate monoglutamates, the degree of polyglutamylation of RBC folates was calculated, which appeared to be fairly constant in samples obtained from these volunteers, ranging from 96.9 to 98.4% (n = 4).

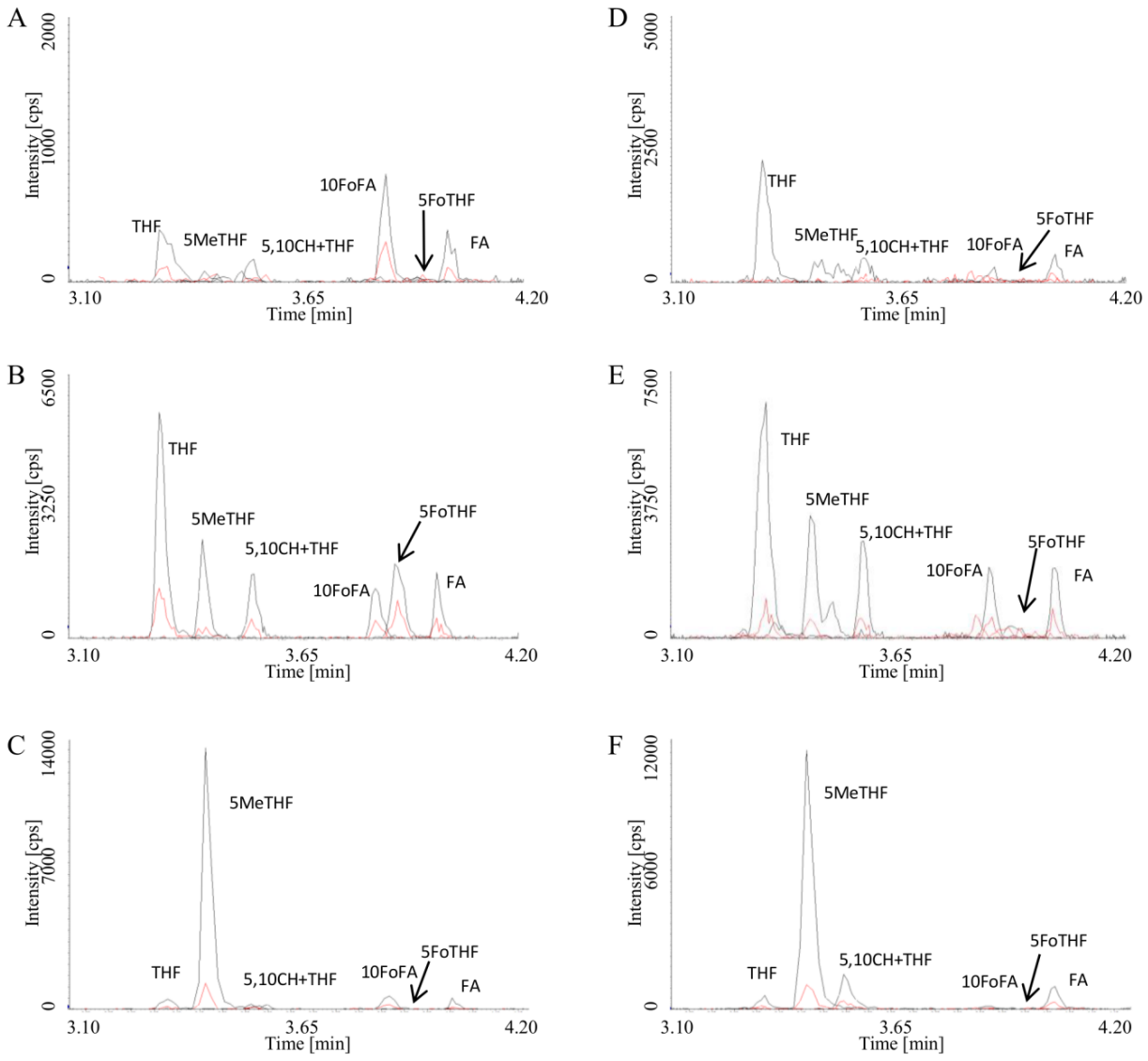


Figure V.5: UPLC®-MS/MS chromatogram (A) of a stripped plasma sample, (B) a stripped plasma sample spiked at lower limit of quantitation (LLOQ) level, (C) a sample of a healthy volunteer, (D) a stripped red blood cell (RBC) sample, (E) a stripped RBC sample spiked at LLOQ level, (F) a RBC sample of a healthy volunteer.

V.6. Conclusion

Due to the similarity between the plasma and RBC analyses, these can be performed simultaneously, thus optimizing workload and sample throughput while minimizing the consumption of solvents and consumables.

Starting from a single sample of 300 µl EDTA-anti-coagulated whole blood, the fully validated analytical method presented here allows to quantitatively determine 6 folate species in plasma and RBC extracts. The validation data showed linearity of the method from 1 to 150 nM for plasma samples and 5 to 1500 nM for RBC samples, encompassing the clinically relevant ranges. Our method includes selective and reproducible ammonia-based RBC lysis, the addition of DTT to all solvents and acidic protein precipitation. In RBCs, both free folate monoglutamates and total folate (monoglutamates + polyglutamates) can be quantified. From this, the degree of polyglutamylation can be calculated. This may provide information on the efficacy of the FPGS enzyme responsible for the intracellular accumulation of folate polyglutamates. Our method can also be used to identify individuals with a polymorphism in the gene encoding the methylenetetrahydrofolate reductase enzyme, based on the determination of individual folate species. A significant difference in folate speciation has been observed in blood samples obtained from these individuals.(Bagley and Selhub 1998) For instance, individuals homozygous for the 677T polymorphism will have lower 5MeTHF concentrations and higher THF and 5,10CH^{+}THF concentrations compared to heterozygotes and 677C homozygotes.

V.7. References

Alter, B.P., Metzger, J.B., Yock, P.G., Rothchild, S.B. and Dover, G.J. 1979. "Selective Hemolysis of Adult Red Blood Cells: An Aid to Prenatal Diagnosis of Hemoglobinopathies." *Blood* 53 (2): 279–87.

Arias, M., Quijano, J.C., Haridas, V., Gutterman, J.U. and Lemeshko, V.V. 2010. "Red Blood Cell Permeabilization by Hypotonic Treatments, Saponin, and Anticancer Avicins." *BBA-Biomembranes* 1798 (6): 1189–96.

Bagley, P.J., and Selhub, J. 1998. "A Common Mutation in the Methylenetetrahydrofolate Reductase Gene Is Associated with an Accumulation of Formylated Tetrahydrofolates in Red Blood Cells." *Proc Natl Acad Sci USA* 95 (22): 13217–20.

Bertino, J.R., Simmons, B. and Donohue, D.M. 1962. "Purification and Properties of the Formate-Activating Enzyme from Erythrocytes." *J Biol Chem* 237: 1314–8.

Chernyshev, A.V., Tarasov, P.A., Semianov, K.A., Nekrasov, V.M., Hoekstra, A.G. and Maltsev, V.P. 2008. "Erythrocyte Lysis in Isotonic Solution of Ammonium Chloride: Theoretical Modeling and Experimental Verification." *J Theor Biol* 251 (1): 93–107.

Chi, L.M. and Wu, W.G. 1991. "Mechanism of Hemolysis of Red Blood Cell Mediated by Ethanol." *Biochim Biophys Acta* 1062 (1): 46–50.

Crider, K.S., Yang, T.P., Berry, R.J. and Bailey, L.B. 2012. "Folate and DNA Methylation: A Review of Molecular Mechanisms and the Evidence for Folate's Role." *Adv Nutr* 3 (1): 21–38.

De Brouwer, V., Zhang, G.F., Storozhenko, S., Van Der Straeten, D. and Lambert, W.E. 2007. "pH Stability of Individual Folates during Critical Sample Preparation Steps in Prevision of the Analysis of Plant Folates." *Phytochem Anal* 18 (6): 496–508.

De Brouwer, V., Storozhenko, S., Van De Steene, J.C., Wille, S.M.R., Stove, C.P., Van Der Straeten, D. and Lambert, W.E. 2008. "Optimisation and Validation of a Liquid Chromatography–tandem Mass Spectrometry Method for Folates in Rice." *J Chromatogr A* 1215 (1–2): 125–32.

European Medicines Agency. 2011. "Guideline on Bioanalytical Method Validation." Edited by Committee for Medicinal Products for Human Use (CHMP).

Fazili, Z., and Pfeiffer, C.M. 2004. "Measurement of Folates in Serum and Conventionally Prepared Whole Blood Lysates: Application of an Automated 96-Well Plate Isotope-Dilution Tandem Mass Spectrometry Method." *Clin Chem* 50 (12): 2378–81.

Fazili, Z., Pfeiffer, C.M., Zhang, M., and Jain, R. 2005. "Erythrocyte Folate Extraction and Quantitative Determination by Liquid Chromatography-Tandem Mass Spectrometry: Comparison of Results with Microbiologic Assay." *Clin Chem* 51 (12): 2318–25.

Fazili, Z., Whitehead, R.D.Jr., Paladugula, N. and Pfeiffer, C.M. 2013. "A High-Throughput LC-MS/MS Method Suitable for Population Biomonitoring Measures Five Serum Folate Vitamers and One Oxidation Product." *Anal Bioanal Chem* 405 (13): 4549–60.

Garbis, S.D., Melse-Boonstra, A., West, C.E. and van Breemen, R.B. 2001. "Determination of Folates in Human Plasma Using Hydrophilic Interaction Chromatography-Tandem Mass Spectrometry." *Anal Chem* 73 (22): 5358–64.

Hannisdal, R., Ueland, P.M. and Svardal, A. 2009a. "Liquid Chromatography-Tandem Mass Spectrometry Analysis of Folate and Folate Catabolites in Human Serum." *Clin Chem* 55 (6): 1147–54.

Hannisdal, R., Ueland, P.M., Eussen, S.J., Svardal, A. and Hustad, S. 2009b. "Analytical Recovery of Folate Degradation Products Formed in Human Serum and Plasma at Room Temperature." *J Nutr* 139 (7): 1415–8.

Herzfeld, J., Seidel, N.E., Taylor, M.P., Droupadi, P.R. and Wang, N.E. 1990. "Gentle Chemical Deoxygenation of Hemoglobin Solutions." *Hemoglobin* 14 (4): 399–411.

Jagerstad, M. and Jastrebova, J. 2013. "Occurrence, Stability, and Determination of Formyl Folates in Foods." *J Agric Food Chem* 61 (41): 9758–68.

Kamen, B.A., and Caston, J.D. 1986. "Properties of a Folate Binding Protein (FBP) Isolated from Porcine Kidney." *Biochem Pharmacol* 35 (14): 2323–9.

Kiekens, F., Blancquaert, D., Devisscher, L., Van Daele, J., Stove, V.V., Delanghe, J.R., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015. "Folates from Metabolically Engineered Rice: A Long-Term Study in Rats." *Mol Nutr Food Res* 59 (3): 490-500.

Kirsch, S.H., Herrmann, W., Geisel, J. and Obeid, R. 2012. "Assay of Whole Blood (6S)-5-CH3-H4folate Using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry." *Anal Bioanal Chem* 404 (3): 895–902.

Laird, P.W. and Jaenisch, R. 1996. "The Role of DNA Methylation in Cancer Genetic and Epigenetics." *Annu Rev Genet* 30: 441–64.

Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. 2003. "Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS." *Anal Chem* 75 (13): 3019–30.

Monch, S., Netzel, M., Netzel, G. and Rychlik, M. 2010. "Quantitation of Folates and Their Catabolites in Blood Plasma, Erythrocytes, and Urine by Stable Isotope Dilution Assays." *Anal Biochem* 398 (2): 150–60.

Nelson, B.C., Pfeiffer, C.M., Margolis, S.A. and Nelson, C.P. 2004. "Solid-Phase Extraction-Electrospray Ionization Mass Spectrometry for the Quantification of Folate in Human Plasma or Serum." *Anal Biochem* 325 (1): 41–51.

Owen, W.E. and W.L. Roberts. 2003. "Comparison of Five Automated Serum and Whole Blood Folate Assays." *Am J Clin Pathol* 120 (1): 121–6.

Pfeiffer, C.M., Zhang, M., Lacher, D.A., Molloy, A.M., Tamura, T., Yetley, E.A., Picciano, M.F. and Johnson, C.L. 2011. "Comparison of Serum and Red Blood Cell Folate Microbiologic Assays for National Population Surveys." *J Nutr* 141 (7): 1402–9.

Philpott, N., Kelleher, B.P., Smith, O.P. and O'Broin, S.D. 2001. "High Serum Folates and the Simplification of Red Cell Folate Analysis." *Clin Lab Haematol* 23 (1): 15–20.

Shane, B. 2010. "Folate Chemistry and Metabolism." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 1–24. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Smith, D.E., Kok, R.M., Teerlink, T., Jakobs, C. and Smulders, Y.M. 2006. "Quantitative Determination of Erythrocyte Folate Vitamer Distribution by Liquid Chromatography-Tandem Mass Spectrometry." *Clin Chem Lab Med* 44 (4): 450–9.

Vuorte, J., Jansson, S.E. and Repo, H. 2001. "Evaluation of Red Blood Cell Lysing Solutions in the Study of Neutrophil Oxidative Burst by the DCFH Assay." *Cytometry* 43 (4): 290–6.

Wang, C., Riedl, K.M. and Schwartz, S.J. 2010. "A Liquid Chromatography-Tandem Mass Spectrometric Method for Quantitative Determination of Native 5-Methyltetrahydrofolate and Its Polyglutamyl Derivatives in Raw Vegetables." *J Chromatogr B* 878 (29): 2949–58.

Wille, S.M.R., Peters, F.T., Di Fazio, V. and Samyn, N. 2011. "Practical Aspects Concerning Validation and Quality Control for Forensic and Clinical Bioanalytical Quantitative Methods." *Accredit Qual Assur* 16 (6): 279–92.

Wright, A.J., Finglas, P.M. and Southon, S. 1998. "Erythrocyte Folate Analysis: A Cause for Concern?" *Clin Chem* 44 (9): 1886–91.

Wright, A.J., Finglas, P.M. and Southon, S. 2000. "Erythrocyte Folate Analysis: Saponin Added during Lysis of Whole Blood Can Increase Apparent Folate Concentrations, Depending on Hemolysate pH." *Clin Chem* 46 (12): 1978–86.

Yetley, E.A., Pfeiffer, C.M., Phinney, K.W., Fazili, Z., Lacher, D.A., Bailey, R.L., Blackmore, S. *et al*. 2011. "Biomarkers of Folate Status in NHANES: A Roundtable Summary." *Am J Clin Nutr* 94 (1): 303S – 12S.

Zeisel, S.H. 2009. "Importance of Methyl Donors during Reproduction." *Am J Clin Nutr* 89 (2): 673S – 7S.

Chapter VI: Rodent feeding trial of folate rice

Based on:

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Author contributions

FK, CPS and WEL designed the research; FK, LD, VS and JRD conducted the research; DB and DVDS provided essential materials; FK analyzed the data and conducted statistical analysis; JVD reviewed the data; FK and CPS wrote the paper, which was revised by DVDS, JVD, WEL, LD, VS and JRD; FK and CPS are responsible for the final content.

Table of contents

VI.1. Abstract

The biological impact of folates from folate rice, a metabolically engineered (biofortified) rice line, rich in folates, was investigated. Its consumption may be helpful to fight folate deficiency. Our objective was to investigate the potential of folate rice to supply the organism with folates and evaluate its biological effectiveness using a rat model.

Five groups of 12 Wistar rats were monitored during a 7/12-week depletion/repletion trial. Animals receiving folate-free diet (0 µg/rat/day) and those additionally receiving wild type rice (on average 0.11 µg/rat/day) suffered from decreased hematocrit and lower folate concentrations in both plasma and red blood cells (RBCs). This resulted in serious morbidity and even lethality during the trial. In contrast, all animals receiving a daily supplement of folate rice or folic acid fortified rice (on average 3.00 µg/rat/day and 3.12 µg/rat/day, respectively) and those receiving a positive control diet (11.4 to 25.0 µg/rat/day), survived. In these groups, the hematocrit normalized, plasma and RBC folate concentrations increased and pronounced hyperhomocysteinemia was countered.

Using an animal model, we demonstrated that biofortified folate rice is a valuable source of dietary folate, as evidenced by folate determination in plasma and RBCs, the alleviation of anemia and counteraction of pronounced hyperhomocysteinemia.

VI.2. Introduction

In 2007, our research consortium presented a metabolically engineered rice variety, known as folate rice, which contains approximately a hundredfold the folate content of wild type rice.(Storozhenko *et al*. 2007) Locally grown folate rice could be a valuable asset to reduce the health burden caused by folate deficiency in poor rural regions where other ways of sustained supplementation are difficult to implement. These regions, like the Shanxi province of China, are confronted with a prevalence of neural tube defects (60.9/10 000 live births; 38% folate deficient individuals) far above that found in more developed regions (±2.9/10 000 live births; <10% folate deficient individuals).(De Steur *et al*. 2010; De Steur *et al*. 2012) In 2010, De Steur *et al.* theoretically investigated the potential health impact and willingness to accept folate rice in China and found that the implementation of this modified crop could have a positive effect on the health and economic burden of folate deficiency. However, an improved nutrient status of a consumer cannot be simply inferred from the intake of food with an increased nutrient content. First, the biological effectiveness of this genetically modified crop needs to be demonstrated, which is a pivotal step towards public acceptance.(De Steur *et al*. 2010; Jeong and Guerinot 2008)

Several studies have investigated the potential health benefits of genetically modified crops with modified nutritional characteristics as functional food. Best known and most thoroughly studied is Golden Rice, in which genes are expressed that lead to a higher provitamin A content.(Beyer *et al*. 2002) A human trial has been conducted with this rice variety, demonstrating bio-accessibility and bio-availability (via retinol in plasma) in human volunteers.(Tang *et al*. 2009) In 2005, Haas *et al*. investigated the potential of a high-iron rice variety to improve the iron intake of Filipino women by measuring serum-ferritin, total body iron and hemoglobin during a 9-month trial with modest results.(Haas *et al*. 2005) Furthermore, Morris *et al*. investigated whether the increase of calcium accumulation in carrots leads to an increase in calcium intake upon consumption in both humans and mice.(Morris *et al*. 2008) Recently, Castorena-Torres *et al*. reported on the folate bio-availability from lyophilized biofortified tomatoes using a murine model, making a comparison with orally dosed synthetic 5-methyltetrahydrofolate (5MeTHF) and folic acid (FA). These authors found that folates from tomato had a comparable bioavailability as synthetic 5MeTHF but were absorbed more slowly into the bloodstream than synthetic FA. FA was also found to be less bioavailable (±50%). (Castorena-Torres *et al*. 2014) However, although demonstrating a proof-of-principle of bio-availability, only one single administration was performed, with only a 12-h follow-up. Much more relevant to support a potential benefit for human health is a long term evaluation, in which repeated administration of biofortified food is performed, meanwhile periodically evaluating the long term RBC folate status, as well as the short term folate status in plasma.(Shane 2010; Visentin *et al*. 2014) This is only possible in a long term study spanning at least one full RBC life cycle, which in rats takes about 67 days.(Kurata *et al*. 1993)

Since folates play a key role in the methylation cycle and the formation of nucleotides, indicators of the disruption of these processes may provide additional information on the folate status of a subject. As folate deficiency has an impact on the cell cycle in any given cell, it also impacts erythropoiesis, leading to anemia.(Koury 2014) Folate deficiency also leads to an increased plasma concentration of homocysteine, as its conversion to methionine is folate-dependent.(Ward *et al*. 1997; Glier *et al*. 2014)

VI.3. Objectives

The aim of this study was to investigate the effectiveness of folates present in genetically engineered folate rice to serve as a supply of folates for the organism. To this end, we analyzed folates in RBCs and plasma and measured hematocrit, overall blood cell profile and homocysteine as indirect markers of folate status during a 7/12-week depletion/repletion feeding trial using rats as a model organism.

VI.4. Materials and methods

4.1. Reagents and materials

The metabolically engineered rice or folate rice used in this study was created in the Nipponbare variety. It was generated by introducing into the rice genome transgenes encoding Arabidopsis GTP cyclohydrolase I (GTPCHI) and aminodeoxychorismate synthase (ADCS) from a single T-DNA locus, hence the acronym GA rice.(Storozhenko *et al*. 2007) This leads to an increased production of the folate building blocks deoxyneopterin and p-aminobenzoic acid, resulting in a hundredfold increased folate concentration over wild type rice seeds. The folate rice used in this study contained on average 1563 µg folates/100g, of which 92% was 5MeTHF, while only 1% was FA (Table VI.1).

To prepare FA fortified rice, wild type rice seeds of the Nipponbare variety underwent a parboiling process in which these seeds were soaked in a FA (Sigma-Aldrich, Schnelldorf, Germany) solution of approximately 120 µg/ml at 70°C for one hour with regular mixing.(Kam *et al*. 2012) After filtration, the rice seeds were spread over a large sheet of tinfoil and left to dry in darkness for two days at room temperature. Thereafter, the seeds were stored at -80°C prior to analysis and the preparation of aliquots for feeding. This FA fortified rice contained on average 1385 µg folates/100g, of which 99% was FA, while only 0.5% was 5MeTHF (Table VI.1).

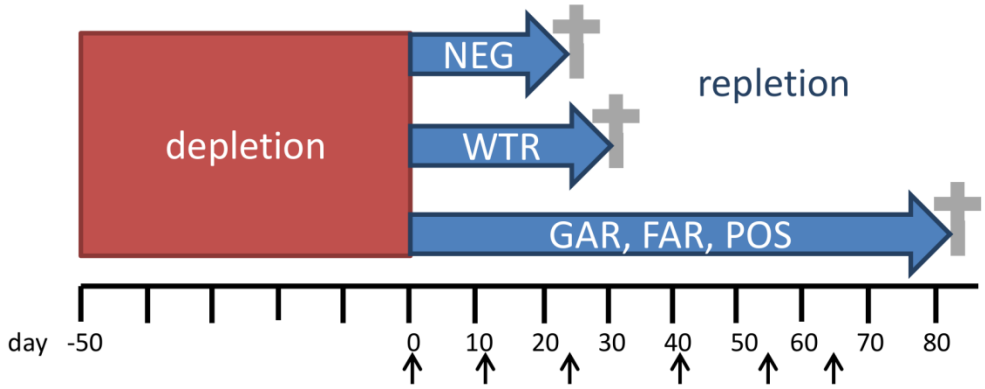
All experiments were approved by the ethical committee of Ghent University (ECD 09/28). Sixty female Wistar Han rats were obtained from Harlan Laboratories (Horst, The Netherlands). These were 3 to 4 weeks old at the start of the experiment. The animals were randomly housed in a room maintained at 23°C and 40% humidity on a 12-h day/night-cycle.

aliquot	daily ration of rice [mg]	folate concentration in rice [µg/100g]						
		THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF	total
WTR1	244	1.0	4.2	19.7	1.7	3.5	16.0	46.0
WTR2	311	1.4	1.6	15.5	1.0	3.7	10.1	33.4
WTR3	232	1.4	3.2	18.8	1.5	3.6	15.1	43.7
average	267	1.3	3.0	18.0	1.4	3.6	13.7	45.0
%/total	/	3.1	7.3	43.9	3.4	8.8	33.5	100
GAR1	230	12.6	15.6	1197	5.7	49.3	32.7	1313
GAR2	221	13.6	10.5	1290	6.3	26.8	26.5	1373
GAR3	208	5.8	15.8	1283	7.2	57.6	67.4	1437
GAR4	164	13.5	13.5	1713	13.8	37.7	39.2	1830
GAR5	173	5.4	18.2	1595	10.7	44.6	60.6	1734
GAR6	176	6.6	15.1	1534	6.1	51.4	74.0	1688
average	197	9.6	14.8	1435	8.3	44.6	50.1	1563
%/total	/	0.6	0.9	91.9	0.5	2.9	3.2	100
FAR1	260	0.2	1139	1.6	0.4	4.7	4.9	1151
FAR2	478	0.1	600.8	7.1	0.5	5.3	7.0	621
FAR3	245	0.1	1196	6.3	0.7	5.6	4.7	1213
FAR4	290	0.1	964.3	3.8	0.3	5.7	13.5	988
FAR5	168	0.7	1802	9.4	1.0	4.8	10.8	1829
FAR6	120	0.1	2488	10.7	0.7	4.2	5.7	2509
average	220	0.2	1365	6.5	0.6	5.1	7.8	1385
%/total	/	<0.1	98.6	0.5	<0.1	0.4	0.6	100

Table VI.1: Folate concentrations in different batches of rice used in the feeding trial. GAR; folate rice group; FAR: folic acid fortified rice group; WTR: wild type rice group

During the 7-week folate depletion phase, chosen to sufficiently reduce effects of previous exposure to a diet rich in folates, animals were housed socially with 6 rats per cage. All animals were fed a folate depleted diet containing 1% succinylsulfathiazole (a sulphonamide, inhibiting folate biosynthesis) to limit the release of folates from the intestinal microbiota (TD.06691, Harlan Laboratories).

At the start of the repletion phase, a block randomization was performed to create 5 treatment groups of 12 animals each. The animals were housed individually to monitor feed intake on a daily basis and to ensure full consumption of administered rice. The negative control group (NEG) continued to receive the negative control diet including succinylsulfathiazole and served as a control for a prolonged lack of folate intake. Prior to euthanasia of the negative control group, pair feeding was maintained: the consumption of feed, provided to this group *ad libitum*, gradually decreased, and determined the amount of feed provided to the other groups, with a minimum of 6 g/animal/day. After euthanasia of the negative control group (from day 24 on), the remaining animals received a restricted amount of diet of approximately 12 g/animal/day (Figure VI.1).



group	diet	rice	average folate intake [µg/day - µg/kg feed/day]
NEG	folate depleted	–	0
WTR	folate depleted	wild type rice	0.11 – 14.1
GAR	folate depleted	biofortified folate rice	3.00 – 311
FAR	folate depleted	folic acid fortified rice	3.12 – 313
POS	standard	–	20.4 – 2000

Figure VI.1: Experimental set-up: the different groups and different phases of the feeding trial are depicted. The crosses indicate the time of euthanasia, at which blood was sampled for analysis of folates in plasma and RBC, homocysteine and vitamin B12 measurement in plasma, hematocrit estimated via dried blood spots (DBS) and hematological analysis of whole blood. The arrows indicate time points at which tail vein blood was obtained for analysis of folates in plasma and RBC and hematocrit prediction via DBS. NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

The wild type rice group (WTR) received the same depleted diet including succinylsulfathiazole. However, these animals additionally received a daily amount of wild type rice (on average 267 mg rice/day or 13 grains/day providing 0.11 µg folate/animal/day) to account for any compound present in the wild type rice elucidating an unknown effect. The actual amount of rice provided to these animals depended on the rice intake of the groups receiving folate rice or folic acid fortified rice (see further) and was calculated as the daily average rice supplementation of these groups. In addition to the folate depleted diet including succinylsulfathiazole, the folate rice group (GAR) additionally received on average 197 mg of folate rice per day (±10 grains), resulting in an intake of 3.00 µg folate/animal/day. Similarly, the folic acid fortified rice group (FAR) continued to receive the folate depleted diet including succinylsulfathiazole and on average 220 mg folic acid fortified rice per day (±11 grains). This resulted in an average folate intake of 3.12 µg/animal/day. As such, the FAR group serves as a control group closely related to the GAR group, in that these animals receive a supplementation of folic acid in an amount nearly identical to that received by the GAR group and also receive intact rice grains. The amount of rice provided to the animals was determined

by three aspects: i) the amount of rice that can be grown on a lab scale is limited; ii) this amount should be sufficient to maintain a viable folate status(Clifford *et al*. 1989; Bailey and Gregory 3rd 1999; House *et al*. 2003); iii) administration of too high amounts may mask subtle differences between groups. From the start of the repletion phase on, the positive control group (POS) received a rodent diet containing 2000 µg folic acid/kg feed resulting in a folate intake of 11.4 to 25.0 µg folate/animal/day, depending on the amount of feed provided and consumed, with an average folate intake of 20.4 µg/animal/day (see Table VI.2). While the FAR group served as a control group for the supplementation of a limited amount of folic acid through the feeding of fortified rice, the POS group served as a reference for the effects of a non-restricted folate intake. Due to the high amount of folate present in this positive control feed, these animals present the clinical parameters associated with an adequate folate status. Optimal erythropoiesis is expected to lead to a normal hematocrit while homocysteine concentrations should be fully reduced to baseline levels due to the high availability of 5-methyltetrahydrofolate in the circulation. Both the folate depleted diet and the positive control diet are standardized AIN-93G diets, similar in carbohydrate, fat and protein content. The letter G indicates that this diet is specially formulated to be used during the growth phase of the rodents' life.

Irrespective of feed limitations, wild type rice, folate rice and folic acid fortified rice were provided to the animals prior to providing the rest of the feed. Non-ground, intact grains were provided separately from the feed in a porcelain bowl to allow evaluation of the bio-accessibility of the rice folates. At all stages we visually confirmed that all animals fully consumed the rice grains provided.

4.2. Blood sampling

Blood sampling was performed in a random order after overnight fasting. After 50 days of folate depletion and at days 11, 24, 41, 55 and 65 of the supplementation period, blood samples of approximately 300 µl were obtained from the lateral tail vein under isoflurane anaesthesia using a 30G needle (BD Microlance™ 3) and a 2-ml disposable syringe (Terumo). Blood samples were immediately transferred to an amber-colored microcentrifuge tube containing 20 µl of a 6% EDTA solution (Sigma-Aldrich). At the time of euthanasia a 1-ml sample was obtained via cardiac puncture using an 18G needle (BD Microlance™ 3) and a 10-ml disposable syringe (Terumo). Blood samples were immediately transferred to a 5-ml tube containing 100 µl of a 6% EDTA solution. Samples were immediately stored on ice in a light-protecting box. At every time point, a dried blood spot (DBS) was collected on Whatman 903 filter paper (Novolab) after the needle was withdrawn from the vein, to determine the haematocrit (HCT) through potassium measurement.(Capiau *et al*. 2013) Figure VI.1 gives a schematic overview of the sampling schedule.

Group	NEG	WTR	GAR	FAR	POS
n (total)	12	12	12	12	12
n (at time of euthanasia)	8	9	12	12	12
animal weight at the start of the repletion period [g]	$204^{\pm 14\ a}$	$207^{\pm 13\ a}$	$199^{\pm 18\ a}$	$210^{\pm 16\ a}$	$211^{\pm 14\ a}$
animal weight at time of death [g]	$154^{\pm 19\ a}$	$175^{\pm 20\ a}$	$254^{\pm 17\ b}$	$253^{\pm 12\ b}$	$241^{\pm 16\ b}$
day of euthanasia (after the 50-day folate depletion period)	24	29	83	83	83
feed consumption on the second day of the repletion period [g/day]	$8.64^{\pm 1.38\ a}$	$9.15^{\pm 1.14\ a}$	$9.86^{\pm 0.18\ a}$	$9.53^{\pm 1.12\ a}$	$9.81^{\pm 0.29\ a}$
feed consumption two days prior to euthanasia [g/day]	$4.1^{\pm 2.7\ a}$	$5.4^{\pm 4.8\ a}$	$11.3^{\pm 1.1\ b}$	$11.7^{\pm 0.3\ b}$	$11.2^{\pm 1.1\ b}$
rice consumption entire supplementation period [mg/day] (min – max)	/	267 (224 - 361)	197 (156 - 243)	221 (112 - 487)	/
folate intake entire supplementation period [µg/rat/day] (min – max)	0	$0.11^{\pm 0.01\ a}$ (0.075-0.120)	$3.00^{\pm 0.07\ b}$ (2.69-3.19)	$3.12^{\pm 0.78\ c}$ (2.34-3.32)	$20.4^{\pm 4.2\ d}$ (11.4-25.0)

Table VI.2: Key experimental parameters; Values indicate mean ± standard deviation; groups with shared letters are not significantly different; Daily feed consumption shown in Figure VI.2. Average folate intake during the entire supplementation period, expressed per kilogram feed, was calculated only when feed intake surpassed 6 g/day; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

4.3. Sample analysis

Plasma and RBC folates were analyzed using a validated in-house developed liquid chromatographic tandem mass spectrometric (LC-MS/MS) method.(Kiekens *et al*. 2015a; Chapter V) Briefly, 500 µl phosphate buffered saline (PBS) was added to 300 µl of whole blood. After centrifugation, the supernatant was collected and L-ascorbic acid, dithiothreitol and ^{13}C-labeled internal standards were added prior to storage at -80°C. The remaining cell pellet was washed three times with PBS after which the RBCs were selectively lyzed using 800 µl of an ammonium carbonate buffer. RBC extracts were treated with rat serum folate conjugase to convert folate polyglutamates to the corresponding monoglutamates. Sample clean-up was performed for both plasma and RBC extracts using reversed phase C_{18} solid phase extraction in a 96-well format. The purified samples were injected onto a Waters Acquity UHPLC system equipped with a Waters HSS T3 C_{18} column and a Vanguard guard-column. Detection of the 6 folate species (tetrahydrofolate, THF; folic acid, FA; 5-

methyltetrahydrofolate, 5MeTHF; 5,10-methenyltetrahydrofolate, 5,10CH^{+}THF; 10-formylfolic acid, 10FoFA and 5-formyltetrahydrofolate, 5FoTHF) was performed using an ABSciex API 4000^{TM} triple quadrupole mass spectrometer in scheduled MRM-mode. The method was validated based on EMA guidelines and the acceptance criteria specified therein. This validation encompassed selectivity, accuracy, precision, linearity, matrix effect and recovery, as well as long term stability during sample storage at -80°C and autosampler stability.

The folate content in both folate rice and FA fortified rice was verified with the same equipment as described above, using an existing validated LC-MS/MS method, with minor modifications.(De Brouwer *et al*. 2010) Four hundred rather than 200 units of α-amylase were added to further reduce the viscosity of the sample. In addition, scheduled multiple reaction monitoring (sMRM) was applied, allowing the acquisition of more data points per analyte. Although a presence, up to 10.9%, of 10-formyldihydrofolate was reported in white rice, its accurate quantification is not possible due to the lack of a pure reference standard.(Pfeiffer *et al*. 1997) When following the mass transitions of this compound, we could not detect it in wild type rice and folic acid fortified rice, while it was present at trace amounts (<1% of total folate) in GA rice. Folate concentrations in rodent diets were determined using the method described in Chapter IV.(Kiekens *et al.* 2015b)

Haematological analysis was performed using a Sysmex XE-5000 Automated Haematology System. Quantification of potassium in DBS extracts (which allows to estimate the HCT) was performed using a Cobas® 8000 Modular Analyser (Roche Diagnostics). Briefly, a 3-mm punch was collected and extracted twice with 50 µl of 2.5 mM of KCl. After centrifugation for 1 minute at 5000 g, respectively 40 and 50 µl of supernatant were collected into an injection cup. Calibrators were prepared by removal or addition of rat plasma, starting from a pool of rat whole blood with a HCT of 0.39, as verified by haematological measurement.(Capiau *et al*. 2013)

Homocysteine and vitamin B12 measurements were performed with a homocysteine Liquid Stable Reagent Kit enzymatic assay (Axis-Shield) and Vitamin B12 immunoassay (Roche Diagnostics) using a Modular-P and Modular Analytics E170 system (Roche Diagnostics), respectively. Because of the limited sample volume, all samples were diluted 3-fold with PBS prior to analysis.(Kellogg *et al*. 2005)

4.4. Statistical analysis

All statistical analyses were performed using IBM SPSS22. Multiple comparisons were performed using the Dunnett's T3 algorithm as nearly all groups show differences in sample size and variance. Results below the limit of quantification were not included into the statistical analysis. When less than 6 results remained, this was mentioned since this compromises the power of the statistics. The treatment groups were compared at the 5% significance level. Where possible the 95% confidence interval was indicated in the graphs.

All box-and-whisker plots, composed of the first, second (=median) and third quartiles as well as the minimal and maximal values, were made in Microsoft Excel®.

VI.5. Results and discussion

Folates present in the diet are transported from the small intestine to the portal circulation. Upon arrival in the liver, they are either polyglutamylated and stored in the hepatocytes or are released into the systemic circulation or the bile.(Steinberg *et al*. 1979) The latter causes an enterohepatic recirculation and reabsorption into the portal circulation.(Kurata *et al*. 1993) Every study evaluating biological efficacy of a food item in laboratory animals has the intrinsic shortcoming that animals can never fully replace humans. With respect to folates, a relevant difference is e.g. that rats release bile into the intestine through a bile duct, causing a difference in the enterohepatic recirculation. Also, in rats the activity of intestinal folate conjugase is lower than that observed in humans. However, since 90% of folates in folate rice are present as monoglutamates, the effect of this limitation in enzymatic deconjugation should be negligible.(Wang *et al*. 1985; McKillop *et al*. 2006; Storozhenko *et al*. 2007) In this study, using a folate-depleted rat model, we determined whether or not folates, present in folate rice are released in the gastrointestinal system and can reach the systemic circulation.

A first factor determining the biological availability of a compound is its bioaccessibility, or its availability for absorption in the GI-tract. This was nicely demonstrated for ferulic acid, the most abundant phenolic acid in wheat, which has very low bioaccessibility from wheat and derived products but high bioaccessibility when added in free form to e.g. flour.(Mateo Anson *et al*. 2009) In addition, in animal studies investigating the biological availability of a compound of interest from a natural crop, these crops are usually ground or lyophilized and the resulting powder is mixed into food pellets. This can possibly influence the liberation of nutrients through the destruction of the natural barriers. In the case of rice, the nutrients contained within the endosperm, such as folates, can easily be released when the rice is ground into a fine powder. This was clearly observed by O'Dea *et al*. in an *in vitro* digestibility study of ground and unground rice. These authors found that after 30 minutes of incubation with pancreatic amylase, ground polished rice was 71.8% hydrolyzed while unground polished rice was only 30.8% hydrolyzed. In unpolished rice, the difference was even greater with a respective hydrolysis rate of 68.2 and 17.6% of ground versus non-ground rice. Since folates are contained within the endosperm, or the starchy interior, of the rice grain, the use of ground rice would affect the speed by which folates are released from the grain.(O'Dea *et al*. 1981; Gregory 3rd 2001) To avoid this potential artefact in this study, we provided the animals with intact rice grains. Full consumption of these rice grains was observed before providing the daily amount of rodent diet to the animal. Following this procedure, the folates are absorbed from the rice grains in a more realistic way.

5.1. Food consumption, animal weight and general health status

Over the 50-day depletion period the animals consumed on average 12.1 grams of folate depleted rodent feed per day, with a minimum of 9.6 and a maximum of 13.8 g/animal/day. From 37 days of folate depletion on, a decreased feed intake was observed in all animals. This feed intake continued to decrease in the negative control and wild type rice group, with a respective average feed consumption of only 4.1 (day 22) and 5.4 (day 27) grams of feed per animal per day (Table VI.2, Figure VI.2). A paired feeding approach was applied, in which the negative control group, which consumed least feed -provided to this group *ad libitum*- determined how much feed the other groups received, with a minimum of 6 g/day. After euthanasia of the negative control group (day 24), the amount of diet given to the remaining animals was augmented again to 12 g/day per animal. The wild type rice group showed a transient increase in feed consumption, before a sharp drop shortly prior to euthanasia (day 29). In the other groups, the food was entirely consumed, although a slight decrease in food consumption was observed during the later stage of the study, possibly due to the fact that the animals neared their mature body weight while a growth diet was provided to them. Animal weight showed strong correlation with feed intake (Figures VI.2 and VI.3). At the time of euthanasia of the negative control group, the average weight of these animals had dropped more than 20%, from 204 to 160 grams (weight of animals that died spontaneously was not taken into consideration). Given the paired feeding approach, also all other groups had lost weight, although less pronounced. After euthanasia of the negative control group, the 12 gram/day diet per animal allowed the average bodyweight to increase gradually to approximately 250 grams at the end of the trial. Neither at the time of euthanasia of the wild type rice group, after 24 days of supplementation ($p \geq 0.06$), nor at the end of the trial, after 83 days of supplementation ($p \geq 0.18$), was there a significant difference in bodyweight of the animals assigned to different treatment groups.

On day 11 of supplementation the first visual symptoms of folate deficiency appeared. Animals from both the group receiving folate depleted diet and the group additionally receiving wild type rice suffered from hemorrhagic lesions at the mouth and front paw region, leading to lethality, starting at day 18. Seven animals died spontaneously during the trial: 4 in the negative control group and 3 in the wild type rice group. In some animals a lesion at the corners of the eyes was observed. For ethical reasons, to limit the distress of the animals, euthanasia was performed on day 24 for the negative control group and on day 29 for the animals receiving wild type rice.

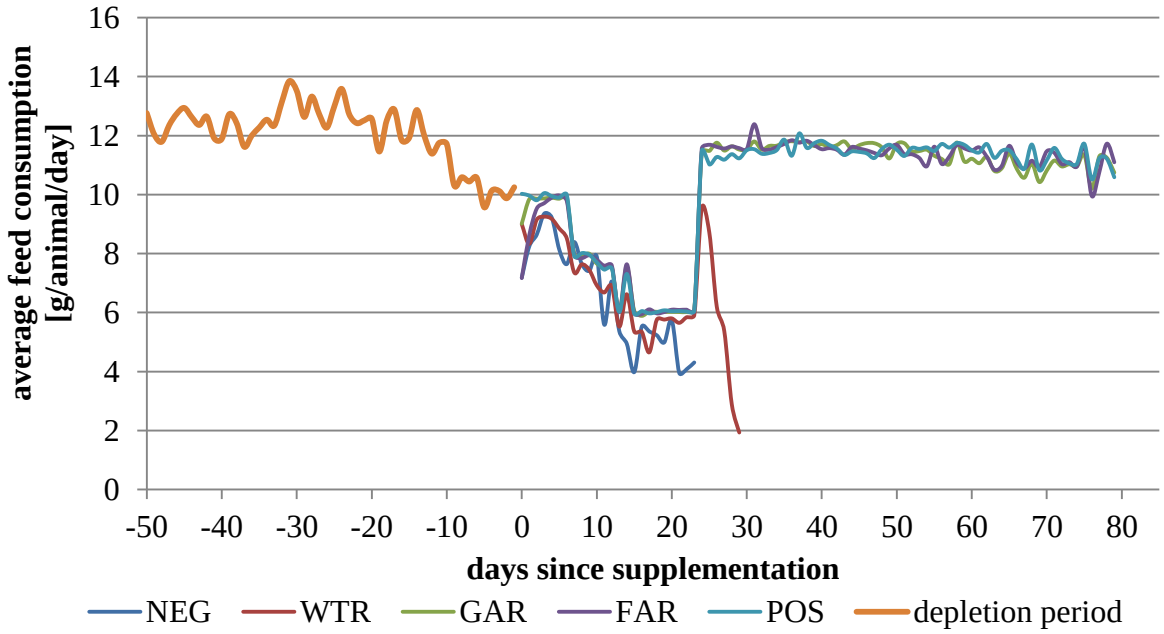


Figure VI.2: Average feed consumption per animal per day; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

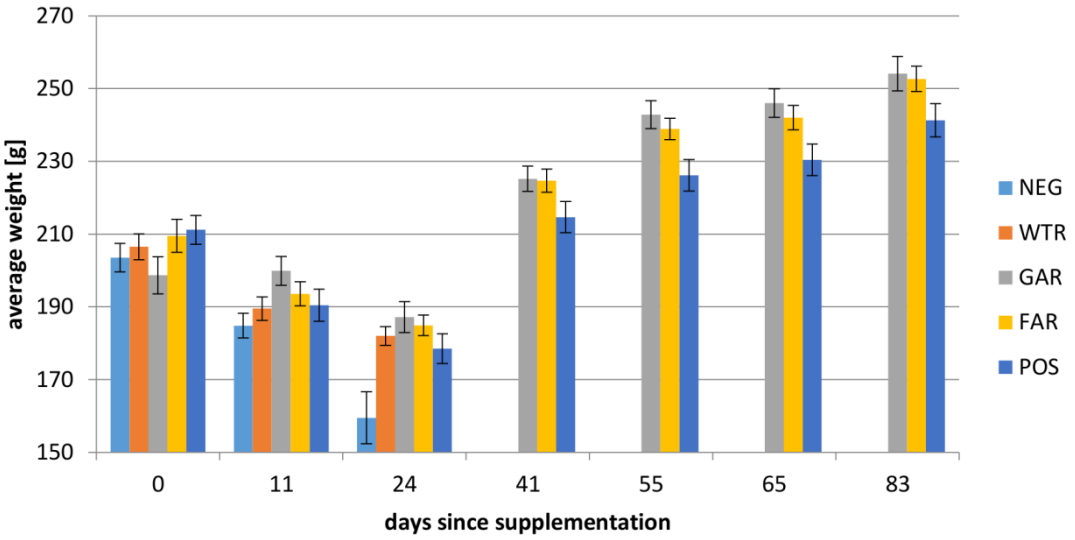


Figure VI.3: Average weight of the animals during the course of the feeding trial; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

Only animals in the negative control and wild type rice group suffered from hemorrhagic skin lesions and natural deaths, which can be linked to the pronounced folate deficiency. The feed intake of these groups gradually decreased down to 33% of the original feed intake. To avoid that differences between the distinct groups would merely reflect differential feed intake, we applied pair feeding, taking food consumption of the negative control group as the reference. With the exception of some animals from the wild type rice group, all other animals fully consumed the limited amount of feed offered to them through pair feeding. After euthanasia

of the negative control group, the amount of feed offered was increased to 12 g/animal/day, after which a steady increase in body weight was observed. Though folate intake is not directly related to animal weight, it can be concluded that severe folate deprivation has an impact on overall health status, which results in a reduced feed intake, leading to a decrease in body weight. These observations are consistent with previous reports on folate-depleted rats.(Aboko-Cole and Lee 1974; Clifford *et al*. 1989)

5.2. Folate status

At the start of the supplementation (i.e. after 50 days of folate depletion) there was no significant difference in RBC folate concentrations between the different treatment groups ($p=1.00$). However, readily at the first blood sampling (day 11), three distinct groups could be distinguished, based upon RBC folate concentrations (Figure VI.4A). In both the negative control group and the wild type rice group a significant decrease in folate status was observed ($p<0.01$), where after 24 days of supplementation, total folate concentrations had dropped to 122 and 172 nM, respectively. In contrast, RBC folate concentrations in the positive control group -receiving approximately 20 µg FA / day- rose to an average of 1093 nM after 24 days and continued to rise to 1884 nM at the end of the trial. In the groups receiving the minimal folate supplementation of approximately 3 µg folate/day, either via folate rice or via FA fortified rice, the total RBC folate concentrations on day 24 were 385 and 271 nM, respectively. For these two groups, RBC folate concentrations stabilized at the end of the trial, respectively yielding concentrations of 451 and 507 nM, with no significant difference between the two groups ($p=0.27$) (Table VI.3) .

group (n)	NEG (8)	WTR (9)	GAR (12)	FAR (12)	POS (12)
plasma folate [nM]	<LLOQ	<LLOQ	$5.4^{\pm1.2\ a}$	$10.1^{\pm2.4\ b}$	$137^{\pm11\ c}$
RBC folate [nM]	$122^{\pm49\ a}$	$143^{\pm33\ a}$	$451^{\pm66\ b}$	$507^{\pm94\ b}$	$1884^{\pm189\ c}$
homocysteine [µM]	$154^{\pm32\ a}$	$155^{\pm45\ a}$	$46.6^{\pm18.0\ b}$	$30.3^{\pm10.6\ b}$	$8.5^{\pm2.7\ c}$
vitamin B_{12} [ng/l]	$503^{\pm213\ a\,b}$	$448^{\pm96\ b}$	$741^{\pm105\ a}$	$691^{\pm121\ a}$	$804^{\pm131\ a}$

Table VI.3: Plasma and RBC analysis; Data obtained at the time of euthanasia; Values indicate mean ± standard deviation; groups with shared letters are not significantly different; <LLOQ, lower than 1 nM; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

When looking at the pattern of folates, 5MeTHF was the main folate species that could be quantified in RBC extracts, constituting 88-97% of total folates (Figure VI.5). At the time of euthanasia of the negative control group and wild type rice group, the amount of non-5MeTHF-folates had risen to 27.5 and 17.3%, respectively. In contrast, towards the end of the trial the fraction of non-5MeTHF-folates declined to 3.3% in the positive control group. In the FA fortified rice and folate rice groups, the non-5MeTHF fraction remained fairly constant throughout the trial and ranged from 6.1 to 9.9%, with a maximum value of 12.7% at 24 days of repletion. Apart from 5MeTHF also THF and $5,10CH^{+}THF$ could be consistently

quantified in RBCs, with respective concentrations of 6.2 to 24.4 and 6.7 to 45.7 nM. FA was only found up until 24 days since the start of the repletion, in concentrations ranging from 5.7 to 20.7 nM (Figure VI.5).

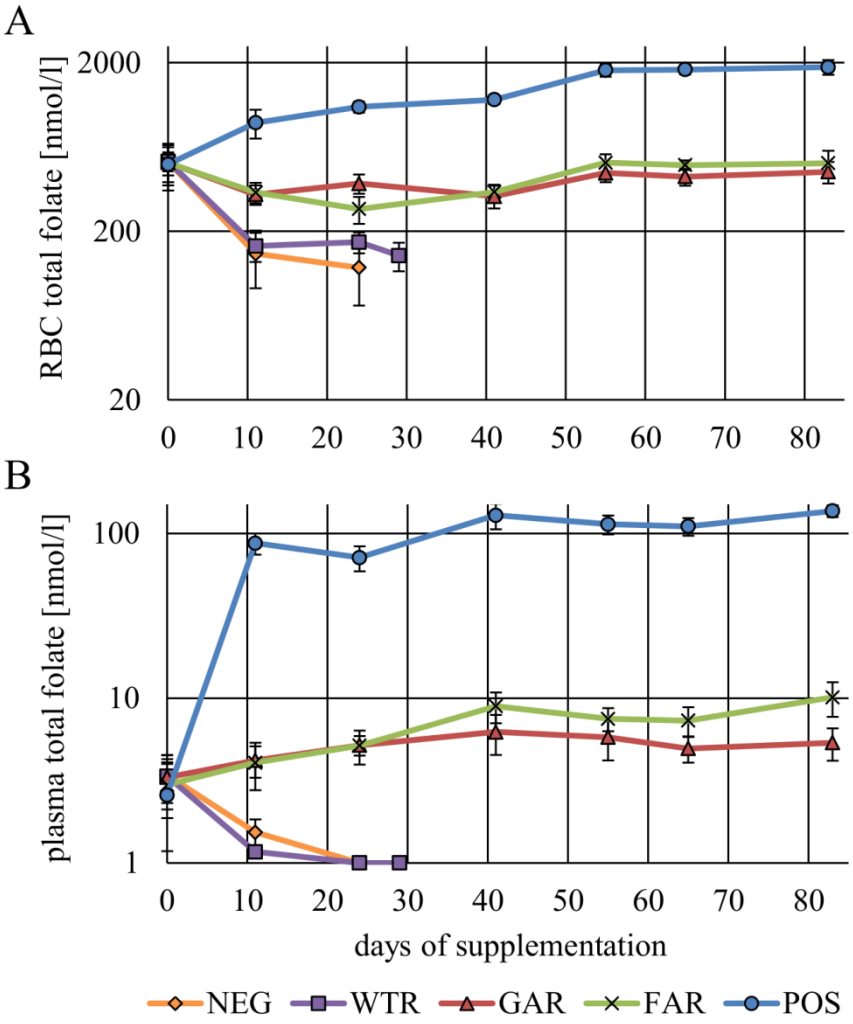


Figure VI.4: Panel A: RBC total folate; panel B: plasma total folate; both represented on a log-scale; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

Similar to the folate concentrations measured in RBC's, three response groups could be distinguished based on the plasma folate concentrations (Figure VI.4B). In both negative control and wild type rice groups the plasma folate concentration was halved after 11 days of rice feeding and was no longer quantifiable after 24 days. In contrast, plasma folate concentrations increased slowly in the groups receiving folate rice or FA fortified rice to respectively 5.2 and 5.1 nM after 24 days of supplementation, which were not significantly different ($p=1.00$). At the end of the trial, the plasma folate concentrations of these groups, resp. 5.4 and 10.1 nM, were significantly different ($p<0.01$) (Table VI.3). In the positive control group the plasma concentration increased rapidly to 71.2 nM after 24 days of supplementation and continued to rise to 137 nM at the end of the trial. During the study FA

could occasionally be quantified in samples of all groups, in a concentration ranging from 1.3 to 3.8 nM (Figure VI.6).

The plasma folate concentrations were clearly related with folate intake. Concentrations in both the negative control and wild type rice group continued to decline after the folate depletion phase of the trial. This shows that the limited amount of folates present in wild type rice is too low to serve as a considerable folate source, since no folates were measurable at the time of euthanasia. The positive control group did show a strong increase in plasma folate concentrations, as could be expected from the large amount of FA present in the diet. After 24 days, plasma folate concentrations in groups receiving folate rice and FA fortified rice were significantly different from both the positive control group, the negative control group and the group receiving wild type rice ($p<0.01$). At the end of the trial (day 83) there was also a significant difference between the groups receiving either folate rice or FA fortified rice, with an almost double plasma folate concentration (Figure VI.4B) in the latter group ($p<0.01$). Several reasons may underlie this result. First, a difference between the GAR and FAR groups could be explained by a somewhat lower bio-availability of natural folates.(Lewis *et al*. 1999) Second, a difference in kinetics between FA and 5MeTHF may also lead to this observation. As described in literature, there is a difference in absorption and absorption rate of FA and food-derived THF species: while FA requires reduction and methylation in the bowel epithelium or in the liver, 5MeTHF does not and is thus readily transferred to the systemic circulation.(Wright *et al*. 2005; Caudill 2010; Castorena-Torres *et al*. 2014) As a result, the plasma response to FA may be slower and, in our case, may still be increased to a higher extent at the time of sampling than the response following ingestion of natural folates (primarily 5MeTHF).(Wright *et al*. 2005) While cut-off values for rodents are lacking, Clifford *et al*. reported on animal weight and serum and liver folate concentrations. These authors found that 125 to 500 µg FA/kg diet resulted in serum folate concentrations of approximately 16 nM, somewhat higher than the concentrations we observed with GA or FA rice supplementation (resp. 5.4 and 10.1 nM), while a higher supplementation led to a drastic increase of the serum folate concentration, similar to what we observed in the positive control group.(Clifford *et al*. 1989) Although FA in serum has been reported when high amounts of FA are consumed (Kelly *et al*. 1997), we could only occasionally quantify FA in plasma samples. As these FA-positive samples appeared in all groups, FA presence was not related to FA intake. These findings are in accordance with literature: while a peak concentration of FA in serum occurs rapidly after intake of FA (< 2h) in both rats and humans (Steinberg *et al*. 1979; Castorena-Torres *et al*. 2014), we performed sampling after overnight starving of the animals, to rule out short-term differences that could be related to very recent feed intake. Similar to plasma, the RBC folate concentrations were not significantly different between the negative control group and the group receiving wild type rice, with respective average RBC folate concentrations measured prior to euthanasia of 121.7 and 143.4 nM ($p=0.33$). These concentrations correspond with a severe depletion of folate stores in these groups. It is clear that this will strongly impair nucleotide synthesis, thus disrupting homeostasis (e.g. normal

erythropoiesis), leading to symptoms of severe folate deficiency and eventually death.(Bills *et al*. 1992; Stabler 2010) In contrast, repletion with a folate-rich positive control diet after the 50-day depletion period resulted in a quick re-establishment of RBC folate concentrations, with an average measured total folate concentration of 1884 nM at the end of the trial. Also the groups receiving folate rice or FA fortified rice were able to maintain an adequate RBC total folate concentration, at the end of the trial stabilizing to a concentration of respectively 450.7 and 507.3 nM. Although these concentrations were not significantly different ($p=0.27$), the lower concentrations in the folate rice group are likely the result of the reported approximate 80% bioavailability of food folates, as compared to FA in humans.(Winkels *et al*. 2007) However, a small part of the difference between these two groups may also originate from the slightly lower average folate intake of the animals in the folate rice group (3.00 compared to 3.12 µg/animal/day; see Table VI.2). The RBC-folate concentrations measured - though not as high as those observed in the positive control group- are above the 340 nM cut-off value for folate deficiency in humans, as suggested by the World Health Organization using homocysteine as a metabolic indicator.(WHO 2012) Also, it is possible that the FA does not penetrate the rice grain completely during the parboiling process. This could also facilitate the release of FA from fortified rice in the bowel.

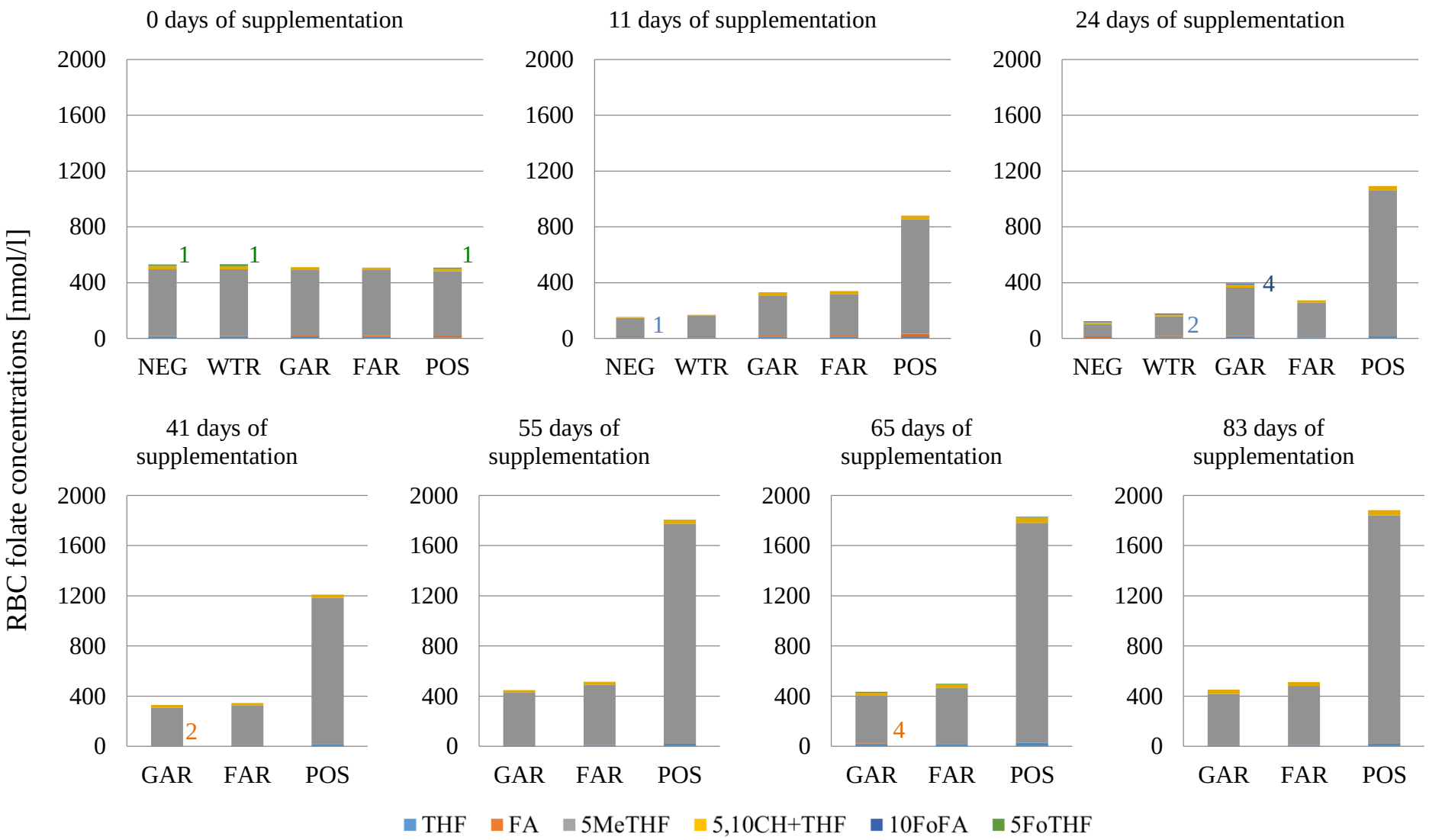


Figure VI.5: Average red blood cell folate speciation with day of sampling specified above; only measurements exceeding the lower limit of quantitation are shown, reducing sample size. When averages represent less than 6 values, n is indicated; all other measurements are averages of measurements from at least half of the animals; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

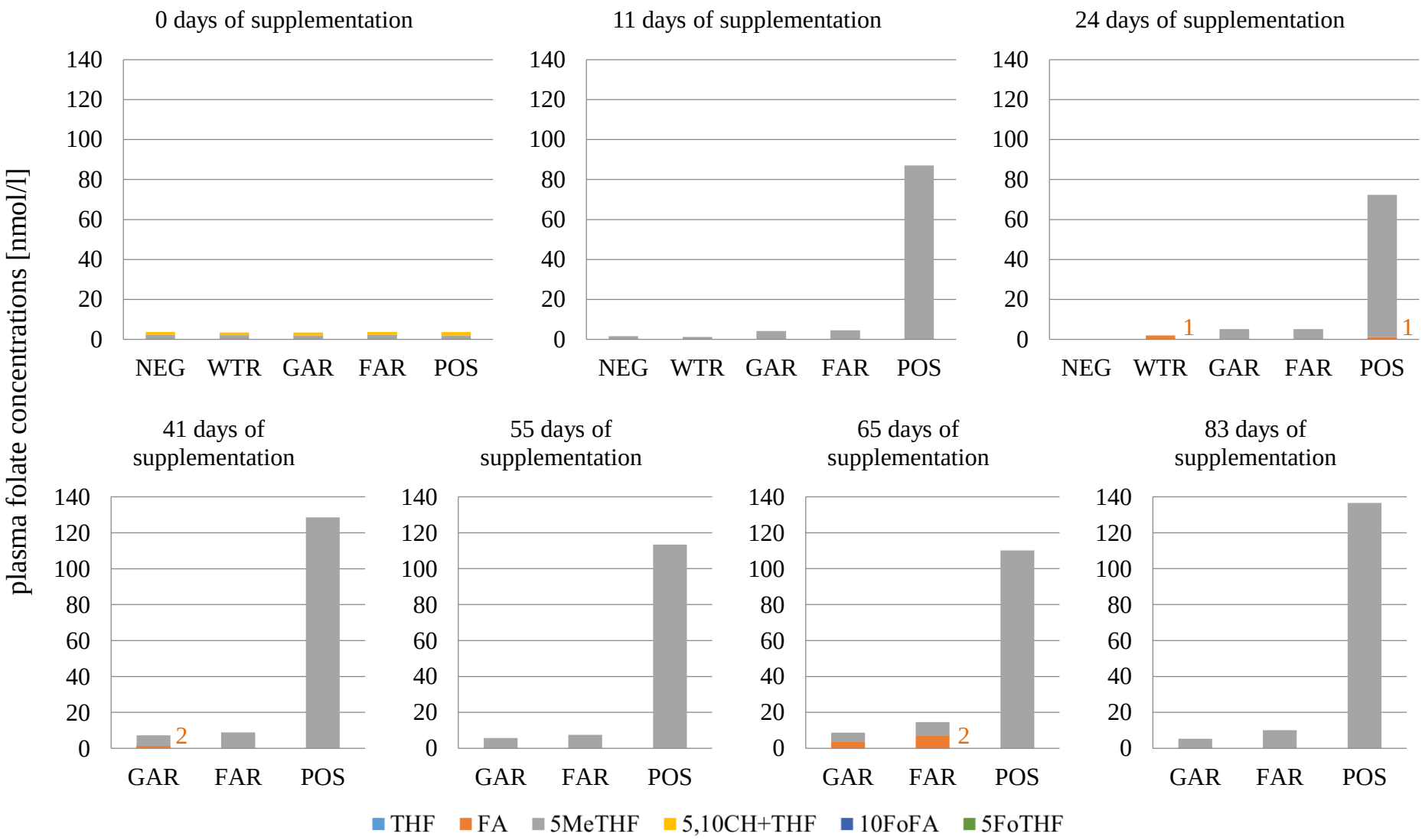


Figure VI.6: Average plasma folate speciation with day of sampling specified above; only measurements exceeding the lower limit of quantitation are shown, reducing sample size. When averages represent less than 6 values, n is indicated; all other measurements are averages of measurements from at least half of the animals; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

5.3. Homocysteine and vitamin B_{12} concentrations

At the time of euthanasia, the average plasma homocysteine concentration in the negative control and wild type rice groups was more than 18 times higher (resp. 154 and 155 vs. 8.5 µmol/l) than in rats receiving the positive control diet containing approximately 2000 µg FA/kg feed (Table VI.3; Figure VI.7). While average plasma homocysteine concentrations in rats receiving a limited folate supplementation of 3 µg folate /day via folate rice or FA fortified rice were significantly lower (resp. 46.6 and 30.3 µmol/l) than those in the negative control and wild type rice group ($p<0.01$), these concentrations remained significantly higher than those in positive control diet animals ($p<0.01$) (Figure VI.8).

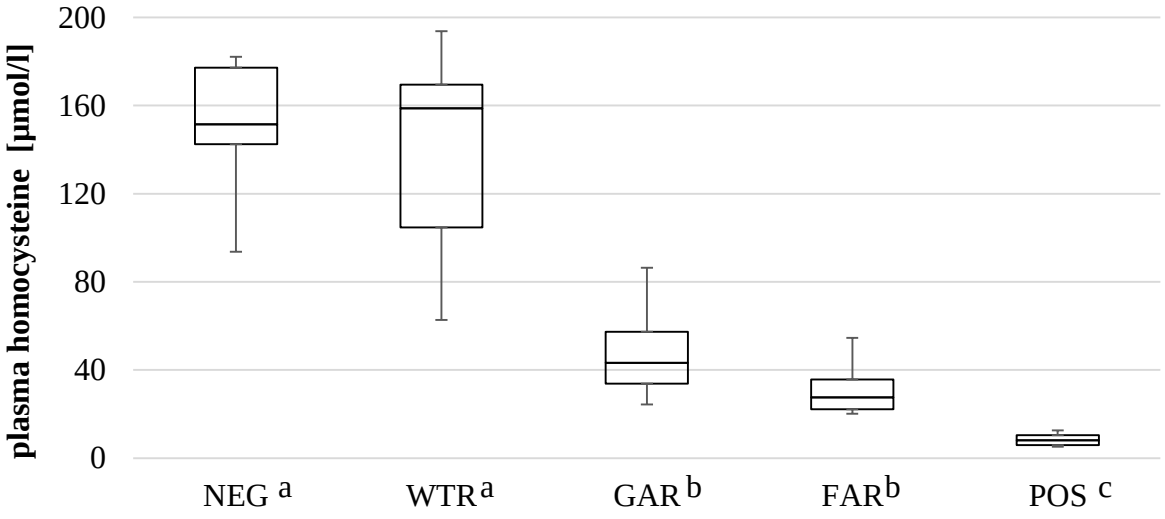


Figure VI.7: Plasma homocysteine concentration at time of euthanasia (Table VI.3); groups with shared letters are not significantly different; NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

Analysis of homocysteine in plasma clearly showed the disruption of the methylation cycle in the negative control and wild type rice groups, with a more than 18 fold higher concentration than in samples obtained from the positive control group. This observation is in line with the report by Duncan *et al*., who observed an inverse correlation between plasma folate and homocysteine concentrations.(Duncan *et al*. 2013) Moreover, the plasma homocysteine concentrations we observed for the folate rice and folic acid fortified rice group (respectively 46.6±18.0 and 30.3±10.6 µmol/l) are in line with those reported by House *et al*. (25.0±2.8 µmol/l), who supplemented rats with a diet containing 250 µg folate/kg during a 4 week repletion trial following 6 weeks of depletion.(House *et al*. 2003) However, also samples obtained from rats receiving folate rice or FA fortified rice had on average 5- resp. 3-fold higher plasma homocysteine concentrations than those observed in the positive control group (Figure VI.7). Similar to what was observed from the RBC folate measurements, the difference between the plasma homocysteine concentrations in the GAR and FAR groups, 46.6 ±18.0 and 30.3 ±10.6 µmol/l respectively, might indicate a lower net absorption of folates from folate rice compared to folic acid fortified rice. Hence, it can be concluded that,

while a 12-week folate supplementation via folate rice (3.00 µg folates/day or 311 µg folate/kg feed) or folic acid fortified rice (3.12 µg folate/day or 313 µg folate/kg feed) is sufficient to prevent or counter prominent homocysteine accumulation in rats, an even higher supplementation is needed to keep (or bring) homocysteine concentrations down to normal levels (about 6.4 µmol/l, as observed by Miller *et al*.), as those observed in our positive control group. (Miller *et al*. 1994)

Although at the time of euthanasia all groups had plasma vitamin B12-concentrations higher than the cut off value of 300 ng/l for deficiency in humans (Klee 2000), lower concentrations were present in the negative control and wild type rice group. Only the latter was significantly lower than the concentrations measured in the folate rice, FA fortified rice and positive control group ($p<0.01$) (Table VI.3; Figure VI.8). The lower vitamin B12 concentrations in the negative control and wild type rice group are likely due to the fact that these animals were sacrificed at the time they had a reduced feed intake.

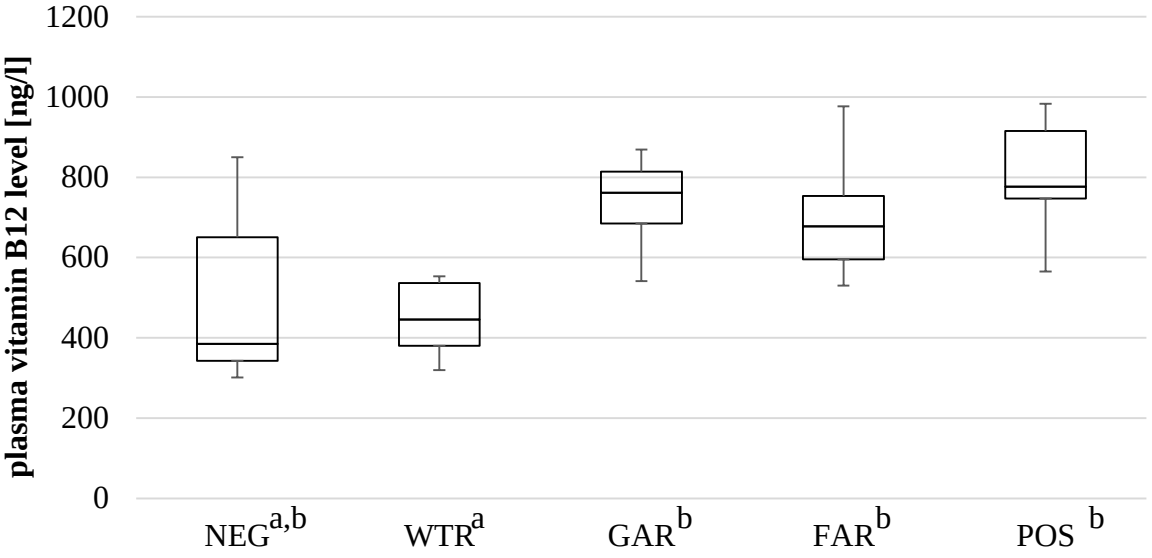


Figure VI.8: Plasma vitamin B12 concentration at time of euthanasia (Table VI.3); groups with shared letters are not significantly different NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

5.4. Dried blood spot hematocrit estimation

After the 50-day depletion period the average HCT estimated via dried blood spot potassium analysis had dropped from the normal value of approximately 0.40 (Harlan Laboratories 2011) to approximately 0.32. While the anemia observed in the folate rice, FA fortified rice and positive control groups quickly normalized, the average HCT of the negative control and wild type rice group continued to decline. As compared to the other groups, the negative control and wild type rice group had a significantly lower estimated HCT of respectively 0.20 and 0.27 after 11 days of supplementation ($p<0.01$) and 0.18 and 0.20 prior to euthanasia at resp. 24 and 29 days after the start of the trial (Figure VI.9A). In contrast, in all other groups, the hematocrit had already normalized after 11 days of supplementation, not yielding

significant differences between these groups ($p \geq 0.76$) and remaining normal throughout the trial, irrespective of the extent of folate supplementation (limited in the GAR and FAR groups and high in the POS group).

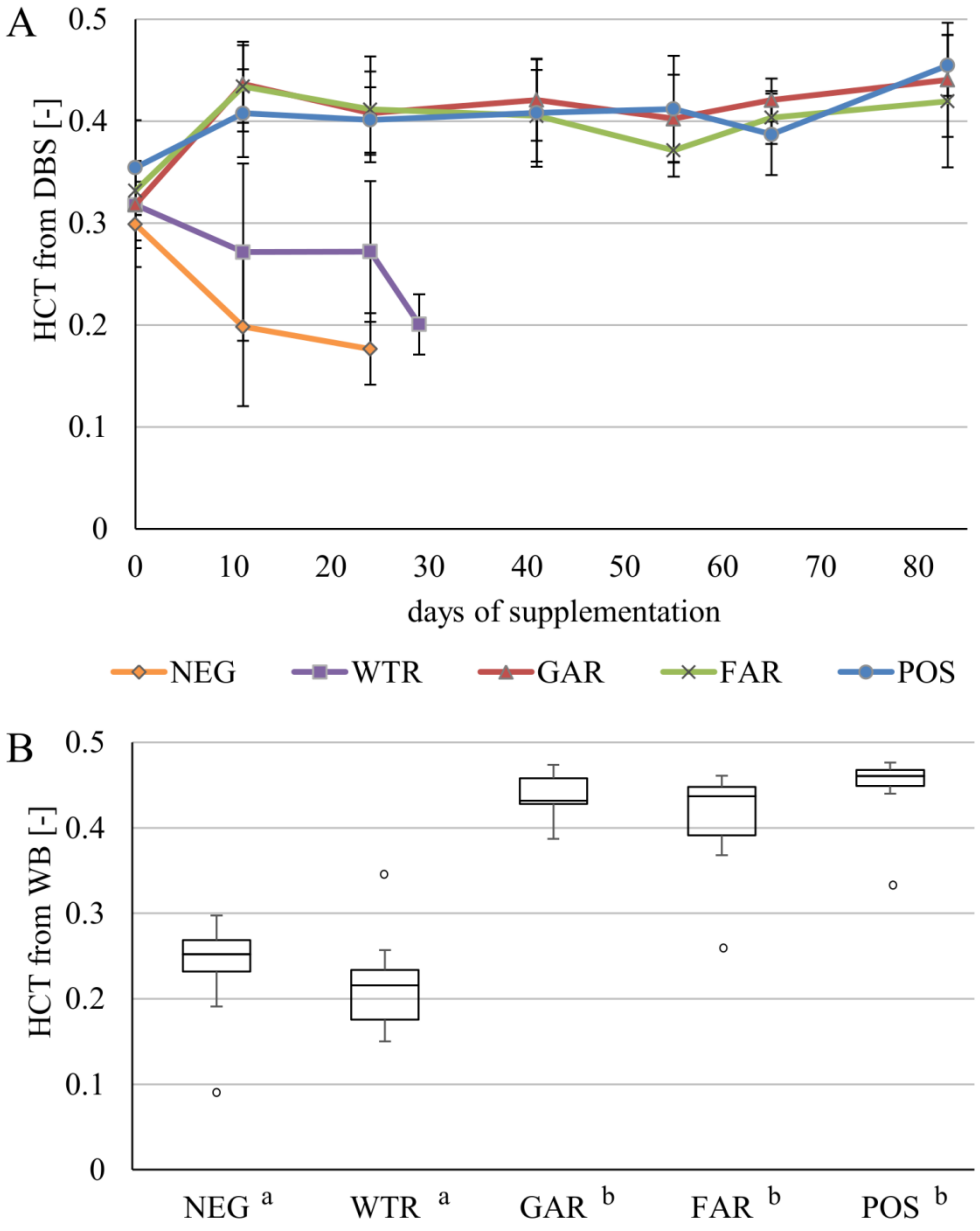


Figure VI.9: Panel A: hematocrit estimation based on DBS-sampling via potassium measurement; panel B: hematocrit measurement in whole blood at time of euthanasia (Table VI.4); groups with shared letters are not significantly different; extreme values are indicated by dots NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

5.5. Hematological analysis

Analysis of whole blood samples obtained at the time of euthanasia confirmed the results obtained via K-based HCT estimation: the average HCT of the negative control and wild type rice group was significantly lower ($p \leq 0.05$) than that of the other groups (Figure VI.9B). The same trend was found for the relative reticulocyte (immature RBCs) count ($p < 0.01$). Only the negative control group had a significantly lower average RBC size, expressed as mean corpuscular volume (MCV), compared to all other groups ($p \leq 0.04$) (Table VI.4).

group (n)	NEG (8)	WTR (9)	GAR (12)	FAR (12)	POS (12)
mean hematocrit from WB [-]	$0.248^{\pm 0.043\ a}$	$0.203^{\pm 0.026 a}$	$0.438^{\pm 0.024\ b}$	$0.410^{\pm 0.058\ b}$	$0.448^{\pm 0.039\ b}$
mean hematocrit from DBS [-]	$0.177^{\pm 0.035\ a}$	$0.201^{\pm 0.030\ a}$	$0.441^{\pm 0.056\ b}$	$0.420^{\pm 0.065\ b}$	$0.455^{\pm 0.030\ b}$
mean corpuscular volume [fl]	$51.7^{\pm 1.4\ a}$	$54.8^{\pm 2.0\ b}$	$57.8^{\pm 2.0\ b}$	$58.3^{\pm 3.5\ b}$	$55.3^{\pm 1.8\ b}$
relative reticulocyte count [‰]	$6.2^{\pm 3.3\ a}$	$7.2^{\pm 5.3\ a}$	$26.0^{\pm 2.7\ b}$	$27.6^{\pm 5.8\ b}$	$22.7^{\pm 3.7\ b}$

Table VI.4: Results obtained following hematological and dried blood spot (DBS) analysis of whole blood at the time of euthanasia; Values indicate mean ± standard deviation, groups with shared letters are not significantly different NEG, negative control group; WTR, wild type rice group; GAR, folate rice group; FAR, folic acid fortified rice group; POS, positive control group.

The HCT measurements clearly show that only the animals receiving a diet supplemented with FA in case of the positive control group and the animals receiving folate or FA fortified rice were able to regain a normal HCT value. Folate deficiency results in the impairment of deoxynucleotide synthesis and the loss of terminally differentiating erythroblasts through apoptosis during the S-phase of the cell cycle. In mature animals, this typically results in the release of immature, large erythroblasts, which can be measured by an increase of MCV, known as megaloblastic anemia. However, the MCV values found for the negative control and wild type rice groups were lower than those found for the other groups, which is in agreement with Koury *et al*., who found that macrocytosis does not accompany anemia in young animals due to their larger folate requirement.(Koury *et al*. 1997) Also, using the Sysmex XE-5000, erythroblasts are not included in the MCV measurement. While no significant difference was observed between the folate rice, FA fortified rice and positive control groups ($p \geq 0.19$), the significantly reduced ($p < 0.01$) relative reticulocyte count of the negative control and wild type rice group clearly indicates an impairment of erythropoiesis and the failure to remediate the onset of the anemia present in these animals. Overall, these findings are in accordance with those of Kim *et al*. who recorded bodyweight, hematological parameters and plasma folate and homocysteine during a 5 week study. (Kim *et al*. 2002)

VI.6. Conclusion

The use of an accepted fortification technique, the fortification of rice with synthetic FA by parboiling, served as a benchmark for synthetic folate-fortified food. From the data gathered here, it is clear that both folate rice and FA fortified rice could sustain a folate status that allowed the animals to grow and remain apparently healthy. The observed difference in plasma folate concentrations is likely the result of a difference in absorption rates between FA present in FA fortified rice and 5MeTHF and other folates present in folate rice. Even the limited folate supplementation of 3 µg folate/day, supplied either via folate rice or via FA fortified rice allowed rapid normalization of HCT values. The resulting folate concentrations were sufficient to counter the major rise in plasma homocysteine concentrations, although not yet optimal, as a result of the limited folate supplementation as compared to normal rat feed. All together, we have provided evidence that folates from folate rice are released in the gastrointestinal system following consumption by rats, reach the systemic circulation, are incorporated in RBCs and are biologically active, as evidenced by folate determination in plasma and RBCs, the alleviation of anemia and counteraction of pronounced hyperhomocysteinemia. Pending confirmation of these findings in a human trial, locally grown folate rice may serve as a source of folate in regions of the world where traditional fortification techniques are not feasible.

VI.7. References

Aboko-Cole, G.F. and Lee, C.M. 1974. "Trypanosoma Lewisi: Body Weight Gains and Food Consumption of Folate-Deficient Rats." *Int J Biochem* 5 (9-10): 703–6.

Bailey, L.B., and Gregory, J.F.III 1999. "Folate Metabolism and Requirements." *J Nutr* 129 (4): 779–82.

Beyer, P., Al-Babili, S., Ye, X., Lucca, P., Schaub, P., Welsch, R. and Potrykus, I. 2002. "Golden Rice: Introducing the Beta-Carotene Biosynthesis Pathway into Rice Endosperm by Genetic Engineering to Defeat Vitamin A Deficiency." *J Nutr* 132 (3): 506S – 10S.

Bills, N.D., Koury, M.J., Clifford, A.J. and Dessypris, E.N. 1992. "Ineffective Hematopoiesis in Folate-Deficient Mice." *Blood* 79 (9): 2273–80.

Capiau, S., Stove, V.V., Lambert, W.E. and Stove, C.P. 2013. "Prediction of the Hematocrit of Dried Blood Spots via Potassium Measurement on a Routine Clinical Chemistry Analyzer." *Anal Chem* 85 (1): 404–10.

Castorena-Torres, F., Ramos-Parra, P.A., Hernandez-Mendez, R.V., Vargas-Garcia, A., Garcia-Rivas, G. and de la Garza, R.I. 2014. "Natural Folates from Biofortified Tomato and Synthetic 5-Methyl-Tetrahydrofolate Display Equivalent Bioavailability in a Murine Model." *Plant Foods Hum Nutr* 69 (1): 57–64.

Caudill, M.A. 2010. "Folate Bioavailability: Implications for Establishing Dietary Recommendations and Optimizing Status." *Am J Clin Nutr* 91 (5): 1455S – 60S.

Clifford, A.J., Wilson, D.S. and Bills, N.D. 1989. "Repletion of Folate-Depleted Rats with an Amino Acid-Based Diet Supplemented with Folic Acid." *J Nutr* 119 (12): 1956–61.

De Brouwer, V., Storozhenko, S., Stove, C.P., Van Daele, J., Van der Straeten, D. and Lambert, W.E. 2010. "Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) for the Sensitive Determination of Folates in Rice." *J Chromatogr B* 878 (3-4): 509–13.

De Steur, H., Gellynck, X., Blancquaert, D., Lambert, W.E., Van Der Straeten, D. and Qaim, M. 2012. "Potential Impact and Cost-Effectiveness of Multi-Biofortified Rice in China." *N Biotechnol* 29 (3): 432–42.

De Steur, H., Gellynck, X., Storozhenko, S., Liqun, G., Lambert, W.E., Van Der Straeten, D. and Viaene, J. 2010. "Health Impact in China of Folate-Biofortified Rice." *Nat Biotechnol* 28 (6): 554–6.

Duncan, T.M., Reed, M.C. and Nijhout, H.F. 2013. "The Relationship between Intracellular and Plasma Levels of Folate and Metabolites in the Methionine Cycle: A Model." *Mol Nutr Food Res* 57 (4): 628–36.

Glier, M.B., Green, T.J. and Devlin, A.M. 2014. "Methyl Nutrients, DNA Methylation, and Cardiovascular Disease." *Mol Nutr Food Res* 58 (1): 172–82.

Gregory J.F.III 2001. "Case Study: Folate Bioavailability." *J Nutr* 131 (4 Suppl): 1376S – 82S.

Haas, J.D., Beard, J.L., Murray-Kolb, L.E., del Mundo, A.M., Felix, A. and Gregorio, G.B. 2005. "Iron-Biofortified Rice Improves the Iron Stores of Nonanemic Filipino Women." *J Nutr* 135 (12): 2823–30.

Harlan Laboratories. 2011. "HsdHanWIST_datasheet_(03-11).pdf." http://www.harlan.com/download.axd/3848f9a445614166814c4b26a31c81e2.pdf?d=HsdHanWIST datasheet (consulted: 01/30/2016).

House, J.D., O'Connor, C.P. and Guenter, W. 2003. "Plasma Homocysteine and Glycine Are Sensitive Indices of Folate Status in a Rodent Model of Folate Depletion and Repletion." *J Agric Food Chem* 51 (15): 4461–67.

Jeong, J. and Guerinot, M.L. 2008. "Biofortified and Bioavailable: The Gold Standard for Plant-Based Diets." *Proc Natl Acad Sci USA* 105 (6): 1777–78.

Kam, K., Arcot, J. and Adesina, A.A. 2012. "Folic Acid Fortification of Parboiled Rice: Multifactorial Analysis and Kinetic Investigation." *J Food Eng* 108 (1): 238–43.

Kellogg, M.D., Parker, R., Ricupero, A. and Rifai, N. 2005. "Evaluation of an Enzymatic Homocysteine Assay for the Hitachi Series Chemistry Analyzer." *Clin Chim Acta;* 354 (1-2): 117–22.

Kelly, P., McPartlin, J., Goggins, M., Weir, D.G. and Scott, J.M. 1997. "Unmetabolized Folic Acid in Serum: Acute Studies in Subjects Consuming Fortified Food and Supplements." *Am J Clin Nutr* 65 (6): 1790–5.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015a. "A Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Selective Analysis of Free and Total Folate in Plasma and Red Blood Cells." *J Chromatogr A* 1398: 20–28.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E., Stove, C.P. 2015b. "Determination of five folate monoglutamates in rodent diets" *J Agric Food Chem* 63:10089-95

Kim, Y.-I., Hayek, M., Mason, J.B. and Nikbin Meydani, S. 2002. "Severe Folate Deficiency Impairs Natural Killer Cell-Mediated Cytotoxicity in Rats." *J. Nutr.* 132 (6): 1361–67.

Klee, G.G. 2000. "Cobalamin and Folate Evaluation: Measurement of Methylmalonic Acid and Homocysteine vs Vitamin B(12) and Folate." *Clin Chem* 46 (8 Pt 2): 1277–83.

Koury, M.J. 2014. "Abnormal Erythropoiesis and the Pathophysiology of Chronic Anemia." *Blood Rev* 28 (2):49–66

Koury, M.J., Horne, D.W., Brown, Z.A., Pietenpol, J.A., Blount, B.C., Ames, B.N., Hard, R. and Koury, S.T. 1997. "Apoptosis of Late-Stage Erythroblasts in Megaloblastic Anemia: Association with DNA Damage and Macrocyte Production." *Blood* 89 (12): 4617–23.

Kurata, M., Suzuki, M. and Agar, N.S. 1993. "Antioxidant Systems and Erythrocyte Life-Span in Mammals." *Comp Biochem Physiol B* 106 (3): 477–87.

Lewis, C.J., Crane, N.T., Wilson, D.B. and Yetley, E.A. 1999. "Estimated Folate Intakes: Data Updated to Reflect Food Fortification, Increased Bioavailability, and Dietary Supplement Use." *Am J Clin Nutr* 70 (2): 198–207.

Mateo Anson, N., van den Berg, R., Havenaar, R., Bast, A. and Haenen, G.R.M.M. 2009. "Bioavailability of Ferulic Acid Is Determined by Its Bioaccessibility." *J Cereal Sci* 49 (2): 296–300.

McKillop, D.J., McNulty, H., Scott, J.M., McPartlin, J.M., Strain, J.J., Bradbury, I., Girvan, J., *et al*. 2006. "The Rate of Intestinal Absorption of Natural Food Folates Is Not Related to the Extent of Folate Conjugation." *Am J Clin Nutr* 84 (1): 167–73.

Miller, J.W., Nadeau, M.R., Smith, J., Smith, D. and Selhub, J. 1994. "Folate-Deficiency-Induced Homocysteinaemia in Rats: Disruption of S-Adenosylmethionine's Co-Ordinate Regulation of Homocysteine Metabolism." *Biochem J* 298 (Pt2): 415-9

Morris, J., Hawthorne, K.M., Hotze, T., Abrams, S.A. and Hirschi, K.D. 2008. "Nutritional Impact of Elevated Calcium Transport Activity in Carrots." *Proc Natl Acad Sci USA* 105 (5): 1431–5.

O'Dea, K., Snow, P. and Nestel, P. 1981. "Rate of Starch Hydrolysis in Vitro as a Predictor of Metabolic Responses to Complex Carbohydrate in Vivo." *Am J Clin Nutr* 34 (10): 1991–3.

Pfeiffer, C.M.,Rogers, L.M. and Gregory, J.F.III 1997. "Determination of Folate in Cereal-Grain Food Products Using Trienzyme Extraction and Combined Affinity and Reversed-Phase Liquid Chromatography." *J Agric Food Chem* 45 (2): 407–13.

Shane, B. 2010. "Folate Chemistry and Metabolism." In *Folate in Health and Disease*, edited by Bailey, L.B. 2nd ed., 1–24. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Stabler, S.P. 2010. "Clinical Folate Deficiency." In *Folate in Health and Disease*, edited by Bailey, L.B. 2nd ed., 409–28. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Steinberg, S.E., Campbell, C.L. and Hillman, R.S. 1979. "Kinetics of the Normal Folate Enterohepatic Cycle." *J Clin Invest* 64 (1): 83–8.

Storozhenko, S., De Brouwer, V., Volckaert, M., Navarrete, O., Blancquaert, D., Zhang, G.F., Lambert, W. and Van Der Straeten, D. 2007. "Folate Fortification of Rice by Metabolic Engineering." *Nat Biotechnol* 25 (11): 1277–9.

Tang, G., Qin, J., Dolnikowski, G.G., Russell, R.M. and M.A. Grusak. 2009. "Golden Rice Is an Effective Source of Vitamin A." *Am J Clin Nutr* 89 (6): 1776–83.

Visentin, M., Diop-Bove, N., Zhao, R. and Goldman, I.D. 2014. "The Intestinal Absorption of Folates." *Annu Rev Physiol* 76: 251–74.

Wang, T.T., Reisenauer, A.M. and Halsted, C.H. 1985. "Comparison of Folate Conjugase Activities in Human, Pig, Rat and Monkey Intestine." *J Nutr* 115 (6): 814–9.

Ward, M., McNulty, H., McPartlin, J., Strain, J.J., Weir, D.G. and Scott, J.M. 1997. "Plasma Homocysteine, a Risk Factor for Cardiovascular Disease, Is Lowered by Physiological Doses of Folic Acid." *Q J Med* 90 (8): 519–24.

WHO. 2012. "Serum and Red Blood Cell Folate Concentrations for Assessing Folate Status in Populations." http://www.who.int/vmnis/indicators/serum_RBC_folate/en/ (consulted: 01/30/2016)

Winkels, R.M., Brouwer, I.A., Siebelink, E., Katan, M.B. and Verhoef, P. 2007. "Bioavailability of Food Folates Is 80% of that of Folic Acid." *Am J Clin Nutr* 85 (2): 465–73.

Wright, A.J., Finglas, P.M., Dainty, J.R., Wolfe, C.A., Hart, D.J., Wright, D.M. and Gregory, J.F.III 2005. "Differential Kinetic Behavior and Distribution for Pteroylglutamic Acid and Reduced Folates: A Revised Hypothesis of the Primary Site of PteGlu Metabolism in Humans." *J Nutr* 135 (3): 619–23.

Chapter VII: Effect of folate status on lymphocyte proliferation and intracellular folate levels

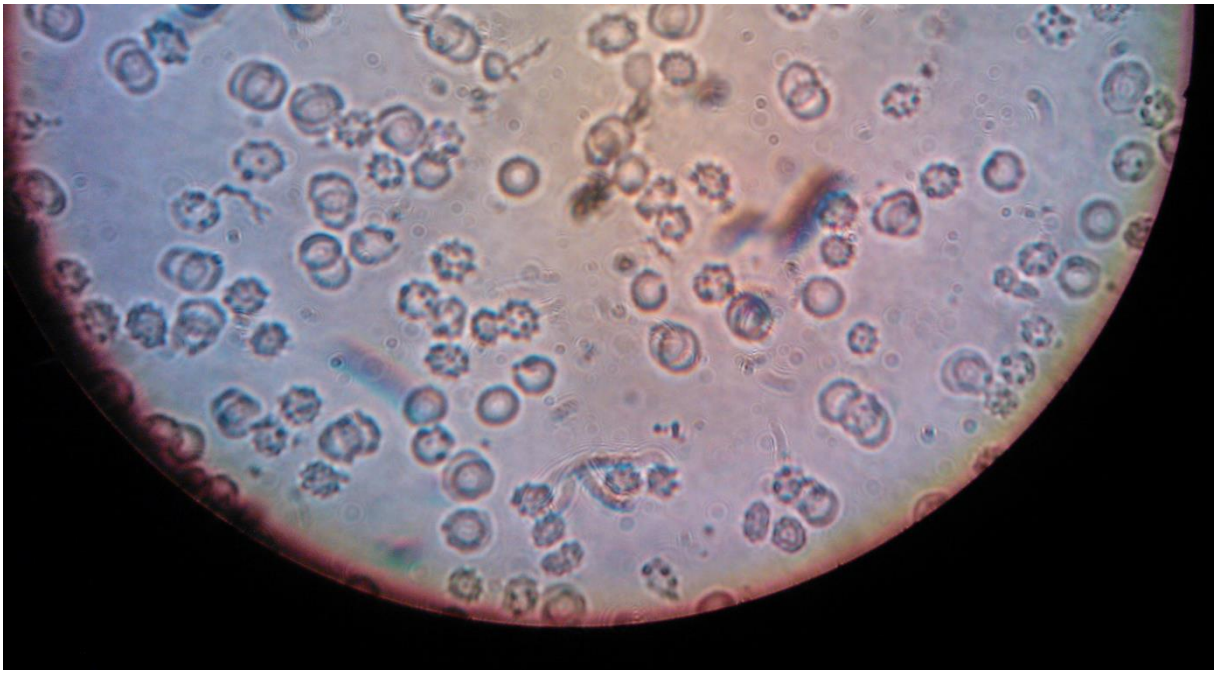
Table of contents

VII.1. Abstract

While following decades of research, the importance of folate status for the health of an individual is evident, the manner in which folates influence health status is often not so clear. Few studies have evaluated the direct impact of folate concentrations in plasma or growth medium on cellular responses.

In 2004, Courtemanche *et al*. reported a significant difference in proliferation behavior between CD4+ and CD8+ T lymphocytes in response to different folate concentrations. As the reason for this difference was not clarified, further research was needed to characterize how folate deficiency affects different immune responses and the proliferation of lymphocytes in particular.

We performed an *in vitro* study, investigating the influence of the folate species used, folic acid or 5-methyltetrahydrofolate (the main species *in vivo*), on the proliferation of CD2+, CD4+ and CD8+ primary T lymphocytes. Besides monitoring cell proliferation, we also determined the folate concentration within these subsets following cultivation. From these results, it was apparent that the dependence of cell replication on folate concentration depends on the folate species used.



VII.2. Introduction

Cellular metabolism relies on a continuous supply of folate, a group of water soluble B-vitamins (B9), to maintain DNA-synthesis and proper cell proliferation. Folate deficiency has been implicated in several health disorders including cancers, neural damage and cardio-vascular disease as a result of hyperhomocysteinemia. To date, only the link with neural tube defects has been formally proven.(Stover 2004; Blom and Smulders 2011; Jagerstad 2012)

Since folate status impacts nucleotide synthesis and therefore cell proliferation, folate deficiency hypothetically impacts the functioning of the immune system by arresting the cell cycle in the S-phase. This was demonstrated by Courtemanche *et al.* through *in vitro* culturing of peripheral blood mononuclear cells (PBMCs). Interestingly, these authors found an inverse relation between the CD4+/CD8+ lymphocyte ratio and folic acid (FA) concentrations *in vitro*.(Courtemanche *et al*. 2004) This was primarily owing to the fact that the proliferation of activated CD8+ cells appeared to be more sensitive to differences in FA concentrations. However, Kishimoto *et al.* investigated the immune function of a patient suffering from hereditary folate malabsorption prior to and after treatment. Apart from a low general lymphocyte proliferation rate due to the morbidly low folate status of the patient, no difference was observed in the relative presence of CD4+ and CD8+ lymphocytes before and after intravenous administration of 12 mg 5FoTHF/day.(Kishimoto *et al*. 2014) In a study investigating the influence of folate supplementation on age-related immune suppression using a rat model, cell proliferation was improved when dietary folate intake was increased, however, no significantly different CD4+/CD8+ ratio was observed between the treatment and the control group.(Field *et al*. 2006) While 5-methyltetrahydrofolate (5MeTHF) has been used as an *in vitro* folate source to investigate the effect of folate status on DNA stability, only the use of high FA concentrations in the growth medium increased cell viability after 9 days of incubation.(Wang and Fenech 2003) In this chapter, we investigated the influence of the folate concentration in the growth medium on the *in vitro* cell proliferation of CD2+, CD4+ and CD8+ lymphocytes and quantified the resulting intracellular folate concentration.

VII.3. Objectives

Based on an *in vitro* study performed by Courtemanche *et al*., we wanted to evaluate whether the intracellular folate concentration correlates with the extracellular folate concentration (i.e. the concentration in the growth medium) or remains constant, which is important to maintain DNA methylation. As such, the relationship between the proliferation rate and the intracellular folate concentration could be evaluated. Also, the use of 5MeTHF instead of FA as a folate source should be evaluated since the former more closely resembles *in vivo* circulating folate speciation.

VII.4. Materials and methods

4.1. Reagents and materials

4.1.1. Cell separation

The basic procedure proposed by Courtemanche *et al.* was upscaled to obtain a larger amount of cells for intracellular folate analysis.(Courtemanche *et al*. 2004) Concentrated buffy coats were obtained from the Belgian Red Cross and treated as described by Meyer *et al.*(Meyer *et a*l. 2005) Briefly, 10 ml of concentrated buffy coat was mixed with 10 ml of sterile PBS. This mixture was then layered carefully on top of 15 ml Ficoll-Paque^{TM} Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Following centrifugation at 445 g for 35 minutes at room temperature, the cells at the interface of the Ficoll and plasma/PBS layer were transferred to a sterile tube. To remove remaining traces of Ficoll, these cells were washed twice with 20 ml of sterile PBS and centrifuged at 200 g for 15 minutes.

4.1.2. Cell culture

Cells were cultured at 37°C and a CO_2 concentration of 5 vol% in 20 ml folate free RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) at an initial density of 10^6 cells/ml. This medium was supplemented with 10% dialyzed fetal bovine serum to sustain cell viability (dFBS, Life Technologies) and Pen-Strept 5000U (Life Technologies) as antibiotic. At the start of the incubation, phytohemagglutinin (PHA, Sigma-Aldrich, Darmstadt, Germany) and interleukin 2 (IL-2, ED 50 range = 0.08-0.5 ng/ml, Life Technologies) were added in concentrations of 22 µg/ml and 0.01 µg/ml, respectively. The medium was refreshed every 3 days, at which time the cells were spun down at 200 g for 5 minutes at 21 °C and 90% of medium was replaced by fresh medium. While fresh IL-2 was added to promote cell proliferation, PHA was only necessary for initial cell activation and was only included at the start of incubation. At the second time of refreshing, the cells were counted and returned to the original density to prevent too high cell densities.

FA (Sigma-Aldrich) or (6S)-5MeTHF (Schircks Laboratories, Jona, Switzerland) were weighed, dissolved and diluted in deoxygenated PBS to a final concentration of 4.4 and 4.6 µg/ml (9.98 and 10.0 µM), respectively. This solution was sterilized using a 0.22 µm syringe filter. Aliquots were stored at -80 °C and discarded after use.

4.1.3. Cell sorting and lysis

Positive cell selection was performed using Easysep^{TM} kits for human cells expressing the CD2, CD4 and CD8 antigens, which were obtained from Stemcell Technologies (Grenoble, France). This selection was performed as prescribed by the manufacturer. Briefly, cells were suspended at a concentration of 10^8 cells/ml in 10/90 v/v% dFBS/PBS. Then, 50 µl/ml (CD4) or 100 µl/ml (CD2 and CD8) of positive selection cocktail was added, followed by incubation at room temperature for 3 (CD4 and CD8) or 5 minutes (CD2). Then, 50 µl/ml of magnetic microparticle suspension was added and incubated for 3 (CD4 and CD8) or 10 minutes (CD2)

at room temperature. After adding 10/90 v/v% dFBS/PBS to a total volume of 2.5 ml, the sample was placed in an Easysep^{TM}-magnet for 3 minutes, after which the supernatant was poured off. The cells were washed twice with 2.5 ml of 10/90 v/v% dFBS/PBS. The supernatant was either collected after each step pending the selection of another cell type or transferred to waste.

The selected cells were washed twice with and resuspended in PBS to remove any extracellular folates due to the presence of dFBS. Cell counting was performed manually using a Bürker counting chamber following staining with trypan blue. For intracellular folate analysis, the cells were lysed using 200 µl of Focus Whole proteome lysis buffer (GBioscience, St. Louis, MO, USA). To this, 250 µl of PBS and 50 µl of 100 mg L-ascorbic acid and 50 mg DL-dithiothreitol (Sigma-Aldrich)/ml, pH 7 was added. The internal standards (ISs, $^{13}C_5$-THF, $^{13}C_5$-FA, $^{13}C_5$-5MeTHF, $^{13}C_5$-5,10CH+THF) were added to the antioxidant solution in a concentration of 0.1 µg/ml.

4.1.4. Extract purification and LC-MS/MS analysis

Further analysis of the samples was performed as described earlier for the analysis of folates in plasma and red blood cells (RBCs).(Kiekens *et al*. 2015; Chapter V) Due to the need for enzymatic polyglutamate deconjugation, the sample treatment procedure for RBCs was applied to the cell extract. The efficacy of the rat serum conjugase (Harlan Laboratories, Horst, The Netherlands) treatment was verified by spiking 5MeTHF di-, tri- and tetraglutamate (Schircks Laboratories) to a mixture of cell lysis buffer, PBS and antioxidant solution and was found not to be hindered by the lysis buffer. However, due to the incomplete removal of endogenous folates from rat serum by treatment with activated charcoal (as described in Chapter V paragraph 4.1), the treatment was performed twice to further remove this folate background and increase sensitivity.

4.1.5. Calibration

Similar to the standard preparation procedure described in Chapter V for plasma folate, the calibration curves were generated by spiking individual folates (tetrahydrofolate (THF), FA, 5MeTHF, 5,10-methenylTHF (5,10CH$^+$THF), 10-formylFA (10FoFA) and 5-formylTHF (5FoTHF)) to a mixture of cell lysis buffer (200 µl), PBS (250 µl) and antioxidant solution (50 µl), matching the sample matrix. Apart from a zero sample (blank + ISs), 7 calibrators were used with concentrations of each folate individually ranging from 0.26 to 26 nmol/l.

4.2. CD2+ proliferation in response to folate status

Following Ficoll separation of PBMCs, $2*10^7$ cells were brought in 20 ml of folate free culture medium supplemented with 0, 10 or 100 nM of either FA or 5MeTHF. To verify the amount of folates in the growth medium, samples were collected after 3 days of incubation.

4.3. Stability of folates in growth media

The chemical stability of FA and 5MeTHF was monitored by spiking either FA or 5MeTHF in a concentration of 100 nM to 20 ml folate free culture medium (in the absence of cells). Duplicate culture flasks were kept for 3 days in the incubator. Samples were collected daily, including one at the start of the experiment.

4.4. CD4+ and CD8+ proliferation in response to folate status

Based on the investigation into the proliferation of CD2+ cells and the evaluation of folate stability in growth medium, two changes were made to the study protocol. First, the folate free sample (0 nM) was omitted since it has limited clinical relevance. Second, 5 or 50 nM 5MeTHF was added daily to replenish this folate species, which was lost due to degradation during incubation. From day 6 to day 9, the folate concentration in the medium was monitored to verify the efficacy of the 5MeTHF replenishment.

Since culturing was performed in duplicate, the cell isolation order was altered between both replicates to reduce the potential influence of incomplete recovery. When CD4+ cells were selected first in one replicate, CD8+ cells were selected first in the other replicate. After selection of the distinct cell subsets, the cells from both replicates were counted and pooled for intracellular folate analysis.

VII.5. Results and discussion

5.1. CD2+ proliferation in response to folate status

As was observed by Courtemanche *et al.*, the FA concentration significantly influences the cell count after 9 days of incubation, as depicted in Figure VII.1A. The addition of 10 nM FA as a source of folate resulted in a 3-fold higher CD2+ cell count compared to cells exposed to folate free medium. However, an additional 15-fold increase in CD2+ cell count was observed for cells exposed to 100 nM of FA, indicating a concentration dependent growth response. This was accompanied by an increase in intracellular folate concentrations. While 5MeTHF was detectable in cells exposed to 10 nM FA folates, the signal was too low to allow for a quantification of its concentration. In the cells exposed to 100 nM FA, both 5MeTHF and FA could be quantified, as depicted in Figure VII.1B. The presence of FA is surprising since this is not a metabolically active compound. Given cell sorting and subsequent washing steps, the presence of FA is unlikely to be the result of contamination with growth medium. One possibility is that FA enters the cells as a result of passive diffusion.

Also when 5MeTHF was present in the growth medium, cell counts significantly exceeded those obtained in the absence of folate (0 nM). Strikingly, cell counts for 10 and 100 nM 5MeTHF were quite similar, lying in the same range as when supplementing the medium with 100 nM FA. The intracellular concentrations did however show a dependence upon extracellular concentrations during incubation. In the cells exposed to 10 nM 5MeTHF, THF

and 5MeTHF could be quantified with THF being the major species. In cells exposed to 100 nM 5MeTHF, 5MeTHF was the major folate species, while THF and 5,10CH^{+}THF were also present.

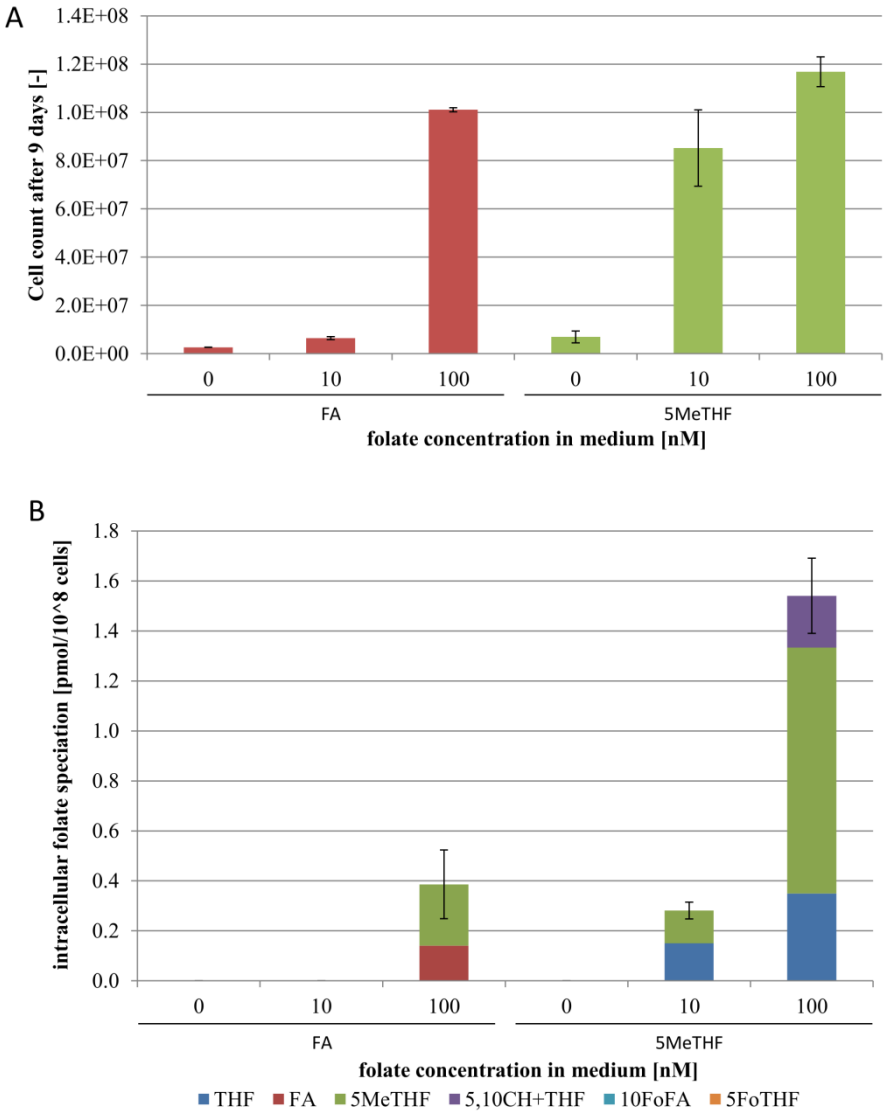


Figure VII.1: A: Total CD2+ cell count and B: Measured intracellular folate species following 9 days of incubation with varying folate content in the growth medium.

5.2. Stability of FA and 5MeTHF during incubation in growth medium

Analysis of the medium at the end of the CD2+ trial revealed a significantly decreased 5MeTHF concentration, 10 times lower than that initially added to the medium. To investigate this further, a stability experiment was conducted, comparing the stability of 5MeTHF and FA during incubation. Daily sampling over a 3 day period revealed that FA remains fairly stable over time, while the 5MeTHF concentration decreases by approximately 50% per day (data depicted in Figure VII.2A). After 3 days, only approximately 10% of the initial 5MeTHF concentration remained. Based on the concentration over the first 48 hours, the area under the curve (AUC) was calculated. For 5MeTHF (2591 nM.h), the AUC was only 57% of that calculated for FA (4525 nM.h).

To remedy this disadvantage of 5MeTHF compared to FA, daily addition of 50% of the original 5MeTHF concentration was evaluated during the CD4+/CD8+ experiment. Over 2 days, the medium was sampled prior to and after addition of fresh 5MeTHF and 6 hours after addition. This data, depicted in Figure VII.2B, shows the 5MeTHF and FA concentration over a 48-hour period. While the average 5MeTHF concentration is still lower than that of FA, its degradation is partly compensated for, avoiding the low 5MeTHF concentrations after 3 days measured during the CD2+ experiment. By replacing the 5MeTHF lost due to degradation, the AUC increased to 3740 nM.h while the AUC of FA (4739 nM.h) remained virtually the same. Relative to the AUC of FA, the AUC of 5MeTHF increased to 79% which is a vast improvement over the 57% obtained when the degradation of 5MeTHF is not compensated for.

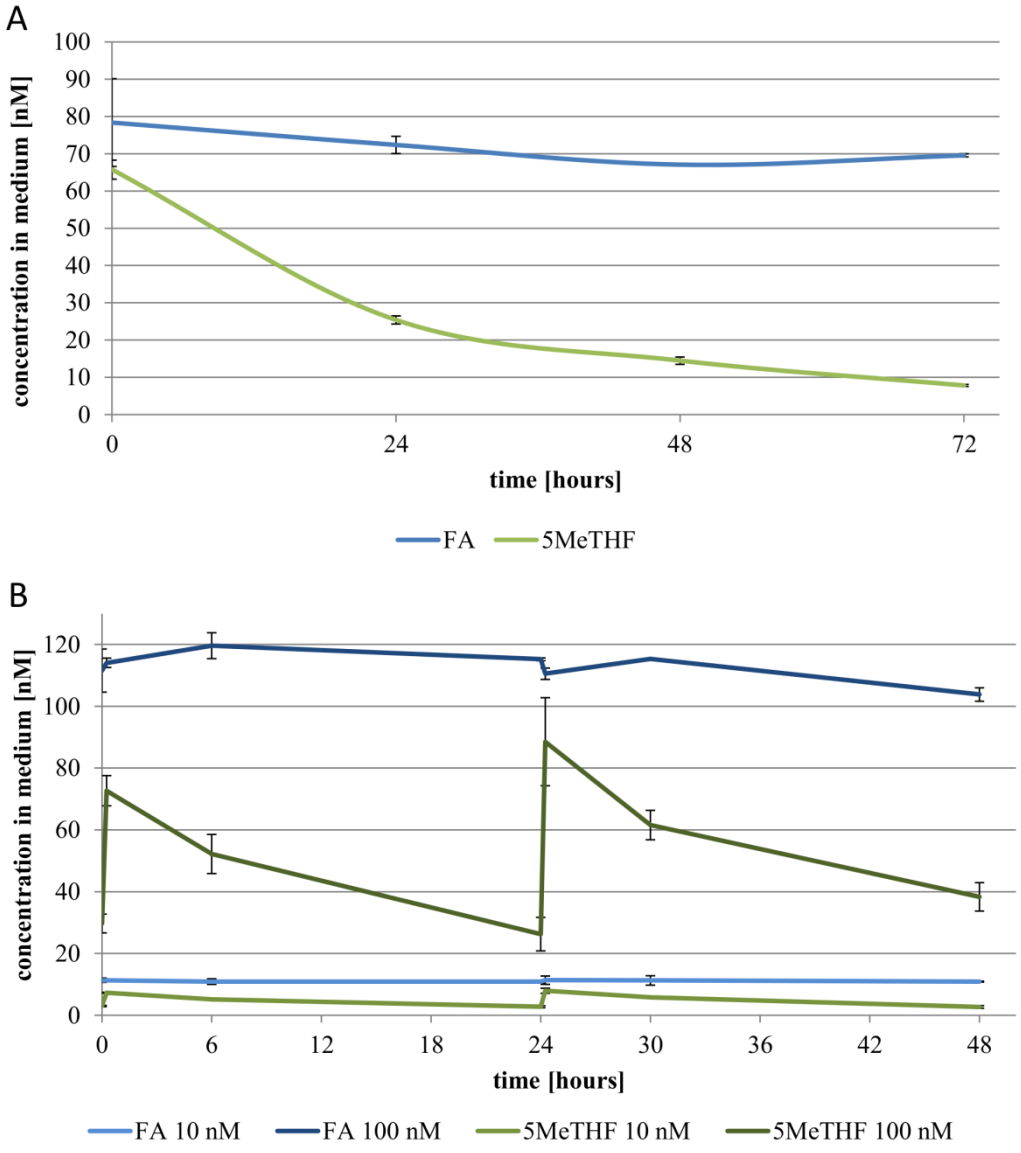


Figure VII.2: Periodic evaluation of FA and 5MeTHF stability in growth medium without (A) and with (B) replacement of degraded 5MeTHF (n=2). Note the difference in scale on the x and y-axes.

The observed instability of 5MeTHF, when not checked and compensated for, may have profound effects on the conclusions drawn from *in vitro* studies. E.g. Wang and Fenech observed a higher cell viability at high FA concentration versus low FA and low or high 5MeTHF concentrations which they related to a preferential uptake of FA compared to 5MeTHF into lymphocytes. However, our findings suggest that their results are likely confounded by the limited stability of 5MeTHF during incubation.(Wang and Fenech 2003)

5.3. CD4+/CD8+ proliferation in response to folate status

Similar to the evaluation of CD2+ cell proliferation, the addition of 100 nM FA to the growth medium resulted in higher cell counts compared to 10 nM FA (Figure VII.3A). Also, as previously observed by Courtemanche *et al.*, the CD8+ cells are most affected by the difference in FA concentration in the growth medium.(Courtemanche *et al*. 2004) This is reflected by a respective CD4+/CD8+ ratio of 0.69 and 0.48 for 10 and 100 nM FA. However, this concentration dependence was not observed when comparing 10 to 100 nM 5MeTHF. The calculated CD4+/CD8+ ratios were 0.46 and 0.53 respectively and were both comparable to the results obtained when cells were incubated with 100 nM FA (cfr. Table VII.1).

		CD4+		CD8+		ratio CD4+/CD8+
	concentration [nM]	average cell count	SD	average cell count	SD	
FA	10	1.49E+07	1.59E+06	2.15E+07	2.44E+06	0.69
	100	2.39E+07	1.06E+06	5.00E+07	1.91E+06	0.48
5MeTHF	10	3.50E+07	6.15E+06	7.65E+07	7.64E+06	0.46
	100	3.24E+07	7.21E+06	6.08E+07	4.45E+06	0.53

Table VII.1: Cell count and ratio of CD4+ and CD8+ cells following 9 days of incubation in response to varying folate levels. SD: standard deviation

One explanation for this phenomenon is that the cells' ability to use FA as an *in vitro* folate source depends on the activity of specific folate carriers (proton coupled folate transporter) and reducing enzymes (dihydrofolate reductase)(Shane 2010), respectively capable of transferring FA to the cytosol and reducing it to THF.

The increased amount of cells per sample provided clearer data concerning the evaluation of intracellular folate concentrations, of which the results are depicted in Figure VII.3B. For cells incubated with FA, similar results were obtained when compared to the analysis of CD2+ cells. Here, some 5MeTHF could be quantified in cells exposed to 10 nM FA, while folate concentrations were much higher in cells exposed to 100 nM FA. Strikingly, the folate concentration is substantially higher in CD8+ cells compared to CD4+ cells. However, the levels in cells exposed to 10 nM FA are too low to make a sound conclusion. Also, in CD8+ cells exposed to 100 nM FA, FA was present at quantifiable levels while this folate species does not participate in folate metabolism.

As can be seen in Figure VII.3B, when 5MeTHF is used as a folate source in the growth medium, the intracellular concentration is directly related to the extracellular concentration for both the CD4+ and CD8+ cells. There is, however, no appreciative difference between CD4+ and CD8+ cells at each respective 5MeTHF concentration level. While THF concentrations also increase when the 5MeTHF concentration in the medium is increased, the bulk of the concentration increase can be attributed to 5MeTHF.

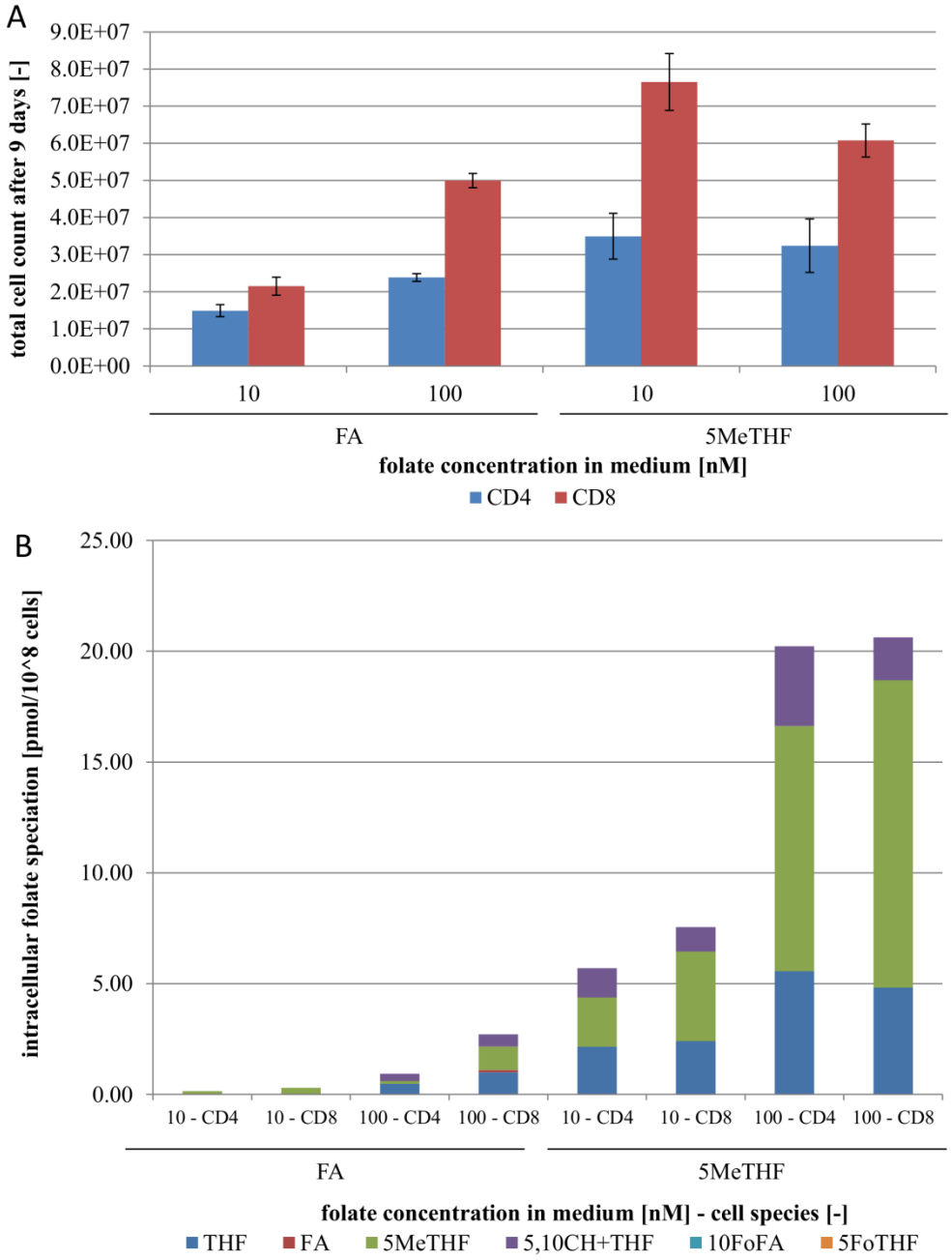


Figure VII.3: A: Total CD4+ and CD8+ cell count (n=2) and B: Measured intracellular folate species following 9 days of incubation with varying folate content in the growth medium (n=1).

VII.6. Conclusion

We were able to successfully replicate the results obtained by Courtemanche *et al.*, by using FA as a folate source during incubation. However, we provide evidence that the obtained results depend on the folate species that is used. The use of FA in cell cultures is easy because of its stability and its lower cost compared to 5MeTHF. However, if the carrier proteins or metabolic enzymes are not or inadequately expressed or if they present a rate limiting step, the results of the study will be influenced by the folate species used during incubation.

While 5MeTHF is the main circulating folate species *in vivo*, its chemical instability poses a problem in *in vitro* experimentation. While not perfect, periodic addition of 5MeTHF to the growth medium can help to stabilize the 5MeTHF concentration. By replacing the degraded 5MeTHF on a daily basis, we were able to show that the relationship between the proliferation rate of CD8+ versus CD4+ cells and the folate status, might not be as dramatic as reported by other authors.

VII.7. References

Blom, H.J. and Smulders, Y. 2011. "Overview of Homocysteine and Folate Metabolism. With Special References to Cardiovascular Disease and Neural Tube Defects." *J Inherit Metab Dis* 34 (1): 75–81.

Courtemanche, C., Elson-Schwab, I., Mashiyama, S.T., Kerry, N. and Ames, B.N. 2004. "Folate Deficiency Inhibits the Proliferation of Primary Human CD8+ T Lymphocytes in Vitro." *J Immunol* 173 (5): 3186–92.

Field, C.J., Van Aerde, A., Drager, K.L., Susan Goruk, S. and Basu, T. 2006. "Dietary Folate Improves Age-Related Decreases in Lymphocyte Function." *J Nutr Biochem* 17 (1): 37–44.

Jagerstad, M. 2012. "Folic Acid Fortification Prevents Neural Tube Defects and May Also Reduce Cancer Risks." *Acta Paediatr* 101 (10): 1007–12.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015. "A Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Selective Analysis of Free and Total Folate in Plasma and Red Blood Cells." *J Chromatogr A* 1398: 20–8.

Kishimoto, K., Kobayashi, R., Sano, H., Suzuki, D., Maruoka, H., Yasuda, K., Chida, N., Yamada, M. and Kobayashi, K. 2014. "Impact of Folate Therapy on Combined Immunodeficiency Secondary to Hereditary Folate Malabsorption." *Clin Immunol* 153 (1): 17–22.

Meyer, T.P.H., Zehnter, I., Hofmann, B., Zaisserer, J., Burkhart, J., Rapp, S., Weinauer, F., Schmitz, J. and Illert, W.E. 2005. "Filter Buffy Coats (FBC): A Source of Peripheral Blood Leukocytes Recovered from Leukocyte Depletion Filters." *J Immunol Methods* 307 (1-2): 150–66.

Shane, B. 2010. "Folate Chemistry and Metabolism." In *Folate in Health and Disease*, edited by L.B. Bailey, 2nd ed., 1–24. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Stover, P.J. 2004. "Physiology of Folate and Vitamin B12 in Health and Disease." *Nutr Rev* 62 (suppl 1): S3–12.

Wang, X. and Fenech, M. 2003. "A Comparison of Folic Acid and 5-Methyltetrahydrofolate for Prevention of DNA Damage and Cell Death in Human Lymphocytes in Vitro." *Mutagenesis* 18 (1): 81–6.

Chapter VIII: Folate polyglutamate analysis

Table of contents

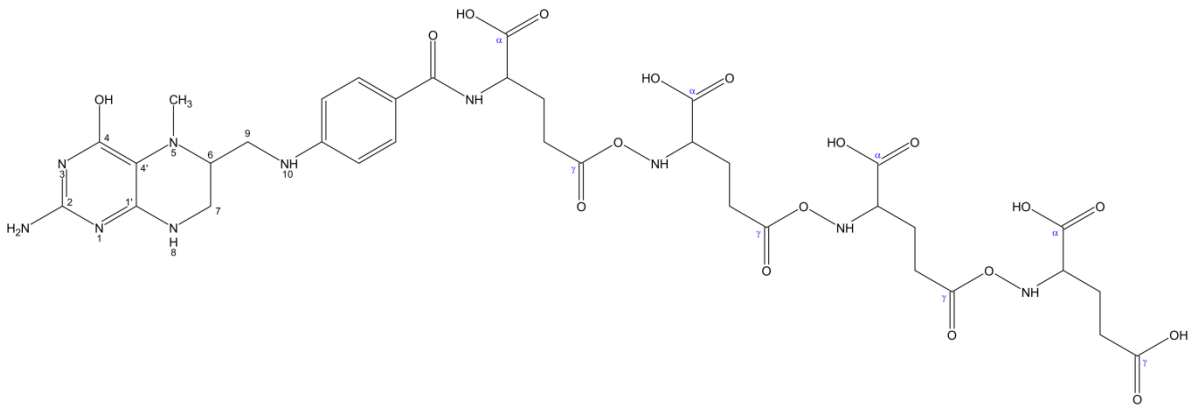
VIII.1. Abstract

Increasing the activity of the folylpolyglutamate synthase enzyme in a plant may result in the presence of a larger folate storage pool, which may increase folate stability during storage of folate-biofortified rice. Determination of polyglutamylation is typically done in an indirect manner, via the quantification of free monoglutamates on the one hand and total folate on the other, the latter following enzymatic hydrolysis of polyglutamates to monoglutamates. From the difference between these measurements, the % polyglutamylated folate can be derived. However, due to analytical and biological variation, the degree of polyglutamylation cannot always be determined with adequate statistical confidence. In addition, another research question is equally relevant: to what extent are these folates polyglutamylated, i.e. what is the length of the polyglutamate chain?

To investigate the feasibility of setting up a method to quantify individual polyglutamates in rice, we developed a method for 5-methyltetrahydropteroylpolyglutamates since this is the major folate species in genetically engineered rice. The sample treatment procedure used for the determination of free and total folate concentrations was evaluated to check for potential hydrolysis of polyglutamates by native γ-glutamylhydrolase enzyme released during sample homogenization.

Since not all polyglutamate standard materials are commercially available, some require in-house synthesis and verification of the final concentration in the obtained standard solution. By modifying an existing method, involving chemical reduction and methylation of commercially available pteroylpolyglutamates, the conversion yield was increased.

Overall, the method can be used for to compare the degree of polyglutamylation between different rice samples. (e.g. to determine the success of genetic engineering). However, method validation was not possible as a result of difficulties verifying the concentration of the standard solutions.



VIII.2. Introduction

Folates do not only differ in oxidation state and the presence of covalently bound substituents, they also differ in length of the glutamate tail, ranging from monoglutamate to octaglutamate species or even longer. Folate polyglutamylation serves a dual purpose. While this process is responsible for the retention of folates within the cells and restricting inter-organellar movement (Matherly and Goldman 2003), it also increases the affinity for folate-dependent enzymes.(Ravanel *et al*. 2004) Given that folate polyglutamates serve as a storage pool, increased polyglutamylation might result in a higher total folate content in the plant material and a reduced folate loss during storage.(Blancquaert *et al*. 2010; Blancquaert *et al*. 2014) As such, one possible strategy to further increase folate concentrations and augment stability during storage is to increase polyglutamylation by overexpressing the folylpolylutamate synthetase (FPGS) enzyme in rice. In Arabidopsis, folate polyglutamates are formed by the action of the FPGS isozymes that are specified by three genes, *FPGS1*, *2* and *3*, which reportedly encode plasmidic, mitochondrial and cytosolic isoforms, respectively.(Mehrshahi et al. 2010) The folate polyglutamate stores can in turn be released through the action of the γ-glutamyl hydrolase (GGH) enzyme. This vacuolar enzyme is able to remove glutamate units and, together with FPGS, is involved in plant folate homeostasis.(Akhtar *et al*. 2010)

For the determination of folate polyglutamylation, several possible strategies exist. Basic determination of the degree of polyglutamylation is possible by analyzing the folate content in 2 (sub)samples. One sample is treated with folate conjugase, to hydrolyze the polyglutamate tail to the corresponding monoglutamate form, allowing measurement of total folate. The other sample is not treated with conjugase enzyme, allowing measurement of free monoglutamates. The difference between the amount of folate monoglutamates in treated and untreated samples is a measure for the degree of polyglutamylation.(De Brouwer *et al*. 2010) Alternatively, by performing acid hydrolysis, the folate polyglutamates can be split into a pterin and a pABA-(poly)glutamate moiety. These pABA-glutamates can then be determined by either LC-MS, LC coupled to electrochemical detection or GC-MS analysis. Using such a method, it is possible to quantify different glutamate chain lengths present in the sample, though information per folate species is lost. (Shane 1982; Santhoshkumar *et al*. 1995; Bagley and Selhub 2000) In 2001 Ndaw *et al.* published an alternative method for the analysis of folate polyglutamates, applying a precolumn conversion of all folates present to 5MeTHPGlu$_n$, with varying polyglutamylation. While limiting the need for a full spectrum of reference standards, this approach also enables the use of fluorescence detection.(Ndaw *et al*. 2001) In 2010 Wang *et al.* omitted the precolumn conversion and measured native 5MeTHPGlu$_n$ using LC-MS/MS to investigate the inhibition of GGH during sample extraction of vegetables.(Wang *et al*. 2010) Other methods have been developed to quantify individual folate mono- and polyglutamate species simultaneously. In the method by van Haandel *et al.*, extrapolated MS settings and response factors are used to obtain a semiquantitative folate profile for individual folate species with varying degree of polyglutamylation since not all are commercially available.(van Haandel *et al*. 2012) As such,

different approaches are available from literature to assess or quantify the degree of polyglutamylation. However, the method of choice depends on the availability of sample material, equipment and the desired result.

VIII.3. Objectives

While an available method for the quantification of free and total folates in rice allowed to derive general polyglutamate data, we investigated here to what extent this method could be adapted for quantitation of individual polyglutamates of 5MeTHF. To achieve this, several key objectives needed to be achieved.

First, the required pure reference standards needed to be obtained or synthesized if commercially unavailable. Next, the adaptability of the LC-MS method for monoglutamate analysis to polyglutamate analysis was investigated. Finally, the sample preparation method should be appropriate and able to prevent hydrolysis of glutamate units during all preparation steps. Given that 5MeTHF is the major folate species in biofortified rice, polyglutamates of this folate species were selected to limit the complexity of method development.

VIII.4. Materials and methods

Whenever possible, all experiments were carried out under subdued light and in glassware for light sensitive samples. All water used was prepared using a Synergy-UV purification system (H_2O-MQ, Merck-Millipore, Darmstadt, Germany).

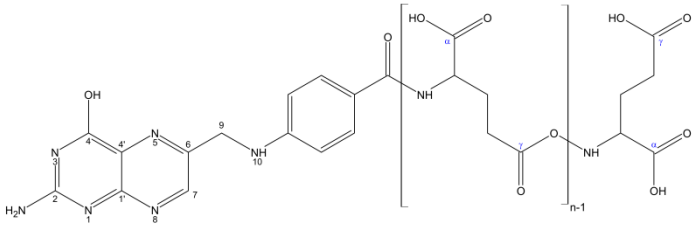
4.1. Preparation of reference materials

While 5MeTHF mono- to tetraglutamates (5MeTHFPteGlu$_{1\text{-}4}$) were available from Schircks Laboratories (Jona, Switzerland), larger polyglutamates were not and required in-house synthesis. As a basis, pteroylpenta-, hexa- and octaglutamate (PteGlu$_{5,6\ \text{and}\ 8}$) could be obtained from Shircks Laboratories. Sodiumborohydride was obtained from Acros Organics (Geel, Belgium). L-Ascorbic acid and formaldehyde 37% were from Sigma-Aldrich (Schnelldorf, Germany). Ammonium acetate and 5N sodium hydroxide were obtained from Merck KGaA (Darmstadt, Germany).

To obtain the corresponding 5MeTHFPteGlu$_n$ species from PteGlu$_n$, a procedure described by Ndaw *et al.* and adapted by Wang *et al.* was used, though with some key modifications.(Ndaw *et al*. 2001; Wang *et al*. 2010) To reduce cost, this procedure was optimized using FA monoglutamate. The different steps of this procedure are presented graphically in Figure VIII.1.

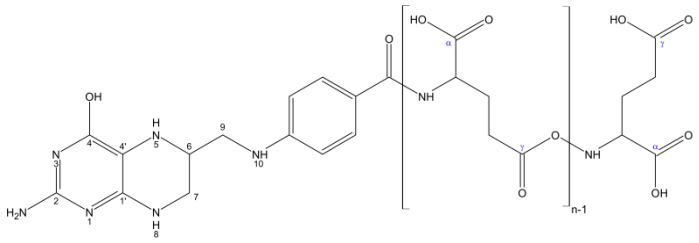
procedure by Wang *et al.*

adapted procedure



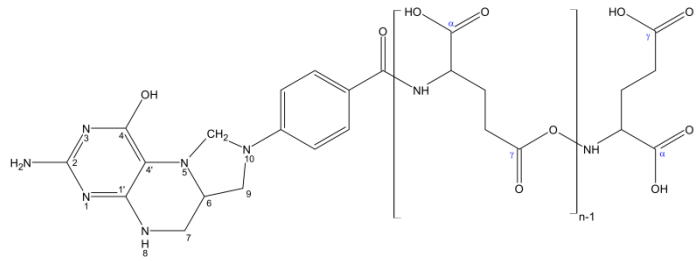
2 mg $PteGlu_n$ + 1 ml of reducing buffer*
+ 4 ml of $NaBH_4$ (3.2M in H_2O)
10 minutes incubation at room temperature
→ pH 7.4 with 5M acetic acid

2 mg $PteGlu_n$ + 1 ml of reducing buffer*
+ 4 ml of $NaBH_4$ (3.2 M; 75 mM NaOH; 0 °C)
20 minutes incubation at room temperature
+ 2x 750 µl 5M acetic acid → pH 7.4



+ 30 µl of 37% formaldehyde
shaking for 30 seconds

+ 60 µl of 37% formaldehyde
shaking for 1 minute



+ 4 ml of $NaBH_4$ (3.2M in H_2O)
20 minutes incubation at room temperature
to 25 ml with reducing buffer*

+ 4 ml of $NaBH_4$ (3.2 M; 75mM NaOH; 0 °C)
40 minutes incubation at room temperature
+ 15 ml reducing buffer* → pH 7.4

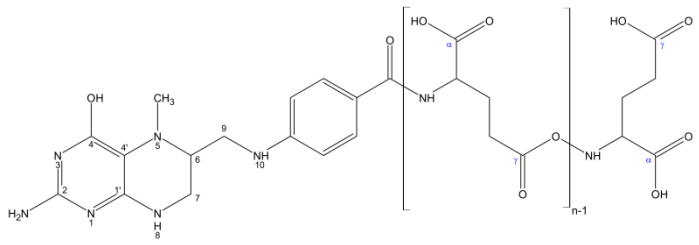


Figure VIII.1: Schematic representation of the procedure to prepare $5MeTHPGlu_n$ from $PteGlu_n$ based on the procedure published by Wang *et al.* 2010. *reducing buffer: 0.1 M ammonium acetate, 1% ascorbic acid, pH= 7.9.

The final procedure was successful in converting FA to 5MeTHF and was also successfully used to convert $PteGlu_{5,6\ and\ 8}$ to $5MeTHFPteGlu_{5,6\ and\ 8}$, respectively. First, 2 mg of standard powder was weighed and transferred to a 30 ml brown glass bottle. Next, 1 ml of reducing buffer (0.1 M ammonium acetate, 1% ascorbic acid, pH= 7.9) was added and the folate powder was dissolved. 1.2 grams of $NaBH_4$ powder was weighed and transferred to a similar glass bottle. To this, 10 ml of 75 mM NaOH was added and the crystals were dissolved. This solution was kept on ice while capped less than hand tight. 5 M acetic acid was prepared by diluting pure acetic acid obtained from Merck. Under a fume hood, 4 ml of $NaBH_4$ solution was added to the standard solution, mixed and allowed to stand for 20 minutes at room temperature. To halt the reaction, 1.5 ml of 5M acetic acid was added in two aliquots to avoid the solution bubbling over. As hydrogen gas was escaping, ignition sources were kept well clear. When the reaction had stopped, the pH was adjusted to 7.4. Then, 60 µl of 37% formaldehyde was added, the bottle was capped and shaken for 1 minute. Next, another 4 ml of the alkaline $NaBH_4$ solution was added and left uncapped for 40 minutes at room temperature. To halt the reaction, 15 ml of reducing buffer was added and the pH was adjusted to 7.4 using 5 M acetic acid. To remove the solid sodium metaborate ($NaBO_2$) formed during the reaction, the solution was filtered using a 0.22 µm syringe filter prior to aliquoting and storage at -80 °C.

4.2. Enzyme preparation

Enzymes α-amylase (Type Ia, 700-1400 U/mg protein; E.C. 3.2.1.1) and protease type XIV (>3.5 U/mg; E.C. 3.4.24.31) were purchased from Sigma Aldrich (Diegem, Belgium). The protease powder was dissolved at a concentration of 5 mg/ml in deionized water. As a source of γ-glutamylhydrolase enzyme (GGH; E.C. 3.4.19.9), non-sterile, non-hemolyzed rat serum was obtained from Harlan Laboratories (Horst, The Netherlands). Both the protease solution and the rat serum were stirred on ice for 1 hour with 100 mg/ml of activated charcoal (Sigma-Aldrich) to remove endogenous folates.(De Brouwer *et al*. 2008) Following removal of the activated charcoal by centrifugation at 4500 g for 15 minutes at 4 °C, the solutions were filtered over a 0.45 µm syringe filter (CA-S 30/0.45, Whatman, GE Healthcare, Little Chalfont, UK), divided into aliquots and frozen at -20 °C.

4.3. Chromatographic separation and detection

4.3.1. HPLC separation of folate polyglutamates to evaluate standard purity

The final method to evaluate the purity of the reference standard concentrations consisted of HPLC-UV analysis using an Agilent Zorbax Eclipse XDB-C_8 (150x4.6 mm; 5 µm, Santa Clara, CA, USA) column and a LaChrom HPLC system from Merck Hitachi (Tokyo, Japan) with a binary pump (type L-7100), autosampler (type L-7200) and interface module (type D-7000). The detector was a DAD-detector also from Merck Hitachi (type LaChrom L-7455). The mobile phase consisted of 0.1% formic acid in H_2O-MQ and acetonitrile at a flow rate of 1.0 ml/minute and a column temperature of 40°C. The injection volume was 50 µl. Gradient elution was performed as described in Table VIII.1. UV detection was performed at a

wavelength of 290 nm, a slit width of 2 nm and a spectral interval of 200 ms. Peak smoothing was switched off while auto zero was performed at injection. All polyglutamates had retention times between 6.3 and 6.6 minutes while ascorbic acid formed part of the solvent peak.

time [min]	0	1.5	4	5	6.5	6.6	9
% H_2O 0.1% HCOOH	95	87	85	5	5	95	95
% ACN 0.1% HCOOH	5	13	15	95	95	5	5

Table VIII.1: Gradient elution settings for HPLC-UV determination of folate polyglutamates

4.3.2. UHPLC separation of folate polyglutamates

Quantitation of folate polyglutamates was performed using a Waters Acquity UPLC®-system equipped with binary pump, refrigerated autosampler and column oven. To assure compatibility with the analysis of folate monoglutamates in rice samples, the same column (Waters HSS T3®, Milford, MA, USA) was used as for monoglutamate analysis. Mobile phases composed of 0.1% MS-grade formic acid in H_2O-MQ and LC-MS-grade acetonitrile (Sigma-Aldrich, Saint Louis, MI, USA and Biosolve, Valkenswaard, The Netherlands) were used at a flow rate of 0.6 ml/minute and a column temperature of 60°C. A sample volume of 10 µl was injected on the column. Following injection, respectively 600 and 800 µl of strong (10/90 v/v% H_2O-MQ/ACN) and weak wash solvent (90/10 v/v% H_2O-MQ/ACN) were used to rinse the needle and prevent carry-over. Samples were stored in an autosampler at 4 °C. Once optimized, gradient elution was performed as described in Table VIII.2.

time [min]	0	0.5	3	5	5.05	6	6.05	9
% H_2O 0.1% HCOOH	98	98	95	89	5	5	98	98
% ACN 0.1% HCOOH	2	2	5	11	95	95	2	2

Table VIII.2: Mobile phase composition of the optimized HSS T3® method

4.3.3. Mass spectrometric detection of folate polyglutamates

The API 4000™ triple quadrupole mass spectrometer was used as detector for UHPLC polyglutamate analysis. The obtained and prepared reference standards were infused at a concentration of 1 µg/ml in 50/50 v/v% H_2O/ACN with 0.1% formic acid. From these experiments, quantifier and qualifier ions were selected and the source parameters were optimized by injecting a 1 µg/ml mix of all polyglutamates using flow injection analysis at 600 µl/min of 90/10 v/v% H_2O/ACN with 0.1% formic acid. The solvent composition of the injected sample was the same as that of the mobile phase. The obtained settings can be found in Table VIII.3. As $5MeTHPteGlu_7$ was not used for early method development, the parameters for this compound were extrapolated from the parameters obtained for $5MeTHPteGlu_6$ and $5MeTHPteGlu_8$.

component parameters						
component	Q1 [amu]	Q3 [amu]	DP [V]	EP [V]	CE [V]	CXP [V]
5MeTHFPteGlu$_1$	460	**313** 180	42	10	30	8
				11	52	6
internal standard	465	**313**	42	10	31	8
5MeTHFPteGlu$_2$	589	**313** 180	66	10	30	4
				10	64	4
5MeTHFPteGlu$_3$	718		72	11	46	8
				10	82	4
5MeTHFPteGlu$_4$	847		84	10	50	4
				11	90	4
5MeTHFPteGlu$_5$	976		96	10	62	4
				10	106	4
5MeTHFPteGlu$_6$	1105		96	10	70	4
				10	114	4
5MeTHFPteGlu$_7$	1234		97	10	82	4
				10	123	4
5MeTHFPteGlu$_8$	1363		98	11	92	4
				13	122	4

source parameters	
collision gas setting	12
curtain gas setting	10
gas 1 [psig]	80
gas 2 [psig]	90
ion spray voltage [V]	2700
source temperature [°C]	600

Table VIII.3: Mass spectrometer settings, quantifier ion is indicated in bold, DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential; V: volt.

4.4. Sample preparation

To analyze the 5MeTHPteGlu$_n$ content in rice samples, 1.5 ml of 50 mM Na_3PO_4, 10 g/l ascorbic acid and 5 g/l DL-dithiothreitol (pH 7.4) including 20 ng/ml of $^{13}C_5$-5MeTHF as internal standard was added to 200 mg of rice grains. Heat treatment for 10 minutes at 99 °C and 650 rpm softened the rice grains prior to homogenization for 60 minutes at 30 Hz. (Retsch MM 301 ball mill, Retsch GmbH, Haan, Germany). After cooling on ice and removal of the milling ball, 20 µl of α-amylase was added and the sample was incubated for 10 minutes at room temperature. Next, 150 µl of protease solution was added and samples were incubated for 60 minutes at 37 °C. To denature the protease, the samples were subjected to heat denaturation in a heating block/shaker for 10 minutes at 99 °C and 650 rpm. Samples were subsequently cooled on ice and divided into two aliquots of 700 µl each, either for free or total folate analysis. Two hundred µl of water was added to the first sample and 200 µl of

charcoal-stripped rat serum was added to the second sample as a source of conjugase, converting poly- to monoglutamates. Both were incubated for 2 hours at 37°C. Finally, heat treatment for 10 minutes at 99 °C and 650 rpm, followed by cooling on ice was performed and 500 µl was transferred to 3kDa cut-off ultrafiltration tubes (Merck-Millipore, Darmstadt, Germany). For LC-MS/MS analysis, 150 µl of filtrate was transferred to inserts while any surplus was used to prepare a 20-fold dilution in IS-phosphate buffer to prepare the calibration curve. The calibration curves used consisted of serially diluted 5MeTHPteGlu$_n$ mixtures ranging from 0.2 ng/ml to 6.25 µg/ml. Following the analysis of monoglutamates in conjugase treated and untreated samples, the samples not treated with conjugase were re-injected for individual polyglutamate quantitation.

VIII.5. Results and discussion

5.1. Preparation of reference materials

We set off by evaluating the procedure described by Wang *et al*. for the quantification of 5MeTHPGlu$_n$. This revealed that approximately 10% of THF remained, when quantifying the reaction product with the LC-MS/MS method for folate monoglutamate determination following deconjugation with rat serum. This result suggested that treatment with formaldehyde to add the methyl function to THF was inadequate. This was resolved by doubling the amount of formaldehyde and doubling the incubation time. Any remaining formaldehyde is later reduced to methanol by the addition of $NaBH_4$. This adaptation increased the formation of 5MeTHPteGlu$_n$ to 99%.

The use of $NaBH_4$ in this reaction is problematic due to the very limited stability of this reagent in aqueous solutions. $NaBH_4$ reacts with water to form $NaBO_2$ and hydrogen gas. As such, extreme care should be taken that any and all ignition sources are removed from the fume hood when performing this procedure. Containers should also be loosely capped to avoid pressure build-up. However, the decomposition of $NaBH_4$ can be slowed down by increasing the pH as can be seen in Table VIII.4.

pH	$NaBH_4$ half life
4.0	0.0037 sec
5.0	0.037 sec
6.0	0.37 sec
7.0	3.7 sec
8.0	36.8 sec
9.0	6.1 min
10.0	61.4 min
11.0	10.2 hours
12.0	4.3 days
13.0	42.6 days
14.0	426.2 days

Table VIII.4: Stability of aqueous $NaBH_4$ solutions, data provided by the Applied Nano Bioscience Centre at Arizona State University

Hydrolysis of $NaBH_4$ in aqueous solution:

$$NaBH_4 + 2H_2O \rightarrow 4\ H_2 + NaBO_2$$

Due to the reactivity of $NaBH_4$, half of this reagent will be lost in about 30 seconds when dissolved in water at neutral pH. As such, the working solution of $NaBH_4$ (3.2 M) was prepared in a 75 mM solution of NaOH. However, this increases the theoretical pH during the reaction from 7.9 to 10.17. Since this was likely to slow down the reduction of $PteGlu_n$ and $5,10CH_2THPteGlu_n$, the incubation time was doubled. The decrease in reactivity was likely countered by the increased stability of the $NaBH_4$ working solution, since evaluation of the reduction steps revealed nearly complete (>99%) formation of the reaction product.

Wang *et al.* used spectrophotometric and LC-UV analysis to verify the final concentration in the solution, relying on the fact that the chromophore group is the same in both mono- and polyglutamates and that the length of the polyglutamate tail would not influence the molar absorption coefficient.(Wang *et al*. 2010) However, spectrophotometric detection is hindered by the overlapping spectra of $5MeTHPGlu_n$ and L-ascorbic acid. Therefore, either L-ascorbic acid should be removed from the reaction medium, thereby risking oxidation of the reduced folates, or chromatographic separation of L-ascorbic acid and folates should be performed. Since ascorbic acid could not be omitted from the reaction medium due to the oxidative sensitivity of folates, spectrophotometric determination was deemed not suitable. Consequently, LC-UV analysis was used to evaluate standard purity.

5.2. Chromatographic separation and detection

5.2.1. HPLC separation of folate polyglutamates to evaluate standard purity

For purity evaluation of polyglutamates using ultraviolet and/or fluorescence detection on an HPLC system, a lower pressure column was needed. The BEH C_{18} (50 x 2.1 mm; 1.7 µm, Waters) and Purospher Star RP18e (150 x 4.6 mm; 5 µm, Merck KGaA) columns gave poor retention and peak shape and were not retained for further optimization. While the Lichrospher 100 (125 x 4.6 mm; 5 µm, Merck KGaA) provided a good separation, though not baseline, peak width was very large. The Zorbax Eclipse XDB-C_8 (150 x 4.6 mm; 5 µm, Agilent) provided good retention and peak shape and was therefore used for UV control of standard concentration. A sample chromatogram can be seen in Figure VIII.2.

This method was verified using the commercially available $5MeTHPGlu_{2\text{-}4}$. However, a significant discrepancy was observed between the calculated and theoretical concentration. Personal communication with Dr. C. M. Pfeiffer and Dr. Z. Fazili of the Centers for Disease Control and Prevention (Atlanta, GA, USA), revealed that the reference materials in powdered form, obtained from Schircks Laboratories, may contain an unknown amount of water, which may account for the difference in results observed during LC-UV evaluation of the standard concentration. Then, the actual concentration, determined using spectrophotometry in the absence of ascorbic acid, can be used to evaluate the performance of the LC-UV verification method.

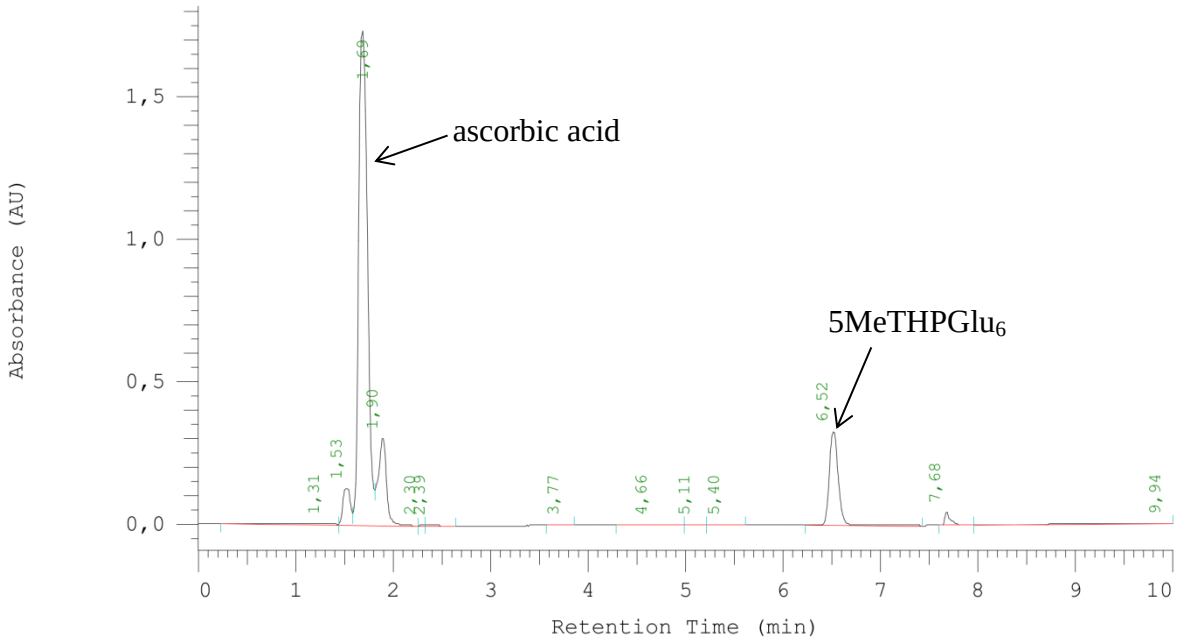


Figure VIII.2: UV chromatogram of 5MeTHPteGlu$_6$

5.2.2. UHPLC separation of folate polyglutamates

Given the availability of an optimized method for the analysis of folate monoglutamates, we attempted to maintain the column and mobile phase of this method and adapt the parameters for polyglutamate analysis.

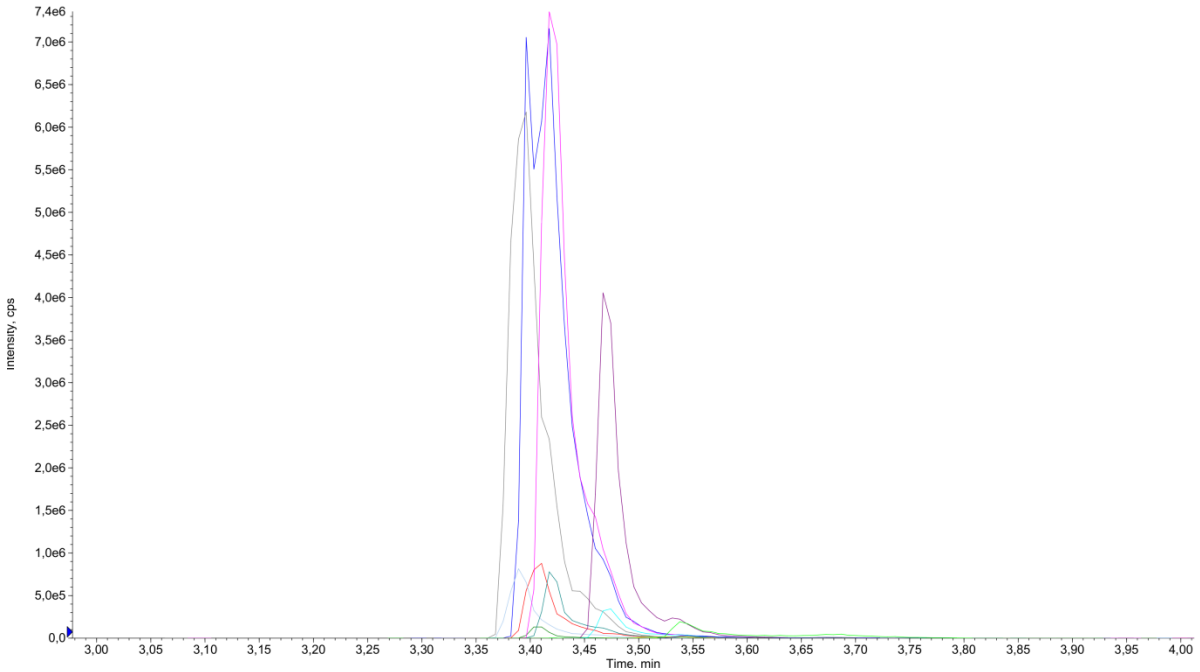


Figure VIII.3: Chromatogram showing 5MeTHPteGlu$_{1,2,3,4\&6}$ using the method of De Brouwer *et al (2008)*.

The HSS T3 column is able to retain the different polyglutamate species using the existing mobile phase. However, using the elution program developed by De Brouwer *et al.(2008)*, significant peak overlap was observed (Figure VIII.3). Since there is approximately 1% cross-talk of larger polyglutamates towards smaller analytes, chromatographic separation is

important to achieve acceptable selectivity. However, some tailing was observed, with peak shape deteriorating as polyglutamate length increased. This decreases method sensitivity.

By altering the gradient elution scheme, the different polyglutamate species could be further separated, though baseline separation was not possible, especially for $5MeTHPteGlu_1$ and $5MeTHPteGlu_2$. In this case, the cross-talk effect will result in an additional signal for $5MeTHPteGlu_1$ originating from $5MeTHPteGlu_2$-ions. As such, the evaluation of cross talk requires extra attention during method validation. Chromatograms for a standard mix, rice and red blood cell (RBC) sample are provided in Figure VIII.4.

5.2.3. Mass spectrometric detection of folate polyglutamates

From the mass spectrometric settings specified in Table VIII.3 it is clear that the declustering potential (DP) reaches its maximal settings for ions larger than tetraglutamates. This voltage is applied to prevent the ions from clustering together. As such, it is possible that cluster formation of larger polyglutamates exceeds the capacity of the ion source, leading to low sensitivity. This problem should be remediated to achieve the lowest possible quantitation limit required for polyglutamate analysis in samples with a low degree of folate polyglutamylation.

5.3. Sample preparation

Wang *et al.* found that the release of GGH-enzymes during sample homogenization caused the deconjugation of polyglutamates prior to LC-MS analysis. These authors proposed a steaming step prior to homogenization to denature the GGH-enzymes and preserve the polyglutamates during homogenization.(Wang *et al*. 2010) In line with this, the boiling step performed prior to homogenization can serve as a means to denature the GGH-enzymes and preserve the folate polyglutamylation in the sample.(De Brouwer *et al*. 2008) This was verified by adding $5MeTHPteGlu_{2\text{-}6}$ to wild type rice samples prior to and after sample preparation. No indication of deglutamylation was observed.

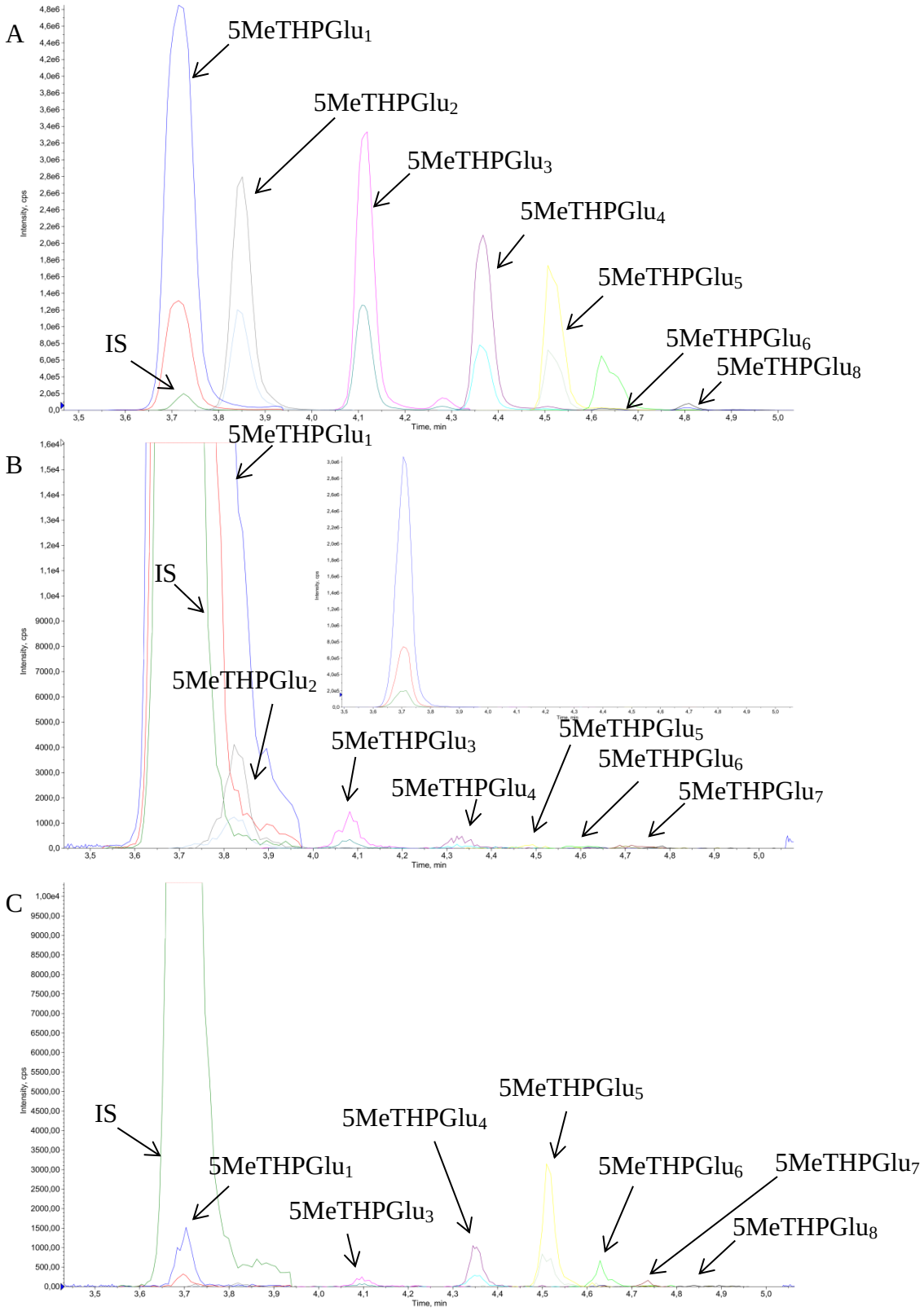


Figure VIII.4: Chromatograms obtained using the optimized T3 method for (A) a standard solution with a concentration of 2 µg/ml per species; (B) a folate rice sample with respective concentrations of 459.9, 4.3, 1.5, 0.9 µg/100g for 5MeTHFPteGlu$_{1\ to4}$, all others <lower limit of quantification; (C) a red blood cell extract

VIII.6. Conclusion

Accurate quantitation of individual polyglutamates was not possible due to the lack of standards with accurate concentrations. As a result of an observed difference between the measured and theoretical concentration for the commercially available 5MeTHPteGlu$_{2-4}$, the LC-UV procedure proved unreliable and should be evaluated further based on purity data for the commercially available 5MeTHPteGlu$_{2-4}$. 5MeTHPteGlu$_{5-8}$ can be reproducibly prepared using the adapted method described in this chapter. However, to use the obtained solutions for calibration, the actual concentration should also be reliably determined using the LC-UV method.

Further optimization of the LC-MS/MS procedure could be possible if cluster formation during ionization can be prevented. This phenomenon limits the sensitivity of the method for folates with larger polyglutamate-tail length. Since the degree of polyglutamylation in rice is limited, utmost sensitivity is required to compare different rice varieties. Yet, when sufficient material is available, comparative profiling may be achieved.

VIII.7. References

Akhtar, T.A., Orsomando, G., Mehrshahi, P., Lara-Núñez, A., Bennett, M.J., Gregory, J.F.III and Hanson, A.D. 2010. "A Central Role for Gamma-Glutamyl Hydrolases in Plant Folate Homeostasis." *Plant J* 64 (2): 256–66.

Bagley, P.J., and Selhub J. 2000. "Analysis of Folate Form Distribution by Affinity Followed by Reversed- Phase Chromatography with Electrical Detection." *Clin Chem* 46 (3): 404–11.

Blancquaert, D., De Steur, H., Gellynck, X. and Van Der Straeten, D. 2014. "Present and Future of Folate Biofortification of Crop Plants." *J Exp Bot* 65 (4): 895–906.

Blancquaert, D., Storozhenko, S., Loizeau, K., De Steur, H., De Brouwer, V., Viaene, J., Ravanel, S., Rébeillé, F., Lambert, W.E. and Van Der Straeten, D. 2010. "Folates and Folic Acid: From Fundamental Research Toward Sustainable Health." CRC *Crit Rev Plant Sci* 29 (1): 14-35.

De Brouwer, V., Storozhenko, S., Stove, C.P., Van Daele, J., Van der Straeten, D. and Lambert, W.E. 2010. "Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) for the Sensitive Determination of Folates in Rice." *J Chromatogr B* 878 (3-4): 509–13.

De Brouwer, V., Storozhenko, S., Van De Steene, J.C., Wille, S.M.R., Stove, C.P., Van Der Straeten, D. and Lambert, W.E. 2008. "Optimisation and Validation of a Liquid Chromatography–tandem Mass Spectrometry Method for Folates in Rice." *J Chromatogr A* 1215 (1–2): 125–32.

Matherly, L.H. and Goldman, D.I. 2003. "Membrane Transport of Folates." *Vitam Horm* 66: 403–56.

Mehrshahi, P., Gonzalez-Jorge, S., Akhtar, T.A., Ward, J.L., Santoyo-Castelazo, A., Marcus, S.E., Lara-Núñez, A., *et al*. 2010. "Functional Analysis of Folate Polyglutamylation and Its Essential Role in Plant Metabolism and Development." *Plant J* 64 (2): 267–79.

Ndaw, S., Bergaentzle, M., Aoude-Werner, D., Lahely, S. and Hasselmann, C. 2001. "Determination of Folates in Foods by High-Performance Liquid Chromatography with Fluorescence Detection after Precolumn Conversion to 5-Methyltetrahydrofolates." *J Chromatogr A* 928 (1): 77–90.

Ravanel, S., Block, M.A., Rippert, P., Jabrin, S., Curien, G., Rébeillé, F. and Douce, R. 2004. "Methionine Metabolism in Plants: Chloroplasts Are Autonomous for de Novo Methionine Synthesis and Can Import S-Adenosylmethionine from the Cytosol." *J Biol Chem* 279 (21): 22548–57.

Santhoshkumar, C.R., Deutsch, J.C., Hassell, K.L., Kolhouse, N.M. and Kolhouse, J.F. 1995. "Quantitation of Red Blood Cell Folates by Stable Isotope Dilution Gas Chromatography-Mass Spectrometry Utilizing a Folate Internal Standard." *Anal Biochem* 225 (1): 1–9.

Shane, B. 1982. "High Performance Liquid Chromatography of Folates: Identification of Poly-Gamma-Glutamate Chain Lengths of Labeled and Unlabeled Folates." *Am J Clin Nutr* 35 (3): 599–608.

van Haandel, L., Becker, M.L., Williams, T.D., Stobaugh, J.F. and Leeder, J.S. 2012. "Comprehensive Quantitative Measurement of Folate Polyglutamates in Human Erythrocytes by Ion Pairing Ultra-Performance Liquid Chromatography/tandem Mass Spectrometry." *Rapid Commun Mass Spectrom* 26 (14): 1617–30.

Wang, C., Riedl, K.M., and Schwartz, S.J. 2010. "A Liquid Chromatography-Tandem Mass Spectrometric Method for Quantitative Determination of Native 5-Methyltetrahydrofolate and Its Polyglutamyl Derivatives in Raw Vegetables." *J Chromatogr B* 878 (29): 2949–58.

Chapter IX: Broader international context, relevance and future perspectives

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IX.1. Achievements of this PhD project

The prime achievement of this PhD project lies in the demonstration of the biological effectiveness of folate rice following its consumption, using an animal model. To achieve this aim, also tools needed to be developed that allow the determination of folates in rodent diets, as well as in red blood cells and plasma. These tools, as well as other achievements of this thesis, are relevant for future development in the field of folate research.

1.1. Determination of folates in rodent diets

While methods for the quantitative determination of folates in several different food matrices existed, no method had been developed to measure individual folate species in rodent diets. Though these diets are commonly used for folate research purposes, the supplier generally only mentions the amount of folic acid (FA) added to the diet. However, when we applied our method to folate deficient diets, we observed that these contained an amount of natural folates originating from various feed ingredients. As such, the development of a method for folate determination provides a tool for future researchers to also account for folate intake from the rodent feed.(Chapter IV, Kiekens *et al.* 2015a)

1.2. Determination of folates in plasma and RBCs

While microbiologic or protein binding assays are widely used for the determination of clinical folate status, emerging LC-MS/MS assays provide an orthogonal determination strategy focusing on exact chemical determination instead of biological activity. While the microbiologic assay currently remains the gold standard method for the determination of biologically active folates, the protein binding assay is susceptible to differences between the folate species present in the sample and the species, mostly FA, used for assay calibration.(Pfeiffer *et al*. 2004)

The accuracy and precision obtained using LC-MS/MS and the possibility to quantify individual folate species has led to an increased interest to implement this technology for clinical folate research. The implementation of this technique in a routine clinical laboratory will depend on the automation of the sample preparation steps and the availability of LC-MS/MS equipment. Due to the ease of the assay and possible automation, protein binding assays are currently used most to determine the amount of folates in patient samples. Comparability of analytical results with other labs is not a major concern for the clinician since the use of reference intervals specific for the assay will suffice to distinguish folate deficient patients from those with an adequate folate status. For scientific research purposes, however, method comparability is necessary to compare findings with those obtained by other research groups and as such, LC-MS/MS is the method of choice in folate research.

Whole blood or red blood cell folate analysis is not yet regularly performed for the determination of long term folate status over the lifetime of the RBCs, i.e. approximately 3 months. This measurement is not influenced by recent folate intake so, contrary to plasma or serum folate determination, a fasting period is not strictly necessary.

Since plasma and red blood cell folate concentrations provide complementary information, respectively for acute and chronic folate deficiency, we opted to develop a method to analyze

both using a very comparable sample treatment procedure to limit the use of resources, i.e. time, equipment and reagents.(Chapter V, Kiekens *et al.* 2015b) If routine clinical analysis using an LC-MS/MS assay is considered, such an approach should make a cost effective procedure achievable. To evaluate our method for clinical folate determination, we successfully participated in a round-robin test organized by Fazili and Pfeiffer of the Centers for Disease Control in Atlanta, GA, USA. These results should be published soon.

1.3. Folate rice feeding trial

Since the folates in folate rice originate from genetic manipulations of the rice genome, resulting in supraphysiological accumulation of folates within the rice grain, it is clear that sound scientific evidence must be provided to prove the usefulness of folate rice as a source of dietary folate. Recently, Castorena-Torres *et al*. investigated the relationship between feeding of a folate-biofortified tomato variety on folate status using a murine model. These authors chose to apply an area under the curve approach. Following a single intake of folates, the area under the plasma response curve was used to calculate the degree to which the folates in this biofortified tomato are bioavailable.(Castorena-Torres *et al.* 2014) Apart from this study, no other study had evaluated the impact of folate-biofortified food on folate status.

We chose a different approach due to the complex nature of folate metabolism *in vivo* and the absence of a zero folate level at the start of the experiment. In contrast to the study on biofortified tomato, we performed a long term feeding study to allow for the necessary time to reach steady-state levels, indicating the ability of folates in folate rice to maintain a certain folate status. More particularly, the blood folate concentrations measured during and at the end of our feeding trial provided the information needed to evaluate the efficacy of folate rice as a source of dietary folate. As folate metabolism is intertwined with various other metabolic cycles, we also monitored other metabolic indicators, i.e. homocysteine and the hematological indices of erythropoiesis. (Chapter VI, Kiekens *et al.* 2015c)

With this long term study in rodents we have demonstrated that the folates from folate rice can be released (i.e. are bio-accessible), are bioavailable (i.e. result in increased folate levels) and have biological effects.

1.4. Lymphocytes and folate status

This study was ignited by a publication of Courtemanche *et al.* These authors hypothesized that if folate deficiency impairs the proliferation of CD8+ T-cells, it could impair the capacity of CD8 cells to eliminate infected or tumor cells. Although these authors found a different response between lymphocyte subsets, more research was needed, as readily indicated by these authors, to investigate the underlying reason for this difference. (Courtemanche *et al*. 2010)

Besides replicating the experimental set-up performed by Courtemanche *et al.* (using FA), we also used 5-methyltetrahydrofolate (5MeTHF), ubiquitously present *in vivo*, as a folate source in the growth medium of isolated immune cells. The addition of this folate species on a daily basis to replace the amount lost due to degradation shows that it is possible to perform *in vitro* experiments using 5MeTHF as a folate source.

We were able to demonstrate that there is a concentration dependence between the intra- and extracellular folate concentration and that cell replication is likely to be less dependent on folate status than was concluded by Courtemanche *et al*.

1.5. Folate polyglutamate determination

The classical approach to determine the degree of polyglutamylation of folates in rice samples is not able to discriminate between folates with a different number of glutamate units.(De Brouwer *et al*. 2010) To precisely evaluate the effect an increased expression of folate polyglutamate synthase enzyme (FPGS) has on polyglutamate content, a new method was developed to quantify individual folate polyglutamate species.

Folate polyglutamylation is a possible factor influencing both folate stability in the food item and bioavailability following consumption. Therefore, a method which allows to quantify individual folate polyglutamate species is useful to investigate the effect of this factor on stability and bioavailability.

IX.2. GMO's in Europe and throughout the world

In the European Union, the view towards the use of genetically modified organisms, commonly known under the acronym GMO's, is rather negative. However, in the United States and China, genetically engineered crops are common. Local GMO legislation tends to reflect the acceptance of the general public of crops which contain genes that would not be present by natural processes.

2.1. European policies on GMO's

The European Food Safety Authority uses the following definition to describe GMO's:

"In recent times, it has become possible to modify the genetic make-up of living cells and organisms using techniques of modern biotechnology called gene technology. The genetic material is modified artificially to give it a new property (e.g. a plant's resistance to a disease, insect or drought, a plant's tolerance to a herbicide, improving a food's quality or nutritional value, increased yield)."
(European Commission. 2016a)

At this time, only one genetically engineered crop has been cultivated in the European Union, the MON 810 maize variety (Figure IX.1). The cultivation license for this maize variety expired in November 2013 and the procedure for renewal is ongoing. By introducing a gene from Bacillus *thuringiensis*, the maize plant produces Bt toxin, which is toxic to butterflies and moths, including the European Maize Borer. The caterpillars of these moths chew tunnels through the stalks of the plants, causing them to fall over. When Bt Maize is cultivated, the Maize Borer Caterpillar dies due to the Bt toxin present in the tissues of the plant. Thus, productivity per hectare is increased. However, increasing resistance to Bt toxin is reducing the effectiveness of this genetic modification.(Tabashnik et al. 2013)

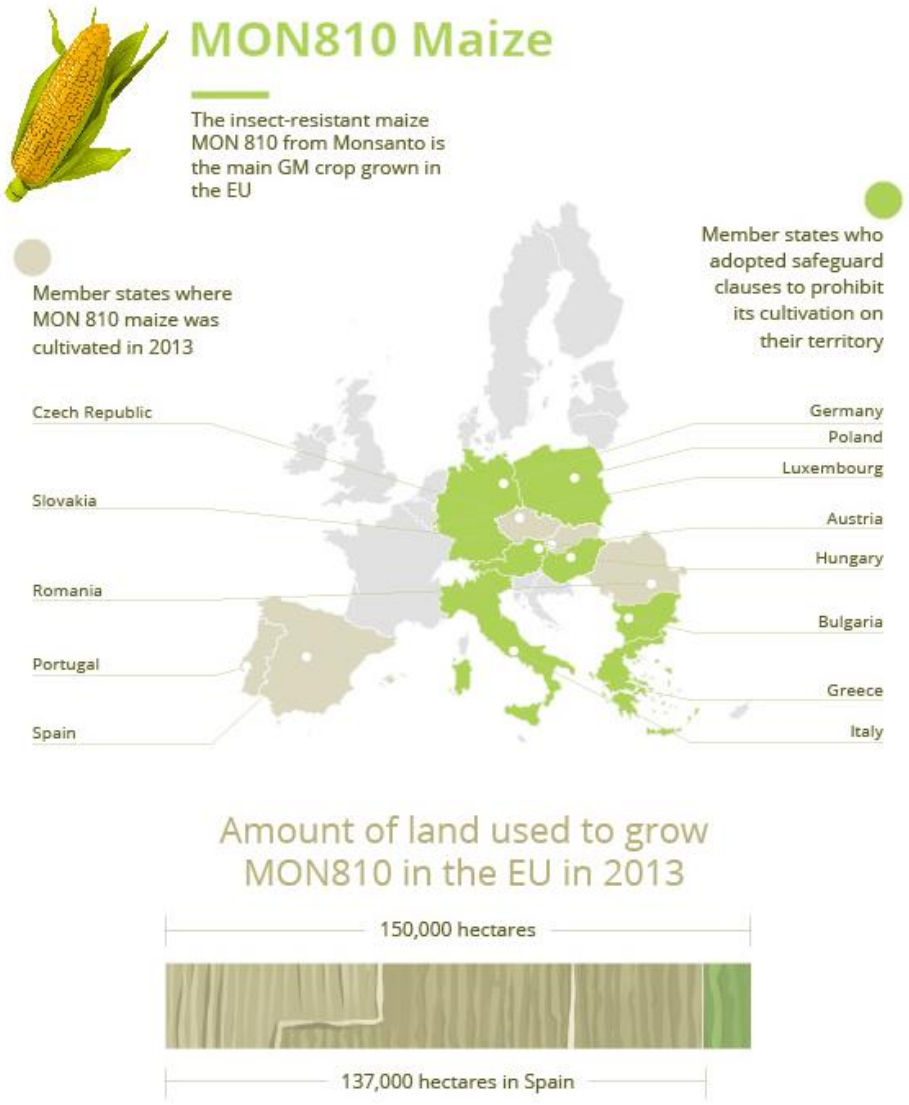

Figure IX.1: MON810 cultivation in the European Union in 2013 (European Parliament Press Service, 2015)

While only one GMO was actually cultivated in the EU, in 2014 57 GMO's were allowed to be imported in the EU. (Vlaams infocentrum land- en tuinbouw, 2014) The genetic modifications made to these GMO's can be found in Table IX.1. Most often, the genetic modification consists of an introduced resistance to herbicides or to lepidopteran (moths and butterflies) or coleopteran (beetles) species. Some genetic modifications result in male sterility and render it impossible for this species to reproduce in the wild. The introduction of drought stress resistance allows to grow these plants in arid areas. (European Commission, 2016b)

gene	**effect**	**plant species**
cp4 epsps	herbicide (glyphosate) resistance	cotton, maize, rape and soybean
pat	herbicide (glufosinate) resistance	cotton, maize, rape and soybean
cry2Ab2	lepidopteran pest resistance	cotton, maize
cry1Ac	lepidopteran pest resistant	cotton, soybean
2mepsps	herbicide (glyphosate) resistance	cotton
cry1F	lepidopteran pest resistance	maize
cry1Ab	lepidopteran pest resistant	maize
cry34Ab1	coleopteran pest resistant	maize
mepsps	herbicide (glyphosate) resistance	maize
cry3Bb1	coleopteran pest resistant	maize
cry1A.105	lepidopteran pest resistant	maize
cry35Ab1	coleopteran pest resistant	maize
cry3A	coleopteran pest resistant	maize
cspB	drought stress resistant	maize
govx247	glyphosate resistant	rape
barnase & barstar	male sterility	rape
gat	herbicide (glyphosate) resistance	soybean
gm-hra	ALS-inhibiting herbicide resistant	soybean
FAD2-1A (fragment) & FAT1-A (fragment)	increased oleic acid production	soybean
DMO	herbicides (dicamba) resistance	soybean
Pj.D6D & Nc.Fed3	stearidonic acid production from linoleic acid	soybean
glycine max-hra	herbicide (acetolactate synthase-inhibiting) tolerance	soybean
acetohydroxyacid synthase	herbicide (imidazoline) tolerance	soybean

Table IX.1: genetic modifications authorized for import in the European Union. (European Commission, 2016b)

2.2. GMO's throughout the world

Worldwide, soy (79% of all cultivated), cotton (70%), maize (32%) and rapeseed (24%) are the 4 most important GMO crops (Table IX.1). In Asia, North and South America, GMO's are commonly cultivated. Western Europe, Russia and Africa are more reluctant in adopting GMO crops (Figure IX.2). (The International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2015)

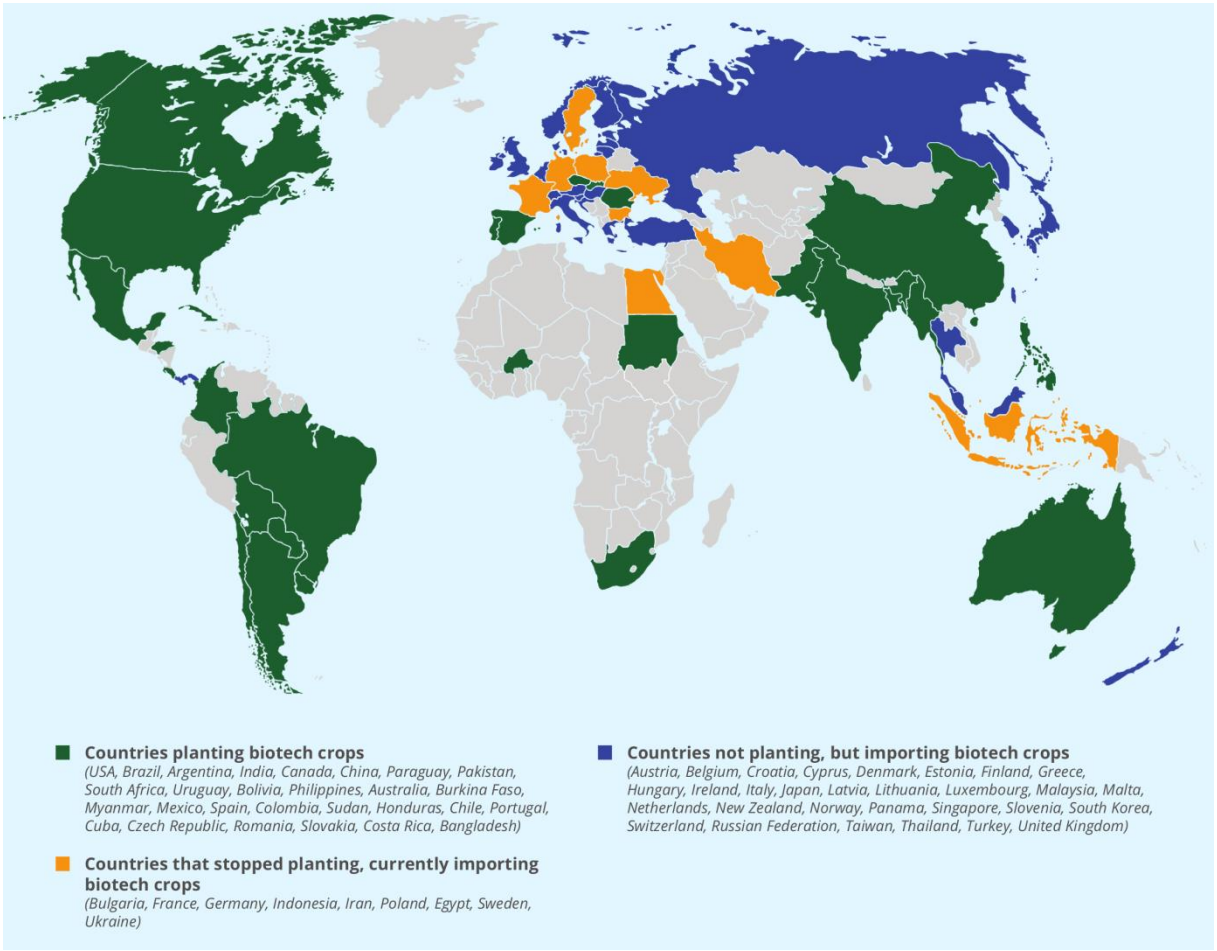

Figure IX.2: GMO's throughout the world (ISAAA, 2015)

Commonly heard advantages of GMO's are the increased food production and the reduced use of insect- and herbicides. Concerns are generally based on the (perceived) risk that these crops may pose for food safety, where views differ, from "demonstrating that GMO's are safe" to "demonstrating that GMO's are not unsafe". Moreover, the use of herbicide- or pesticide-resistant crops may allow the use of certain pesticides, which by itself may be a point of criticism, as exemplified by a study of Bøhn *et al.*, demonstrating glyphosate accumulation (0.4 to 8.8 mg/kg) in a glyphosate resistant soy following application of this chemical during the growth process. This poses a concern given that glyphosate is under scrutiny for possible carcinogenicity. (Bøhn *et al.* 2014) As the use of GMO's continues, research is being conducted to investigate the impact of GM crops on human health. Well-conducted, objective studies are important for public acceptance of GM crops, amongst which folate rice.

IX.3. Folate rice, a recipe for success!

3.1. Folate rice and folate status

In this PhD project we were able to demonstrate that folate rice is able to supply folates to rats consuming it. Even though, in absolute amounts, the folate supplementation used in our study was minimal because of limitations in available folate rice, the rats remained apparently healthy during the long term feeding trial. This was in strong contrast to the animals receiving wild type rice. While the latter animals showed clear signs of folate deficiency and some even perished during the trial, the animals receiving a supplement in the form of folate rice thrived. A similar result was observed for the feeding strategy using FA fortified rice. This shows that folate rice is able to provide similar results when compared to traditional fortification strategies. (Chapter VI, Kiekens *et al.* 2015c)

3.2. Economic aspects of folate rice use

Apart from the investigation into the biological effect of the consumption of folate rice, the economic impact of the introduction of folate rice on a population with a high prevalence of folate deficiency has been investigated. De Steur *et al*. calculated the effect of folate rice consumption on the health status in China. While rice consumption is lower in the northern regions which are confronted with a high impact of folate deficiency compared to southern regions, the required folate intake can still be achieved and a significant reduction of the health burden related to folate deficiency is possible. Based on a disability-adjusted life years approach, implementing folate rice in China would save 37 to 82% of the health burden related to folate deficiency.(De Steur *et al*. 2010) The results obtained in our rodent study, demonstrating the adequacy of folate rice, lend further support to the health claims made by De Steur *et al.* (Chapter VI, Kiekens *et al.* 2015c)

However, public perception of bioengineered food items can be negative, which may impact public acceptance of folate rice. Public acceptance and willingness-to-pay were investigated using a technique of experimental auctions of folate rice. While the nutritional effect of folate rice is valued positively by the consumers in the Shanxi province in Northern China, educated consumers tend to be more negative towards bioengineered rice. The willingness to pay a premium for the perceived higher quality rice demonstrates that genetic engineering is accepted by a public in need for folate fortification.(De Steur *et al*. 2012)

3.3. Recent developments of folate rice

The usefulness of folate rice also depends on the stability of the folates present in this biofortified rice variety during storage after harvest. From a stability experiment it was concluded that approximately 50% of folates were lost following 4 months of storage at temperatures of 21 and 28 °C. Only 40% of folates present at the time of harvest were still present after 8 months of storage. Also, a generation effect was inferred following analysis of rice seeds from different generations of this rice species. While in a rice line (GA9.15), up to 1700 µg of folates were present per 100 g of fresh weight in the fourth generation, this concentration decreased to 700 µg / 100 g fresh weight in the seventh generation.(Blancquaert *et al*. 2015)

A possible solution for the stability issue and the generation effect was to increase either the degree of folate polyglutamylation, through the increase of folylpolyglutamate synthase (FPGS) expression, or to introduce the genes necessary for the expression of folate binding protein (FBP) in the rice plant. The increased expression of the FPGS enzyme did not succeed in augmenting the degree of polyglutamylation due to a concurrent increase of the γ-glutamylhydrolase enzyme expression opposing the function of FPGS. The introduction of FBP in the rice genome resulted in lines with a higher and more stable folate concentration. (Blancquaert *et al*. 2015)

IX.4. What the future might bring

Further biofortification of staple crops might extend the use of these food items to more than just a source of energy. By adding different vitamins and nutrients, these functional foods can support the health status of the individual consuming it. For instance, by further engineering of the rice genome, a rice species could be created with an increased concentration of other B-vitamins such as thiamin (B1). Also a combination with iron biofortification might prove beneficial, especially to counteract anemia. Following this approach, true functional foods could be created that provide the consumer with a sufficient nutrient intake, irrespective of the food diversity available to this consumer. While, apart from extreme cases, a low nutrient status may not be all that significant in a clinical context, an optimal intake of essential vitamins should increase the overall health status of the population by optimizing *in vivo* metabolism and biosynthesis. By doing so, many illnesses can be prevented before requiring medical attention.

Though it is unlikely that LC-MS/MS will completely replace the microbiologic and binding assays used for clinical folate measurement, these higher order assays will be very useful to evaluate the systematic differences between different assays to achieve comparability of assay results. Since an evaluation of the folate status of different populations depends on the comparability of reference values (not biased by systematic differences), the importance of certified reference materials, verified using LC-MS/MS, will only increase. LC-MS/MS is definitely required when quantitative information on the individual folate monoglutamate and polyglutamate species is needed. As such, it is possible to study the enzymes taking part in folate metabolism in greater detail, thus allowing to obtain the most information possible from complex experiments.

Although our understanding of folates as an essential nutrient is rapidly growing, much is still to be learned on the actual function of different folate monoglutamate and polyglutamate species. For instance, the effect that low folate status has on immune response has not been thoroughly investigated. While the use of FA is common practice in folate research, this may result in biased results, as FA is not an appropriate folate source for *in vitro* studies. In this thesis, we demonstrated that cells exposed to FA behave differently than cells exposed to 5MeTHF (Chapter VII). While this is an important finding, our understanding of intracellular processes involving folate remains limited. With the gradual and ongoing increase in LC-MS/MS sensitivity, it should be possible to further characterize intracellular folate

metabolism. This could be helpful to relate the cause and onset of certain illnesses to folate status. A causal relation between folate status and illnesses remains largely elusive to this day.

IX.5. Conclusion

The importance of nutrient intake for an optimal overall health status is gaining interest due to the distribution of the knowledge gained following decades of scientific research. While the quality of the western diet is improving and fruits and vegetables rich in nutrients are promoted by the government, an optimal nutrient status may not easily be achieved. Especially prior to and during pregnancy an additional folate intake is necessary to minimize the risk for neural tube defects. FA supplementation, either as a pill or as food fortification, can provide the additional folate intake needed to achieve an adequate folate status. However, not in all parts of the world preventive healthcare or food fortification on an industrial scale is available to the general public. In this respect, biofortified folate rice can provide a source of dietary folate which can be farmed locally.

One of the steps towards public acceptance of this genetically modified crop is the proof that the folates accumulated within the rice grains are effectively biologically available and that folate rice helps to maintain an adequate folate status. Using a rat model and by providing the rice on a daily basis during a long term feeding trial with periodic monitoring of short and long term folate status and other biological indices, we were able to demonstrate that folate rice is able to serve as an adequate source of dietary folate.

IX.6. References

Blancquaert, D., Van Daele, J., Strobbe, S., Kiekens, F., Storozhenko, S., De Steur, H., Gellynck, X., Lambert, W.E., Stove, C.P. and Van Der Straeten, D. 2015. "Improving Folate (vitamin B9) Stability in Biofortified Rice through Metabolic Engineering." *Nat Biotechnol* 33 (10): 1076–8.

Bøhn, T., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., Primicerio, R. 2014. "Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans." Food Chem 153: 207-15

Castorena-Torres, F., Ramos-Parra, P.A., Hernández-Méndez, R.V., Vargas-García, A., García-Rivas, G. and de la Garza, R.I.D. 2014. "Natural Folates from Biofortified Tomato and Synthetic 5-methyl-tetrahydrofolate Display Equivalent Bioavailability in a Murine Model." *Plant Foods Hum Nutr* 69: 57–64

Courtemanche, C., Elson-Schwab, I., Mashiyama, S.T., Kerry, N. and Ames, B.N. 2004. "Folate Deficiency Inhibits the Proliferation of Primary Human CD8+ T Lymphocytes in Vitro." *J Immunol* 173 (5): 3186–92.

De Brouwer, V., Storozhenko, S., Stove, C.P., Van Daele, J., Van der Straeten, D. and Lambert, W.E. 2010. "Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) for the Sensitive Determination of Folates in Rice." *J Chromatogr B* 878 (3-4): 509–13.

De Steur, H., Gellynck, X., Storozhenko, S., Liqun, G., Lambert, W.E., Van Der Straeten, D. and Viaene, J. 2010. "Health Impact in China of Folate-Biofortified Rice." *Nat Biotechnol* 28 (6): 554–6.

De Steur, H., Gellynck, X., Feng, S., Rutsaert, P. and Verbeke, W. 2012. "Determinants of Willingness-to-Pay for GM Rice with Health Benefits in a High-Risk Region: Evidence from Experimental Auctions for Folate Biofortified Rice in China." *Food Qual Prefer* 25 (2): 87–94.

European Commission. 2016a. "Genetically Modified Organisms." http://ec.europa.eu/food/plant/gmo/index_en.htm (Consulted: 04/26/2016)

European Commission. 2016b. "EU Register of authorised GMOs." http://ec.europa.eu/food/dyna/gm_register/index_en.cfm (Consulted: 05/01/2016)

European Parliament Press Service. 2015. "Eight things you should know about GMOs." http://www.europarl.europa.eu/news/en/news-room/20151013STO97392/Eight-things-you-should-know-about-GMOs (Consulted: 05/01/2016)

The International Service for the Acquisition of Agri-biotech Applications (ISAAA). 2015. "19 Years of Biotech Crops in the World." http://www.isaaa.org/resources/infographics/19yearsofbiotechcrops/19%20Years%20of%20Biotech%20Crops%20in%20the%20World.pdf (Consulted: 05/01/2016)

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove C.P. 2015a. "Determination of Five Folate Monoglutamates in Rodent Diets." *J Agric Food Chem* 63(45):10089-10095

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015b. "A Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Selective Analysis of Free and Total Folate in Plasma and Red Blood Cells." *J Chromatogr A* 1398: 20–8.

Kiekens, F., Blancquaert, D., Devisscher, L., Van Daele, J., Stove, V.V., Delanghe, J.R., Van Der Straeten, D., Lambert, W.E. and Stove, C.P. 2015c. "Folates from metabolically engineered rice: a long-term study in rats." *Mol Nutr Food Res* 59(3):490-500.

Pfeiffer, C.M., Fazili, Z., McCoy, L., Zhang, M. and Gunter, E.W. 2004. "Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay." *Clin Chem* 50 (2): 423–32.

Tabashnik, B.E., Brévault, T. and Carrière, Y. 2013. "Insect resistance to Bt crops: lessons from the first billion acres." *Nat Biotechnol* 31 (6): 510-21.

Vlaams infocentrum land- en tuinbouw. 2014. "Oppervlakte ggo's is sinds 1996 vergroot met factor 100"
http://www.vilt.be/Oppervlakte_ggos_is_sinds_1996_vergroot_met_factor_100
(Consulted: 04/28/2016)

Chapter X: Summary and Conclusions

In this thesis, an *in vivo* study is described that aimed at investigating the nutritional value of folate rice, a bioengineered rice species with an increased folate content compared to wild type rice. However, to obtain the data necessary for a complete evaluation of folate status, a method for folate determination in small blood samples was developed and validated. Also a method to assess the presence of folates in rodent diets was developed to exclude constituents of these diets as a confounding factor.

As described in **Chapter IV**, the development of a method for the analysis of folates in rodent diets proved more challenging than initially expected. The synthetic nature of the rodent diets resulted in the release of oils and fats during sample extraction, which complicated the manipulation of the samples. Though the separation of these oils and fats using an organic water-immiscible solvent was evaluated, it provided no improvement. The pH was of key importance for the optimal deconjugation of folate polyglutamates. While a pH of 7.4 is optimal for the determination of folates in rice, for rodent diet, the optimal pH was 6.0. As such, a pH adjustment was introduced as part of the tri-enzyme treatment. Also, a solid-phase extraction (SPE) procedure was introduced to remove interfering peaks and assure method selectivity. By opting for an orthogonal separation technique using a strong-anion exchange sorbent, the remaining triglycerides could be removed prior to the analysis of the samples using a reversed phase analytical column. This method, once fully validated based on the guideline of the Food and Drug Administration, was published in the Journal of Agricultural and Food Chemistry.

Similarly, a method for the analysis of folates in plasma and red blood cells (RBCs) was developed based on the method for folates in rice. However, an all-new sample preparation procedure was needed. The limited amount of whole blood (±300 µl) available from the periodic sampling of laboratory animals necessitated the use of one aliquot of whole blood to measure both the plasma and the RBC folate. To achieve this, a washing procedure was devised. First, the plasma fraction is removed after which the RBCs are washed 3 times and then lysed using a selective ammonia-based buffer. The sample clean-up procedure, including deconjugation for RBC extracts, protein precipitation and SPE was similar for both matrices. This allowed simultaneous analysis of both plasma and RBC samples. To increase sample throughput, the SPE-procedure was adapted from a 12-port manifold to a 96-well system. This method, as described in **Chapter V** and published in Journal of Chromatography A, was fully validated based on the guidelines for method validation of the European Medicine Agency. This method was also used during a collaborative study with C.M. Pfeiffer and Z. Fazili of the Centers for Disease Control and Prevention (Atlanta, GA, USA) to assess method comparability in the first ever LC-MS/MS based round-robin test for folates (unpublished data).

To evaluate the bioavailability and biological efficacy of folates from folate rice, a rodent study was performed. Devised as a long term feeding trial, the animals received either wild type, folate or folic acid fortified rice on a daily basis for a period of 12 weeks (cfr. **Chapter VI**). This allowed for the stabilization of the RBC folate levels and allowed to evaluate the efficacy of prolonged folate rice consumption. Also, the rice was provided as intact grains to avoid artefacts when the rice grains would be ground to a powder and incorporated in the rodent diet. As such, daily rice rations were provided to the animals in porcelain bowls, a procedure, though uncommon, that allowed visual confirmation that the entire ration of rice was consumed. During the trial, the animals in the negative control group (only receiving a folate free diet) and the animals in the wild type rice group (receiving both wild type rice and a folate free diet) fared less well than animals in the other groups. Following the death of some animals in these affected groups, the remaining animals were euthanized and the samples were collected as would be the case later on for the groups with folate supplemented diets. At the end of the feeding trial, none of the animals receiving folate rice suffered from folate deficiency as evidenced by normal plasma and RBC folate levels, moderate plasma homocysteine levels and a normal hematology. As such, we concluded that folate rice as a food source is able to provide folates to the organism.

As can be observed from the results obtained during the feeding trial, 5-methyltetrahydrofolate (5MeTHF) is the main circulating folate species in the bloodstream and not folic acid (FA). However, common understanding of the effect of folate status on the functioning of the immune system relies on *in vitro* studies using FA as a folate source (i.e. Courtemanche *et al.* 2004 *J Immunol* 173 (5): 3186-3192.). In **Chapter VII**, we investigated the difference in both cell proliferation and intracellular folate concentrations in response to differences in folate concentrations and the folate species used as a folate source. Due to the limited stability of 5MeTHF during incubation, daily replenishment of this folate species was necessary to maintain the desired folate levels during the 9-day incubation period. While the observations of Courtemanche *et al*. could be replicated using FA as a folate source, the proliferation of CD2+, CD4+ and CD8+ lymphocytes proved less susceptible to concentration differences when 5MeTHF was used as a folate source.

Finally, the quantitative determination of individual folate polyglutamate species was investigated. The chromatography and mass detection described in **Chapter VIII** was optimized for the determination of 5-methyltetrahydropteroylpolyglutamates ($5MeTHPGlu_n$). Given the lack of commercially available reference standards, the chemical synthesis of these compounds was optimized. However, although the confirmation of the standard using an HPLC-UV procedure did not match the theoretical concentrations of commercially available $5MeTHPGlu_n$, it has since been clarified that the commercial folates in powdered form contain crystalline water due to their hygroscopic nature. Therefore, this procedure should be re-evaluated after measurement of the purity of the commercial polyglutamate standards.

As described in this thesis, we demonstrated that folate rice serves as a valuable source of dietary folate and that it may have the potential of reducing the prevalence of folate deficiency in regions where traditional folic acid fortification is not feasible. While the methods described in this thesis were purposely developed for the bioavailability trial, these can be used in a wider research context. Especially the method for folate determination in plasma and red blood cells provides several improvements over existing methods.

Curriculum vitae

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Education and work experience

1999 – 2005
Industrial Sciences – Vrij Technisch Instituut Aalst

2005 – 2008
Bachelor in Industrial Engineering (Chemistry) – KaHo Sint-Lieven (KULeuven)

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Master in Industrial Engineering (Chemistry) – KaHo Sint-Lieven (KULeuven)

2009 – 2010
Master in Environmental Management and Sanitation – Ghent University

2010 – 2015
PhD at the Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University

A1 publications as first author

- Folates from metabolically engineered rice: a long-term study in rats.
  Kiekens, F., Blancquaert, D., Devisscher, L., Van Daele, J., Stove, V.V., Delanghe, J.R., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  Molecular Nutrition & Food Research; 2015 ;59(3):490-500

  impact factor (year): 4.603 (2014)
  category (ranking): Food Science & Technology (4/123) D1/Q1

- A validated ultra-high-performance liquid chromatography-tandem mass spectrometry method for the selective analysis of free and total folate in plasma and red blood cells.
  Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove C.P.

  Journal of Chromatography A; 2015; 1398:20-28

  impact factor (year): 4.169 (2014)
  category (ranking): Chemistry, Analytical (6/74) D1/Q1

- Determination of Five Folate Monoglutamates in Rodent Diets.
  Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove C.P.

  Journal of Agricultural and Food Chemistry; 2015; 63(45):10089-10095

  impact factor (year): 2.912 (2014)
  category (ranking): Agriculture, Multidisciplinary (2/56) D1/Q1

- Determination of clinical folate status: practical considerations.
  Kiekens, F., Blancquaert, D., Van Daele, J., De Steur, H., Gellynck, X., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  To be submitted to: Journal of Chromatography B

A1 publications as co-author

- Folate Profiling in Potato (Solanum tuberosum) Tubers by Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry.
  Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W.E. and Stove C.P.

  Journal of Agricultural and Food Chemistry. 2014; 62(14):3092–3100

  impact factor (year): 2.912 (2014)
  category (ranking): Agriculture, Multidisciplinary (2/56) D1/Q1
  Food Science & Technology (13/123)
  Chemistry, Applied (11/72)

- Improving folate (vitamin B9) stability in biofortified rice through metabolic engineering.
  Blancquaert, D., Van Daele, J., Strobbe, S., Kiekens, F., Storozhenko, S., De Steur, H., Gellynck, X., Lambert, W.E., Stove, C.P. and Van Der Straeten, D.

  Nature Biotechnology. 2015; 33(10):1076-1078

  impact factor (year): 41.514 (2014)
  category (ranking): Biotechnology and Applied Microbiology (2/163) D1/Q1

- Degradation and interconversion of plant pteridines during sample preparation and ultra-high performance liquid chromatography-tandem mass spectrometry.
  Van Daele, J., Blancquaert, D., Kiekens, F., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  Food Chemistry. 2016; 194:1189-1198

  impact factor (year): 3.391 (2014)
  category (ranking): Food Science & Technology (8/123) D1/Q1

Proceedings

- Development of a chromatographic method for the analysis of folates in rodent diets.
  Kiekens, F., Van Daele, J., Blancquaert, D., Storozhenko, S., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  Pteridines. 2012; 23(1):165

  impact factor (year): 0.520 (2012)
  category (ranking): Biophysics (69/72) Q4

Oral Presentations

- 15th International Symposium on Pteridines and Folates
  Development of a chromatographic method for the analysis of folates in rodent diets comprising biofortified rice
  Kiekens, F., Van Daele, J., Blancquaert, D., Storozhenko, S., Van Der Straeten, D., Lambert, W.E. and Stove C.P.

  Antalya, Turkey, 2012

- 1st Caparica Christmas Conference on Sample Treatment
  Sensitive and selective determination of folates in plasma and red blood cell extracts: pitfalls and possibilities
  Kiekens, F. and Stove, C.P.

  Lisbon, Portugal, 2014

Poster and Short Presentation

- 2nd International Vitamin Conference
  Development of an UPLC®-MS/MS method for the analysis of folates in rodent diets comprising biofortified rice
  Kiekens, F., Van Daele, J., Blancquaert, D., Storozhenko, S., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  Copenhagen, Denmark, 2012

Poster Presentation

- 3rd International Vitamin Conference
  Demonstration of bioaccessibility, bioavailability and biological activity of natural folates from metabolically engineered rice: a long-term study in rats
  Kiekens, F., Devisscher, L., Blancquaert, D., Van Der Straeten, D., Lambert, W.E. and Stove, C.P.

  Washington D.C., USA, 2015

Educational work

- Support of practical courses Toxicology: 2010 - 2015
  overseeing practical experiments and teaching a session on sample preparation

- Support of demonstrations for courses Chemical Criminalistics: 2010 - 2015
  providing an introduction in sample preparation methodology and the principle of chromatography

- Support for Bachelor Project: 2013
  tutor and point of contact for Bachelor Project sessions

- Support of Master dissertations:
  Benoit Louage – 2010 - 2011 – "*Ontwikkeling Staalvoorbereiding voor Folaatbepaling in Plasma en Rode Bloedcellen via LC-MS/MS.*"

  Rozemien Lemey – 2011 - 2012 – "*Ontwikkeling Staalvoorbereiding voor Bepaling van 5-Methyltetrahydrofolaat Polyglutamaten in Rode Bloedcellen via LC-MS/MS.*"

  Ekaterina Ulyanava – 2011 - 2012 – "*Methodevalidatie voor de Bepaling van Folaten in Knaagdiervoeder*"