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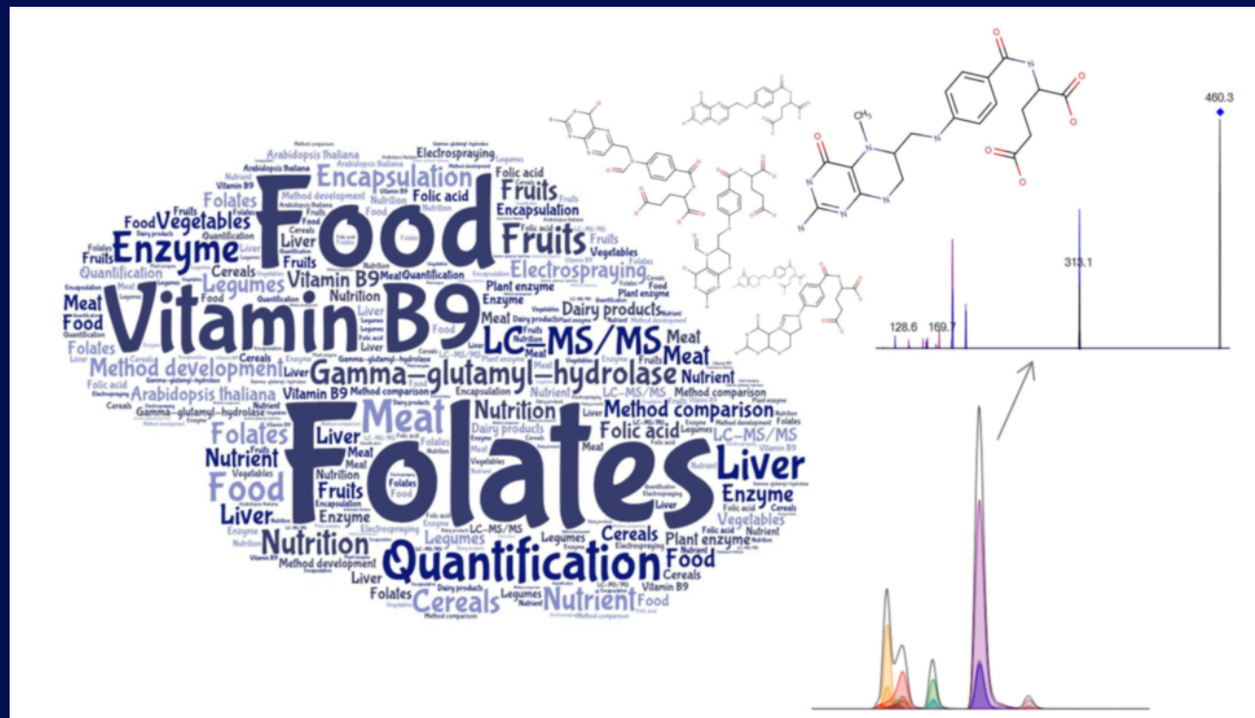

Petra Ložnjak
PhD Thesis
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PREFACE

This thesis presents the results from three years research which was conducted as a PhD project entitled "Naturally occurring folates in food – Quantification and possible food fortification strategy". The research was performed at the Research group for Bioactives – Analyses and Application, National Food Institute at the Technical University of Denmark from 1st of November 2015 until 28th of February 2019. The project was funded by Danish Veterinary and Food Administration and Technical University of Denmark.

The project was carried out under supervision of Senior Scientist Jette Jakobsen and co-supervision of Senior Scientist Anette Bysted, and it included two external research stays. During the first external research stay at the School of Engineering and Science, Institute of Technology and Higher Education, Monterrey, Mexico (October 2016), the work was supervised by Associate Professor Rocío Isabel Díaz de la Garza. The second external research stay was conducted at the Chair of Analytical Food Chemistry, Technical University of Munich, Germany and was supervised by Professor Michael Rychlik (May-September 2017).

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are no limits and that I should always try. Thank you for all your support and for believing in me. I sincerely thank to my sisters and brothers in law for having faith in me, to my nieces and nephew for reminding me how important it is to be happy, full of love and always smile, to Mario's family for all the support they gave to me…and to Mario...well, there are no words enough to thank you. You were always there. You were there in all my ups and downs. Thank you for being my part of Croatia throughout this period and for living our Danish adventure. With you and because of you, I made it possible, I could feel your support in every single moment and I am extremely glad to have you by my side. Thank you.

SUMMARY

A group of biologically active compounds with various chemical structures called folate is well known by its major role in the transport of one-carbon groups in numerous metabolic reactions in humans. It takes part in core intracellular cycles such as in the synthesis of nucleic acids, in production, methylation and regeneration of DNA, in remethylation of homocysteine and synthesis of various amino acids. Due to its crucial role in cell production, inadequate folate intake can lead to occurrence of various chronic diseases such as neural tube defects, anemia, certain types of cancer and cognitive degenerative diseases. As it is synthesized by plants and microorganisms, folate is widely present in nature. However, due to its minor concentrations in food, sensitivity and complexity of its chemical structure, and complexity of food matrices, folate analysis in food is challenging. Three main steps included in folate quantification are extraction, deconjugation and detection. Deconjugation or removal of polyglutamyl tail from the biologically active part of the molecule by the use of ɣ-glutamyl hydrolase (GGH) is a bottle-neck in folate analysis. Numerous studies have been performed in order to obtain reliable data on folate content, but there are many discrepancies in these data, depending on the natural variation of folate occurrence, but also on the method used for folate analysis. So far, a GGH of animal origin is mainly used for folate deconjugation. Microbiological assay as the only standardized method for folate detection in food is incapable of distinguishing among various folate forms, which have different stability and presumably bioavailability. Therefore, the main hypothesis in this PhD project is that the use of a recombinant plant origin GGH can be a starting point for the standardization of the deconjugation step by providing fast and effective deconjugation in various food matrices, with possibility of distinguishing among biologically active folate forms. The overall aim was to establish research based documentation for a new, accurate and precise chemical method for quantification of folate forms, and further investigate a new strategy to produce folate fortified food products.

Therefore, the main focus was on the development of a rapid, sensitive and reproducible method for folate quantification in various groups of foods. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) detection, was chosen as a method for analysis of various folate forms due to its selectivity and sensitivity. The developed LC-MS/MS method was validated for the quantification of folate forms such as tetrahydrofolate, formyl folate forms (10-formylfolic acid, 5,10-methenyltetrahydrofolate, 5-formyltetrahydrofolate), folic acid, 5-methyltetrahydrofolate and 10-methylfolic acid using three $^{13}C_5$-labeled internal standards. A single-enzyme extraction provided satisfactory deglutamylation with enzyme activity being >95% after 1 hour of incubation.

Validation parameters of trueness at 80-120% in spiked samples and certified reference materials, and a precision <10% were met in all compounds of interest except tetrahydrofolate. In the future, quantification of tetrahydrofolate as the least stable folate form should be performed by the use of a corresponding isotopically labeled internal standard.

The single-laboratory validated method using recombinant GGH of plant origin was compared to microbiological assay and another LC-MS/MS method using the animal origin GGH in a round robin study on a large and diverse sample set. Food groups of fruits, vegetables, legumes, cereals, dairy products, meat and offal represented a sample set of 89 samples analyzed for the folate content. A significantly lower constant bias of 17% ($p \leq 0.05$) was observed when the method using plant origin GGH was compared to microbiological assay. This indicates that folate daily intake calculated from the national food composition databases that normally contain data obtained by the use of microbiological assay, is overestimated. On the other side, comparison to another LC-MS/MS method using animal origin GGH evidenced superiority of deconjugation activity of plant origin GGH in plant matrices. Especially in the groups of fruits, vegetables and cereals constant bias of 25% was shown ($p \leq 0.05$). The reason for the difference is hypothesized to be due to inhibition of the GGH of animal origin in the plant food matrices. The superior activity of the GGH of plant origin over the commonly used GGH of animal origin encourage the incorporation of the new GGH for future standardization of an accurate, precise, and specific method for folate vitamers.

Food groups of offal, vegetables, legumes and certain fruits were shown to be sources rich in folate. 5-methyltetrahydrofolate was the most abundant folate form in the mentioned groups and the second most abundant in food groups of cereals, meat and dairy products after formyl forms. However, folate distribution varied within the food group and between different varieties of the same foodstuffs. Even though they were not considered as folate sources, food groups of cereals and dairy products contribute to the folate intake among Danes due to their wide consumption. Therefore, a fortification strategy to increase the folate content in products made from flour by fortification of flour with biologically active folate form 5-methyltetrahydrofolate was studied. Due to its instability, 5-methyltetrahydrofolate was incorporated into food system in the forms of nanocapsules, where it was protected by carbohydrate matrix produced by the electrospraying encapsulation. A significant improvement in the stability of 5-methyltetrahydrofolate was achieved via encapsulation, even though its incorporation into food system did not confirmed the need for encapsulation, indicating the importance of future investigation of this fortification strategy.

RESUMÉ

Folat er en gruppe af biologisk aktive forbindelser med forskellige kemiske strukturer. Folat er velkendt for dets vigtige rolle i transporten af et-karbon-grupper i adskillige metaboliske reaktioner i mennesker. Folat tager del i vigtige intra-cellulære cyklusser f.eks. i syntesen af nukleinsyrer, i produktion, metylering og regenerering af DNA samt i remetylering af homocystein og syntese af forskellige aminosyrer. På grund af dets afgørende rolle i celleproduktionen kan et utilstrækkeligt indtag af folat føre til en række kroniske sygdomme såsom neuralrørsdefekter, anæmi, visse typer af kræft og kognitiv nedbrydende sygdomme. Da folat syntetiseres af planter og mikroorganismer, er det meget udbredt i naturen, men på grund af dets lave koncentrationer i fødevarer, sensitiviteten og kompleksiteten af dets kemiske struktur samt kompleksiteten af fødevarematricerne er folatanalyser i fødevarer en udfordring. De tre hovedtrin i bestemmelsen af indholdet af folat er ekstraktion, dekonjugering og detektion. Dekonjugering eller fjernelse af polyglutamyl-halen fra den biologisk aktive del af molekylet ved hjælp af ɣ-glutamylhydrolase (GGH) er en flaskehals i folatanalyser. Der er udført adskillige studier for at opnå pålidelige indhold af folat, men der er mange uoverensstemmelser i disse data afhængigt af både den naturlige variation i forekomsten af folat og metoden, der anvendes til folatanalyser. Indtil nu har GGH af animalsk oprindelse hovedsageligt været brugt til dekonjugering af folat. Den mikrobiologisk analyse, som er den eneste standardmetode til bestemmelse af folat i fødevarer, kan ikke skelne mellem de forskellige folatformer, der har forskellig stabilitet og formentlig også forskellig biotilgængelighed. Hovedhypotesen i dette ph.d.-projekt er derfor, at brugen af et rekombinant GGH af vegetabilsk oprindelse er et udgangspunkt for standardisering af dekonjugeringstrinnet, fordi det giver en hurtig og effektiv dekonjugering af forskellige fødevarematricer med mulighed for at skelne mellem biologisk aktive folatformer. Det overordnede formål var at etablere forskningsbaseret dokumentation for en ny, nøjagtig og præcis kemisk metode til kvantificering af folatformer samt undersøge en ny strategi til produktion af folat-berigede fødevarer.

Hovedvægten blev derfor lagt på udvikling af en hurtig, følsom og reproducerbar metode til bestemmelse af indholdet af folat i forskellige fødevaregrupper. Væskekromatografi koblet til tandem-massespektrometri (LC-MS/MS) detektion blev valgt som metode til analyse af forskellige folatformer på grund af dens selektivitet og følsomhed. Den udviklede LC-MS/MS metode blev valideret til kvantificering af folatformerne: tetrahydrofolat, formyl-folatformer (10-formyl-folsyre, 5,10-metenyl-tetrahydrofolat, 5-formyl-tetrahydrofolat), folsyre, 5-metyl-tetrahydrofolat og 10-metyl-folsyre. Tre $^{13}C_5$-mærkede interne standarder blev brugt til denne validering. Enkelt-enzym-ekstraktion gav tilfredsstillende deglutamylering. Enzymaktiviteten var >95% efter en times inkubation. Valideringsparametrene korrekthed på 80-120% i spikede prøver og i certificerede referencematerialer

samt præcision på <10% blev opnået for alle relevante forbindelser undtagen for tetrahydrofolat. I fremtiden bør en tilsvarende isotop-mærket intern standard bruges til kvantificering af tetrahydrofolat, der er den mindst stabile folatform.

Metoden med recombinant GGH fra planter (valideret på et enkelt laboratorium) blev sammenlignet med den mikrobiologiske metode og en anden LC-MS/MS metode, der benytter GGH af animalsk oprindelse. Metodesammenligningen blev udført i et round robin studie med et stort udvalg af forskellige fødevarer. Indholdet af folat blev analyseret i 89 prøver udvalgt fra fødevaregrupperne med frugter, grøntsager, bælgfrugter, cerealier, mejeriprodukter, kød og indmad. Der blev observeret en signifikant lavere konstant bias på 17% ($p \leq 0.05$) ved anvendelse af metoden med GGH fra planter i forhold til den mikrobiologiske metode. Dette indikerer en overestimering af det daglige indtag af folat beregnet fra nationale fødevaredatabaser, hvor der normalt findes data tilvejebragt ved anvendelse af den mikrobiologiske metode. På den anden side viste sammenligning med en anden LC-MS/MS metode med GGH af animalsk oprindelse evidens for forbedret dekonjugeringsaktivitet af GGH af vegetabilsk oprindelse i plantematricer. Specielt i grupperne med frugter, grøntsager og cerealier hvor der blev fundet en konstant bias på 25% ($p \leq 0.05$). Grunden til denne forskel kan skyldes inhibering af GGH fra animalsk oprindelse i planteføde vare matricer. Den øgede aktivitet af GGH af vegetabilsk oprindelse i forhold til aktiviteten af den almindeligt anvendte GGH af animalsk oprindelse tilskynder inkorporering af den nye GGH i fremtidig standardisering af en nøjagtig, præcis og specifik metode til bestemmelse af folatformer.

Det blev vist, at fødevaregrupperne med indmad, grøntsager, bælgfrugter og enkelte frugter er rige folatkilder. 5-metyl-tetrahydrofolat var den hyppigst forekommende folatform i de nævnte grupper og den næst hyppigste i fødevaregrupperne med cerealier, kød og mejeriprodukter efter formylformerne, dog varierede folatfordelingen inden for fødevaregrupperne og mellem forskellige slags af de samme fødevarer. Selvom de ikke betragtes som folatkilder, bidrager fødevaregrupperne med cerealier og mejeriprodukter til danskernes indtag af folat på grund af det store indtag af fødevarerne. Som følge heraf blev der undersøgt en berigelsesstrategi for at øge indholdet af folat i produkter lavet af mel ved berigelse af mel med den biologisk aktive folatform 5-metyl-tetrahydrofolat. Da 5-metyl-tetrahydrofolat er ustabilt, blev det inkorporeret i fødevaresystemet i form af nano-kapsler, så det var beskyttet af kulhydratmatricen produceret ved elektrospray-indkapsling. Ved indkapslingen blev der opnået en signifikant forbedring i stabiliteten af 5-metyl-tetrahydrofolat, selvom dets inkorporering i fødevaresystemet ikke bekræftede behovet for indkapsling. Dette indikerer vigtigheden af fremtidige undersøgelser af denne berigelsesstrategi.

LIST OF PUBLICATIONS

The thesis is based on the publications listed below.

Paper I: **Ložnjak P.**, García-Salinas, C., Díaz de la Garza, R.I., Bysted, A., Jakobsen, J. (2019) The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1594, 34-44. doi: 10.1016/j.chroma.2019.02.037

Paper II: **Ložnjak P.**, Striegel, L., Diaz de la Garza, R., Rychlik, M., Jakobsen, J. (2019) Quantification of folate in food using deconjugase of plant origin combined with LC-MS/MS: A method comparison of a large and diverse sample set.
Manuscript submitted to *Food Chemistry*

Paper III: **Ložnjak P.**, Bysted, A., Jakobsen, J. Folate distribution in commonly consumed foodstuffs analyzed by LC-MS/MS and its importance for estimated folate intake.
In preparation to be submitted to *Journal of Food Composition and Analysis*

Paper IV: **Ložnjak P.**, García-Moreno, P. J., Mendes, A. C., Fallahasghari, Z., Jakobsen, J. Electrospraying encapsulation of L-5-methyltetrahydrofolic acid for food applications. In preparation to be submitted to *Journal of Functional Foods*

OTHER PUBLICATIONS (not used in this Thesis)

Ložnjak, P., Jakobsen, J. (2018) Stability of vitamin D_3 and vitamin D_2 in oil, fish and mushrooms after household cooking, *Food Chemistry*, 254, 144-149. doi: 10.1016/j.foodchem.2018.01.182

CONFERENCE CONTRIBUTIONS

Ložnjak, P., Jakobsen, J., Bysted, A., Diaz de la Garza, R. I., Rychlik, M. (2018) The use of a plant enzyme provides a fast, sensitive and reproducible LC-MS/MS method for folate analyses in food. Oral presentation at 5th International Vitamin Conference. Sydney, Australia.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
Asc	Ascorbic acid
ATP	Adenosine triphosphate
AOAC	Association of Official Analytical Chemists
BAL	2,3-dimercapto-1-propanol
C1	One-carbon
CHES	N-cycloexyl-2-aminoethanesulfonic acid
CRM	Certified reference material
DAD	Diode array detector
DFE	Dietary folate equivalent
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dTMP	Deoxythymidine monophosphate
DTT	Dithiothreitol
EAR	Estimated average requirement
ECD	Electrochemical detector
ESI	Electrospray ionization
FAO	Food and Agriculture Organization (USA)
FBP	Folate-binding protein
FDA	Food and Drug Administration (USA)
FDM	Fat in dry matter
GGH	ɣ-glutamyl hydrolase
GPE	Green peas extract
HEPES	N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MA	Microbiological assay
MCE	β-mercaptoethanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MST	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
m/z	Mass to charge ratio
NIST	National Institute of Standards and Technology

NTDs	Neural tube defects
RBC	Red blood cells
RDA/RDI/RI	Recommended daily allowance/Recommended daily intake/Recommended intake
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAX	Strong anion exchange
SEM	Scanning electron microscopy
SHMT	Serine hydroxymethyltransferase
SIDA	Stable isotope dilution analysis
SPE	Solid phase extraction
TCEP	Tris-(2-carboxyethyl)phosphine hydrochloride
TYMS	Thymidylate synthase
UL	Upper level (tolerable upper intake level)
UPLC	Ultra-pressure liquid chromatography
USDA	U.S. Department of Agriculture
UV	ultraviolet
WHO	World Health Organization
5-FU	5-fluorouracil

Folate abbreviations:

HCO-H_4folates	Formyl folate forms
H_2folate	Dihydrofolate
H_4folate	Tetrahydrofolate
L-5-CH_3-H_4folate	L-5-methyltetrahydrofolate, synthetic form
PteGlu	Folic acid, Pteroylglutamic acid
PteGlu7	Pteroylheptaglutamic acid
5-HCO-H_4folate	5-formyltetrahydrofolate
5-CH_3-H_4folate	5-methyltetrahydrofolate
5,10-CH^+=H_4folate	5,10-methenyltetrahydrofolate
5,10-CH_2-H_4folate	5,10-methylenetetrahydrofolate
10-HCO-H_4folate	10-formyltetrahydrofolate
10-HCO-PteGlu	10-formylfolic acid
10-CH_3-PteGlu	10-methylfolic acid

TABLE OF CONTENTS

Chapter 1: Introduction

Folate or B_9 vitamin is a generic term for a group of compounds that act as cofactors in many metabolic reactions in humans. They play a key role in one carbon-metabolism (Fekete et al., 2012; McGarel et al., 2015). Together with B_6 and B_{12} vitamins they act as cofactors in the synthesis, methylation, and reparation of DNA. They regenerate methionine from homocysteine, reduce homocysteine concentration in organism and consequently reduce the risk of chronic diseases and developmental disorders including Alzheimer's disease, cardiovascular diseases and dementia. Their indispensable role is the prevention of neural tube defects (NTDs) that include abnormalities of the brain, spine and spinal cord which occur in fetus during the first month of gestation period (Saini et al., 2016). Due to numerous beneficial roles, an adequate folate intake is extremely important. Even though it is widely distributed among foods, such as in green leafy vegetables, legumes, offal, certain fruits, grains and fermented dairy products natural folate is extremely sensitive to various conditions such as temperature, light and oxygen. Therefore, people whose habits do not emphasize plant foods and whose diet are based on the processed food normally do not have an adequate folate intake (Delchier et al., 2016).

Due to the complexity of the folate's structure, which affects their stability and presumably bioavailability, the methods for folate quantification from food are complex. There are discrepancies in the folate content in food analyzed by different methods, due to the various parameters, where the use of deconjugase enzyme and detection system plays crucial roles. The enzyme of animal origin is normally used in enzymatic deconjugation of folate extracts, which can be inhibited by the plant food matrix, resulting in inaccurate and imprecise folate values (Bhandari & Gregory, 1990). Furthermore, microbiological assay, as the only standard method for folate detection in food is being laborious, costly and time-consuming, it does not enable separation and quantification of various folate forms present in food, and it is very dependent of the microorganism and the calibrant used for analysis (Arcot & Shrestha, 2005; Quinlivan et al., 2006). The data in food composition databases are obtained by the use of microbiological assay, which analyzes only total folate content. On the other side, the use of chromatographic methods enables separation and quantification of various folate forms and provides information on the stability and bioavailability of folate, opening broad possibilities in the research of folate forms. Improvement of folate quantification and data collection is a challenge in most developed countries, especially in Europe, as there is no mandatory folate fortification in these countries (European Union, 2002, 2006).

The hypothesis for this project was that the use of a recombinant plant origin enzyme can serve as a basis for the standardization of deconjugation step in folate analysis in terms of enzyme effectiveness, by providing fast and complete folate deglutamylation in various food matrices. The overall aim was to establish a research based documentation for the use of a novel plant origin enzyme which would be an essential part in future folate analysis. Therefore, a special emphasis was placed on the development and validation of a specific, sensitive and reproducible LC-MS/MS method for quantification of various naturally occurring folate forms in food by the use of a recombinant plant origin enzyme (*Arabidopsis thaliana*). Furthermore, a method comparison study was performed on a large sample set, where newly developed method was compared to another LC-MS/MS method using an animal origin enzyme and to the microbiological assay, as the only standard method for folate analysis in food. The presumption was that the folate content analyzed by microbiological assay is overestimated and therefore, the final part of the project was to find the fortification strategy to increase the content of folate in food.

To answer the hypothesis, this PhD project was divided into the following parts:

Part 1: Development and validation of LC-MS/MS method using plant origin deconjugase for extraction of naturally occurring folates in various food matrices (Paper I)

Part 2: Analysis of a large and diverse sample set, and method comparison to another validated LC-MS/MS method using animal origin deconjugase and to microbiological assay (Paper II, III)

Part 3: Development of the nanocapsules containing L-5-CH_3-H_4folate using electrospraying as encapsulation technique and their incorporation into food matrix (Paper IV)

The thesis is structured as follows: The background information of folate is presented in Chapter 2. Chapter 3 is a summary of the experimental part of the thesis that is attached in Papers I-IV. Chapter 4 provides brief information on folate analysis in food and presents additional information on method development that was not included in Paper I. Chapter 5 is a general discussion on method comparison studies and folate content in food based on the data presented in Papers II and III. Chapter 6 is presenting a discussion on folate fortification in food with references to Paper IV. Chapter 7 is a general conclusion about the new knowledge obtained in this thesis, whereas suggestions for future perspectives are given in Chapter 8.

Chapter 2: Folate

This section provides essential background information on folate. It describes nomenclature and chemistry of folate, as well as metabolism and bioavailability. The information on the folate deficiency and dietary intake of folate are included.

2.1. Discovery and significance of folate

In 1931, Lucy Wills discovered an anti-anemia factor by using yeast extract to treat macrocytic anemia in pregnant women (Wills, 1931). It took 10 years until that curative agent was isolated from spinach leaves, defined as an acid and received the name *folic acid* from Latin word *folium*, meaning leaf (Mitchell et al., 1941). Even though originally named as *folic acid*, nowadays naturally occurring folate forms are named *folate*, whereas *folic acid* is a term used for the synthetic form of the vitamin. Folate is a group of essential compounds synthesized by plants and microorganisms, since their biosynthesis pathways are absent in animals (Saini et al., 2016). They play a key role in association with vitamins B_6 and B_{12} in the synthesis, reparation and methylation of DNA, and also as a cofactor in numerous metabolic reactions. Together with these vitamins, folate is involved in two major inter-related cycles: methylation cycle and DNA biosynthesis cycle (Crider et al., 2012). They are responsible for homocysteine remethylation which is catalyzed by the vitamin B_{12}-dependent enzyme methionine synthase. When folate status is poor, the ability of the cell to remethylate cellular homocysteine is very low and this results in increased plasma homocysteine levels, which presents an increased risk of various chronic diseases (Fan et al., 2017). In DNA biosynthesis cycle, folate plays a key role in one-carbon metabolism, enabling the transfer of one-carbon units during the synthesis of purine and pyrimidine precursors of nucleic acids, required for normal cell division, growth and production of cells (McGarel et al., 2015). Due to their connection with vitamin B_{12} and B_6, it is very hard to access the level of subclinical deficiency. Plasma levels of folate are inversely related to plasma homocysteine levels at concentrations <40 mM, suggesting a link between folate intake and reduced risk of vascular diseases (Scaglione & Panzavolta, 2014; Voutilainen et al., 2001; Blom & Smulders, 2011). Numerous studies have shown that mothers who have inadequate folate intake are at higher risk of giving birth to a child with NTDs which include complex congenital malformations of the central nervous system resulting from failure of the neural tube closure during embryogenesis (Imbard et al., 2013; Pitkin, 2007; De Wals et al., 2007). Both deficiency and abundance or over-

supplementation of folic acid, in addition to other conditions, may contribute to breast carcinogenesis at different stages of tumor development or in different neoplastic or tumor phenotypes (Stolzenberg-Solomon et al., 2006), whereas folate deficiency can contribute to neurological damage and cause Alzheimer's disease (Snowdon et al., 2000).

2.2. Nomenclature and chemistry of folate

Folate is a generic term for a group of compounds that exhibit similar chemical characteristics and biological activity, but they differ in chemical structure. The basic structure is composed of pteridine ring (2-amino-4-hydroxy-6-methylpterin) that is connected by methylene bridge to para-aminobenzoate which is linked to one or more (2-9) L-glutamic acid residues with ɣ-peptide bond (Figure 1).

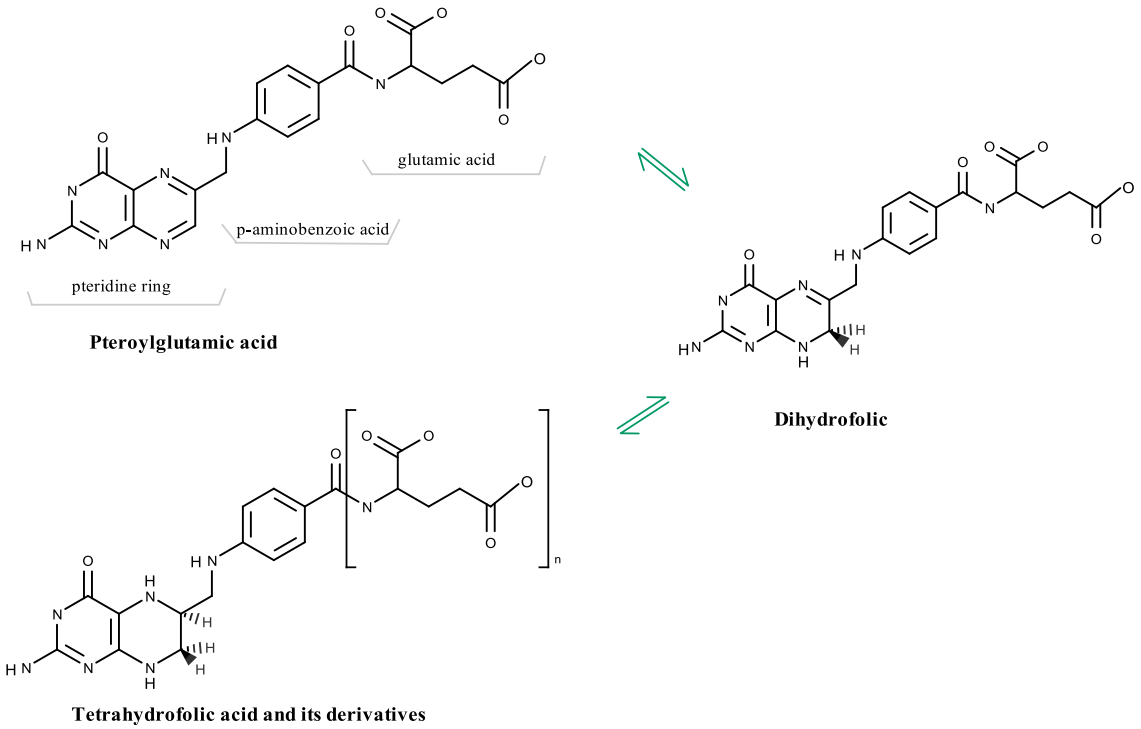

Figure 1 Structure of folate, showing different oxidation states

In 1944, Keresztesy introduced a word *vitamer* as any of a number of chemical compounds that have similar chemical structure, and comparable biological activity within the same vitamin family (Keresztesy, 1944). Due to the complex structure, and possible differences at three sites of a structure, there are a large number of folate forms, or possible vitamers. Firstly, there are three possible oxidation states of the pteridine ring. It can be fully oxidized, as it is in the synthetic form of folate *folic acid*, partially reduced at 7,8-position (H_2folate) or fully reduced. Fully reduced form is called 5,6,7,8-tetrahydropteroylglutamic acid or tetrahydrofolate (H_4folate) according to IUPAC (Quinlivan et al., 2006). Secondly, naturally occurring biologically active tetrahydrofolate forms can be substituted with either methyl (CH_3), formyl (HCO), or formimino (CH=NH) group on the N^5 or N^{10} position of the pteridine ring, or N^5 and N^{10} can be linked to form 5,10-methenyl- (5,10-CH^+=) or 5,10-methylenetetrahydrofolate (5,10-CH_2-) as shown on Figure 2 (Jägerstad & Jastrebova, 2013).

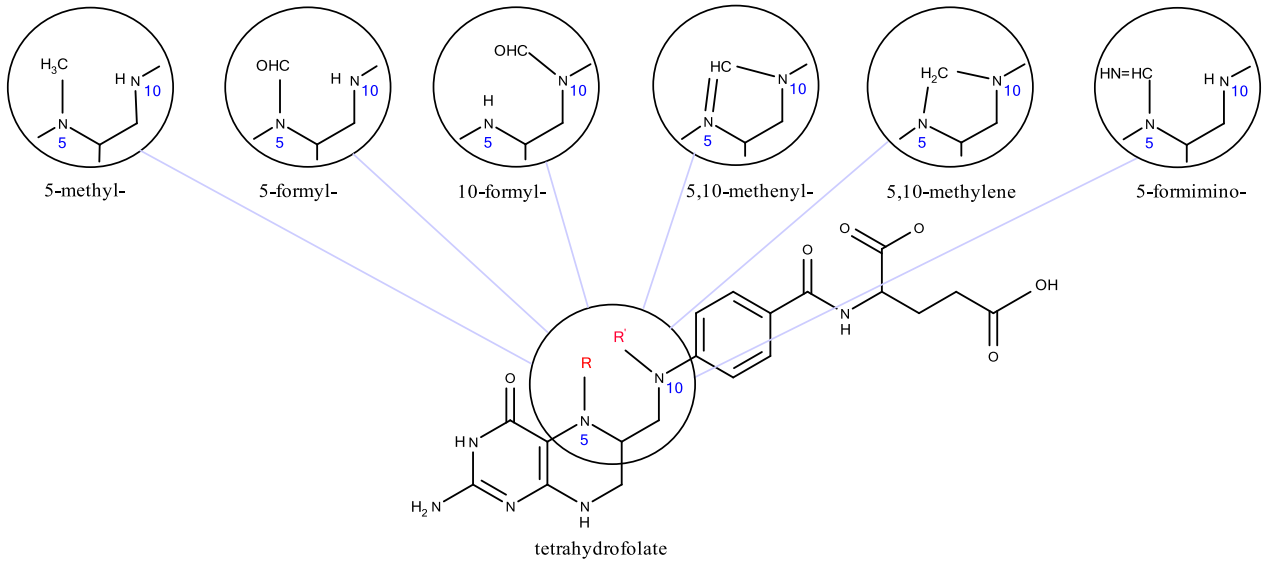

Figure 2 C_1 units carried by tetrahydrofolate - biologically active forms of folate

Thirdly, the polyglutamyl chain of folate is usually composed of up to 8 glutamate residues (Ndaw et al., 2001), even though lengths of up to 10 or 14 glutamate residues were also recorded (Garratt et al., 2005). All these structural differences contribute to the wide number of naturally occurring folate forms, which can result in more than 250 different folate forms.

Folate forms are sensitive to various environmental conditions such as exposure to light, oxygen, high temperatures and the changes in pH values. Due to the various substituents on N^5 and N^{10} positions, their stability in water solutions changes depending on the conditions mentioned above, and it decreases as follows: 5-HCO-H_4folate > 5-CH_3-H_4folate > 10-HCO-H_4folate > H_4folate (Delchier et al., 2016). At acidic pH values, 10-HCO-H_4folate, 5-HCO-H_4folate and 5-NH=CH-H_4folate are cyclized to 5,10-CH^+=H_4folate, where the rate of interconversion depends on the extraction buffer and its pH. At physiological pH values, 5,10-CH_2-H_4folate dissociates to formaldehyde and H_4folate, which results in joint quantification of these folate forms during folate analysis which is normally performed using these extraction conditions (e.g. extraction at pH 7.85) (Quinlivan et al., 2006). At pH 10, a little amount of 5,10-CH^+=H_4folate is present, as the equilibrium between 5,10-CH=H_4folate and 10-HCO-H_4folate is shifted toward 10-CHO-H_4folate (Quinlivan et al., 2006). Folic acid is the oxidized synthetic form of folate which is only found in fortified foods, supplements and pharmaceuticals and it shows greater stability than the reduced folate forms. It does not exist in nature, even though oxidation of naturally occurring folate to folic acid is seen in stored and cooked foods (Forssén et al., 2000).

2.3. Folate metabolism

Folate forms play a crucial role in fundamental cellular processes, such as biosynthesis of nucleic acids, transport of methyl group and amino acid metabolism. In other words, they are responsible for the normal function of three important intracellular cycles in humans; folate cycle, methylation cycle and DNA biosynthesis cycle (Scaglione & Panzavolta, 2014). Dietary folate forms have no coenzyme activity until they are reduced to H_4folate via H_2folate in two reduction steps catalyzed by the enzyme dihydrofolate reductase (DHFR). H_4folate is the active form of folate that carries C_1 units and therefore plays an important role in thymine synthesis, serine/glycine metabolism and methionine/S-adenosyl methionine (SAM) regeneration. SAM is the universal methyl group donor and the second most common enzymatic cofactor in human metabolism after adenosine triphosphate (ATP) (Zhang & Zheng, 2016; Ducker & Rabinowitz, 2017). H_4folate is metabolized in the reversible reaction by the B_6-dependent enzyme serine hydroxymethyltransferase (SHMT) to generate glycine and 5,10-CH_2-H_4folate (Gregory et al., 2000). 5,10-CH_2-H_4folate is a hub of the folate pathway, since it can lead to 4 different outcomes, as shown on Figure 3:

1) It can be processed back to H_4folate by SHMT, depending on the amount of glycine and serine.
2) It can be processed back to H_2folate by the enzyme thymidylate synthase (TYMS) during the production of nucleic pyrimidine base deoxythymidine monophosphate (dTMP) in DNA synthesis.
3) It can go towards the methylation cycle, by converting to 5-CH_3-H_4folate by the action of B_2-dependent enzyme methylenetetrahydrofolate reductase (MTHFR) that is then utilized to methionine if the cell needs SAM.
4) It can go towards the production of 5,10-methenyl-H_4folate and 10-HCO-H_4folate which in further metabolic processed take part in purine synthesis.

Therefore, intracellular folate metabolism is at a branch of two major inter-related metabolic cycles: DNA biosynthesis cycle and methylation cycle. The role of 5,10-CH_2-H_4folate is dependent on the cell requirements and on the state of vitamins B_2, B_6 and B_{12} as they are cofactors in folate cycle. 5-CH_3-H_4folate is a methyl donor for a homocysteine remethylation to form methionine. This reaction is of a particular physiological importance because methionine is the substrate for SAM synthase. SAM plays a major role in biosynthetic processes including phosphatidylcholine, creatine, and polyamine synthesis (Mudd et al., 2007; McBreairty et al., 2013), whereas phosphatidylcholine synthesis is the largest source of S-adenosylhomocysteine (SAH) (Stead et al., 2006). Therefore, SAM/SAH balance is extremely important in order to control the methylation of DNA and phospholipid synthesis. The synthesis of methionine from 5-CH_3-H_4folate is catalyzed by the B_{12}-dependent methionine synthase (MST) (Ducker & Rabinowitz, 2017). If a B_{12} deficiency occurs, the production of SAM is reduced, and the content of homocysteine and 5-CH_3-H_4folate is increased, which results in inhibition of MTHFR. Furthermore, the activated H_4folate pool is decreased and the ability of the cell to produce methionine, SAM and dTMP is very low, which results in folate deficient state. On the other side, the reduction of synthetic folic acid to H_4folate does not require MTHFR and MST, because of what folic acid may mask the signs of vitamin B_{12} deficiency. Therefore, plasma homocysteine levels reflect cellular folate and vitamin B_{12} use, and are indirect indicator of their levels (Ma et al., 2017).

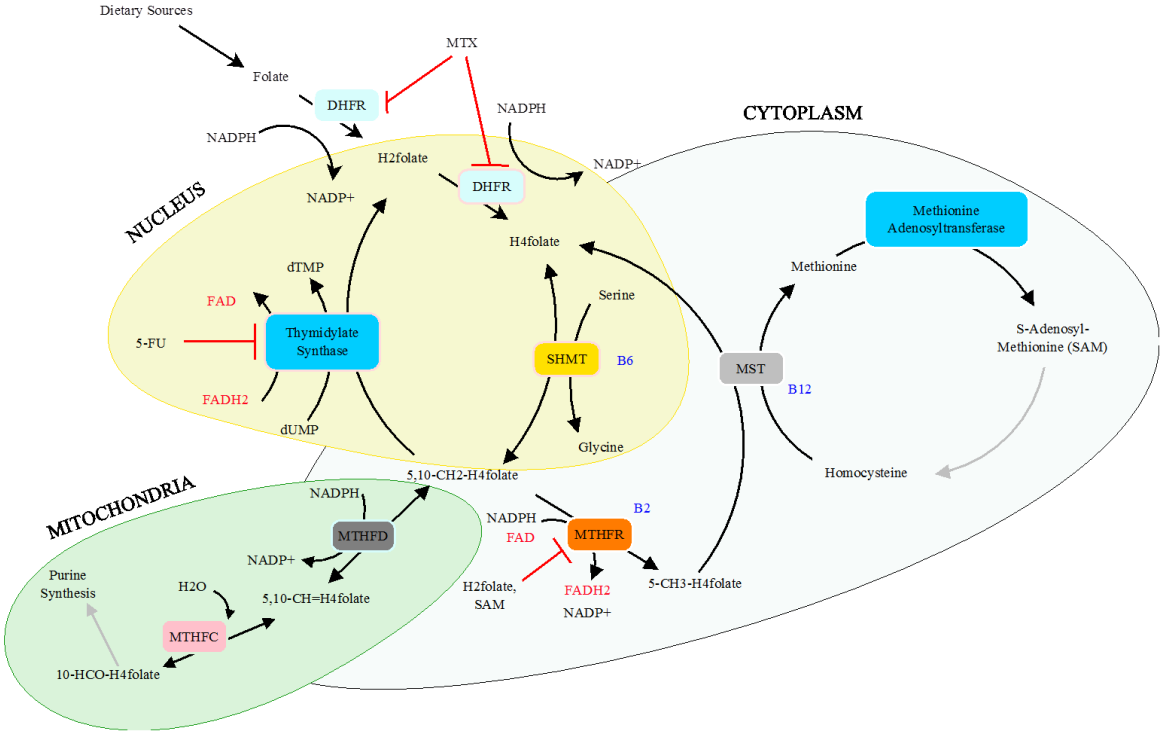

Figure 3 Intracellular folate metabolic pathways. Modified from Scaglione & Panzavolta (2014).

2.4. Absorption and bioavailability

Naturally occurring folate forms, which mainly exist in their polyglutamylated form, are absorbed in the jejunum of the small intestine as monoglutamyl forms. Therefore, their polyglutamyl chain has to be removed by intestinal GGH, following reduction and methylation in the enterocyte (Halsted, 1989). The deglutamylation is catalyzed by a GGH placed on the jejunal brush border membrane, whereas the GGH from pancreatic solution can also contribute to deglutamylation (Gregory, 2001). There are two possible folate absorption systems. The first and the most common one is a carrier mediated mechanism known as a *saturable transport*, where folate forms are bound to membrane-associated folate-binding proteins (FBP), following the transport across the brush-border membrane. The second one is a passive diffusion, which occurs only when the intraluminal concentration of folate is very high (>10 µM/L), and it is called *non-saturable diffusion*. It is normally caused by the consumption of fortified food and/or folate supplementation, while it is hardly activated by dietary folate intake (Brouwer et al., 2001). The brush border GGH is zinc-dependent and is active while the lumen pH is 6.5-7. Therefore, if there is a zinc deficiency and/or a more acidic pH, the GGH's activity is lower and consequently the folate absorption also (Pfeiffer et al., 1997).

After absorption, folate forms enter the hepatic portal vein in monoglutamylated form mostly present as 5-CH_3-H_4folate. Absorbed folate is transported to liver, where approximately a half of the body pool of folate forms is stored, and 10-20% absorbed during the first-pass. The rest is transported via circulation to cells in other body tissues (Ohrvik & Witthoft, 2011). When stored, folate forms are in polyglutamylated form, where addition of glutamyl residues traps them within the cell.

There are several factors that may hinder the absorption of naturally occurring folate forms, which could be divided into two groups: pre-absorptive and post-absorptive variables (Gregory et al., 2005). Pre-absorptive variables include:

1) Entrapment of folate forms in food matrix, or partial release from cellular structures.
2) Gastric instability of folate forms in the low pH environment of gastrointestinal tract.
3) Inability of GGH to deglutamylate folate forms due to the alteration of the pH from optimal.
4) Portion size of the ingested food.

Some dietary constituents such as organic acids (citrates, malates, ascorbates and formats) can contribute to the inhibition of GGH (Wei & Gregory, 1998). However, these factors are irrelevant in the case of folic acid as it is synthetic monoglutamate form which is less sensitive to the variability in conditions of gastrointestinal tract. On the other side, post-absorptive factors influence both naturally occurring folate forms and folic acid, as they are specific to the individual, and include:

1) Individual's status of vitamins (e.g. B_{12}, B_6, B_2).
2) Genetic polymorphism.
3) Individual's variation in the ability to metabolize folic acid ingested in large amounts.
4) Drug/alcohol interactions.

Chemo-therapeutic drugs, e.g. 5-fluorouracil (5-FU) or methotrexate (MTX) as shown on Figure 3, inhibit enzymes responsible for tumor cell growth, but simultaneously unable the cell division of the beneficial cells (Caudill, 2010; Gregory et al., 2005). The factors mentioned above are also affecting folate bioavailability as these two terms are tightly connected. There are several definitions of bioavailability, but the most commonly used one was defined by Jackson (1997), who defined it as "a fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage". It is a concept associated with the efficiency of absorption and a metabolic utilization of an ingested nutrient. There are many approaches that have yielded information regarding folate bioavailability, such as *in vivo* models (animal and human studies), and

in vitro methods. The assessment of urinary folate is also used since folate is excreted from the body mostly by urine and in lower extent by feces (Gregory, 2001). It is estimated that 0.3-0.8% of the folate pool is excreted per day, which means that folate from the diet is absorbed effectively and stored in tissues (Ohrvik & Witthoft, 2011). The use of animal studies is discontinued because of differences between the intestinal deconjugation mechanisms between animals (chickens and rats) and humans (Gregory, 2001). Human studies use the area under the curve (AUC) approach for assessment of folate bioavailability. The AUC approach is based on the assumption that with standardized study design, the post dose folate concentrations in plasma/serum sampled over time correspond to the folate fraction that was absorbed from a single dose. By comparing the sample doze with an oral reference dose of supplemental folate, which is normally folic acid, the information of relative folate absorption is obtained (Ohrvik & Witthoft 2011). However, Wright et al., (2010) recommended the use of 5-CH_3-H_4folate as a reference folate when food folate bioavailability is being estimated, since their comparison with folic acid showed that the estimated relative bioavailability of food folate is lower than the one of folic acid when used as a reference. The possible cause of that could be due to the differences in the absorption between folic acid and 5-CH_3-H_4folate. As mentioned before, 5-CH_3-H_4folate is absorbed as a monoglutamyl form, whereas folic acid has to pass two metabolic steps in order to be converted to biologically active form H_4folate and an additional one to be converted to 5-CH_3-H_4folate. 20 years ago, the use of isotopically labeled internal standards increased sensitivity in folate quantification, by being used with chromatographic detection, which enabled quantification of different folate forms. The use of isotopic methods using stable isotope-labeled folate forms, combined with the AUC approach and chromatographic methods showed to be the most specific and precise, enabling detection of a very low individual folate concentrations in plasma/serum (Gregory, 2001).

Folate bioavailability is highly variable and mostly incomplete, due to many factors that can affect it. The overall absorption of dietary folate is estimated to 50%, but it can vary from 10-90% (Combs Jr. & McClung, 2017). Some minor differences in the bioavailability of various oxidation forms of folate are possible, but also in the length of the polytlutamyl chain (Gregory et al., 1992). A relative bioavailability of long chain (n = 5-7) polyglutamyl folate forms ranges from ~50 to 100%, with an average of ~75% when compared to monoglutamyl (Gregory, 1995). Therefore, it is considered that the increased polyglutamyl chain reduce the bioavailability. Sauberlich et al. (1987) reported that folate bioavailability in a typical mixed diet was no more than 50% relative to folic acid in a formula diet, which led to conclusion that naturally occurring food folate forms have a lower

bioavailability than folic acid (Caudill, 2010). However, it should be recognized that not all food sources of folate exhibit poor bioavailability, since 60-90% of folate bioavailability relative to folic acid was found in fruits and vegetables (Brouwer et al. 1999; Winkels et al. 2007).

The terms *bioaccessibility* and *bioefficacy* are also described in order to describe *bioavailability*. Bioaccessibility is typically based on *in vitro* studies and refers to the fraction of a nutrient that is released from food and is available for intestinal absorption (Carbonell-Capella et al., 2014). *In vitro* studies are more rapid and easier than *in vivo* studies for assessing folate bioavailability and the main hypothesis in this approach is that the components of food would reduce the bioavailability of polyglutamyl folate forms by inhibiting deconjugation (Gregory, 2001). Bioefficacy is the proportion of ingested nutrient that is converted to its active form (Brouwer et al., 2001). Bioavailability differs widely between investigated foodstuffs. Mönch et al. (2015, 2016) conducted a short-term human study and reported folate bioavailability for spinach, wheat germ and two different Camembert cheeses being 73%, 33%, and 9% and 68%, respectively. Ringling and Rychlik (2017) used *in vitro* model to study folate bioaccessibility from the same foodstuffs and concluded that the results correlate well with the results for bioavailability from human study. Comparison of two different types of Camembert cheeses indicates that folate's distribution and the composition of the foodstuff affect the bioavailability and bioaccessibility of folate forms, as they have different composition of microbial cultures and different folate distributions in the foodstuff. Furthermore, Camembert cheese in which folate bioavailability was 9% has 51% of H_4folate, and contained most of its folate forms (80%) in the rind, which may hinder folate accessibility during gastrointestinal ingestion. The H_4folate presented 19% of the folate content in the other Camembert cheese showing higher bioavailability, whereas more than 60% of folate could be found in the soft dough matrix of the cheese (Mönch et al., 2016). As H_4folate had lower bioavailability than other monoglutamate forms, and it was completely lost during the *in vitro* simulation, this could be an explanation for a lower folate bioavailability from this Camembert cheese. The bioavailability of 5-CH_3-H_4folate, as the major naturally occurring folate form (Gregory, 2012), was dependent on the food matrix, as it was shown in this study.

In 1998, the Institute of Medicine introduced the use of *dietary folate equivalents* (DFE) in order to adjust for the differences in bioavailability of various folate forms in the USA (Institute of Medicine, 1998). DFE are defined as: naturally occurring food folate (µg) + 1.7 x of synthetic folate (µg), which represents the adjustment for the greater contribution of synthetic folate due to its

generally greater bioavailability. The factor 1.7 was calculated from the ratio of 85% and 50%, which are recognized as the relative bioavailability of folic acid consumed by food (Pfeiffer et al., 1997) and dietary folate (Sauberlich et al., 1987), respectively. In the other words, the bioavailability of naturally occurring food folate is 60% that of folic acid when consumed with a meal (50/85x100). However, previously mentioned studies showed that folate bioavailability is dependent on many factors. The C_1 substitution and the length of the polyglutamyl chain should be minor factors, however, the food matrix and entrapment of folate could be the major factor influencing bioavailability and bioefficacy, together with the amount of folic acid that is consumed (Brouwer et al., 2001). Therefore, a further research should be done on the methods assessing bioavailability the same as the development of the specific analytical methods which will enable a proper quantification of naturally occurring folate forms, since the reliable method for folate determination is a priority.

2.5. Folate deficiency and health outcomes

Due to the essential role that folate plays in fundamental cellular processes its adequate intake is of a high importance for human health. An adequate folate intake reduces the risk of many illnesses, while the suboptimal intake causes various conditions that lead to the development of severe chronic diseases. Folate status is most often assessed by measuring folate concentrations in the plasma, serum or red blood cells, and combining them with the data of total homocysteine concentration and unmetabolized folic acid in order to confirm folate deficiency (Sobczynska-Malefora & Harrington, 2018). The most common laboratory test used for the evaluation of folate status is determination of the folate in plasma/serum. However, circulating folate levels are strongly affected by the recent intake, and can be changed by one folate-rich meal. Furthermore, it seems that in about 5% cases where serum folate concentration is normal, there is still a possibility of folate deficiency (Galloway & Rushworth, 2003). Therefore, the determination of a folate in red blood cells (RBC) is a stronger indicator of a folate status, since it is not affected by the changes in dietary intake. Folate content in RBC is fixed during the erythropoiesis, so the low values of the results that present 4 months period indicate folate deficiency, which was defined as <6.8 nmol/L and <340 nmol/L for serum/plasma and RBC folate concentrations, respectively (Sobczynska-Malefora & Harrington, 2018). Folate deficiency is a huge problem in many parts of the world and it is mostly caused by an inadequate folate intake. However, an increased need for cell division

caused by conditions such as pregnancy, lactation and puberty, pathological conditions, anti-folate drugs or some other metabolic inhibitors may also be the cause of folate deficiency (Bailey et al., 2015). Several genetic mutations named polymorphisms that occur in enzymes controlling folate absorption, transport and metabolism may have an effect on folate status and health. A polymorphism of a high importance for folate metabolism is the 677C→T base substitution in the gene that encodes the enzyme MHTFR that catalyzes the reduction of 5,10-CH_2-H_4folate to 5-CH_3-H_4folate, which is connected to 70% lower MHTFR enzyme activity for homozygotes (T/T) and 35% lower activity for heterozygotes (C/T). The activity of MHTFR enzyme is also affected by the 1298A→C polymorphism, but to a lesser extent (Chango et al., 2000; Tamura & Picciando, 2006). Another important polymorphism in folate and methylation cycles is 2756A→G, which reduces the activity of MST, disabling the conversion of homocysteine to methionine (Coppedè et al., 2013). This mutation causes the increased homocysteine concentration in blood, which is a risk factor for development of various diseases such as NTDs, cancer, cardiovascular diseases and neurological disorders, and is connected to all-cause mortality risk (Fan et al., 2017). There is an assumption that polymorphisms may lead to the occurrence of NTDs, even though no consistent evidence for this statement has been found (De Marco et al., 2002; Coppedè et al., 2013; O'Leary et al., 2005; Yan et al., 2012; Yu et al., 2014).

NTDs such as; spina bifida, encephalocele, and anencephaly, are a group of birth disorders that include a set of serious developmental defects connected to inability of a closure of neural tube (Naderi & House, 2018). They occur in the first month of pregnancy when the neural tube is formed. Therefore, an adequate folate intake is not only important for mother's, but also for infant's health. During pregnancy, folate requirements are increased to enable a rapid cell replication and growth of fetal, placental and maternal tissue (Tamura & Picciando, 2006). Food and Drug Administration (FDA) authorized food fortification with folic acid in the USA and Canada, which became mandatory in 1998, in order to reduce the prevalence of NTDs caused by the inadequate intake of naturally occurring folate forms (Food and Drug Administration, 1996), that resulted in significant reduction of NTDs occurrence rates (Imbard et al., 2013; Pitkin, 2007).

Despite playing an important role in reduction of NTDs, an inadequate dietary folate intake, and high homocysteine concentration are also associated with the risk of several cancers, such as leukemia, colorectal, breast, and prostate cancer (Bailey et al., 2015; Pieroth et al., 2018). Folate deficiency may disturb the synthesis of thymidylate and purines, which results in incorporation of

uracil into DNA, which destabilizes DNA. Furthermore, it affects methylation of DNA, which affects gene expression and causes carcinogenesis (Hu et al., 2016). However, anti-folate drugs such as 5-FU or MTX derivatives are used in chemotherapy to prevent the replication and growth of cancer cells, simultaneously disabling the function of folate metabolism. Therefore, the supplementation by folic acid is recommended in the cancer treatment, but precautious should be taken related to the dose, as it seems that the dose, the folate form and timing of folate supplementation determine the efficiency of the therapy (Sanderson et al., 2007). Intake achieved by high doses of supplemental folic acid have been associated with an increased risk of colorectal, breast and prostate cancer (Sanderson et al., 2007; Stolzenberg-Solomon et al., 2006; Wien et al., 2012), even though the previously mentioned MTHFR gene polymorphism could also play a role in development of breast cancer (Zhang et al., 2015).

The most common consequence of folate deficiency is a megaloblastic anemia, which is caused by inadequate nucleic acid synthesis and impaired cellular division, where red blood cells do not fully mature and stay large. As mentioned, folate plays a key role in the methylation cycle, where it converts homocysteine to methionine. Folate deficiency results in hyperhomocysteinemia, which could be also caused by polymorphisms on MST genes or vitamin B_{12} deficiency as B_{12} acts as a co-factor for MST. Hyperhomocysteinemia has been related to an increased risk of hypertension, cardiovascular diseases and cerebrovascular diseases (Blom & Smulders, 2011; Bailey et al., 2015). In addition, polymorphisms on MHTFR gene 677T→C and TYMS 3/3T→C, affect homocysteine and folate responses to folate intake, and present a high risk for development of cardiovascular diseases and ischemic stroke, but also of neurological disorders such as Alzheimer's disease and vascular dementia (Vijayan et al., 2016; Smith & Refsum, 2016). Ma et al. (2017) reported that low blood levels of folate and vitamin B_{12} and high levels of homocysteine were associated with Alzheimer's disease and mild cognitive impairment in older Chinese population.

Due to the tight connection with vitamin B_{12} in folate and methylation cycle, the deficiency of vitamin B_{12} can lead to folate deficiency by enabling conversion of 5-CH_3-H_4folate to H_4folate, which is called "methyl trap", since folate is "trapped" in the form of 5-CH_3-H_4folate (Sauer & Wilmanns, 1977). The lack of a biologically active folate form H_4folate disables the production of 5,10-CH_2-H_4folate that plays a role in many metabolic pathways. However, when the folate intake is adequate and vitamin B_{12} is deficient, there is a constant supply of folate which enables production of red blood cells and DNA, which can mask vitamin B_{12} deficiency (Moll & Davis,

2017). Therefore, it is of a high importance to measure the status of both vitamins in order to be sure that a proper type of deficiency is diagnosed as the cases of masking folate deficiency by B_{12} deficiency have also been reported (Antony, 2017).

2.6. Folate recommendations and intake

Requirements for folate are changing during the life time depending on the age, sex, special conditions such as pregnancy or lactation, and other physiological changes. Moreover, gene's polymorphisms of the key enzymes involved in folate biosynthetic pathways can also contribute to the change in dietary recommendations of individuals. If the differences in bioavailability of various folate forms would also be taken into consideration, it is becoming complicated to establish dietary recommendations. Worldwide countries have specified dietary recommendations for folate intake. The Institute of Medicine (1998) defined the most relevant for the folate intake as; estimated average requirement (EAR), recommended dietary intake/allowance (RDI/RDA/RI), and tolerable upper intake level (UI).

EAR: The intake that would meet the requirements of 50% of the population.

RDA/RDI/RI: Based on the EAR and corrected for the population variance. It is the average daily dietary intake level that is sufficient to meet requirements of ~98% of the population (NNR 2014).

UL: "Maximum daily intake levels at which no risk of adverse health effects is expected for almost all individuals in the general population, including sensitive individuals, when nutrient is consumed over long periods of time" (Institute of Medicine 2000).

Worldwide established recommendations for dietary intake of folate (µg/day) are shown in Table 1. Denmark is using Nordic Nutrient Recommendations, where the recommended folate intakes for children are 80 µg/day (2-5 years), 130 µg/day (6-9 years) and 200 µg/day (10-13 years), adjusted on the basis of bodyweight. The folate intake for adults is 300 µg/day, whereas women of reproductive age and women in pregnancy and lactation are recommended to have an intake of 400 µg and 500 µg of folate per day, respectively. Danish average dietary folate intake of 350 µg/10 MJ is the highest among Nordic countries, even though is shown to be inadequate related to the reference values for heterogeneous groups (450 µg/10 MJ) (NNR, 2014).

Table 1 Dietary recommendations of dietary folate (µg/day) for adults

	Nordic countries[1]	United Kingdom[2]	Germany, Switzerland, Austria[3]	The Netherlands[4]	South--east Asia[5]	United States and Canada[6]	FAO/ WHO[7]	Australia, New Zealand[8]
	µg/day of folate					µg/day of DFE		
Category**	RI	RNI	RI	RDA/AI	RDA	RDA/AI	RNI	RDA/AI
Males	300	200	300	300	400	400	400	400
Females	300 (400)*	200	300 (400)*	300	400	400	400	400
Pregnancy	500	300	550	400	600	600	600	600
Lactation	500	260	450	400	500	500	500	500

*recommendation for women of a reproductive age

**Units used to express recommended dietary intake recommendations differ worldwide. DFE, dietary folate equivalent; RI, recommended intake; RNI, recommended nutrition intake; RDA, recommended dietary allowance; AI, adequate intake.

[1]Nordic Nutrition Recommendations, 2014; [2]Department of Health, 1991; [3]Krawinkel et al., 2014; [4]Health Council of The Netherlands, 2008; [5]Barba & Cabrera, 2008; [6]Institute of Medicine, 1998; [7]World Health Organization and Food and Agriculture Organization of the United Nations, 2004; [8]Australian Government Department of Health and Aging, 2006

In 1998, the mandatory food fortification of cereal grain products was implemented in the United States, Canada and Costa Rica, followed by many other countries, which reduced significantly occurrence of NTDs (Crider et al., 2011). However, the increased intake of folic acid by fortified food in combination with dietary folate intake and supplementation opened new challenges, since possible adverse effects of excessive folic acid consumption occurred. A dose greater than 280 µg of folic acid is enough to saturate the hepatic biotransformation of folic acid and result in appearance of "unmetabolized folic acid" (Tam et al., 2012). Institute of Medicine defined UL of 1000 µg/day of folic acid for adults, whereas there is no UL for naturally occurring food folate forms. Adverse health effects were observed in the combination of high level of folate in blood (≥45.3 nmol/L) with poor vitamin B_{12} status. Reynolds (2016) concluded that the UL for folic acid is set too high as adverse neurological effects have been observed from the long-term exposure to folic acid at doses of 500-1000 µg per day in the presence of B_{12} deficiency. The European Union sat recommendations in order to reduce the prevalence of folate deficiency, but no mandatory fortification was introduced (European Union, 2002, 2006). At the moment, there is still no consensus about the use of folic acid as some researchers suggest reduction of the doses (Dolin et al., 2018; Pitkin, 2007), while the others find that there is no need for having UL and that

mandatory food fortification should be introduced, such as in the United Kingdom (Wald et al., 2018; Morris et al., 2016).

Institute of Medicine (1998) defined the EAR for folate as a 320 µg/day. In 1997, a large Dutch study summarized folate intake of European countries and reported the intake of 291 µg/day for men and 247 µg/day for women, with a wide variation (de Bree et al., 1997). If the EAR is reliable, these findings would mean that 20 years ago there was an obvious folate deficiency within the Europe, as the actual intake was lower than EAR. In 2013, the International Life Sciences Institute (ILSI) evaluated prevalence of low micronutrient intakes of different European countries (Mensink et al., 2013). They used UK's recommendations as a reference for folate intake in which RNI was defined as 200 µg/day as is shown in Table 1. The mean folate intake from the base diet ranged from 156 µg/day (The Netherlands, 19-30 years) to 299 µg/day (Denmark, 18-60) for women and from 199 µg/day (The Netherlands, over 60 years) to 333 µg/day (Poland, 18-60 years) for men, which led to a conclusion that the folate intake is adequate. However, if the NNR would be taken as a reference, the mean folate intake would be inadequate for women of reproductive age. Tetens et al. (2011) evaluated the intake of micronutrients from the diet and from supplements in a representative sample of the Danish adult population. The medians of a dietary intake of non-users of supplements among males (18-49 years) and females (18-49 years) was 311 µg/day and 264 µg/day, respectively, indicating that the dietary intake of women in a reproductive age was inadequate. However, >50% of the subjects from the study were classified as users of supplements, and their folate intake was adequate. Moreover, Danes with special types of diet, such as vegetarian or vegan, have folate intakes ranging from 758 µg/day (men) to 601 µg/day (women) (Kristensen et al., 2015), which exceeded NNR of 300 µg/day. However, it is very challenging to compare intakes between the countries as there are variations between the methods used for the determination of the folate content in national food databases.

2.7. Food sources

Folate is synthesized by plants and microorganisms, and it is therefore present in most of foodstuffs (Saini et al., 2016; Hanson & Gregory, 2011). Contents of selected food sources are presented in Table 2. The data shows that yeast and offal, including mainly liver, are the best folate sources, which is expected since yeast is known by folate production and liver is the folate storage organ in mammals (Korhola et al., 2014; Ohrvik & Witthoft, 2011). From the foodstuffs of plant origin, green leafy vegetables, spices and herbs, and legumes are the best folate sources, followed by

certain fruits such as berries and citrus. Moreover, tropical fruits and their products are shown to be great sources of folate with total folate content ranging up to 440 µg/100 g of fresh weight (Akilanathan et al., 2010; Striegel et al., 2018).

Table 2 Selected food sources of folate. Data are taken from Frida – Danish Food Composition Databank (Revision 3a, https://frida.fooddata.dk)

Food group	**Foodstuff**	**Folate content (µg/100 g)**
Yeast	Yeast, dried	4000
	Yeast, extract, Marmite	1010
	Yeast, baker's, compressed	1000
Offal	Liver, ox, raw	2300
	Liver, pig, raw	1000
	Liver, cod, raw	300
	Pate, liver	252
	Kidney, ox, raw	80
Vegetables	Spinach, raw	220
	Cauliflower, all varieties, raw	165
	Brussels sprouts, raw	130
	Carrot, raw	46
Fruits	Strawberries, raw	117
	Avocado, raw	93
	Orange, raw	46
	Raspberry, raw	44
Legumes	Peas, chick, dry, raw	180
	Nut, pea, oil-roasted and salted	124
	Walnuts, dried, raw	66
Spices and herbs	Curry powder	154
	Dill, raw	116
	Chives, raw	80
	Coriander, leaf, raw	62
Cereals	Rice, brown, raw	53
	Rice, polished, raw	31
	Bulgur	21

Table 2 Selected food sources of folate. Data are taken from Frida – Danish Food Composition Databank (Revision 3a, https://frida.fooddata.dk) (*continued*)

Dairy and egg products	Egg, yolk, raw	51
	Yoghurt, plain, whole milk	21
	Milk, whole, 3.5% fat	11
	Egg, egg white, raw	7
Meat and fish products	Chicken, flesh and skin, raw	24
	Tuna, raw	15
	Herring, raw	10
	Roast beef, sliced	9

Even though yeast, offal and tropical fruits are high in folate, their consumption is lower than the one of fruits and vegetables, and consequently their contribution to the folate intake too.

Dietary survey conducted from 2011-2013 showed that the food groups of vegetables, breads and cereals, and fruits contribute the most to the dietary folate intake among Danish population (Pedersen et al., 2015). Even though there is no mandatory fortification of cereals grain products in Europe, and these products are not considered as folate-rich foodstuffs, they contribute significantly to the dietary folate intake among Danes, showing the importance of consumption on the intake.

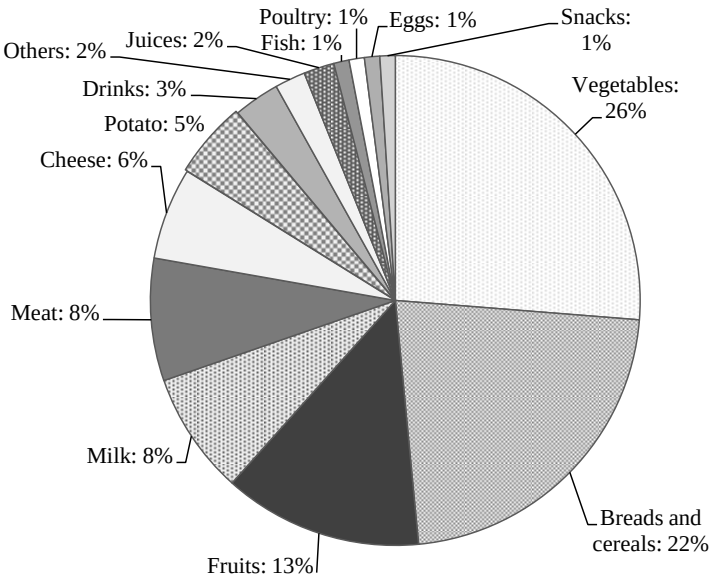

Figure 4 Contribution of various food groups to the folate intake among Danish population (Pedersen et al., 2015)

Furthermore, raw foodstuffs normally have higher folate content than processed ones, due to its instability to various experimental conditions. Delchier et al. (2016) reviewed an effect of various cooking processes such as blanching, steaming, cooking in boiling water, freezing, canning and juicing on the folate's stability in fruits and vegetables. The highest losses of folate were recorded after canning where 65% and 77% of folate was lost in spinach and chickpeas, respectively. On the other side, no significant reduction was found for steaming and microwaving of peas. Leaching was also described as a mechanism of folate loss via diffusion which occurs when foodstuffs are flushed with liquid (mostly water) during processing and folate forms were found in covering liquid (Delchier et al., 2014). It mostly occurs in dry legumes which have to stand in water before cooking. Folate content was analyzed in raw, cooked and malted pseudocereals, such as quinoa, amaranth and buckwheat. Total folate content expressed as folic acid equivalent was 309 ± 8 µg/100 g, 228 ± 24 µg/100 g and 153 ± 12 µg/100 g of dry weight of raw quinoa, amaranth and buckwheat, respectively. Boiling and steaming reduced the total folate content by 58% and 22%, respectively, whereas a 10-15% increase was observed in quinoa. Moreover, malting significantly increased total folate content in amaranth and buckwheat by 21% and 27%, respectively, while no significant change was observed in quinoa. The retention of folate vitamers was also studied and differences in stability of various folate forms were observed, depending on the foodstuff and processing procedure (Motta et al., 2017). Therefore, even though some foodstuffs are considered as good folate sources, their consumption, folate retention in specific food matrix during specific processing procedure and other previously mentioned factors will determine whether their contribution to the dietary intake is significant.

Chapter 3: Summary of papers describing the studies performed

This PhD thesis is based on four research papers presenting the work performed on quantification of naturally occurring folate forms in various groups of foods and potential fortification strategy. A short summary of the papers is given here. It is recommended to read the research papers before continuing with reading the thesis.

Paper I. The aim of the study was to develop and validate a rapid, sensitive and reproducible method for quantification of six naturally occurring folate forms, such as; tetrahydrofolate, 5,10-methenyl-tetrahydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, folic acid and 5-methyltetrahydrofolate in food. The method was based on the use of recombinant plant origin deconjugase for folate deglutamylation. Folate forms were extracted from the food matrix by using a combination of a heat treatment and single-enzyme deconjugation, followed by the solid phase cleanup and detection by liquid chromatography-tandem mass spectrometry using electrospray ionization (LC-ESI-MS/MS). Three $^{13}C_5$-labeled internal standards were used for the quantification, which resulted in acceptable calibration curve (R^2>0.99 and trueness 85-115%), a limit of quantification at 0.1 µg/100 g, trueness at 80-120% in spiked samples, and a precision <10%. An interconversion of unstable formyl folate forms was also studied, and their joint quantification as a sum of folic acid equivalent was proposed.

Paper II. The aim of the study was to compare the newly developed and validated method from the **Paper I** with the microbiological assay as the only standard method for folate determination in foodstuffs and another LC-MS/MS method using the deconjugase enzyme of animal origin such as rat serum and chicken pancreas. Both LC-MS/MS method included single-enzyme extraction, whereas microbiological assay used tri-enzyme extraction by human plasma as deconjugase. A round robin comparison was performed in which 89 samples representing food groups of fruits, vegetables, legumes, cereals, dairy products, meat and offal were analyzed for folate content. The significant bias ($p \leq 0.05$) of 17% lower and 25% higher results was observed if the newly developed LC-MS/MS method using the enzyme of plant origin was compared to microbiological assay or another LC-MS/MS method, respectively. Interestingly, no difference in the folate content analyzed in legumes, which are considered as a complex matrix, was found between the three methods. This round robin comparison gave the insight in the performance of our newly developed method and showed that it could serve as a proper substitute to the currently standardized microbiological assay.

Paper III. The aim of the study was to examine folate distribution in commonly consumed raw materials from food groups of fruits, vegetables, legumes, cereals, dairy products, meat and offal and their contribution to the daily dietary intake. 89 food samples were analyzed for the folate content by the method developed in **Paper I**. Foodstuffs from food groups of offal (829 – 1897 µg/100 g), vegetables (71 – 162 µg/100 g), legumes (73 – 115 µg/100 g) and certain fruits such as strawberries (79 – 94 µg/100 g) were shown to be "sources rich in folate" providing the highest folate intake and contributing to the recommended daily intake of up to 40% per serving, whereas 1 serving of liver exceeded the recommended daily intake (400-600%). 5-CH_3-H_4folate was the most abundant folate form (>80%) in fruits, vegetables and certain legumes, whereas it was the second most abundant in liver and dairy products after formyl forms. Folate distribution can vary within the same food group and between different varieties of the same foodstuff. Commonly consumed foodstuffs such as meat (0.6 – 6 µg/100 g), cereals (4 – 41 µg/100 g), dairy products (6 – 41 µg/100 g) and certain fruits as banana (20 – 22 µg/100 g), apple (0.7 – 5 µg/100 g) and tomato (12 – 24 µg/100 g) are not good sources of folate. However, due to the increased consumption of these foodstuffs, they also contribute to the daily intake of folate.

Paper IV. The aim of this study was to develop an effective encapsulation technique for stabilization of synthetic 5-methyltetrahydrofolate (L-5-CH_3-H_4folate). The encapsulation of L-5-CH_3-H_4folate using combination of carbohydrates, such as glucose syrup and pullulan as biopolymers was tested. The encapsulation process was optimized, producing capsules with 100% of L-5-CH_3-H_4folate recovered from biopolymer solution. Stability study of produced capsules was performed by storing the capsules at room temperature, in the dark for 21 day. The use of ascorbic acid as antioxidant was also tested, which did not indicate any significant improvement of the stability of the capsules. However, encapsulation enabled oxidative stability of the capsules, as the stability of the free L-5-CH_3-H_4folate used as a control started to significantly decrease after day 7, decreasing for 40% in 21 day, whereas no difference was observed in folate content in the capsules from day 0 until day 21. A further step of incorporating the encapsulated L-5-CH_3-H_4folate in food matrix was performed, where all-purpose flour was enriched by 150 µg/100 g of L-5-CH_3-H_4folate, in either free or encapsulated form and was used for preparation of buns. The buns were baked at 200°C/7.5 min and stored at room temperature in the dark for 9 days. No significant difference was observed in the oxidative stability of encapsulated folate, being 100%. However, the stability of free L-5-CH_3-H_4folate was significantly lower on day 9, decreasing from 100% to 88%. However, a further work should be done in order to test efficiency of this protective technique.

Chapter 4: Quantification of folate in food

In order to examine the efficiency of the use of enzyme of plant origin for quantification in various food products, the analytical method for folate quantification has been developed and validated. This chapter describes the relevant background information for the choice of analytical method used in ***Paper I, II, III*** *and* ***IV****. Section 4.1. presents the general information related to folate quantification in food. Section 4.2. summarizes development of LC-ESI-MS/MS, as folate quantification method, and it includes some unpublished results generated during the method development. Detailed description is given in* ***Paper I****.*

4.1. Folate analysis

The differences in the behavior of various folate forms and the entrapment of the naturally occurring folate forms in the food matrix present a challenge in folate determination. However, due to the important role that folate plays in human health, a proper method for the quantification of folate in food is of a high importance in order to know more about folate consumption and intake.

Folate analysis is challenging due to their low concentrations in food and their different stabilities during heat, light and oxygen exposure. There are three main steps in folate analysis, such as extraction, deconjugation and quantification as it is shown on Figure 5. Depending on the detection method, a purification step may also be included (Quinlivan et al., 2006; Jägerstad & Jastrebova, 2013). Extraction is the first step in folate analyses in which folate is liberated from food matrix by the use of heat, various enzymes, or their combination. Deconjugation is the following step in which folate polyglutamates are reduced to monoglutamyl forms by the use of GGH enzymes, which is followed by folate quantification using microbiological or chemical assays. It is important to have an appropriate method for folate quantification, as can be shown in the case of Konings et al. (2001) where folate intake estimated by HPLC method was ~25% lower than the one estimated by microbiological assay, which illustrates how differences in the method's performance can influence the final outcome of the study. However, data obtained by HPLC have not been used in food composition database, as MA is more widely used than HPLC. Therefore, the data obtained by MA provide easier comparison among various national food composition databases.

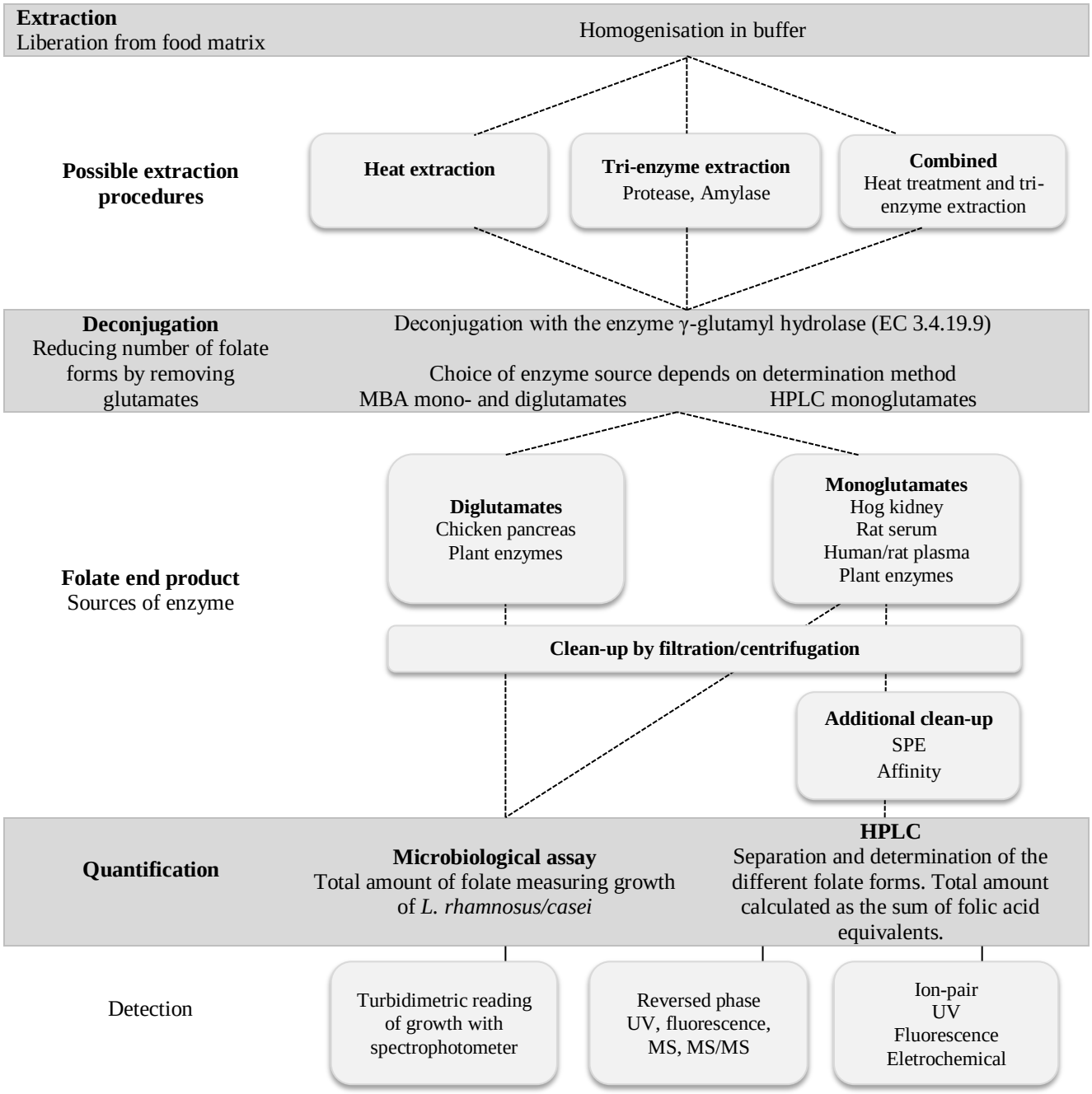

Figure 5 Schematic overview of analytical methods for folate determination in foods modified from (Quinlivan et al., 2006; Delchier et al., 2016; Arcot & Shrestha, 2005)

Furthermore, folate may be entrapped in complex food matrix which reduces their extraction rate. In order to be analyzed, food folate forms have to be extracted from food matrix which includes exposure to temperature change in several heating and cooling steps during the extraction. Moreover, low pH values (2-3) are used during sample purification and in mobile phases in liquid chromatographic (LC) methods, which causes interconversion of some folate forms due to their different stability at different pH (de Brouwer et al., 2007; Kirsch et al., 2010; Quinlivan et al., 2006; Ringling & Rychlik, 2013). The scheme of interconversion reactions is shown on Figure 6. These changes affect folate stability and they have to be taken into consideration during extraction and quantification of folate forms. For instance, folic acid and 5-CH_3-H_4folate are shown to be relatively stable at pH 2-10 with and without heat treatment, whereas 5,10-CH^+=H_4folate and 5-HCO-H_4folate are relatively stable without heat treatment. However, if heated, 5,10-CH^+=H_4folate and 5-HCO-H_4folate are not stable at pH 3-9 and pH < 3, respectively, which makes folate analysis very complex (de Brouwer et al., 2007).

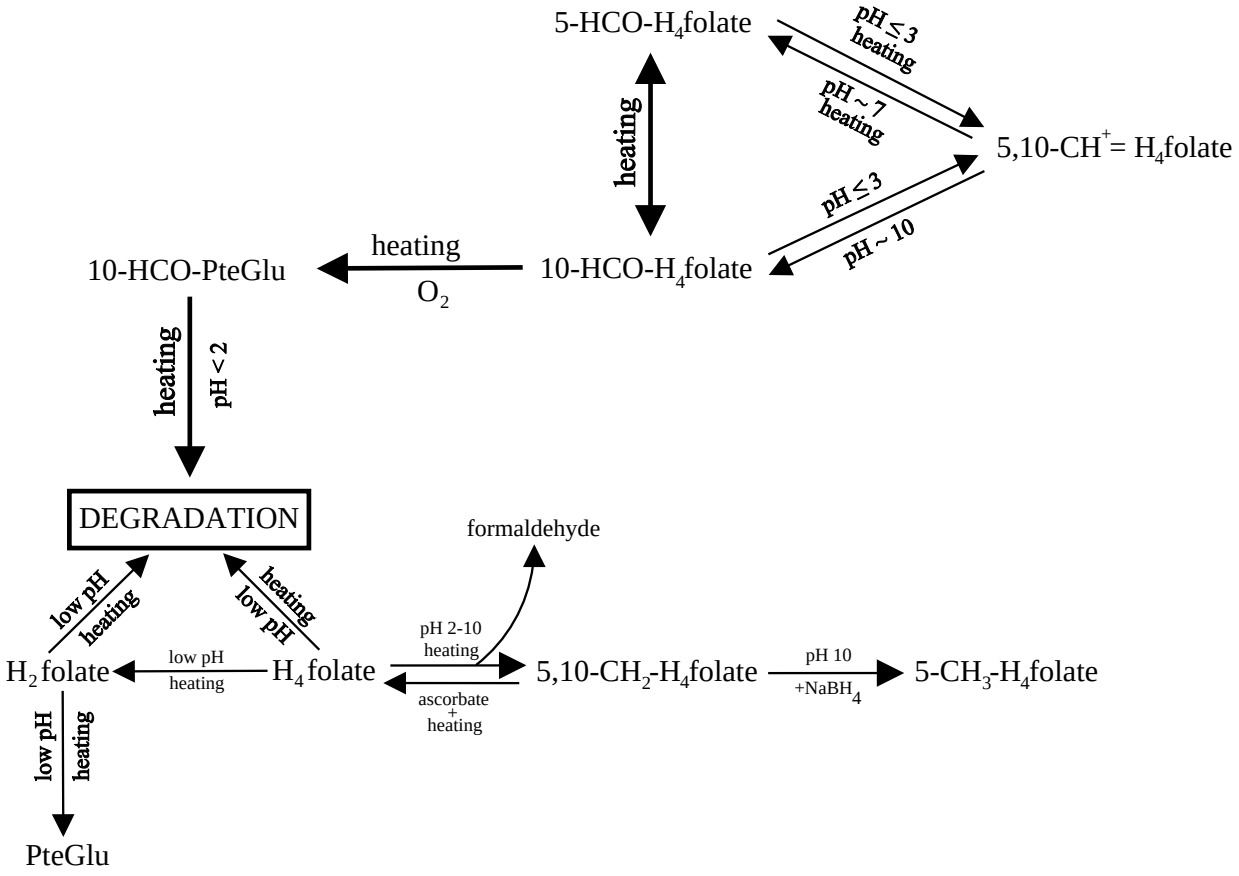

Figure 6 Scheme of folate interconversion and degradation under various conditions modified from (de Brouwer et al., 2007; Quinlivan et al., 2006)

4.1.1. Sample extraction and purification

Folate extraction involves liberation of folate from food matrices by heat treatment in buffers containing reducing agents. Neutral or slightly acidic or alkaline pH of the buffers such as; acetate, phosphate, MES and HEPES/CHES buffer is normally used (Delchier et al., 2016). Ascorbic acid (Asc) is the most commonly used antioxidant, combined with other reducing agents such as β-mercaptoethanol (MCE), dithiothreitol (DTT), 2,3-dimercapto-1-propanol (BAL) or 2-thiobarbituric acid (TBA) (Patring et al., 2005). Recently, the use of odorless and more powerful reducing agent tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) has also been reported (Bhandari et al., 2018). Patring et al. (2005) studied the antioxidant effectiveness of mentioned reducing agents in yeast extracts. They suggested the use of BAL as a reducing agent of choice in folate analysis, because it enables H_4folate stability during heating, storage and freezing/thawing. However, MCE is still very commonly used reducing agent (Vishnumohan et al., 2017; García-Salinas et al., 2016; Luo et al., 2017; Zou et al., 2019). The combination of heat treatment and tri-enzyme extraction has been suggested as the best extraction procedure in folate analyses (Hyun & Tamura, 2005). Tri-enzyme treatment includes treatments with protease in order to release folate from the matrix containing proteins, α-amylase to reduce carbohydrates and GGH to remove polyglutamyl tail in order to reduce folate to monoglutamyl form, which is necessary prior the quantification by chemical analysis as shown at Figure 5. During this procedure, folate forms are exposed to a couple of heat treatments due to inactivation of enzyme activity. Furthermore, various GGH enzymes (E.C 3.4.19.9.) have different pH optima, which also defines some steps in folate analyses and affect their stability and interconversion, as shown in Figure 6. GGHs are obtained from several sources and are mainly of animal origin such as rat plasma/serum, human plasma, hog kidney, and chicken pancreas (Jägerstad & Jastrebova, 2013). Chicken pancreas is able to reduce folate forms only to a diglutamyl forms, and it is therefore not suitable if used alone in chromatographic procedures which quantify monoglutamyl forms. Therefore, it is used in the combination with some other GGHs in order to improve deconjugation (Ringling & Rychlik, 2013). The optimal activity of rat plasma and chicken pancreas is at neutral pH, whereas hog kidney deconjugases have optimum activity at pH 4.5-5 (Arcot & Shrestha, 2005). GGHs of animal origin contain some endogenous folate forms which have to be either removed by purification steps or it has to be corrected for endogenous content of folate. The activity of GGHs varies depending on the enzyme and food components that may inhibit their activity. Rat serum/plasma is a very commonly used GGH as it is easy to prepare it and it is commercially available (De Brouwer et al., 2010; Kiekens et al., 2015; Luo et al., 2017;

Striegel et al., 2018). It is highly recommended to test the activity of each GGH batch by the use of polyglutamated substrate, such as pteroyltriglutamic acid (Patring et al., 2005). It was reported that the enzymes of animal origin may be inhibited by the plant food matrix (Wei & Gregory, 1998). In 2013, Ramos-Parra et al. published results on the use of a plant origin recombinant GGH produced from *Arabidopsis thaliana*, which enabled complete deglutamylation of folate within 1 hour (Ramos-Parra et al., 2013; Orsomando et al., 2005). Recently, the same deconjugation efficiency was shown to be accomplished by the use of recombinant commercially available human GGH in different berries and berry juice (Zou et al., 2019). Even though tri-enzyme treatment was suggested as an extraction procedure of choice, some studies showed that there is no difference between tri-enzyme and single-enzyme treatment (Ringling & Rychlik, 2017a; Zou et al., 2019).

If chromatographic methods are used as detection, sample cleanup is preferable to avoid as much as possible interferences during chromatographic measurement. Solid-phase extraction (SPE) is a very common purification strategy which often includes strong anion exchange (SAX) or affinity chromatography using folate binding protein (FBP) (García-Salinas et al., 2016; Ringling & Rychlik, 2013). SAX is used to purify negatively charged acids which bound to positively charged groups of the cartridge sorbent that are positively charged under all conditions, and the purification is done by changing the pH of washing and elution solutions. Affinity chromatography using FBP is more specific for folate analyses, as it consists of FBP covalently bound to a solid support, usually agarose beads (Quinlivan et al., 2006). Their high specificity enables excellent purification lowering significantly limits of detection (LOD) if compared to SAX, even though using SAX results in sufficient LOD (Freisleben et al., 2003a). However, agarose-FBP columns are not commercially available, which makes their use laborious and expensive. Moreover, they do not bind equally to all folate forms, as 5-HCO-H_4folate showed lower affinity to the FBP that the other folate forms (Kariluoto et al., 2001).

4.1.2. Detection methods

Detection methods used for folate quantification can be divided in three main groups; microbiological assay (MA), ligand-binding methods using FBP, and chemical assays. Chemical assays present high pressure liquid chromatography (HPLC) methods coupled to various detectors such as ultraviolet (UV), fluorescence, electrochemical, mass spectrometer (MS) or tandem mass spectrometer (MS/MS). Chemical assays provide higher sensitivity, specificity and precision when compared to MA.

4.1.2.1. Microbiological assay

Microbiological assay is the only standardized method for folate determination in food (DeVries et al., 2005; EN14131, 2003). It is based on the bacterial growth, *Lactobacillus rhamnosus* (ATCC7469), also known as *L. casei*, determined by turbidimetric detection using UV detector. Quantification of folate using tri-enzyme extraction has been the method of choice for a long time, even though is tedious, time-consuming, and require special expertise (Tamura, 1998). Furthermore, the microbial response can be affected by the presence of some other compounds, such as; thymidine, amino acids, purines, and pyrimidines, which can give inaccurate results (Koontz et al., 2005). Previous studies reported that the response of *L. rhamnosus* is not the same to all folate vitamers, showing the highest response for 5-HCO-H_4folate, followed by the response for 10-HCO-H_4folate, PteGlu, 5-CH_3-H_4folate and H_4folate (Weber et al., 2011). Moreover, microbiological assay is dependent on the calibrant used for quantification, which was reported by Ringling and Rychlik (2017a), where the significant difference in the folate content in wheat germs, chickpeas and mung beans was found depending on the use of 5-HCO-H_4folate or PteGlu as a calibrant. However, standard methods for folate quantification by the use of MA normally use folic acid as a calibrant, which should provide comparable results. Today microbiological assay is still used as a reference method, even though it is possible that the microbial growth is affected by non-folate compounds in the sample. Hence validated, precise and accurate chemical methods are warranted to form the bases for future standardization.

4.1.2.2. Chemical methods

In contrast to microbiological assay that quantifies only total folate content; chemical methods enable quantification of various folate forms due to the separation on chromatographic columns and sensitive detection systems. Chromatographic methods are coupled to detector such as UV, fluorescence, ECD or MS, as shown in Table 3, which differ in their sensitivity and specificity. UV detection was the most common detection system for a long time, but it was not specific and sensitive enough to detect low limits of all naturally occurring folate forms. Therefore, it was often combined with some other more sensitive detectors, such as fluorescence detector. On the other side, native fluorescence detection is less sensitive than UV detection for some folate forms, whereas folic acid cannot be detected as it is not fluorescent. However, folic acid can be analyzed if irradiated with intense UV-irradiation (λex/em = 280/440 nm), which also increases fluorescence of

5-CH_3-H_4folate and 5-HCO-H_4folate (λex/em = 280/360 nm) (Martín Tornero et al., 2017; Arcot & Shrestha, 2005).

In the last 20 years, the use of MS detection enabled fast and sensitive separation of various folate compounds which was troublesome due to small differences in their ionic characters. MS gave a new dimension of mass-to-charge ratio (*m/z*) which enables more accurate, precise and sensitive detection of naturally occurring folate forms. However, the challenge in such a complex chemical analysis has been the lack of a suitable internal standard that could compensate for losses during folate extraction, sample cleanup and for the matrix effect. In 2001, Pawlosky et al. used for the first time stable isotope dilution (SIDA) method for quantification of 5-CH_3-H_4folate using $^{13}C_5$-5-CH_3-H_4folate as internal standard. Two years later, $^{2}H_4$-labeled internal standards were introduced for quantification of PteGlu, 10-HCO-H_4folate, H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate, which presented a start of new era in folate analysis (Freisleben et al., 2003b). Isotopically labeled internal standards are widely used since they ensure more accurate quantification (Ringling & Rychlik, 2013; Striegel et al., 2018; Vishnumohan et al., 2017; Zou et al., 2019). Recently, a UPLC-MS/MS method using three $^{13}C_5$-labeled internal standards was approved as an AOAC-method for determination of PteGlu, 5-CH_3-H_4folate, 5-HCO-H_4folate and total folate content expressed as PteGlu equivalent in infant formula and adult nutritionals (Bhandari et al., 2018). The optimum folate analysis would include a proper labeled internal standard for each vitamer form.

4.2. The development and validation of an LC-ESI-MS/MS for folate in food

A highly specific and sensitive LC system, coupled to triple quadrupole mass spectrometer (MS/MS), was chosen as the detection principle for folate quantification in various groups of foods. The specific parts of the method development are discussed in the upcoming sections.

4.2.1. Sample preparation

A proper sample preparation and purification are very important in order to make sure that samples are free from interferences that can affect the quantification of the analyte from interest due to the low unit mass resolution (4000 Th) and low mass accuracy (100 ppm) of triple quadrupoles if compared to other mass analyzers (de Hoffmann & Stroobant, 2007). As folate forms are extremely sensitive to various environmental conditions that cause their degradation or interconversion, a special precaution should be taken to prevent folate degradation by exposure to light and oxygen. Therefore, all analyses were performed under yellow light, in brown glass, or protected by UV

absorbing film (EN14131, 2003). Furthermore, the change in the temperature and formation of large ice crystals which are mostly extracellular causes damages to the tissue and possibly folate degradation during freezing and thawing cycles (Ninagawa et al., 2016). Therefore, instant freezing by the use of liquid nitrogen was chosen as a freezing technique which enables immediate freezing of the samples to temperatures < -80°C. Prior the analyses, the samples were homogenized while frozen using a coffee grinder which enabled production of a fine powder and consequently the use of a low amount of sample for the chemical analysis. Ascorbic acid and MCE were used as antioxidants (**Paper I**) and nitrogen-flushing was used in order to remove oxygen from sample bags during sampling or extracts during chemical analyses.

Folate extraction is normally composed of the combination of several heating steps and the use of enzymes that enable liberation of folate from food matrices (Jägerstad & Jastrebova, 2013; Quinlivan et al., 2006). Due to the thermal instability of folate forms, it is preferable to use as less as possible heating/cooling steps. The composition and the pH of the extraction buffer are playing an important role in the stability of folate. Phosphate buffer pH 6-7 is one of the most commonly used buffers, even though pH 4.5 has also been used depending on the use of enzyme and the pH of its optimal activity (Czarnowska-Kujawska et al., 2017; Tyagi et al., 2015; de Brouwer et al., 2007). The use of the deconjugase enzyme plays an important role in folate analyses. As various deconjugases have various pH optima, that should be taken into consideration during the method development (Arcot & Shrestha, 2005). Chicken pancreas converts polyglutamyl folate forms to diglutamate, whereas rat serum ensures the necessary transition to monoglutamate. Rychlik et al. (2007) reported the activity of rat serum incubated overnight alone and with addition of chicken pancreas being 42% and 96% respectively. The recombinant enzyme of plant origin was produced by genetic engineering from *Arabidopsis thaliana* (Orsomando et al., 2005), whose optimal activity is at pH 6. In 2013, Ramos-Parra et al. published the comparison of deconjugation using the enzyme of plant origin and rat plasma, which showed superiority of plant enzyme over animal enzyme in terms of the efficiency (Ramos-Parra et al., 2013). Plant origin enzyme provided 100% deconjugation within 1 hour by using very low amounts of enzyme solution in food matrices such as tomato fruit, black bean seeds, and alfalfa sprouts, whereas the enzyme of animal origin provided 55%, 28% and 57% deconjugation in these foodstuffs, respectively.

Table 3 HPLC methods for folate determination in food samples and biological tissue samples

Sample	Extraction buffer	Enzyme treatment	Purification	Column	Chromatographic conditions	Detection	Reference
Food samples	75 mM K_2HPO_4 + 52 mM ascorbic acid/ascorbate mixture, 0.1% MCE, pH 6.0	Single enzyme treatment, D: hog kidney + chicken pancreas	SPE, SAX (quaternary amine)	Shandon Hypersil ODS column (3 µm, 120 x 4.6 mm) and Spherisorb ODS column (5 µm, 250 x 4.6 mm)	Gradient: ACN/30 mM phosphate buffer (pH 2.2)	Fluorescence ex/em 290/356 nm and 360/460 nm (UV 290 nm)	Vahteristo et al., (1996)
Rat liver, rat and mouse brain	50 mM potassium tetraborate, 1% sodium ascorbate, pH 9.2	Single enzyme treatment, D: chicken pancreas	Affinity chromatography (Bovine milk FBP)	250 x 4.6 mm Betasil Phenyl	A: 28 mM K_2HPO_4 + 60 mM H_3PO_4 in H_2O B: 28 mM K_2HPO_4 + 60 mM H_3PO_4 in 20:80 ACN:H_2O C: 25 mM K_3PO_4, pH 7.0 in 5:95 ACN:H_2O D: H_2O	Four-channel electrochemical detector	Bagley & Selhub, (2000)
Rye varieties	50 mM HEPES/CHES buffer, 2% ascorbate, 10 mM MCE, pH 7.85	Tri-enzyme treatment, D: hog kidney (4h in total)	Affinity chromatography (Bovine milk FBP)	Shandon Hypersyl ODS column (3 µm ,150 mm x 4.6 mm)	Gradient: ACN/20 mM phosphate buffer	Fluorescence ex/em 290/356 nm and 360/460 nm (DAD 290 nm)	Kariluoto et al., (2001)
Rice	50 mM phosphate buffer, 1% Asc, 0.5% DTT, pH 7.5	Tri-enzyme treatment, D: not specified	Ultrafiltration using 5 kDa Millipore filter	Acquity HSS T3 column (1.8 µm, 150 mm x 2.1 mm)	A: 0.1% formic acid in H_2O B: 0.1% formic acid in acetonitrile	UPLC-ESI-MS/MS	V. De Brouwer et al., (2010)
Bread, wheat germs, spinach, Camembert cheese, vegetable mix	200 mM MES buffer, 2% ascorbic acid, 0.2 mol/L MCE, pH 5.0	Single enzyme treatment, D: rat serum + chicken pancreas	SPE, SAX (quaternary amine)	C_{18} BDS, 3 µm, 150 x 3.2 mm)	A: 0.1% acetic acid in H_2O B: 0.1% acetic acid in ACN	LC-ESI-MS/MS	Ringling & Rychlik, (2013)

Table 3 HPLC methods for folate determination in food samples and biological tissue samples (*continued*)

Tomato	50 mM potassium phosphate, 1% ascorbic acid, 0.5% MCE and 1 mM calcium chloride, pH 4.5	Tri-enzyme treatment D: rat plasma	Ultrafiltration using 10 kDa cut-off membrane filter	Luna C_{18} column (5 µm, 250 x 4.6 mm)	A: 0.1% formic acid in H_2O B: acetonitrile	UPLC-MS/MS	Tyagi et al., (2015)
Avocado, banana, papaya, tomato	50 mM HEPES/CHES buffer, 2% ascorbic acid, 10 mM MCE, pH 7.8	Dienzyme treatment for high starch samples, D: recombinant plant GGH	Affinity chromatography using FBP	Prodigy ODS (5 µm, 150 x 3.2 mm)	A: 28 mM K_2HPO_4, 59 mM H_3PO_4 B: 75% A, 25% ACN	LC-Four-channel electrochemical detector	García-Salinas et al., (2016)
Lettuce, spinach, pak choi and rice leaf	50 mM phosphate buffer, 1 % ascorbic acid, 0.1% BAL, pH 6.7	Single enzyme treatment, D: rat serum + chicken pancreas	Ultrafiltration using 0.22 µm PVDF hydrophilic membrane filters	Acquity UPLC BEH, C_{18} column, (1.7 µm, 50 x 2.1 mm)	A: 0.1% formic acid in H_2O B: 0.1% formic acid in acetonitrile	UPLC-MS/MS	Shohag et al., (2017)
Berries and berry juices	100 mM ammonium acetate, 1% ascorbic acid, 0.2% MCE, pH 6.0	Single enzyme treatment, D: recombinant human GGH	SPE (Oasis HLB)	HILIC column (2.6 µm, 100 x 4.5 mm)	A: 0.1% formic acid in H_2O B: 0.1% formic acid in acetonitrile	UPLC-ESI-MS/MS	Zou et al., (2019)

4.2.2. Electrospray ionization

Ones when extracted and purified, sample extracts are in a liquid phase. In order to be analyzed by MS/MS they have to be transferred to gas phase. Nowadays, the universal ionization source for LC-MS/MS analysis is electrospray ionization (ESI). It is a soft ionization technique that transfers ions from a liquid to gas phase by applying a high voltage (normally 2-5 kV) without changing the chemical structure of the analyzed compounds.

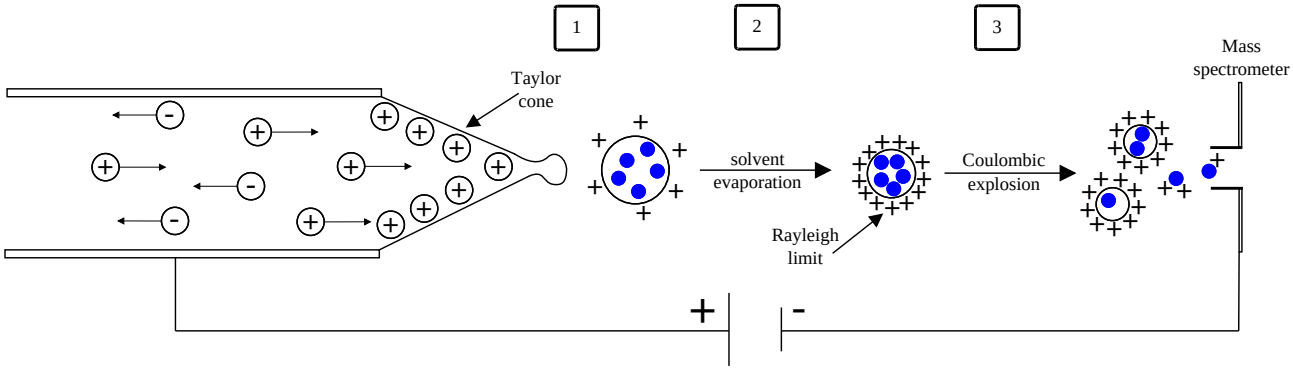

Figure 7 Scheme of the positive mode electrospray ionization modified from Cech & Enke (2001)

The applied voltage provides the electric gradient that is required to separate charge at the surface of the liquid. Positively charged ions are drawn to the capillary tip in the case that negative electric field is created and *vice versa*. Once when field strength is high enough, there will be a "Taylor cone" formed at the capillary tip that will release a jet of droplets with an excess of charge on the surface (Figure 7-1). As the droplets are moving towards the entrance to mass spectrometer, the solvent evaporates (Figure 7-2), and at a certain point reaches Rayleigh limit, when the Coulombic forces between ions exceed the surface tension of the solvent (Figure 7-3). At that point, charged gas-phase ions are formed and ready to be analyzed for mass-to-charge (*m/z*) ratio by entering mass spectrometer (Cech & Enke, 2001; Kebarle & Verkerk, 2009). For folate analyses, ESI is a perfect ionization source since folate vitamers are polar compounds, present in their ionic form in the solution, and it is normally used in folate analysis (Striegel et al., 2018; Zou et al., 2019; Vishnumohan et al., 2017). Despite the power of coupling LC and MS, ESI has some drawbacks in terms of its sensitivity to matrix effects that can occur in analysis of complex samples, such as food (Glish & Vachet, 2003).

4.2.3. LC-MS/MS

Triple quadrupole is typically used mass analyzer for MS/MS analysis that consists of two quadrupole mass filters and a collision cell that is placed in between mass filters. MS/MS technique involves two stages of MS. In the first stage, ions of desired *m/z* called *precursor or parent ions* are isolated from the ion beam entering mass analyzer from ion source. They pass first mass filter, enter the second quadrupole called *collision cell*, where they collide with a collision gas, normally nitrogen, and fragment to various *product ions*. These product ions are analyzed in the second MS stage in second mass filter, and passed to detector. An important advantage of MS/MS detection is that two MS analyses can be independent variable, increasing the sensitivity and specificity of the chromatographic analysis (Glish & Vachet, 2003). The sensitivity is lowest in the scan mode in which all ions produced are passed to the detector, and the highest in SRM (selected reaction monitoring), where single ion is selected and passed into collision cell and a few product ions are selected in the second mass filter for detection. The principle of triple quadrupole work and various MS/MS modes are presented in Figure 8. In SRM mode, signal to noise ratio (S/N) is very good as this principle excludes all other compounds present in the sample, and reduces the background noise. During the method development, the use of one more ion transition was included in the MS/MS method as a qualifier, which shows the peak purity and is used for a confirmation of the analyte of interest. Combining this evidence with retention time and a proper peak shape enables a proper quantification of specific folate forms (**Paper I**).

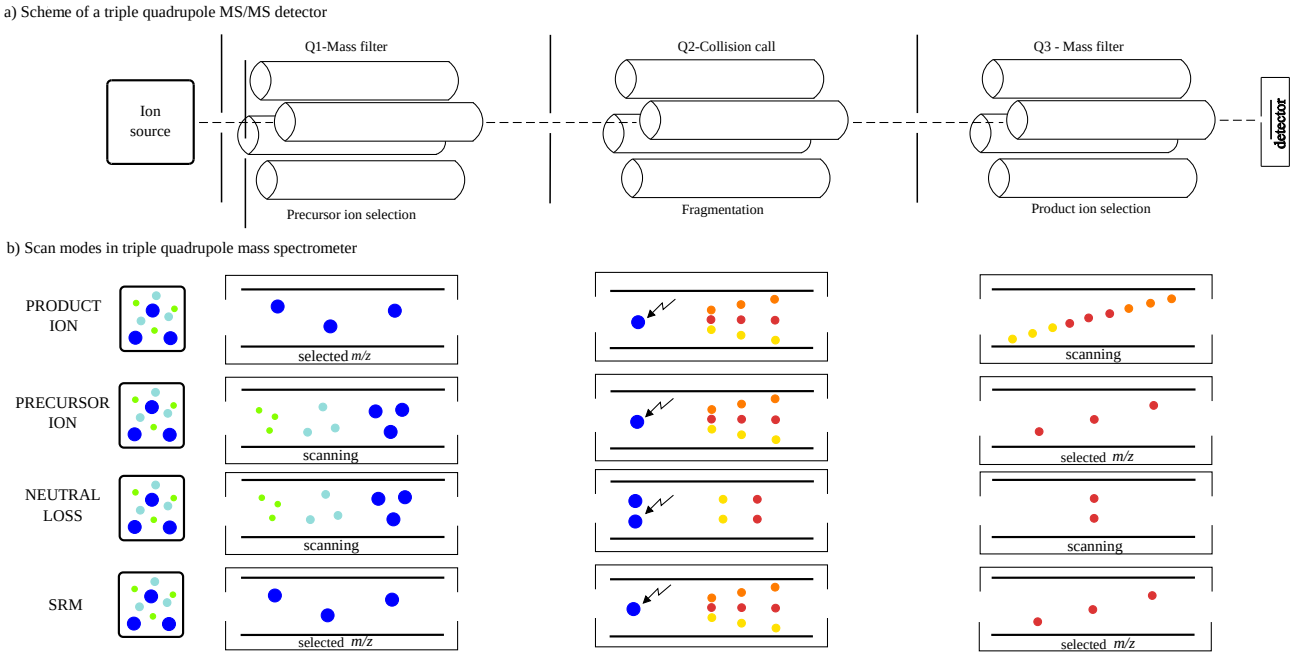

Figure 8 Scheme of the principle of work of MS/MS modified from Glish & Vachet (2003); de Hoffmann & Stroobant (2007). Green and blue circles show different precursor ions entering first triple quadrupole from the ion source, whereas yellow, orange and red circles present product ions obtained after the collision of precursor ions with collision gas in collision cell.

4.2.4. Quantification of folate in food

The use of plant origin enzyme served as a starting point for this PhD project, and the aim was to test its performance in various food matrices. Its activity was tested immediately after the production in papaya fruits, where folate content was analyzed using electrochemical detection, as it is shown at Figure 9.

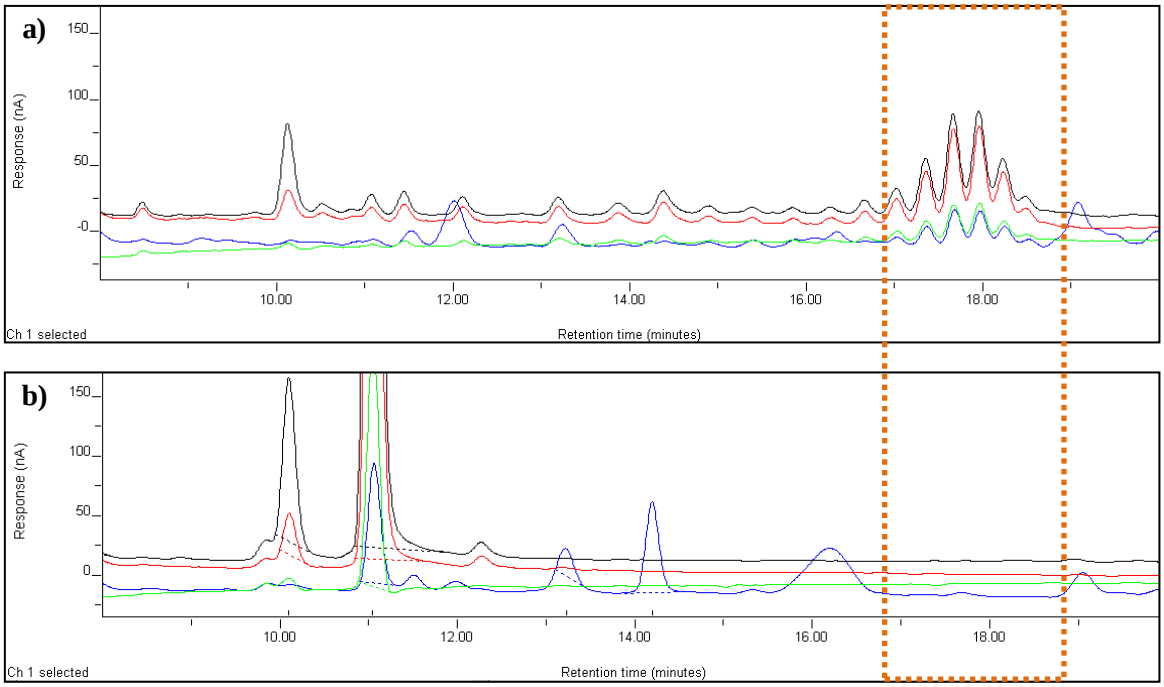

Figure 9 The plant enzyme activity analyzed in papaya sample by the use of electrochemical detection. a) Folate polyglutamates signals detected in the marked area before the use of plant enzyme. b) Folate polyglutamates signals absent after the use of plant enzyme.

The method from Ramos-Parra et al. (2013) contained the pH adjustment step, since they used 50 mM HEPES/CHES buffer, pH 7.9 and tri-enzyme extraction. De Brouwer et al. (2007) studied the stability of nine monoglutamate folate vitamers, among the others H_4folate, 5,10-CH^+=H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, PteGlu and 5-CH_3-H_4folate, and reported that they are mostly stable at pH values between 4 and 8 at 37°C, except H_4folate that was shown to be unstable at low pH values. However, if heated, formyl forms such as 5,10-CH^+=H_4folate interconvert to 5-HCO-H_4folate, as previously mentioned. Due to the stability of the majority of folate forms, and the optimal activity of the GGH of plant origin, folate extraction was performed at pH 6. Therefore, pH adjustment step was avoided by using phosphate extraction buffer of pH 6, which reduced the time of the analyses. In order to test the enzyme activity, green peas, representing a complex food matrix, was used (**Paper I**), and the satisfactory enzyme activity was reported already after half an hour of incubation at 37°C by using 100 µg of enzyme per gram of sample, which was equivalent to approximately 50 µL of the enzyme solution, depending on the purity of obtained batch, as is shown on Figure 10.

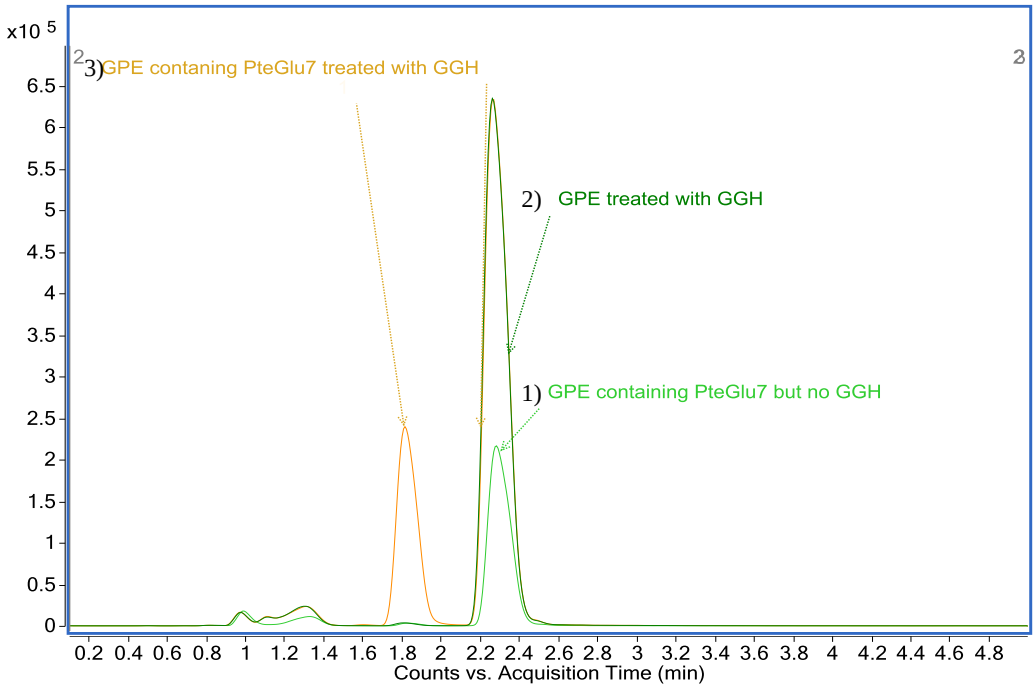

Figure 10 Deconjugation of pteroylheptaglutamic acid (PteGlu7) in green peas extract (GPE) by the use of plant enzyme (GGH). 1) GPE containing PteGlu7 without GGH treatment; 2) GPE without PteGlu7 treated with GGH; 3) GPE containing PteGlu7 treated with GGH. Yellow peak is PteGlu detected after conversion of PteGlu7 due to the GGH treatment.

The use of SAX was included in the purification step (**Paper I**) in order to reduce the contamination of analytical column and the ionization source by interfering substances such as pigments, and thereby to reduce interferences during the ionization in LC-MS/MS analyses and consequently to improve the response of the instrument to various folate forms. SPE is suitable for routine analyses, as it takes less time, reduces organic solvent consumption and enables automated purification of a higher number of samples (Majors, 2010). The use of cut-off filters instead of SPE was also reported (Shohag et al., 2017). Zhang et al. (2018) optimized folate extraction from seeds using 10 kDa cut-off filters at 4°C for 30 min. Testing the performance of cut-off filters as a purification step of the more complex samples would be beneficial for this method, to make it even shorter.

During folate extraction, two heating/cooling steps were used. In the first one, samples were heated in order to denaturate proteins and liberate folate vitamers from food matrix, whereas in the second they were heated in order to stop deconjugase activity. As folate forms are sensitive compounds that interconvert and degrade during the sample extraction and chemical analysis, it is beneficial to use

the internal standard which improves their quantification. Internal standard is useful in multiple sample preparation chemical analysis because it compensates for everything what happens with the compound of interest, such as loss, signal variation etc. In folate analyses the use of $^{13}C_5$ and $^{2}H_4$ isotopically labeled internal standards made an improvement in folate analyses, but the costliness of the internal standards makes them impossible to use for all compounds. Three internal standards were used in this method, such as $^{13}C_5$-PteGlu, $^{13}C_5$-5-HCO-H_4folate and $^{13}C_5$-5-CH_3-H_4folate (**Papers I, II, and III**).

The purpose was to develop a sensitive and precise method for simultaneous quantification of six folate monoglutamates that are naturally occurring in food. Therefore, the optimization of LC and MS/MS conditions was performed as described in **Paper I**. A C18 analytical column and a gradient elution composed of eluent A (2.5 mM ammonium formate in H_2O:methanol (95:5)) and eluent B (2.5 mM ammonium formate in methanol) was chosen for chromatographic conditions, whereas the optimized ion source parameters are shown in Table 4. The optimization of fragmentation was also performed in order to establish multiple reactions monitoring method (MRM) for simultaneous analysis of various folate forms. The table presenting MRM transitions included in the method is shown in **Paper I**, whereas the fragmentation of each folate form is presented on Figure 11.

Table 4 Optimized ion source parameters used in ESI-MS/MS

Drying gas temperature	225 °C
Drying gas flow	11 mL/min
Nebulizer	40 psi
Capillary voltage	3000 V
Sheath gas temperature	400°C
Sheath gas flow	12 mL/min

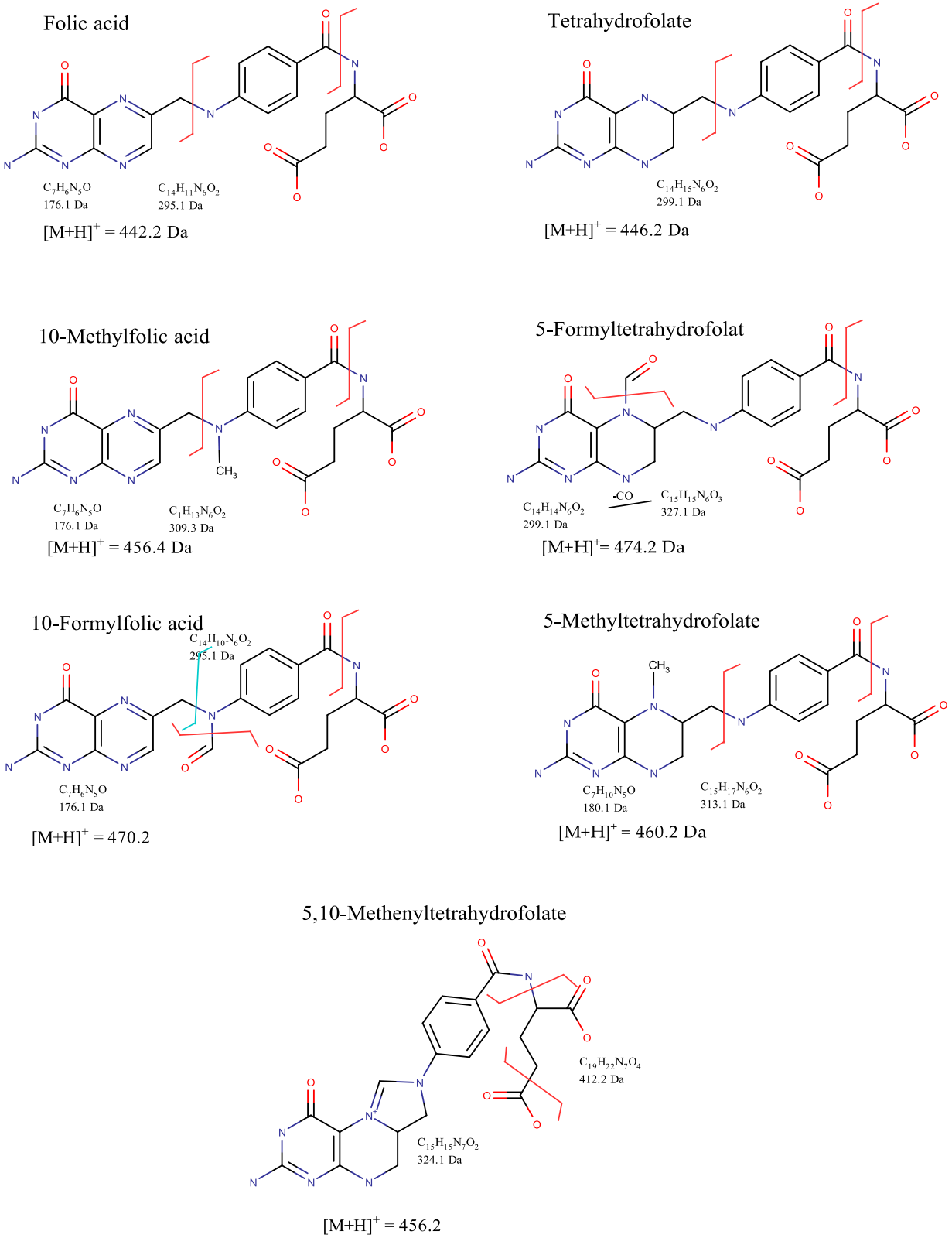

Figure 11 Fragmentation of various folate forms results in product ions that were used for MRM transitions included in the LC-MS/MS method.

The use of methanol as the organic solvent increased instrument's response and enabled faster analysis time by shortening the analysis to 9 min as it is shown on Figure 13, which is 8 minutes less than if it was analyzed by acetonitrile as the organic solvent, though this is a commonly used procedure in folate analysis (Vishnumohan et al., 2017; Striegel et al., 2018).

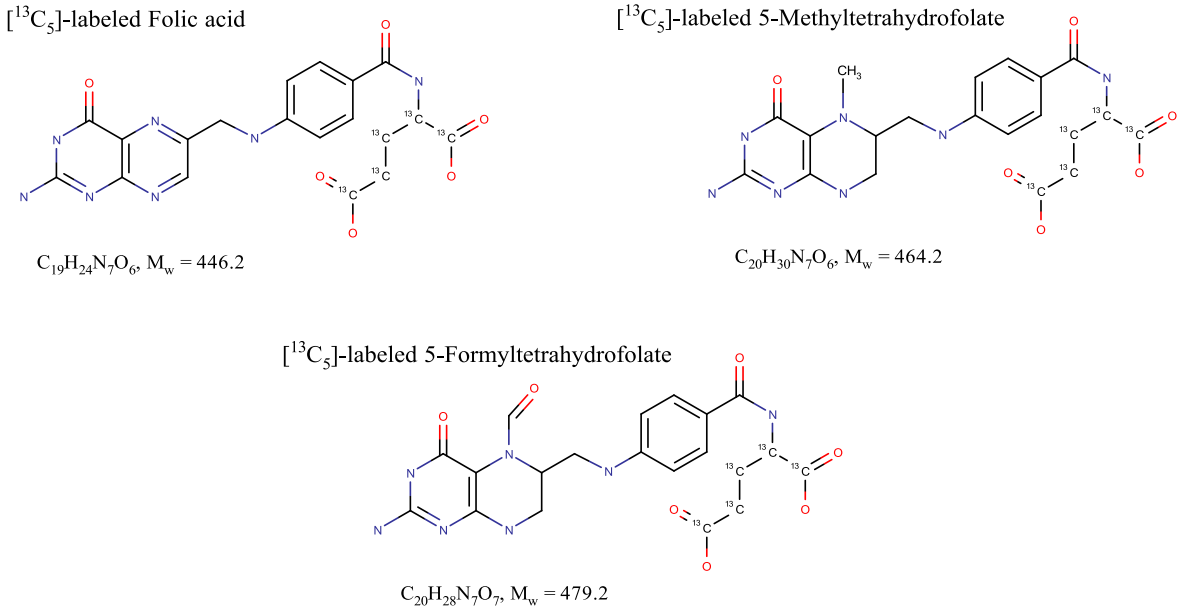

Figure 12 $^{13}C_5$-labeled internal standards used for quantification of folate

The internal standards used in this method are illustrated in Figure 12. However, due to the costliness of isotopically labeled internal standards, we did not have a proper internal standard for the quantification of the most sensitive folate form, H_4folate, and for 5,10-CH^+=H_4folate that is interconverting at acidic pH if heated (de Brouwer et al., 2007).Only one internal standard for formyl forms was included, hence the complete separation of formyl forms wasn't performed in order to keep these compounds close to their corresponding internal standard, $^{13}C_5$-labeled 5-HCO-H_4folate. As shown on Figure 13, H_4folate and formyl forms were co-eluting together with the internal standard. Even though the baseline separation was not achieved, the selectivity of the MRM mode was sufficient. Moreover, using methanol increased the signal for 22 – 188x for all compounds.

Gregory (2012) recommended joint quantification of 10-HCO-H_4folate and 5,10-CH^+=H_4folate, due to the rapid conversion of 10-HCO-H_4folate. Its final oxidation product, 10-HCO-PteGlu, was included into this method. After testing the interconversion between formyl forms, it was concluded

that due to the interconversion, and acidic environment during SPE and in the mobile phase, the quantification of the sum of formyl forms is recommended. It would not be a drawback in nutritional interpretation, as these folate forms exhibit comparable bioactivity (Gregory, 2012).

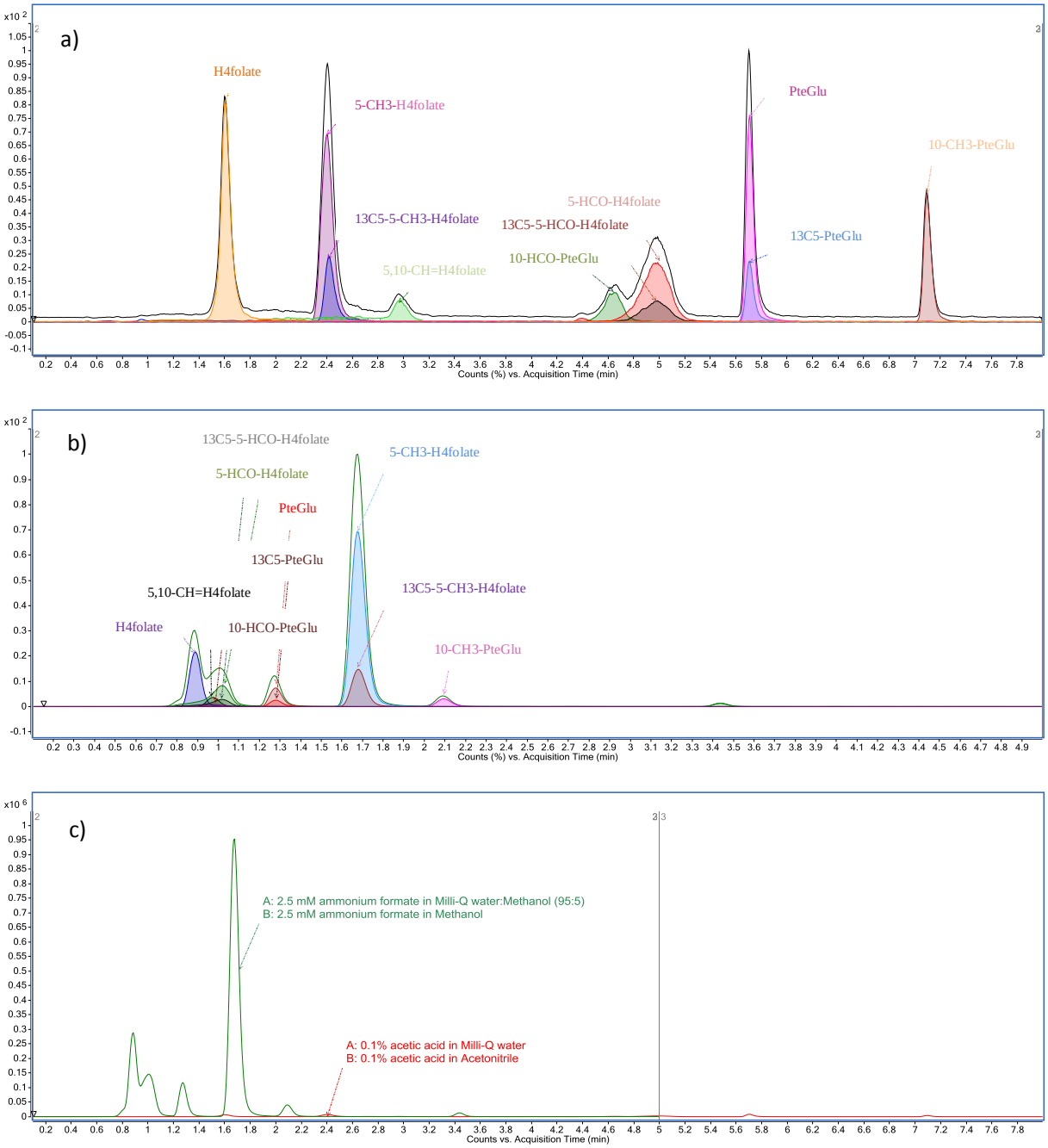

Figure 13 The chromatograms of folate standards analyzed by either acetonitrile (a) or methanol (b) as an eluent B. Methanol was used as an eluent B, due to the increased sensitivity (c)

The method was validated in terms of linearity (R^2>0.99 and trueness 85-115%), a limit of quantification at 0.1 µg/100 g, trueness 80-120% in spiked samples and certified reference materials, and a precision <10%. The precision in quantification of H_4folate was not satisfactory, ranging from 7-34%, which was due to the lack of a labeled H_4folate internal standard. To access trueness of the method, certified reference materials (CRM) representing various food matrices were analyzed (**Paper I**). When analyzing NIST 1845a using newly developed LC-MS/MS method, a total folate content quantified as PteGlu equivalent was 68 ± 5 µg/100 g (n = 7), whereas the 5-CH_3-H_4folate content was 43 ± 3 µg/100 g, which is lower than the certified folate amount 130 ± 7 µg/100 g obtained by microbiological assay. 10-HCO-PteGlu was the second most abundant folate form in eggs (15 ± 2 µg/100 g), followed by PteGlu (7 ± 1 µg/100 g) and H_4folate (1 ± 1 µg/100 g). Camara et al. (2013) quantified 5-CH_3-H_4folate in CRM NIST 1845a using tri-enzyme extraction by rat serum and LC-MS/MS detection. Their 5-CH_3-H_4folate value was 84 ± 4 µg/100 g (n = 2) which was also lower than the microbiological certified value. The results of folate distribution and quantification in eggs obtained in this project were in accordance with the results by Vahteristo et al. (1997), except for the content of 5-CH_3-H_4folate, which ranged from 140-150 µg/100 g in former study. They reported egg yolk as a difficult matrix, reflecting low recoveries for analyzed folate forms (49-67%). However, by using just single-enzyme extraction by hog kidney (pH 4.9, 37°C, 2 h), they managed to quantify high amount of 5-CH_3-H_4folate monoglutamate. In order to improve 5-CH_3-H_4folate extraction, 47 U/g of sample of protease was added during the extraction step to the Whole Egg Powder (CRM NIST 1845a) when it was analyzed by newly developed method. Moreover, the pteroylpentaglutamic acid was also added to study the enzyme activity. 76 ± 1% of pteroylpentaglutamic acid was deconjugated and transferred to monoglutamyl form PteGlu, whereas the use of protease did not show an increase of folate content being 69 ± 1 µg/100 g. Therefore, the enzyme activity of plant origin deconjugase seems to be inhibited by the food matrix present in whole egg powder. The mechanism of inhibition might be related to the presence of free fatty acids (Pande & Mead, 1968).

Through the whole method development and optimization, the unlabeled standard compound 10-CH_3-PteGlu was also used and its quantification was validated. However, this compound was not found in any food products that were analyzed for the folate content. Furlani & Godoy (2007) published it as the main folate form found in mushrooms, which were not part of this project. 10-CH_3-PteGlu was quantified using the MRM transitions *m/z* 456.4→309.3 and *m/z* 456.4→109.1 as a quantifier and a qualifier, respectively. $^{13}C_5$-5-CH_3-H_4folate was used as an internal standard for

its quantification. Method validation showed that the relative recovery of 10-CH_3-PteGlu for 10 ng, 500 ng and 900 ng was 104%, 81% and 80%, respectively. Testing matrix effect by the method of Matuszewski et al. (2003) showed that there was no matrix effect on 10-CH_3-PteGlu. However, the signal of its internal standard was slightly suppress, which would not provide accurate quantification of this compound because only internal standard was affected.

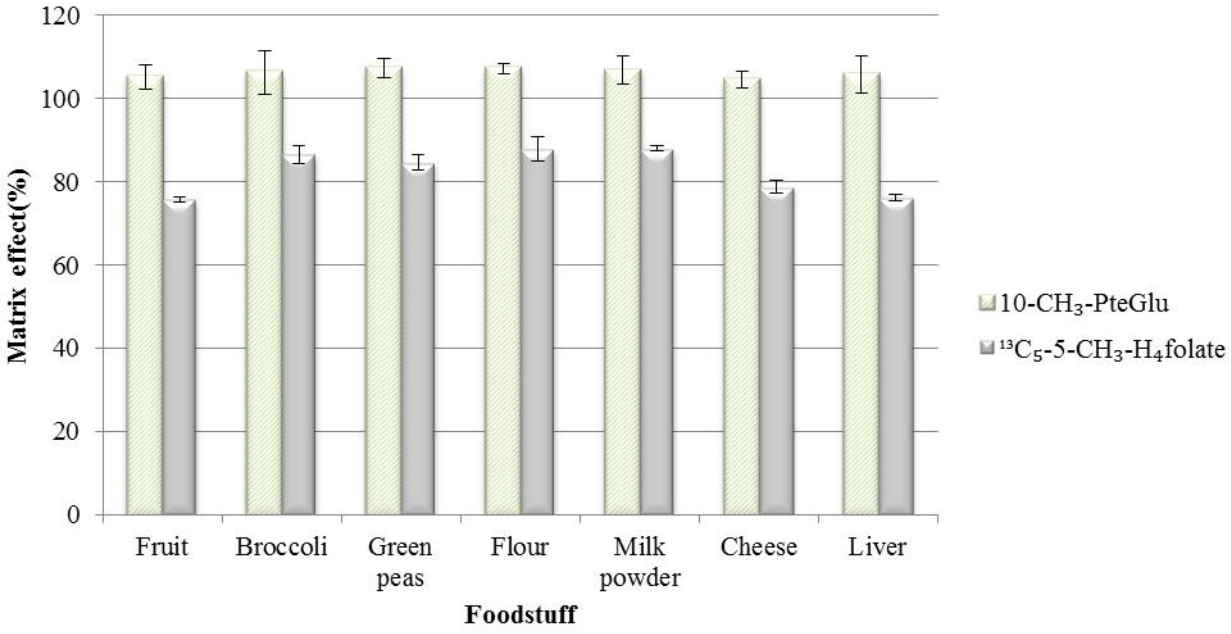

Figure 14 Matrix effect (%) of 10-CH_3-PteGlu compared to the corresponding internal standard's matrix effect ($^{13}C_5$-5-CH_3-H_4folate)

On the other hand, 10-CH_3-PteGlu was mentioned as an oxidation product that behave as folic acid antagonist, and plays as an enzyme inhibitor in folate metabolism (Hochster & Quastel, 1963). Therefore, it should not be present in food and its quantification could be related to oxidation processes during the analysis.

Chapter 5: Folate analysis of a large and diverse sample set

Once the LC-MS/MS method using the enzyme of plant origin was developed and validated, its performance was compared to the two other methods performed in other laboratories. A large sample set of various food samples from groups of fruits, vegetables, legumes, cereals, dairy products, meat and offal was included in the round robin method comparison study. The results obtained from these analyses were also used to study folate distribution within foodstuffs and making the correlation to the impact of folate content and distribution on folate intake.

5.1. Method comparison studies

Differences among methods used for folate analysis in food result in unreliable data of folate content which should be the starting point in any creation of nutritional guidance or epidemiological evidences. Existing folate data in food-composition tables are mainly obtained by the use of microbiological assay as the only standard method for folate determination in food. However, these data are unreliable and nowadays could be considered as being incomplete due to the knowledge about different behavior and bioavailability of various folate forms (Ringling & Rychlik, 2017b). The development of chromatographic methods still requires their comparison to microbiological assay as the only standard method for folate analysis in food. In 2001, Konings et al. compared HPLC using fluorescence and diode array detection in selected items from food groups of vegetables, fruits, bread, dairy products and meat products with microbiological assay. Total folate content was approximately 25% lower if analyzed by chromatographic method than the amounts recorded in various national food composition tables obtained by microbiological assay. In 2005, Koontz et al. compared total folate content analyzed by microbiological assay in four experienced laboratories that perform routine folate analysis in the USA. Various types of food have been analyzed revealing that only results for macaroni and pizza as folic acid-fortified foodstuffs had lower inter-laboratory variation being 9-11% versus >45% for foodstuffs such as fresh strawberries, frozen spinach, orange juice, frozen meat and dried pinto beans. These data indicated that the results of analysis unfortified foods should be taken with caution in terms of accuracy and precision which is crucial for the countries which do not mandate food fortification by folate such as the countries of European Union. The last and the biggest international inter-laboratory method comparison study of folate analysis in food was published in 2005 by Puwastien et al.. Three test materials representing various food types and matrices such as soybean flour, fish powder and breakfast cereals were

analyzed in 26 laboratories worldwide. Tri-enzyme extraction was recommended as the method of choice, however only 9 laboratories used it in their folate analysis. Furthermore, 20 laboratories used microbiological assay, whereas the others implemented HPLC-UV, LC-MS and radio-binding assay. Among 17 laboratories that used microbiological assay using *L. casei* as detection method, the inter-laboratory coefficient of variations for soybean flour, fish powder and breakfast cereals were 24%, 35% and 24%, respectively. These findings indicate the complexity of folate quantification which still hasn't been resolved as there are many newly developed chromatographic methods analyzing folate content published in recent years. With a purpose of establishing valid data for the performance of the plant origin GGH in folate deconjugation when using LC-MS/MS detection system, a round robin method comparison study has been performed.

5.1.1. Round robin method comparison study

In order to examine GGH's performance in various food matrices by various detection methods, three different laboratories have been involved in the study. A large sample set composed of 89 different samples divided to 7 food groups has been analyzed by LC-MS/MS method using an animal-origin single-enzyme extraction step at Technical University of Munich (Germany) and microbiological assay using tri-enzyme extraction step in accredited laboratory Eurofins Steins Laboratory in Vejen (Denmark). The setup of the study was described and results were presented in **Paper II**. Food groups of fruits, vegetables, legumes, cereals, dairy products, meat and offal have been analyzed and compared using the Bland-Altman statistical approach. Significantly lower mean bias of 17% ($p \leq 0.05$) was reported when LC-MS/MS method using plant enzyme was compared to the microbiological assay, indicating a possible overestimation of folate values reported in food composition databases. However, significantly higher constant bias of 25% ($p \leq 0.05$) was obtained when the two LC-MS/MS methods using GGHs of different origin were compared, indicating that the GGH of plant origin is superior in folate deconjugation. When analyzing the differences between food groups, it was observed that food groups of fruits, vegetables and cereals contribute significantly ($p \leq 0.05$) to the bias between the two LC-MS/MS methods indicating that plant food matrix may inhibit the activity of animal origin GGH. However, further investigations have to be done in order to confirm this statement. No significant difference was found in food group of legumes that was considered as a complex food matrix when the newly developed LC-MS/MS using plant origin GGH was compared to microbiological assay or LC-MS/MS using animal origin GGH as a combination of chicken pancreas and rat serum. These results suggest that there is no

need for three enzyme treatment in complex food matrices such as legumes, even though the test of enzyme activity is highly recommendable prior to analysis of any new food matrix. Moreover, these results were in accordance to Zhang et al. (2018) who reported no need for tri-enzyme treatment during the extraction of legumes such as common bean, lentils, chickpeas and peas. Furthermore, they used fast sample purification by cut-off filters prior to UPLC-MS/MS analysis which decreased the time of analysis and showed to be a good method to be implemented in other food matrices.

5.1.2. Strengths and weaknesses of round robin comparison

A large and diverse sample set used in method comparison study is strength of this method comparison. It provided a good overview of the plant-origin GGH's activity in various types of sample. Statistical analysis performed in **Paper II** showed possible overestimation of the results obtained by microbiological assay which could be due to various factors. Bacterial growth caused by the use of some non-folate nutrients is one of them, but also the differences in bacterial growth based on the variability of response depending on the calibrant used for folate quantification. Moreover, the differences in the preference of the enzyme to various folate forms could also be one of the reasons for discrepancies in the results. Ramos-Parra et al. (2013) reported differences in the preference of rat plasma enzyme which completely deconjugated only PteGlu triglutamate within 30 min, whereas only 85% of 5-HCO-H_4folate triglutamate was deconjugated within 1 hour. However, in this sample set, no pattern related to the enzyme preferences towards specific folate forms was observed.

Any bigger multi-laboratory study should have a quality control of analytical measurements by analyzing reference materials, preferably certified reference materials. The lack of the use of reference materials in all three tested methods is a weakness of the performed round robin. The accuracy of the used methods was tested by analyzing certified reference materials by the LC-MS/MS method using plant enzyme and microbiological assay, whereas it was omitted during LC-MS/MS analysis using animal origin GGH. Table 5 shows the comparison of the results obtained by analyzing certified reference materials. The results from the two methods were in accordance with the certified values for the reference materials, except for NIST 1845a when using the plant enzyme and the LC-MS/MS analysis, which was already discussed in Section 4.2.4.

Table 5 Certified reference materials samples analyzed by LC-MS/MS using plant origin enzyme and microbiological assay. Values of the folate content are expressed as PteGlu equivalent (μg/100 g).

Sample	Certified value	CRM Method	Total folate LC-MS/MS	Enzyme	Method	Reference
BCR 487 Pig Liver	1330 ± 130	MA	1291**	D (AtGGH2)	LC-MS/MS	Ložnjak et al. (2019)
			1390*	Creon capsules# + D (human plasma)	MA	CEN (2003)
NIST 1845a Whole Egg	130 ± 7	MA	67 ± 5***	D (AtGGH2)	LC-MS/MS	Ložnjak et al. (2019)
			110*	Creon capsules# + D (human plasma)	MA	CEN (2003)
NIST 1546a Meat Homogenate	None	None	1.0 ± 0.3***	D (AtGGH2)	LC-MS/MS	Ložnjak et al. (2019)
			5.7*	Creon capsules# + D (human plasma)	MA	CEN (2003)
NIST 1549a Whole Milk	None	None	19 ± 1***	D (AtGGH2)	LC-MS/MS	Ložnjak et al. (2019)
			21*	Creon capsules# + D (human plasma)	MA	CEN (2003)

MA, microbiological assay; D, deconjugase

* n=1, ** n=2, *** n=4

#Creon capsules: lipase, amylase and protease

5.2. Folate content and its impact to daily intake in analyzed foodstuffs

In 1992, Gregory et al. studied relative bioavailability of various folate forms and reported that differences exist in the bioavailability of monoglutamyl folate forms indicating that PteGlu has the highest bioavailability followed by 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate. Biologically active folate monoglutamates play immense role in human metabolism as presented in Section 2.3.. However, 5-HCO-H_4folate is not directly used as a C_1-donor. 5-HCO-H_4folate represents 50% of the formyl pool of mitochondria and presumably serves as a storage folate form, which can be converted to various folate derivatives that are afterwards used in C_1 reactions (Orsomando et al. 2005). Another possible function is the regulation of folate-dependent enzymes (Gorelova et al. 2017). Numerous studies have been performed as previously mentioned in Section 2.4. that emphasized the need of having a sensitive and specific chromatographic method for folate determination in food. Folate content and distribution in food varies depending on the natural

variability in the foodstuff, connected to physiological state at the harvesting or production stage of the foodstuffs and the accuracy of the analytical methods used for folate quantification (Delchier et al., 2016). Folate content and distribution in the foodstuffs analyzed by round robin study and their possible impact on folate intake are described in **Paper III**. The distribution of analyzed folate vitamers in 25 foodstuffs divided to 7 food groups is shown on Figure 15.

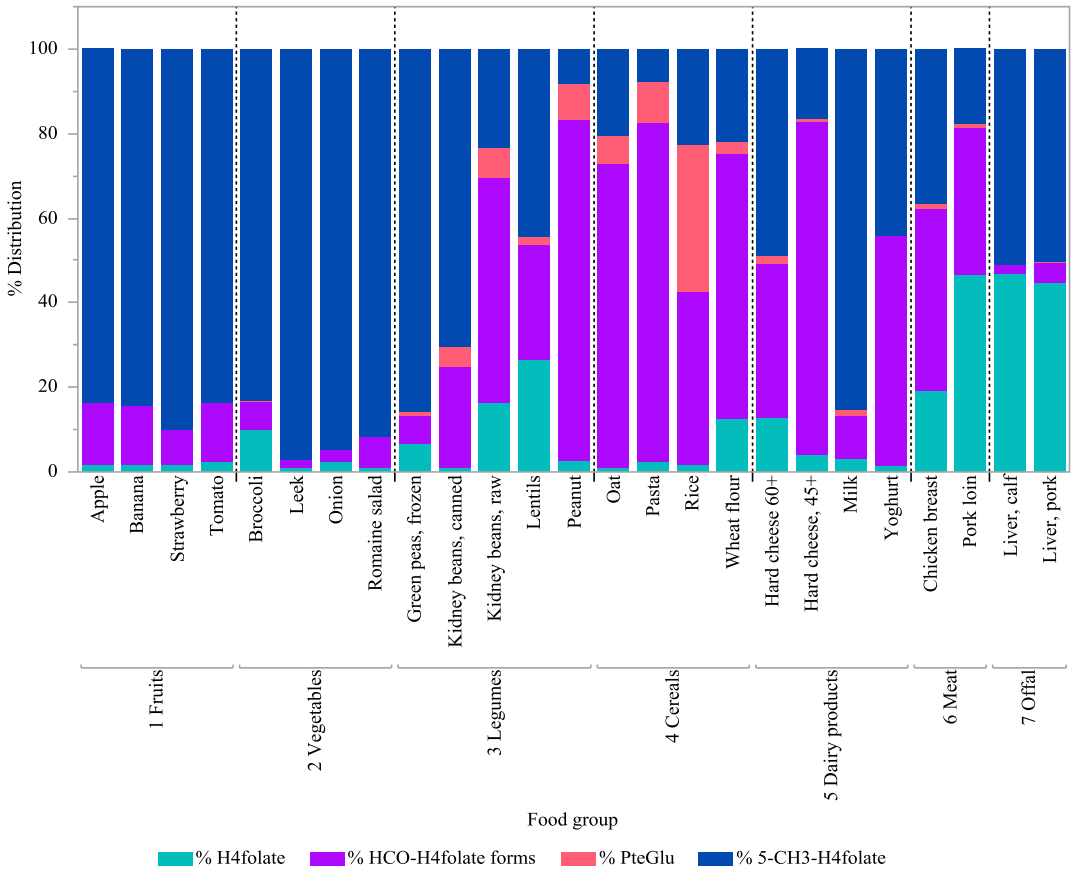

Figure 15 Folate distributions within 25 foodstuffs in seven analyzed food groups

From seven analyzed food groups, food group of offal represented by two different types of liver, calf liver and offal liver, was shown to be the best folate source contributing by >500% to the RDI for adults. Folate content in analyzed liver samples ranged from 829 until 1897 µg/100 g, with 5-CH_3-H_4folate and H_4folate as the most abundant folate forms (Appendix, Table A7). However, as the mean consumption of offal consumed by the Danish population is low, though with a large standard deviation (**Paper III**), the food groups of vegetables contributes the most to the daily folate intake among Danes. Therefore, ~13 g of romaine salad (71 – 162 µg/100 g) that is average consumption among Danish women ensures >25% of RDI for adults. Moreover, >80% of the folate forms found in vegetables is 5-CH_3-H_4folate, which is biologically active form, that can exhibit

high bioavailability depending on the food matrix (Ringling & Rychlik, 2017b). Fruits also contribute to the daily folate intake in a significant way, due to a wide consumption. Bananas containing 21 µg/100 g (n = 2) of folate contribute to almost the same extent as strawberries containing 89 ± 6 µg/100 g (n = 4), providing ~15% of the daily intake.

The most abundant folate form in fruits is 5-CH_3-H_4folate, the same as in vegetables (Figure 15, Appendix, table A1). Similar pattern in the contribution to the daily intake is visible in consumption of cereals and dairy products which highly contribute to the total daily folate intake even though their content ranges from 7 ± 4 µg/100 g (n =4) in dry parboiled rice to 37 µg/100 g (n = 2) in wheat flour, and from 6 ± 1 µg/100 g in milk to 37 ± 6 µg/100 g in high fat content cheese (Appendix, Tables A4 and A5). Patring et al. (2009) studied folate content and distribution in Norwegian and Swedish bread and reported that wholegrain flours such as wholegrain wheat flour and wholegrain rye flour contain higher folate content than sifted flour of the same grains. That is in accordance to new data presented in this thesis where wholegrain wheat flour contained 37 µg/100 g (n = 2), whereas sifted wheat flour contained 12 µg/100 g (n = 2). Mean total folate content in dry legumes ranged from 73 µg/100 g in lentils to 115 µg/100 g in dry kidney beans. Sen Gupta et al. (2013) reported the mean folate content in lentils being 225 µg/100 g, which resulted in providing a significant amount of the RDI of dietary folate (54-73%) for adults. Legumes are complex matrix containing high protein content (18-36%) and fiber content (21-52%) that may hinder folate bioavailability from this sources (Sen Gupta et al. 2013). Moreover, legumes have to be processed prior consumption that causes leaching or other types of folate degradation (Dang et al., 2000; Hefni & Witthöft, 2014).

In brief, 5-CH_3-H_4folate was the most abundant folate form in food groups of fruits, vegetables, offal and partially legumes, followed by HCO-H_4folate forms (Figure 15). As previously mentioned, it is hard to make a connection to bioavailability as it is very dependent on various factors, especially the type and condition of food matrix. Gregory et al. (1992) reported that PteGlu as a stable, synthetic monoglutamate form has the highest bioavailability among folate monoglutamates. However, it is considered that PteGlu should not be found in the distribution of naturally occurring folate forms. This study showed that indeed no PteGlu was detected in food groups of fruits and vegetables, whereas minor amounts were detected in food groups of legumes and cereals, mostly in foodstuffs where HCO-H_4folate forms were the predominant ones. This indicates that PteGlu found in the distribution of naturally occurring folate forms might be the

product of oxidative degradation of HCO-H_4folates during quantification analysis or in food matrix prior to analysis, as it was already quantified in minor amounts in previous studies (Patring et al., 2009; Edelmann et al., 2013).

5.3. Round robin: A model for choice of the sample

Due to the many performed analyses during round robin study, it is possible to propose the use of some samples as test materials in a larger inter-laboratory study. It is always recommended to analyze various groups of food due to the differences in food matrix. Furthermore, keeping the wide concentration range is also desirable to examine if the differences in concentration affect the quantification of folate forms. Following foodstuffs (*Italic*) are proposed as test materials for future method comparison studies:

- Fruits: *Strawberries* have a high content of folate, mainly 5-CH_3-H_4folate and serve as a good test material due to a very easy homogenization of the sample. Strawberries were used in inter-laboratory comparison study by Koontz et al. (2005) and in the presented comparison study. Comparable values for all three used methods.
- Vegetables: A food group of vegetables was troublesome due to the large difference in deglutamylation if using GGHs of different origin (**Paper II**). Furthermore, it was hard to obtain reproducible results for microbiological assays in broccoli samples which could be due to discrepancies in enzyme activities, but also due to differences in representative sample. Broccoli was sampled by making representative sample from broccoli flower clusters which may not be unique in their folate content. Due to the possible discrepancies in preparation of representative samples, a special focus has to be put on the sampling procedure, to make sure that the uniformity of representative samples is achieved. Furthermore, vegetables such as *spinach* or *brussels sprouts* might be more appropriate to keep the uniformity of sampling.
- Legumes: *Lentils* are perfect sample for method comparison study, especially for the comparison of folate distribution as they contain all folate forms. Furthermore, this sample is dry which makes it more stable to environmental changes that may occur during the storage and freeze-thaw cycles. However, the highest agreement of the three used methods in this food group was in the *frozen green pea*s sample which could be also considered as a good test material in a method comparison study.

- Cereals: Formyl forms are the most abundant ones in the food group of cereals. Processed cereals products such as pasta, parboiled rice and refined wheat flour contain very low folate amounts and therefore are not considered as a good choice of test materials in inter-laboratory study due to the various limits of quantification that may affect collection of comparable values. However, whole grain cereals such as rye grain, *oat grain*, barley grain and wholegrain breads have high folate content, and mainly HCO-H_4folate forms and therefore would be considered as a good choice for comparison.
- Dairy products: *Milk* as a food matrix is homogeneous liquid and it could therefore serve as a good test material for lower folate content (<10 µg/100 g). All dairy products are homogeneous, but the use of cheese includes possible problems in extraction step due to the high content of fat per dry matter.
- Meat and offal: As almost no folate was found in in pork loin, *chicken breast* would be recommended as a good test material from the food group of meat. On the other side, food group of offal such as *liver* is one of the richest folate sources due to its storing role in mammals. Therefore, any liver sample would be excellent test material in order to test upper limits of folate quantification.

Chapter 6: Encapsulation of 5-methyltetrahydrofolate using electrospraying

This chapter gives a brief overview of the encapsulation technique used for the stabilization of L-5-methyltetrahydrofolate, a synthetic form of the naturally occurring biologically active form 5-methyltetrahydrofolate. Electrosprayed capsules containing L-5-methyltetrahydrofolate coated by food grade carbohydrates glucose syrup and pullulan were produced. Two stability studies were performed in order to examine capsule performance during storage and during heat processing if applied to food system.

6.1. Encapsulation by electrospraying

High awareness of consumers about the healthy lifestyle increased their demand for foods with beneficial effect on health. Food industry is searching for new technologies in order to increase the production of novel food products and delivery systems that would enhance stability of sensitive bioactive compounds, such as vitamins and antioxidants, and their controlled releasing in the gastrointestinal tract. Encapsulation is a technology for packing various materials in the micro- and nano-structures via entrapment of the material of interest (core material) with another substance (wall material) (Jacobsen et al., 2018). In the last 20 years, various micro- and nano-encapsulation techniques have been developed and improved, such as spray drying, freeze drying, emulsification, coacervation, liposome preparation etc. (Ghorani & Tucker, 2015). Each of these techniques has advantages and disadvantages, but the main drawback for application in prevention of sensitive bioactive compounds in food systems is the use of high temperatures or organic solvents. Despite causing degradation of the sensitive compounds, it also causes toxicity problems due to the residuals of the used organic solvents (López-Rubio & Lagaron, 2012). Therefore, it is desirable that the selected wall material is food grade, which results in a wide use of natural biopolymers such as proteins and carbohydrates. The choice of biopolymer material is also connected to its purpose and future use, in order to ensure that the encapsulated compound will be delivered to the right place in gastrointestinal tract. Carbohydrates are commonly used as delivery systems for various bioactive compounds as they can bind and entrap various hydrophilic and hydrophobic food ingredients, providing a good resistance to high temperature if compared to lipids or proteins which might melt or denaturate (Fathi et al., 2014).

Encapsulation by electrospraying is a technique that has been accepted by the food industry because of the potential to produce high quality delivery systems without the use of organic solvents by

protecting sensitive bioactive compounds from exposure to high temperatures as it is performed at room temperature (Jacobsen et al., 2018). It has the same principle of work as its "sister technique" electrospinning and very similar to the principle of work of electrospraying ionization which was described previously in Section 4.2.2. In brief, high voltage electrostatic field is used to charge the surface of biopolymer solution, thereby inducing the ejection of charged droplets (electrospraying) or jet (electrospinning) towards the nearest lower potential point which is a collector (Ghorani & Tucker, 2015). The difference between electrospraying and electrospinning is in the difference in the concentration of biopolymer solutions. In electrospraying, low biopolymer concentrations are used, the jet is destabilized and it forms capsules, whereas high biopolymer concentrations are used in electrospinning, resulting in formation of fibers. Similar to the principle described in the Section 4.2.2., charged droplets formed by electrospraying solidify through solvent evaporation, and may be collected at the end of the process (Ghorani & Tucker, 2015). Particle size distribution obtained by this encapsulation process is usually narrow, and particles are smaller than the ones obtained by e.g. spray drying (Pérez-Masiá et al., 2015). Having narrow particle size distribution and smaller particle size is desirable due to the higher absorption efficiency of the nanoparticles in the body and easier incorporation to food matrix (Ezhilarasi et al., 2013). However, in order to obtain desirable nanocapsules, processing parameters such as solution properties, processing conditions and ambient conditions have to be optimized, as shown in Table 6.

Table 6 Processing parameters in electrospraying (Ghorani & Tucker 2015)

Electrospraying parameters	
Solution properties	Viscosity
	Polymer concentration
	Molecular weight of polymer
	Electrical conductivity
	Elasticity
	Surface tension
Processing conditions	Applied voltage
	Distance from needle to collector
	Volume feed rate
	Needle diameter
Ambient conditions	Temperature
	Humidity
	Atmospheric pressure

6.2. Previous work on encapsulation of 5-methyltetrahydrofolate

As previously mentioned food fortification by folic acid might mask symptoms of B_{12} deficiency, and have to undergo some additional metabolic steps in order to be converted to the biologically active form. Therefore, a new strategy for food fortification has been sought. 5-CH_3-H_4folate is the most abundant natural biologically active folate form. Therefore, its use as a supplement or food fortifier is preferable, if compared to folic acid (Obeid et al., 2013). However, 5-CH_3-H_4folate is more sensitive to environmental conditions than PteGlu and therefore its addition to food is troublesome, due to the low storage stability and instability during processing.

Former research has been performed on the possibility to increase the folate stability by encapsulation and its incorporation to food system. Shrestha et al., (2012a) studied thermal stability of PteGlu and 5-CH_3-H_4folate in various liquid models such as milk, soymilk, starch-water and water during boiling and autoclaving reporting that the thermal stability of 5-CH_3-H_4folate was lower in almost all studied matrices, resulting in the 17% loss of PteGlu and 70% loss of 5-CH_3-H_4folate. In addition, they tested spray drying as an encapsulation technique that would prevent 5-CH_3-H_4folate from degradation, where 60% of encapsulation efficiency of the process when using the combination of pectin and sodium alginate (P80:A20) for coating was obtained. Encapsulation process increased the stability of 5-CH_3-H_4folate during heating at 130 °C from 79% to 94% (Shrestha et al., 2012a). However, in the next study conducted, when the same capsules were incorporated into biscuits, no protective effect was seen on the stability of 5-CH_3-H_4folate at temperatures >200 °C, and <8.5% of 5-CH_3-H_4folate was retained (Shrestha et al., 2012b). Liu et al. (2013) encapsulated L-5-CH_3-H_4folate by modified starch and using spray drying. They fortified flour by the free-L-5-CH_3-H_4folate and produced capsules, and performed pilot plant and bakery bread baking study obtaining <80% of L-5-CH_3-H_4folate recovery after baking. Furthermore, they studied the stability of L-5-CH_3-H_4folate in baked breads for 7 days of storage and reported that encapsulation improved the stability, especially if sodium ascorbate was added as antioxidant. However, retention of 60% was reported after 3 days of storage in bakery baked bread. As previously mentioned, the bioavailability of 5-CH_3-H_4folate differs from the one of PteGlu, which resulted in a study examining the bioavailability of L-5-CH_3-H_4folate. Green et al. (2013) performed a randomized, placebo-controlled trial to assess the bioavailability of wheat rolls fortified with encapsulated L-5-CH_3-H_4folate or equimolar folic acid, and wheat rolls containing no added folate (placebo) during 16 weeks by measuring an increase in plasma folate concentrations.

Even though the study was too short to assess erythrocyte folate concentration, it showed comparable increase in blood folate concentration with folic acid when compared to placebo. These findings confirmed the use of L-5-CH_3-H_4folate as a supplemental folate form.

6.3. Proposed fortification strategy

Encapsulation of L-5-CH_3-H_4folate by the use of electrospraying has been proposed as a novel encapsulation approach in production of capsules with a purpose of incorporation into food system. Due to the lack of time and an intention to test performance of coating system in encapsulation of a vitamin, a previously described carbohydrates based coating system has been used (García-Moreno et al., 2018; Hermund et al., 2019). A biopolymer solution used for the encapsulation was composed of glucose syrup (15%) and pullulan (4%). Glucose syrup is a common and low-cost wall material, whereas pullulan enables good protection from humidity when compared to some other polymer materials such as whey protein concentrate (García-Moreno et al., 2018; López-Rubio et al., 2012). In order to stabilize L-5-CH_3-H_4folate, ascorbic acid (Asc) was added as antioxidant to one of the biopolymer solutions. The concentration used was 1% of Asc in the produced capsules. Optimization and characterization of the capsules was performed as described in **Paper IV**. Processing parameters used during electrospraying slightly differed from the ones used in the previous study (García-Moreno et al., 2018) being 0.006 mL/min and 20 kV for flow rate and voltage, respectively. Furthermore, surfactant Tween-20 was added to the process to reduce surface tension and enable stabilization of the process related to formation of the droplets (Pérez-Masiá et al., 2014). The optimized electrospray process resulted in production of the capsules without ascorbic acid (*Capsules – Asc*) and capsules with ascorbic acid (*Capsules + Asc*). The morphological characterization revealed that capsule size was equal to 0.72 ± 0.41 μm and 0.55 ± 0.34 μm for *Capsules – Asc* and *Capsules + Asc*, respectively, which was in accordance with the previous study using the same biopolymers indicating that the change in the core material did not affect electrospraying process (García-Moreno et al., 2018). The both types of the optimized capsules contained 1% of L-5-CH_3-H_4folate.

Upon the production, the storage experiment was conducted in order to examine capsules stability in very mild storage conditions in dark and at room temperature (22 °C), as it is described in **Paper IV**, whereas the experimental design is shown in Figure 16. The use of free L-5-CH_3-H_4folate served as a control.

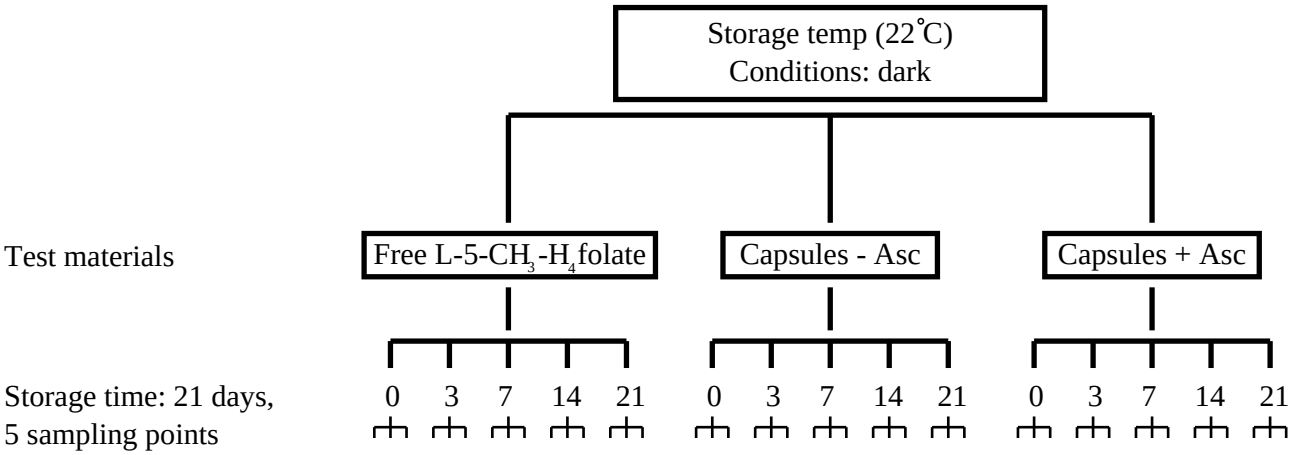

Figure 16 The experimental design for the storage stability study performed in capsules. Three analytical replicated were sampled at each sampling point.

Stability of the folate was calculated by Eq. 1 and it was expressed as recovery of L-5-CH_3-H_4folate. Results presented in Table 7 indicate that encapsulation by electrospraying enhanced stability of L-5-CH_3-H_4folate during 21 days of storage, as a significant difference to the stability of free L-5-CH_3-H_4folate was observed after day 7.

$$Recovery\ of\ 5-CH_4-H_4folate\ (\%) = \frac{concentration\ of\ 5{-}CH_3{-}H_4\text{folate}_{\ quantified}}{concentration\ of\ 5{-}CH_3{-}H_4folate_{added}} * 100 \quad [1]$$

Table 7 Recovery of L-5-CH_3-H_4folate during 21 days storage at room temperature (22°C)

Day	Free L-5-CH_3-H_4folate	Capsules - Asc	Capsules + Asc
0	102 ± 6^{ax}	93 ± 0.3^{ay}	102 ± 1^{ax}
3	97 ± 4^{abx}	88 ± 0.4^{bcy}	98 ± 1^{ax}
7	88 ± 5^{bx}	85 ± 0.5^{cx}	96 ± 2^{ay}
14	75 ± 3^{cx}	94 ± 2^{ay}	102 ± 2^{az}
21	61 ± 2^{dx}	91 ± 3^{aby}	95 ± 7^{ay}

Different black letters abc indicate significant difference ($p \leq 0.05$) in the specific solution between days, whereas red letters xyz indicate significant difference ($p \leq 0.05$) between the treatment groups.

As capsules showed satisfactory stability of >85%, a possibility of food application and effectiveness if exposed to processing conditions was examined. Flour is a widely consumed foodstuff used in the preparation of numerous foods, and as it is already mandated for fortification in some countries, as previously mentioned, it was decided to be used as a food matrix for fortification. The sufficient amount of capsules was produced for fortification and added to all-purpose flour in order to have 150 µg of L-5-CH_3-H_4folate/100 g of flour. The amount aimed for is recommended for the fortification of flour in the US (Food and Drug Administration, 1996). However, the purpose of the study was to test the protective effect offered by the capsules. The use of a very sensitive method enabled quantification of L-5-CH_3-H_4folate, at the low level present in flour. Recovery was calculated as retention of L-5-CH_3-H_4folate in buns after heat processing by Eq. 2, so small differences in the added amounts were not crucial, but desirable. The experimental design of the second stability study is shown in Figure 17.

$$\%Retention = \left(\frac{\mu g\ 5\text{-}CH_3\text{-}H_4\text{folate}\ \ per\ 100\ g\ of\ buns * amount\ of\ buns}{\mu g\ 5\text{-}CH_3\text{-}H_4\text{folate}\ \ per\ 100\ g\ of\ dough * amount\ of\ dough}\right) * 100 \qquad [2]$$

The uniformity of the distribution of added L-5-CH_3-H_4folate, either in a free form or in capsules, was performed as described in **Paper IV**. The results of the second storage experiment performed in buns after baking (220 °C, 7.5 min) are shown in Table 8.

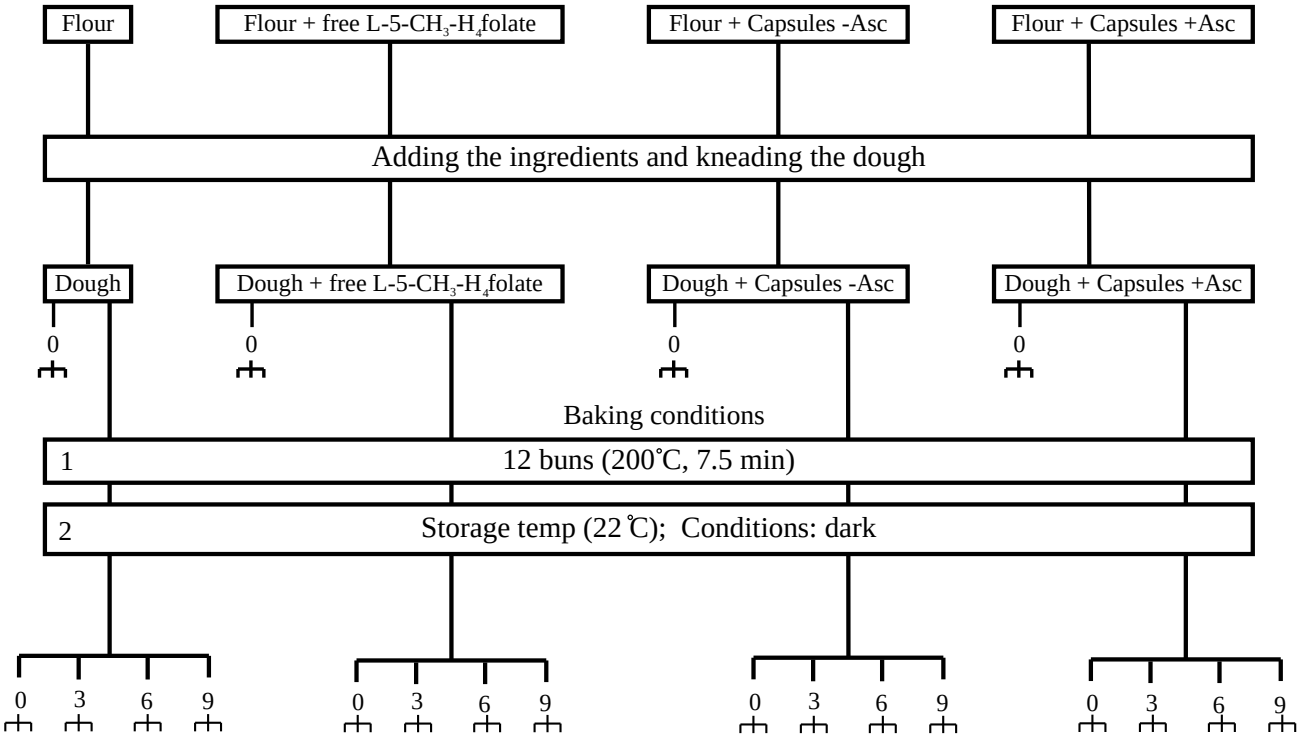

Figure 17 The design of the storage experiment in buns. It included retention during baking and stability during storage for 0, 3, 6, and 9 days. Three analytical replicates were sampled at each sampling point. *indicate dough samples sampled for the calculation of the retention.

Table 8 Recovery of L-5-CH_3-H_4folate in buns after baking (Day 0) and after 3, 6 and 9 days of storage at room temperature (22 °C)

Day	Endogenous 5-CH_3-H_4folate	Free L-5-CH_3-H_4folate	Capsules - Asc	Capsules + Asc
0	46 ± 0.4^{ax}	100 ± 1^{ay}	89 ± 1^{az}	78 ± 6^{au}
3	47 ± 3^{ax}	93 ± 1^{aby}	73 ± 9^{bz}	85 ± 7^{ayz}
6	43 ± 1^{ax}	98 ± 7^{aby}	78 ± 5^{abz}	85 ± 6^{ayz}
9	45 ± 2^{ax}	88 ± 4^{by}	80 ± 2^{aby}	85 ± 4^{ay}

Different black letters abc indicate significant difference ($p \leq 0.05$) in the specific solution between days, whereas red letters uxyz indicate significant difference ($p \leq 0.05$) between treatment groups.

Addition of ascorbic acid did not improve the stability of L-5-CH_3-H_4folate, as no significant difference was found between these two capsules after any of the two performed storage studies. Even though significant protective effect ($p \leq 0.05$) of the capsules on the stability of L-5-CH_3-

H_4folate was observed during the first stability study, no significant protection was observed during baking (day 0), where free L-5-CH_3-H_4folate was shown to be more stable. Endogenous L-5-CH_3-H_4folate represents 5-CH_3-H_4folate present in buns that is naturally found in flour and other used ingredients for the dough preparation. It is interesting to see that >50% of the endogenous folate was lost during the heating at 200°C which confirms the fact that the used L-5-CH_3-H_4folate is already protected from oxidation. In addition, our findings are similar to studies investigating encapsulated 5-CH_3-H_4folate and green tea extract (Shrestha et al. (2012b), Gómez-Mascaraque et al. (2017)). Both studied the stability of the produced capsules and the protective effect of the capsules to 5-CH_3-H_4folate and in green tea extract. In their studies, encapsulation also had protective effect on the bioactives following capsules production and during the characterization of the capsules. However, when they implemented the capsules to food system, no protective effect of the encapsulation was shown.

Even though electrospraying has the potential to enable protection of L-5-CH_3-H_4folate using the carbohydrates-based coating material, it is important to acknowledge a few limitations of the study. As previously mentioned, due to the costliness of the analytical standard of the naturally occurring compound, a synthetic form L-5-CH_3-H_4folate was used for encapsulation as it was seen in previous studies (Liu et al., 2015; Liu et al., 2013). However, L-5-CH_3-H_4folate was more stable during heating and had superior recovery over the recovery of the produced capsules. The ~80% recovery of produced capsules may occur also during the oxidation during electrospraying processing, as the second stability study in buns was performed after the protective effect from oxidation of the capsules was confirmed by the first stability study in capsules. Therefore, two different batches of the capsules were used in the two storage experiments which will increase the uncertainty when comparing the results from the two studies. Buns, as a chosen food matrix, do not have long storage life. In order to test if storage stability in food matrix would be the same as in the capsules, prolonged study with the food matrix with longer shelf life should be done. As previously mentioned, significant difference ($p \leq 0.05$) in the stability of free L-5-CH_3-H_4folate was observed after day 7 of the storage, which is in accordance to the results seen in the stability in buns. The recovery of free L-5-CH_3-H_4folate (88%) on day 9 was significantly different from its recovery on day 0 (100%), whereas recovery of encapsulated L-5-CH_3-H_4folate remained constant during 9 days of storage of the buns. In agreement with former study, these results indicate that the application of the newly developed delivery system to food systems could become a successful fortification strategy.

Chapter 7: Conclusions

The main hypothesis of this PhD project was that the recombinant plant origin GGH can serve as a basis for the standardization of deconjugation step in various food groups. A sensitive and specific chemical analytical method using LC-MS/MS as a detection tool for analysis of seven folate vitamers was developed in order to study the hypothesis. Due to the low stability of folate forms and mainly their interconversion, a method development showed to be a challenging task with a lot of obstacles. However, the newly developed method is shown to be an efficient analytical tool that provided new information about the activity of a recombinant plant origin GGH and folate content in many foodstuffs. When compared to commonly used animal-origin GGHs the use of plant origin GGH enabled a fast deconjugation step using only single-enzyme extraction, which significantly reduced the duration of folate analysis in the lab. The newly developed method was in a round robin study compared with a microbiological assay and another LC-MS/MS method using the animal origin GGH by analyzing a large and diverse sample set composed of 89 food samples. Data obtained from round robin study provided the information about possible overestimation of folate content if using a microbiological assay, and on the superior ability of plant origin GGH to deconjugate folate in plant matrices such as fruits, vegetables and cereals, as it was already reported that animal origin GGHs may be inhibited in plant matrices. No significant difference was observed in the complex food matrix of legumes between the compared methods, indicating that single-enzyme extraction is sufficient for folate deglutamylation within 1 h, if the plant origin GGH was used. Moreover, the data obtained by analyzing a large and diverse sample set in this PhD project are ready to be implemented into the Danish Food Composition Databank and provide new information for creation of future nutritional guidelines, as they contain information on the distribution of biologically active folate forms within various food matrices. 5-CH_3-H_4folate is the most abundant folate form in food groups that are considered as rich sources of folate, whereas formyl forms prevail in food groups of cereals, dairy products and meat. It was shown that just one serving of liver from the food group of offal may provide >500% of the folate RDI (300 µg/day) for adults, whereas food groups of legumes, vegetables and certain fruits such as strawberries represents food rich in folate by containing >79 µg/100 g of total folate content in food. However, a high consumption of food groups of cereals and dairy products results in their contribution to the total folate intake despite being poor folate sources. Therefore, a fortification strategy of incorporating encapsulated 5-CH_3-H_4folate into all-purpose flour was examined. The encapsulation by electrospraying showed to be a successful way of preserving naturally occurring 5-CH_3-H_4folate

from oxidative degradation in newly produced capsules. However, the incorporation of the produced capsules into food matrix such as buns did not show any protective effects during heat processing, indicating that further investigation is needed to utilize this as a future fortification strategy.

Chapter 8: Future perspectives

Method development requires time and lot of work, especially in the analysis of such a complex compounds as folate vitamers. The developed methods showed the limitation in the quantification of H_4folate, therefore the next version of the method is recommended to include a labeled H_4folate. Furthermore, there were some difficulties in complete quantification of 5-CH_3-H_4folate in NIST 1845a Whole Egg Powder by the use of plant-origin GGH, where other combination of deconjugase is needed e.g. of animal-origin deconjugase such as rat serum or recombinant human GGH. Improvement of the method could also be to introduce the use of cut-off filters for sample purification in order to scale down the extraction volumes and provide even less use of valuable pure recombinant enzyme, while at the same way having faster sample purification. This step would make this method user friendly and easy to use in routine analysis.

The method was also validated for quantification of 10-CH_3-PteGlu that was reported to be the most abundant form in mushrooms. Therefore, a future work on this type of foods could be done in order to examine if this compound is occurring in a significant amount in mushrooms and possibly test if there is any nutritional benefit of this compound or it is hazardous and is presenting oxidation product. The single-laboratory validation and the results from the round robin study funds the basis for a future inter-comparison study to assess reproducibility for this new method using the plant origin GGHs. The expected acceptable results will form the base for a new standardized method. Commercial availability seems to be the only drawback which has to be overcome to establish the use of plant-origin GGH as a standard part of folate analysis. The results from this project showed that the plant-origin GGH is an enzyme of choice in terms of superiority in enzyme efficiency, enabling short analysis-time and reproducible deconjugation of biologically active folate forms in complex food samples. Furthermore, 17% lower folate content obtained when analyzed by this method indicates that the dietary folate intake is 17% lower than is stated. This difference results in an inadequate folate intake among the Danish population which means that there will be a need for updated nutritional guidelines.

Having a sensitive method that may lower its limits of quantification to pg/g levels as a detection tool, gives a good starting point in future of folate analysis in blood or biological tissue samples which would enable to study folate bioaccessibility and bioavailability from various food matrices.

The use of encapsulation as a strategy for preservation of biologically active folate forms showed to be successful, even though future work has to be performed in order to examine the appropriate food systems for incorporation of newly produced capsules as no protective effect from capsulation was observed in the used food system of buns baked with all-purpose flour. Moreover, it would be recommendable to use pure analytical standard of 5-CH_3-H_4folate for encapsulation as the commonly used L-5-CH_3-H_4folate, Metafolin form is already protected and produced as a folate form intended to be used in supplementation, therefore masking the real instability of naturally occurring folate forms in food.

REFERENCES

Akilanathan, L., Vishnumohan, S., Arcot, J., Uthira, L., & Ramachandran, S. (2010). Total folate: Diversity within fruit varieties commonly consumed in India. *International Journal of Food Sciences and Nutrition*, 61(5), 463–472.

Antony, A.C. (2017). Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests. *Nutrition Reviews*, 75(8), 600–615.

Arcot, J. & Shrestha, A. (2005). Folate: Methods of analysis. *Trends in Food Science and Technology*, 16(6–7), 253–266.

Australian Government Department of Health and Aging, National Health and Medical Research Council (2006). *Nutrient reference values for Australia and New Zealand including recommended dietary intakes.*, Canberra (Australia).

Bagley, P.J. & Selhub, J. (2000). Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrochemical detection. *Clinical Chemistry*, 46(3), 404–411.

Bailey, L. B., Stover, P. J., Mcnulty, H., Fenech, M. F., Gregory, J. F., Mills, J. L., Pfeiffer, C. M., Fazili, Z., Zhang, M., Ueland, P. M., Molloy, A. M., Caudill, M. A., Shane, B., Berry, R. J., Bailey, R. L., Hausman, D. B., Raghavan, R., Raiten, D. J. (2015). Biomarkers of nutrition for development— Folate review 1–5. *The Journal of Nutrition*, *145*(7), 1636–1680.

Barba, C.V.C. & Cabrera, M.I.Z. (2008). Recommended dietary allowances harmonization in Southeast Asia. *Asia Pacific Journal of Clinical Nutrition*, 17(S2), 405–408.

Bhandari, S.D. & Gregory, J.F. (1990). Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity. *American Journal of Clinical Nutrition*, 51(1), 87–94.

Bhandari, S.D., Gao, M. & Szpylka, J. (2018). Total folate in infant formula and adult nutritionals by trienzyme extraction and LC-MS/MS quantitation: A multilaboratory testing study, Final Action 2011.06. *Journal of AOAC International*, 101(6), 1881–1894.

Blom, H.J. & Smulders, Y. (2011). Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *Journal of Inherited Metabolic*

Disease, 34, 75–81.

Brouwer, I.A., Van Dusseldorp, M., West, C. E., Meyboom, S., Thomas, C. M. G., Duran, M., Van Het Hof, K H., Eskes, T. K. A. B., Hautvast, J., G., A., J., & Steegers-Theunissen, R. P. M. (1999). Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. *The Journal of Nutrition*, 129(6), 1135–1139.

Brouwer, I.A., Van Dusseldorp, M., West, C. E., & Steegers-Theunissen, R. P. M. (2001). Bioavailability and bioefficacy of folate and folic acid in man. *Nutrition Research Reviews*, 14, 267–293.

Camara, J.E., Lowenthal, M.S. & Phinney, K.W. (2013). Determination of fortified and endogenous folates in food-based Standard Reference Materials by liquid chromatography-tandem mass spectrometry. *Analytical and bioanalytical chemistry*, 405(13), 4561–8.

Carbonell-Capella, J.M., Buniowska, M., Barva, F. J., Esteve, M. J., & Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety*, 13(2), 155–171.

Caudill, M.A. (2010). Folate bioavailability: Implications for establishing dietary recommendations and optimizing status. *American Journal of Clinical Nutrition*, 91(5), 1455–1460.

Cech, N.B. & Enke, C.G. (2001). Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrometry Reviews*, 20(6), 362–387.

Chango, A., Boisson, F., Barbé, F., Quilliot, D., Droesch, S., Pfister, M., Fillon-Emery, N., Lambert, D., Frémont, S., Rosenblatt, D. S., & Nicolas, J. P. (2000). The effect of C677T and A1298C mutations on plasma homocysteine and 5, 10-methylenetetrahydrofolate reductase activity in healthy subjects. *British Journal of Nutritionutrition*, (83), 593–596.

Combs Jr., G.F. & McClung, J.P. (2017). Folate. In *The vitamins, Fundamental aspects in nutrition and health*. London, 400–428.

Coppedè, F., Bosco, P., Lorenzoni, V., Migheli, F., Barone, C., Antonucci, I., Stuppia, L., Romano, C., & Migliore, L. (2013). The MTR 2756A>G polymorphism and maternal risk of birth of a

child with Down syndrome: A case-control study and a meta-analysis. *Molecular Biology Reports*, 40(12), 6913–6925.

Crider, K.S., Yang, T. P., Berry, R. J., & Bailey, L. B. (2012). Folate and DNA methylation: A review of molecular mechanisms and the evidence for folate's role. *Advances in Nutrition: An International Review Journal*, 3(1), 21–38.

Crider, K.S., Bailey, L.B. & Berry, R.J. (2011). Folic acid food fortification—Its history, effect, concerns, and future directions. *Nutrients*, 3, 370–384.

Czarnowska-Kujawska, M., Gujska, E. & Michalak, J. (2017). Testing of different extraction procedures for folate HPLC determination in fresh fruits and vegetables. *Journal of Food Composition and Analysis Analysis*, 57, 64–72.

Dang, J., Arcot, J. & Shrestha, A. (2000). Folate retention in selected processed legumes. *Food Chemistry*, 68(3), 295–298.

Danish Food Composition Databank (Revision 3a, https://frida.fooddata.dk) (assessed 10.01.2019).

De Bree, A., Van Dusseldorp, M., Brouwer, I. A., Van Het Hof, K. H., & Steegers-Theunissen, R. P., M. (1997). Folate intake in Europe: recommended, actual and desired intake. *European Journal of Clinical Nutrition*, 51(10), 643–660.

De Brouwer, V., Zhang, G.-F., Storozhenko, S., Van Der Straeten, D., & Lambert, W. E. (2007). pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates. *Phytochemical Analysis*, 18, 496–508.

De Brouwer, V., Storozhenko, S., Stove, C. P., Van Daele, J., Van Der Straeten, D., & Lambert, W. E. (2010). Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the sensitive determination of folates in rice. *Journal of Chromatography B*, 878(3–4), 509–513.

Delchier, N., Herbig, A.-L., Rychlik, M., & Renard, C. M. G. C. (2016). Folates in Fruits and Vegetables: Contents, processing, and stability. *Comprehensive Reviews in Food Science and Food Safety*, 15(3), 506–528.

Delchier, N., Ringling, C., Maingonnat, J.-F., Rychlik, M., Renard, C. M. G. C. (2014).

Mechanisms of folate losses during processing: Diffusion vs. heat degradation. *Food Chemistry*, 157, 439–447.

Department of Health, (1991). *Dietary reference values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Commitee on Medical Aspects of Food Policy*, London: HMSO.

De Marco, P., Calevo, M. G., Moroni, A., Arata, L., Merello, E., Finnell, R. H., Zhu, H., Andreussi, L., Cama, A., & Capra, V. (2002). Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population. *Journal of Human Genetics*, 47(6), 319–324.

de Hoffmann, E. & Stroobant, V. (2007). *Mass spectrometry: Principles and application* 3rd ed. J. W. Ltd, ed.,

DeVries, J.W., Rader, J. I., Keagy, P. M., & Hudson, C. A. (2005). Microbiological assay tri-enzyme procedure for total folates in cereals and cereal foods: Collaborative study. *Journal of AOAC International*, 88(1), 5–15.

De Wals, P., Tairou, F., Van Allen, M. I., Uh, Lowry, B., S.-H., Sibbald, B., Evans, J. A., Van den Hof, M. C., Zimmer, P., Crowley, M., Fernandez, B., Lee, N. S., & Niyonsenga, T. (2007). Reduction in neural-tube defects after folic acid fortification in Canada. *New England Journal of Medicine*, 357(2), 135–142.

Dolin, C.D., Deierlein, A.L. & Evans, M.I. (2018). Folic acid supplementation to prevent recurrent neural tube defects: 4 milligrams is too much. *Fetal Diagnosis and Therapy*, 44(3), 161–165.

Ducker, G.S. & Rabinowitz, J.D. (2017). One-carbon metabolism in health and disease. *Cell Metabolism*, 27–42.

Edelmann, M., Kariluoto, S., Nyström, L., & Piironen, V. (2013). Folate in barley grain and fractions. *Journal of Cereal Science*, 58(1), 37–44.

EN14131, 2003. Foodstuffs – Determination of folate by microbiological assay. *European Standard, European Committee for Standardization*.

European Union (2002). Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food

supplements. *Official Journal of the European Communities*, 183, 51–57.

European Union (2006). Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. *Official Journal of the European Union*, L404, 26–38.

Ezhilarasi, P.N., Karthik, P., Chhanwal, N., & Anandharamakrishnan, C. (2013). Nanoencapsulation techniques for food bioactive components : A review. *Food and Bioprocess Technology*, 6, 628–647.

Fan, R., Zhang, A. & Zhong, F. (2017). Association between homocysteine levels and all-cause mortality: A dose-response meta-analysis of prospective studies. *Scientific Reports*, 7(1), 1–9.

Fathi, M., Martin, A. & McClements, D.J. (2014). Nanoencapsulation of food ingredients using carbohydrate based delivery systems. *Trends in Food Science and Technology*, 39, 18–39.

Fekete, K., Berti, C., Trovato, M., Lohner, S., Dullemeijer, C., Souverein, O. W., Cetin, I., & Desci, T. (2012). Effect of folate intake on health outcomes in pregnancy: A systematic review and meta-analysis on birth weight, placental weight and length of gestation. *Nutrition Journal*, 11(1), 1–8.

Food and Drug Administration, 1996. Food standards: Amendment of standards of identity for enriched grain products to require addition of folic acid. *Federal Register*, 61(151), 8781–8797.

Forssén, K.M., Jägerstad, M. I., Wigertz, K., & Witthöft, C. M. (2000). Folates and dairy products: A critical update. *Journal of the American College of Nutrition*, 19(2), 100S–110S.

Freisleben, A., Schieberle, P. & Rychlik, M. (2003a). Comparison of folate quantification in foods by high-performance liquid chromatography – fluorescence detection to that by stable isotope dilution assays using high-performance liquid chromatography – tandem mass spectrometry. *Analytical Biochemistry*, 315, 247–255.

Freisleben, A., Schieberle, P. & Rychlik, M. (2003b). Specific and sensitive quantification of folate vitamers in foods by stable isotope dilution assays using high-performance liquid chromatography-tandem mass spectrometry. *Analytical and bioanalytical chemistry*, 376(2), 149–56.

Furlani, R.P.Z. & Godoy, H.T., 2007. Contents of folates in edible mushrooms commercialised in the city of Campinas, São Paulo, Brazil. *Ciência e Tecnologia de Alimentos*, 27(2), 278–280.

Galloway, M. & Rushworth, L. (2003). Red cell or serum folate? Results from the National Pathology Alliance benchmarking review. *Journal of Clinical Pathology*, 56(12), 924–926.

García-Salinas, C., Ramos-Parra, P.A. & Díaz de la Garza, R.I. (2016). Ethylene treatment induces changes in folate profiles in climacteric fruit during postharvest ripening. *Postharvest Biology and Technology*, 118, 43–50.

García-Moreno, P.J., Damberg, C., Chronakis, I. S., & Jacobsen, C. (2017). Oxidative stability of pullulan electrospun fibers containing fish oil: Effect of oil content and natural antioxidants addition. *European Journal of Lipid Science and Technology*, 1600305, 1–11.

García-Moreno, P.J., Pelayo, A., Yu, S., Busolo, M., Lagaron, J. M., Chronakis, I. S., & Jacobsen, C. (2018). Physicochemical characterization and oxidative stability of fish oil-loaded electrosprayed capsules: Combined use of whey protein and carbohydrates as wall materials. *Journal of Food Engineering*, 231, 42–53.

Garratt, L.C., Ortori, C. A., Tucker, G. A., Sablitzky, F., Bennett, M. J., & Barrett, D. A. (2005). Comprehensive metabolic profiling of mono- and polyglutamated folates and their precursors in plant and animal tissue using liquid chromatography/negative ion electrospray ionisation tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 19(17), 2390–2398.

Ghorani, B. & Tucker, N. (2015). Fundamentals of electrospinning as a novel delivery vehicle for bioactive compounds in food nanotechnology. *Food hydrocolloids*, 51, 227–240.

Glish, G.L. & Vachet, R.W. (2003). The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery*, 2(2), 140–150.

Gómez-Mascaraque, L.G., Hernández-Rojas, M., Tarancón, P., Tenon, M., Feuillère, N., Vélez Ruiz, J. F., & Fiszman, S. (2017). Impact of microencapsulation within electrosprayed proteins on the formulation of green tea extract-enriched biscuits. *LWT - Food Science and Technology*, 81, 77–86.

Gorelova, V., Ambach, L., Rébeillé, F., Stove, C., Van Der Straeten, D. (2017). Folates in Plants: Research Advances and Progress in Crop Biofortification. *Frontiers in Chemistry*, 5, 21.

Green, T.J., Liu, Y., Dadgar, S., Li, W., Böhni, R., & Kitts, D. D. (2013). Wheat rolls fortified with microencapsulated L-5-methyltetrahydrofolic acid or equimolar folic acid increase blood folate concentrations to a similar extent in healthy men and women. *The Journal of Nutrition*, 143(6), 867–871.

Gregory, J.F., Bhandari, S. D., Bailey, L. B., Toth, J. P., Baumartner, T. G., & Cerda, J. J. (1992). Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *American Journal of Clinical Nutrition*, 55(6), 1147–1153.

Gregory, J. F. (1995). The bioavailability of folate. Chapter in; Folate: Nutritional and clinical perspectives, (L. Bailey, ed.), M. Dekker Publ., 195-235.

Gregory, J.F., Cuskelly, G. J., Shane, B., Toth, J. P., Baumgartner, T. G., & Stacpoole, P. W. (2000). Primed, constant infusion with [2H3]serine allows in vivo kinetic measurement of serine turnover, homocysteine remethylation, and transsulfuration processes in human one-carbon metabolism. *American Journal of Clinical Nutrition*, 72(6), 1535–1541.

Gregory, J.F. (2001). Case study: Folate bioavailability. *The Journal of Nutrition*, 131(4), 1376–1382.

Gregory, J.F., Quinlivan, E.P. & Davis, S.R. (2005). Integrating the issues of folate bioavailability, intake and metabolism in the era of fortification. *Trends in Food Science and Technology*, 16(6–7), 229–240.

Gregory, J.F. (2012). Accounting for differences in the bioactivity and bioavailability of vitamers. *Food and Nutrition Research*, 56, 1–11.

Gupta, D. S., Thavarajah, D., Knutson, P., Thavarajah, P., McGee, R. J., Coyne, C. J., & Kumar, S. (2013). Lentils (*Lens culinaris* l.), a rich source of folates. *Journal of Agricultural and Food Chemistry*, 61(32), 7794–7799.

Halsted, C.H. (1989). The intestinal absorption of dietary folates in health and disease. *Journal of the American College of Nutrition*, 8(6), 650–658.

Hanson, A.D. & Gregory, J.F. (2011). Folate Biosynthesis, turnover, and transport in Plants. *Annual Review of Plant Biology*, 62(1), 105–125.

Health Council of The Netherlands (2008). *Towards an optimal use of folic acid*, The Hague (The Netherlands).

Hefni, M. & Witthöft, C.M. (2014). Folate content in processed legume foods commonly consumed in Egypt. *LWT - Food Science and Technology*, 57(1), 337–343.

Hermund, D., Jacobsen, C., Chronakis, I. S., Pelayo, A., Yu, S., Busolo, M., Lagaron, J. M., Jónsdottir, R., Kristinsson, H. G., Akoh, C. C., & García-Moreno, P. J. (2019). Stabilization of fish oil-loaded electrosprayed capsules with seaweed and commercial natural antioxidants: effect on the oxidative stability of capsule-enriched mayonnaise. *European Journal of Lipid Science and Technology*, 1–10.

Hochster, R.M. & Quastel, J.H. (1963). *Metabolic inhibitors: A comprehensive treatise* Volume I., New York: Academic Press Inc.

Hu, J., Juan, W.Y. & Sahyoun, N.R. (2016). Intake and biomarkers of folate and risk of cancer morbidity in older adults, NHANES 1999-2002 with medicare linkage. *PLoS ONE*, 11(2), 1–11.

Hyun, T.A. & Tamura, T. (2005). Trienzyme extraction in combination with microbiologic assay in food folate analysis: An updated review. *Experimental biology and medicine*, 230(7), 444–454.

Imbard, A., Benoist, J.-F. & Blom, H.J. (2013). Neural tube defects, folic acid and methylation. *International Journal of Environmental Research and Public Health*, 10, 4352–4389.

Institute of Medicine (1998). *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*, Washington, DC: The National Academies Press.

Institute of Medicine (2000). Using the tolerable upper intake level for nutrient assessment of groups. In *Dietary Reference Intakes: applications in dietary assessment*. Washington, DC, 113–126.

Jackson, M.J. (1997). The assessment of bioavailability of micronutrients: introduction. *European journal of clinical nutrition*, 51 Suppl 1, S1-2.

Jacobsen, C., García-Moreno, P. J., Mendes, A. C., Mateiu, R. V., & Chronakis, I. S. (2018). Use of

electrohydrodynamic processing for encapsulation of sensitive bioactive compounds and applications in food. *Annual Review of Food Science and Technology*, *9*(1), 525–549.

Jägerstad, M. & Jastrebova, J. (2013). Occurrence, stability, and determination of formyl folates in foods. *Journal of agricultural and food chemistry*, 61(41), 9758–9768.

Kariluoto, M.S., Vahteristo, L.T. & Piironen, V.I. (2001). Applicability of microbiological assay and affinity chromatography purification followed by high-performance liquid chromatography (HPLC) in studying folate contents in rye. *Journal of the science of Food and Agriculture*, 81, 938–942.

Kebarle, P. & Verkerk, U.H. (2009). Electrospray: From ions in solution to ions in the gas phase, what we know now. *Mass Spectrometry Reviews*, 28, 898–917.

Keresztesy, J.C. (1944). The water-soluble vitamins. *Annual Review of Biochemistry*, 13(1), 367–410.

Kiekens, F., Van Daele, J., Blancquaert, D., Van Der Straeten, D., Lambert, W. E., & Stove, C. P. (2015). Determination of Five Folate Monoglutamates in Rodent Diets. *Journal of Agricultural and Food Chemistry*, 63(45), 10089–10095.

Kirsch, S.H., Knapp, J.-P., Herrmann, W., & Obeid, R. (2010). Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, 878(1), 68–75.

Konings, E. J. M., Roomans, H. H. S., Dorant, E., Goldbohm, R. A., Saris, W. H. M., & van den Brandt, P. A. (2001). Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *American Journal of Clinical Nutrition*, *73*(4), 765–776.

Koontz, J. L., Phillips, K. M., Wunderlich, K. M., Exler, J., Holden, J. M., Gebhardt, S. E., & Haytowitz, D. B. (2005). Comparison of total folate concentrations in foods determined by microbiological assay at several experienced U.S. commercial laboratories. *Journal of AOAC International*, *88*(3), 805–813.

Korhola, M., Hakonen, R., Juuti, K., Edelmann, M., Kariluoto, S., Nystörm, L., Sontag-Strohm, T., & Piironen, V. (2014). Production of folate in oat bran fermentation by yeasts isolated from

barley and diverse foods. *Journal of applied microbiology*, 117(3), 679–689.

Krawinkel, M.B., Strohm, D., Weissenborn, A., Watzl, B., Eichholzer, M., Bärlocher, K., Elmadfa, I., Leschik-Bonnet, E., & Heseker, H. (2014). Revised D-A-CH intake recommendations for folate: how much is needed? *European Journal Of Clinical Nutrition*, 68, 719.

Kristensen, N.B., Madsen, M.L., Hansen, T.H., Allin, K.H., Hoppe, C., Fagt, S., Lausten, M.S., Gøbel, R.J., Vestergaard, H., Hansen, T., Pedersen, O. (2015). Intake of macro- and micronutrients in Danish vegans. *Nutrition Journal*, 14, 1–10.

Liu, Y., Green, T. J., Wong, P., & Kitts, D. D. (2013). Microencapsulation of L‑5-Methyltetrahydrofolic Acid with Ascorbate Improves Stability in Baked Bread Products. *Journal of Agricutural and Food Chemistry*, 61, 247-254.

Liu, Y., Green, T. J., & Kitts, D. D. (2015). Stability of microencapsulated L-5-methyltetrahydrofolate in fortified noodles. *Food Chemistry*, 171, 206–211.

López-Rubio, A., Sanchez, E., Wilkanowicz, S., Sanz, Y., & Lagaron, J. M. (2012). Electrospinning as a useful technique for the encapsulation of living bifidobacteria in food hydrocolloids. *Food hydrocolloids*, 28(1), 159–167.

López-Rubio, A. & Lagaron, J.M. (2012). Whey protein capsules obtained through electrospraying for the encapsulation of bioactives. *Innovative Food Science and Emerging Technologies*, 13, pp.200–206.

Ložnjak, P., García-Salinas, C., Díaz de la Garza, R. I., Bysted, A., & Jakobsen, J. (2019). The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*. (Accepted, In Press)

Luo, S., Duan, H., Zou, Y., Qiu, R., & Wang, C. (2017). Quantification of total folate, folate species and polyglutamyl folate distribution in winged beans (Psophocarus tetragonolobus (L) DC) from different cultivars and growth stages by ultra-high performance liquid chromatography tandem mass spectrometry. *Journal of Nutritional Science and Vitaminology*, 63(1), 69–80.

Ma, F., Wu, T., Zhao, J., Ji, L., Song, A., Zhang, M., & Huang, G. (2017). Plasma homocysteine and serum folate and vitamin B_{12} levels in mild cognitive impairment and alzheimer's disease:

A case-control study. *Nutrients*, 9(7), 1–11.

Majors, R.E. (2010). Solid-Phase Extraction. In J. Pawliszyn & H. L. Lord, eds. *Handbook of sample preparation*. John Wiley & Sons, Inc.

Martín Tornero, E., Espinosa-Mansilla, A. & Durán Merás, I. (2017). High-performance liquid chromatography with fast-scanning fluorescence detection and post-column on-line photoderivatization for the analysis of folic acid and its metabolites in vegetables. *Microchemical Journal*, 133, 333–345.

Matuszewski, B.K., Constanzer, M.L. & Chavez-Eng, C.M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC−MS/MS. *Analytical Chemistry*, 75(13), 3019–3030.

McBreairty, L.E., McGowan, R. A., Brunton, J. A., & Bertolo, R. F. (2013). Partitioning of [Methyl-3H]methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. *Journal of Nutrition*, 143(6), 804–809.

McGarel, C., Pentieva, K., Strain, J. J., & McNulty, H. (2015). Emerging roles for folate and related B-vitamins in brain health across the lifecycle. *Proceedings of the Nutrition Society*, 74(1), 46–55.

Mensink, G.B.M., Fletcher, R., Gurinovic, M., Huybrechts, I., Lafay, L., Serra-Majem, I., Szponar, L., Tetens, I., Verkaik-Kloosterman, J., Baka, A., & Stephen, A. M., (2013). Mapping low intake of micronutrients across Europe. *British Journal of Nutrition*, 110(4), 755–773.

Mitchell, H.K., Snell, E.E. & Williams, R.J. (1941). The concentration of "folic acid." *Journal of the American Chemical Society*, 63(8), 2284.

Moll, R. & Davis, B. (2017). Iron, vitamin B12and folate. *Medicine (United Kingdom)*, 45(4), 198–203.

Mönch, S., Netzel, M., Netzel, G., Ott, U., Frank, T., & Rychlik, M. (2015). Folate bioavailability from foods rich in folates assessed in a short term human study using stable isotope dilution assays. *Food and Function*, 6(1), 242–248.

Mönch, S., Netzel, M., Netzel, G., Ott, U., Frank, T., & Rychlik, M. (2016). Pilot study on folate

bioavailability from a camembert cheese reveals contradictory findings to recent results from a human short-term study. *Frontiers in Nutrition*, 3, 1–5.

Morris, J.K., Rankin, J., Draper, E. S., Kurinczuk, J. J., Springett, A., Tucker, D., Wellesley, D., Wreyford, B., & Wald, N. J. (2016). Prevention of neural tube defects in the UK: A missed opportunity. *Archives of Disease in Childhood*, 101(7), 604–607.

Motta, C., Delgado, I., Matos, A. S., Gonzales, G. B., Torres, D., Santos, M., Chandra-Hioe, M. V., Arcot, J., & Castanheira, I. (2017). Folates in quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus sp.*) and buckwheat (*Fagopyrum esculentum*): Influence of cooking and malting. *Journal of Food Composition and Analysis*, 64(August), 181–187.

Mudd, S.H., Brosnan, J. T., Jacobs, R. L., Stabler, S. P., Allen, R. H., Vance, D. E., & Wagner, C. (2007). Methyl balance and transmethylation fluxes in humans. *American Journal of Clinical Nutrition*, 85(1), 19–25.

Naderi, N. & House, J.D. (2018). Recent Developments in Folate Nutrition. *Advances in Food and Nutrition Research*, 83, 195–213.

Ndaw, S., Bergaentzlé, M., Aoudé-Werner, D., Lahély, S., & Hasselmann, C. (2001). Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates. *Journal of Chromatography A*, 928, 77–90.

Ninagawa, T., Eguchi, A., Kawamura, Y., Konishi, T. (2016). A study on ice crystal formation behavior at intracellular freezing of plant cells using a high-speed camera. *Cryobiology*, 73(1), 20–29.

NNR (2014). Nordic Nutrition Recommendations. *Nordic Nutrition Recommendations 2012. Nordic Council of Ministers.*

O'Leary, V.B., Mills, J. L., Parle-McDermott, A., Pangilinan, F., Molloy, A. M., Cox, C., Weiler, A., Conley, M., Kirke, P. N., Scott, J M., Brody, L C., and Members of the Birth Defects Research Group (2005). Screening for new MTHFR polymorphisms and NTD risk. *American Journal of Medical Genetics*, 138 A(2), 99–106.

Obeid, R., Holzgreve, W. & Pietrzik, K. (2013). Is 5-methyltetrahydrofolate an alternative to folic

acid for the prevention of neural tube defects? *Journal of Perinatal Medicine*, 41(5), 469–483.

Ohrvik, V.E. & Witthoft, C.M. (2011). Human folate bioavailability. *Nutrients*, 3(4), 475–490.

Orsomando, G., Díaz de la Garza, R., Green, B. J., Peng, M., Rea, P A., Ryan, T. J., Gregory, J. F., Hanson, A. D. (2005). Plant γ-glutamyl hydrolases and folate polyglutamates: Characterization, compartmentation, and co-occurrence in vacuoles. *Journal of Biological Chemistry*, 280(32), 28877–28884.

Pande, S. V. & Mead, J.F. (1968). Inhibition of enzyme activities by free fatty acids. *The Journal of Biological Chemistry*, 243(23), 6180–6185.

Patring, J., Wandel, M., Jägerstad, M., & Frølich, W. (2009). Folate content of Norwegian and Swedish flours and bread analysed by use of liquid chromatography-mass spectrometry. *Journal of Food Composition and Analysis*, 22(7–8), 649–656.

Patring, J.D.M., Johansson, M. S., Yazynina, E., & 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. *Analytica Chimica Acta*, 553, 36–42.

Pawlosky, R.J., Flanagan, V.P. & Pfeiffer, C.M. (2001). Determination of 5-methyltetrahydrofolic acid in human serum by stable-isotope dilution high-performance liquid chromatography–Mass Spectrometry. *Analytical Biochemistry*, 298(2), 299–305.

Pedersen, A.N., Christensen, T., Matthiessen, J., Knudsen, V.K., Rosenlund-Sørensen, M., Biltoft-Jensen, A., Hinsch, H.-J., Ygil, K.H., Kørup, K., Saxholt, E., Trolle, E., Søndergaard, A.B., Fagt, S. (2015). Danskernes kostvaner 2011-2013.

Pérez-Masiá, R., López-Nicolás, R., Periago, M. J., Ros, G., Lagaron, J M., & López-Rubio, A. (2015). Encapsulation of folic acid in food hydrocolloids through nanospray drying and electrospraying for nutraceutical applications. *Food Chemistry*, 168, 124–133.

Pérez-Masiá, R., Lagaron, J.M. & López-rubio, A. (2014). Surfactant-aided electrospraying of low molecular weight carbohydrate polymers from aqueous solutions. *Carbohydrate Polymers*, 101, 249–255.

Pfeiffer, C.M., Rogers, L. M., Bailey, L. B., & Gregory, J. F. (1997). Absorption of folate from

fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual- label stable-isotope protocol. *American Journal of Clinical Nutrition*, 66(6), 1388–1397.

Pieroth, R., Paver, S., Day, S., & Lammersfeld, C. (2018). Folate and its impact on cancer risk. *Current Nutrition Reports*, 7(3), 70–84.

Pitkin, R.M. (2007). Folate and neural tube defects. *The American Journal of Clinical Nutrition*, 85(1), 285–288.

Puwastien, P., Pinprapai, N., Judprasong, K., & Tamura, T. (2005). International inter-laboratory analyses of food folate. *Journal of Food Composition and Analysis*, *18*(5), 387–397.

Quinlivan, E.P., Hanson, A.D. & Gregory, J.F. (2006). The analysis of folate and its metabolic precursors in biological samples. *Analytical Biochemistry*, 348, 163–184.

Ramos-Parra, P. A., Urrea-López, R. & Díaz de la Garza, R.I. (2013). Folate analysis in complex food matrices: Use of a recombinant Arabidopsis γ-glutamyl hydrolase for folate deglutamylation. *Food Research International*, 54(1), 177–185.

Reynolds, E.H., (2016). What is the safe upper intake level of folic acid for the nervous system? Implications for folic acid fortification policies. *European Journal of Clinical Nutrition*, 70(5), 537–540.

Ringling, C. & Rychlik, M. (2013). Analysis of seven folates in food by LC-MS/MS to improve accuracy of total folate data. *European Food Research and Technology*, 236, 17–28.

Ringling, C. & Rychlik, M. (2017a). Origins of the difference between food folate analysis results obtained by LC – MS/MS and microbiological assays. *Analytical and bioanalytical chemistry*, 409, 1815–1825.

Ringling, C. & Rychlik, M. (2017b). Simulation of food folate digestion and bioavailability of an oxidation product of 5-methyltetrahydrofolate. *Nutrients*, 9(969), 1–19.

Rychlik, M., Englert, K., Kapfer, S., & Kirchhoff, E. (2007). Folate contents of legumes determined by optimized enzyme treatment and stable isotope dilution assays. *Journal of Food Composition and Analysis*, 20(5), 411–419.

Saini, R.K., Nile, S.H. & Keum, Y.S. (2016). Folates: Chemistry, analysis, occurrence, biofortification and bioavailability. *Food Research International*, 89, 1–13.

Sanderson, P., Stone, E., Kim, Y.-I., Mathers, J. C., Kampman, E., Downes, C. S., Muir, K. R., & Baron, J. A. (2007). Folate and colo-rectal cancer risk. *British Journal of Nutrition*, 98(6), 1299–1304.

Sauberlich, H.E., Kretsch, M. J., Skala, J. H., Johnson, H. L., & Taylor, P. C. (1987). Folate requirement and metabolism in nonpregnant women. *The American Journal of Clinical Nutrition*, 46(6), 1016–1028.

Sauer, H. & Wilmanns, W. (1977). Cobalamin dependent methionine synthesis and methyl-folate-trap in human vitamin B12 deficiency. *British Journal of Haematology*, 36(2), 189-198.

Scaglione, F. & Panzavolta, G. (2014). Folate, folic acid and 5-methyltetrahydrofolate are not the same thing. *Xenobiotica*, 44(5), 480–488.

Shohag, M.J.I., Yang, Q., Wei, Y, Zhang, J., Khan, F. Z., Rychlik, M., He, Z., & Yang, X. (2017). A rapid method for sensitive profiling of folates from plant leaf by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometer. *Journal of Chromatography B*, 1040, 169–179.

Shrestha, A.K., Arcot, J. & Yuliani, S., (2012a). Susceptibility of 5-methyltetrahydrofolic acid to heat and microencapsulation to enhance its stability during extrusion processing. *Food Chemistry*, 130(2), 291–298.

Shrestha, A.K., Arcot, J., Dhital, S., & Crennan, S. (2012b). Effect of biscuit baking conditions on the stability of microencapsulated 5-methyltetrahydrofolic acid and their physical properties. *Food and nutrition sciences*, 3, 1445–1452.

Smith, A.D. & Refsum, H. (2016). Homocysteine, B vitamins, and cognitive deficit in the elderly. *Annual Review of Nutrition*, 36, 211–239.

Snowdon, D. A., Tully, C. L., Smith, C. D., Perez Riley, K., & Markesbery, W. R. (2000). Serum folate and the severity of atrophy of the neocortex in Alzheimer disease: findings from the Nun Study. *American Journal of Clinical Nutrition*, 71(4), 993–998.

Sobczynska-Malefora, A. & Harrington, D.J. (2018). Laboratory assessment of folate (Vitamin B9) status. *Journal of Clinical Pathology*, 71(11), 949–956.

Stead, L.M., Brosnan, J. T., Brosnan, M. E., Vance, D. E., & Jacobs, R. L. (2006). Is it time to reevaluate methyl balance in humans? *American Journal of Clinical Nutrition*, 83(1), 5–10.

Stolzenberg-Solomon, R.Z., Chang, S.-C., Leitzmann, M. F., Johnson, K. A., Johnson, C., Buys, S. S., Hoover, R. N., & Ziegler, R. G. (2006). Folate intake, alcohol use, and postmenopausal breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. *American Journal of Clinical Nutrition*, 83, 895–904.

Striegel, L., Chebib, S., Dumler, C., Lu, Y., Huang, D., & Rychlik, M. (2018). Durian fruits discovered as superior folate sources. *Frontiers in Nutrition*, 5(114), 2–6.

Striegel, L., Chebib, S., Netzel, M.E., & Rychlik, M. (2018). Improved stable isotope dilution assay for dietary folates using LC-MS/MS and its application to strawberries. *Frontiers in Chemistry*, 6, 1–10.

Tam, C., O'Connor, D. & Koren, G. (2012). Circulating unmetabolized folic acid: relationship to folate status and effect of supplementation. *Obestetrics and Gynecology International*, 1–17.

Tamura, T. (1998). Determination of food folate. *The Journal of Nutritional Biochemistry*, 9(5), 285–293.

Tamura, T. & Picciando, M.F. (2006). Folate and human reproduction. *American Journal of Clinical Nutrition*, 83, 993–1016.

Tetens, I., Biltoft-Jensen, A., Spagner, C., Christensen, T., Gille, M.-B., Bügel, S., & Rasmussen, L. B. (2011). Intake of micronutrients among Danish adult users and non-users of dietary supplements. *Food and Nutrition Research*, 55, 1–8.

Tyagi, K., Upadhyaya, P., Sarma, S., Tamboli, V., Sreelakshmi, Y., & Sharma, R. (2015). High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato. *Food chemistry*, 179, 76–84.

Vahteristo, L.T., Ollilainen, V., Koivistoinen, P. E., & Varo, P. (1996). Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid

chromatography. *Journal of Agricultural and Food Chemistry*, 44(2), 477–482.

Vahteristo, L.T., Ollilainen, V. & Varo, P. (1997). Liquid chromatographic determination of folate monoglutamates in fish, meat, egg, and dairy products consumed in Finland. *Journal of AOAC International*, 80(2), 373–378.

Vijayan, M., Chinniah, R., Ravi, P. M., Sivanadham, R., Joseph, A. K. M., Vellaiappan, N. A., Krishnan, J. I., & Karuppiah, B. (2016). MTHFR (C677T) CT genotype and CT-apoE3/3 genotypic combination predisposes the risk of ischemic stroke. *Gene*, 591(2), 465–470.

Vishnumohan, S., Pickford, R. & Arcot, J. (2017). Naturally occurring folates in selected traditionally prepared foods in Southern India. *Journal of Food Science and Technology*, 54(13), 4173–4180.

Voutilainen, S., Rissanen, T. H., Virtanen, J., Lakka, T. A., & Salonen, J. T. (2001). Low dietary folate intake is associated with an excess incidence of acute coronary events: The Kuopio ischemic heart disease risk factor study. *Circulation*, 2674–2681.

Wald, N.J., Morris, J.K. & Blakemore, C. (2018). Public health failure in the prevention of neural tube defects: Time to abandon the tolerable upper intake level of folate. *Public Health Reviews*, 39(2), 1–11.

Weber, W., Mönch, S., Rychlik, M., & Stengl, S. (2011). Quantitation of vitamins using microbiological assays in microtiter formats. In *Fortified foods with vitamins: Analytical concepts to assure better and safer products*. 37–64.

Wei, M.-M. & Gregory, J.F. (1998). Organic acids in selected foods inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *Journal of agricultural and food chemistry*, 46(211–219).

Wien, T.N., Pike, E., Wisløff, T., Staff, A., Smeland, S., & Klemp, M. (2012). Cancer risk with folic acid supplements: A systematic review and meta-analysis. *BMJ Open*, 2(1), 1–13.

Wills, L., (1931). Treatment of "pernicious anaemia of pregnancy" and "tropical anemia." *The British Medical Journal*, 1, 1059–1064.

Winkels, R.M., Brouwer, I. A., Siebelink, E., Katan, M. B., & Verhoef, P. (2007). Bioavailability of

food folates is 80% of that of folic acid. *American Journal of Clinical Nutrition*, 85(2), 465–473.

World Health Organization and Food and Agriculture Organization of the United Nations (2004). *Report of a joint FAO/WHO Expert Consultation. Folate and folic acid: human vitamin and mineral requirements*, Rome (Italy).

Wright, A.J.A., King, M. J., Wolfe, C. A., Powers, H. J., & Finglas, P. M. (2010). Comparison of (6S)-5-methyltetrahydrofolic acid v. folic acid as the reference folate in longer-term human dietary intervention studies assessing the relative bioavailability of natural food folates: Comparative changes in folate status following a 16-week placebo-controlled study in healthy adults. *British Journal of Nutrition*, 103(5), 724–729.

Yan, L., Zhao, L., Long, Y., Zou, P., Ji, G., Gu, A., Zhao, P. (2012.) Association of the Maternal MTHFR C677T Polymorphism with Susceptibility to Neural Tube Defects in Offsprings: Evidence from 25 Case-Control Studies. *PLOS ONE*, 7(10), e41689.

Yu, Y., Wang, F., Bao, Y., Lu, X., Quan, L., & Lu, P. (2014). Association between MTHFR gene polymorphism and NTDs in Chinese Han population. *International Journal of Clinical and Experimental Medicine*, 7(9), 2901–2906.

Zhang, H., Jha, A. B., Warkentin, T. D., Vandenberg, A., & Purves, R. W. (2018). Folate stability and method optimization for folate extraction from seeds of pulse crops using LC-SRM MS. *Journal of Food Composition and Analysis*, 71, 44–55.

Zhang, J. & Zheng, Y.G. (2016). SAM/SAH analogs as versatile tools for SAM-dependent methyltransferases. *ACS Chemical Biology*, 11(3), 583–597.

Zhang, X.F., Liu, T., Li, Y., & Li, S. (2015). Association between MTHFR 677C/T and 1298A/C gene polymorphisms and breast cancer risk. *Genetics and Molecular Research*, 14(4), 16425–16430.

Zou, Y., Duan, H., Li, L., Chen, X., & Wang, C. (2019). Quantification of polyglutamyl 5-methyltetrahydrofolate, monoglutamyl folate vitamers, and total folates in different berries and berry juice by UHPLC – MS/MS. *Food Chemistry*, 276, 1–8.

APPENDICES

Table A1: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Fruits*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Apple	F3A1	0.00	0.34	0.00	2.08	2.42
Apple	F3A2	0.00	0.19	0.00	5.14	5.33
Apple	F3A3	0.00	0.18	0.00	0.49	0.67
Apple	F3A4	0.23	0.42	0.00	2.58	3.23
Banana	F2B1	0.00	3.35	0.00	18.82	22.17
Banana	F2B2	0.70	2.54	0.00	16.86	20.10
Strawberry	F1S1	0.00	12.48	0.00	66.89	79.37
Strawberry	F1S2	1.46	3.79	0.00	85.81	91.06
Strawberry	F1S3	3.35	8.95	0.00	81.59	93.89
Strawberry	F1S4	1.99	3.43	0.00	84.80	90.23
Tomato	F4T1	0.00	3.39	0.00	9.04	12.43
Tomato	F4T2	0.00	0.79	0.00	11.67	12.46
Tomato	F4T3	0.97	1.92	0.00	20.68	23.57
Tomato	F4T4	1.01	2.50	0.00	13.05	16.56

Table A2: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Vegetables*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Broccoli	V1B1	18.76	6.37	0.82	94.21	120.2
Broccoli	V1B2	12.35	14.30	0.00	135.5	162.2
Broccoli	V1B3	0.00	7.38	0.00	103.6	111.0
Broccoli	V1B4	22.24	7.38	0.00	94.99	124.6
Leek	V3L1	1.11	2.96	0.00	96.54	100.6
Leek	V3L2	0.00	0.89	0.00	118.0	118.9
Leek	V3L3	0.35	2.11	0.00	95.33	97.79
Leek	V3L4	3.62	1.40	0.00	115.6	120.7
Romaine salad	V2LS1	0.45	4.73	0.00	65.88	71.06
Romaine salad	V2LS2	0.00	1.32	0.00	81.81	83.14
Romaine salad	V2LS3	1.72	10.13	0.00	117.7	129.5
Romaine salad	V2LS4	3.50	20.87	0.00	137.7	162.1
Onion	V4O1	0.00	0.27	0.00	26.18	26.46
Onion	V4O2	0.00	0.45	0.00	17.14	17.58
Onion	V4O3	0.35	1.15	0.00	18.00	19.50
Onion	V4O4	1.34	0.41	0.00	15.29	17.04

Table A3: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Legumes*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Green peas, frozen	B4GP1	3.63	7.09	1.07	85.09	96.88
Green peas, frozen	B4GP2	4.20	1.43	0.99	74.00	80.63
Green peas, frozen	B4GP3	7.53	7.51	1.21	67.67	83.92
Green peas, frozen	B4GP4	8.24	7.14	0.66	72.21	88.25
Kidney beans, dry	B1KB1	19.48	59.71	8.60	26.91	114.7
Kidney beans, canned	B2KB1	0.00	23.38	2.06	26.34	51.77
Kidney beans, canned	B2KB2	0.00	1.47	0.98	20.53	22.98
Kidney beans, canned	B2KB3	0.11	4.30	1.67	21.75	27.82
Kidney beans, canned	B2KB4	1.32	9.06	1.97	21.81	34.17
Lentils, red, dry	B3L1	19.26	21.97	2.09	38.62	81.94
Lentils, red, dry	B3L2	14.70	20.40	1.83	38.03	74.95
Lentils, green, dry	B3L3	30.08	23.99	1.60	37.09	92.76
Lentils, green, dry	B3L4	25.46	19.62	1.11	29.65	75.84
Peanuts	C4P1	1.17	72.01	6.22	8.15	87.55
Peanuts	C4P2	1.76	73.34	3.35	6.78	85.22
Peanuts	C4P3	2.46	56.07	9.09	5.55	73.17
Peanuts	C4P4	3.42	59.94	9.57	6.96	79.89

Table A4: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Cereals*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Oat, big grain	C3OM1	0.00	18.65	1.60	4.79	25.04
Oat, small grain	C3OM2	0.37	19.57	2.37	7.76	30.07
Oat, small grain	C3OM3	0.32	19.66	2.42	7.19	29.58
Oat, big grain	C3OM4	0.33	22.64	1.59	3.86	28.43
Pasta, dry	C2P2	0.00	10.17	1.11	0.70	11.98
Pasta, dry	C2P1	0.67	9.07	1.36	1.74	12.84
Pasta, dry	C2P3	0.17	11.25	1.01	0.35	12.78
Pasta, dry	C2P4	0.28	8.02	1.28	0.91	10.50
Rice, parboiled, dry	C5R1	0.00	0.57	1.94	1.96	4.47
Rice, parboiled, dry	C5R2	0.37	6.42	2.87	4.69	14.36
Rice, parboiled, dry	C5R3	0.06	2.69	2.15	0.14	5.04
Rice, parboiled, dry	C5R4	0.19	2.79	2.16	0.56	5.70
Wheat flour	C1WF1	0.72	6.16	0.60	3.54	11.02
Wheat flour, wholegrain	C1WF2	3.94	22.12	0.89	4.76	31.71
Wheat flour, wholegrain	C1WF3	6.03	25.70	1.77	7.95	41.45
Wheat flour	C1WF4	2.33	7.07	0.00	2.65	12.04

Table A5: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Dairy products*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Hard cheese, 45+, Danbo	D3HC1	0.00	22.38	0.00	3.53	25.91
Hard cheese, 45+, Danbo	D3HC2	2.15	15.52	0.64	4.89	23.20
Hard cheese, 45+, Danbo	D3HC3	1.01	17.51	0.00	4.58	23.10
Hard cheese, 45+, Danbo	D3HC4	1.24	33.09	0.00	5.07	39.40
Hard cheese, 60+, Brie	D4HC1	0.00	13.44	0.00	22.51	35.96
Hard cheese, 60+, Brie	D4HC2	6.72	21.07	1.54	9.49	38.82
Hard cheese, 60+, Brie	D4HC3	7.74	9.23	2.02	22.10	41.09
Hard cheese, 60+, Brie	D4HC4	5.01	9.37	0.00	15.72	30.10
Milk, 1.5% fat	D2M1	0.00	1.40	0.00	5.24	6.63
Milk, 1.5% fat	D2M2	0.40	0.16	0.00	5.17	5.73
Milk, 1.5% fat	D2M3	0.00	1.01	0.49	5.45	6.95
Milk, 1.5% fat	D2M4	0.36	0.16	0.00	5.46	5.97
Yoghurt, 1.5% fat	D1Y1	0.00	6.83	0.00	3.68	10.51
Yoghurt, 1.5% fat	D1Y2	0.42	5.66	0.00	7.21	13.29

Table A6: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Meat*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Chicken breast	M2C1	1.39	1.15	0.00	3.60	6.13
Chicken breast	M2C2	1.14	3.16	0.23	1.72	6.25
Chicken breast	M2C3	0.90	3.27	0.00	1.35	5.52
Chicken breast	M2C4	0.74	1.92	0.00	1.24	3.90
Pork tenderloin	M1P1	0.64	0.00	0.00	0.36	1.00
Pork tenderloin	M1P2	0.41	0.09	0.02	0.11	0.63
Pork tenderloin	M1P3	0.25	1.40	0.00	0.15	1.80
Pork tenderloin	M1P4	0.57	0.54	0.00	0.11	1.23

Table A7: The content of folate forms and total folate content expressed as PteGlu equivalent analyzed by newly developed LC-MS/MS method using plant origin GGH in food groups of *Offal*. Data are means of analytical duplicates (n=2).

Foodstuff	Sample ID	H_4folate (µg/100g)	HCO-folate forms (µg/100g)	PteGlu (µg/100g)	5-CH_3-H_4folate (µg/100g)	Total folate (µg/100g)
Calf liver	O1LB1	483.0	20.96	0.00	1112	1616
Calf liver	O1LB2	1237	53.09	1.64	605.4	1897
Pork liver	O2LP1	655.8	69.71	1.18	687.6	1414
Pork liver	O2LP2	371.8	36.02	0.00	420.7	828.5