Analytical and Nutritional Aspects of Folate in Cereals

Madelene Johansson
Faculty of Natural Resources and Agricultural Sciences
Department of Food Science
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2005

Acta Universitatis Agriculturae Sueciae
2005:7

Cover image of the folic acid molecule with kind permission by Dr C. B. Martin, Lamar University, Beaumont, Texas, USA

ISSN 1652-6880
ISBN 91-576-7006-4

Tryck: SLU Service/Repro, Uppsala 2005

Abstract

Johansson, M. 2005. *Analytical and Nutritional Aspects of Folate in Cereals.* Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7006-4

A good folate status before conception and during early pregnancy protects against child birth defects, particularly neural tube defects. According to dietary surveys, only a few percent of Swedish women reach the recommended daily intake of folate (400 µg). Swedish authorities are therefore discussing the introduction of mandatory folic acid fortification. A prerequisite for a decision is access to reliable data on folate in cereals since cereal food is both a major contributor to dietary folate intake and a common vehicle for fortification.

Despite much progress in development of HPLC methods for folate, there is still a need for improved selectivity when analysing cereal samples with low level of folate. Ten silica-based stationary phases were compared and alkyl-bonded phases were found to be best for the separation of individual folate forms in terms of selectivity and peak shape. Best selectivity was achieved on an Aquasil C_{18} column.

Optimising the sample purification prior to quantification can also improve the HPLC method. Reversed-phase sorbents, *e.g.* phenyl-endcapped and cyclohexyl-endcapped, were successfully used to purify folate from food extracts. By combining anion-exchange and reversed-phase sorbent, clean extracts of complex food samples were obtained.

Several gluten-free products were analysed for folate content after deconjugation with rat serum using a validated HPLC method. Trienzyme treatment was found to be unnecessary for these samples. Gluten-free products contain considerably less folate than their gluten-containing counterparts and should be considered for folic acid fortification.

Studies on stability of added folic acid during the baking procedure showed an overage of approximately 20% to be necessary to achieve targeted levels. Another important issue in deciding the appropriate fortification level is the extent to which the added folate is absorbed. In a short-term human study using volunteers with ileostomy, mean apparent absorption from folic acid-fortified bread was estimated to be 80%. In a 3-month intervention study, it was observed that a daily additional amount of 166 µg folic acid from fortified white bread was sufficient to significantly increase the folate status in women. Based on these two human studies it is concluded that bread is a suitable vehicle for folic acid fortification.

Keywords: folic acid, 5-CH_3-H_4folate, HPLC, SPE purification, enzyme treatment, folate absorption, fortification, gluten-free products, wheat breakfast rolls

Author's address: Madelene Johansson, Department of Food Science, P.O. Box 7051, Swedish University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden. E-mail address: Madelene.Johansson@lmv.slu.se

Till Ola & Nils

Sammanfattning

Folat är ett B-vitamin som bl.a. levererar byggstenar till kroppens arvsmassa (DNA) och som därför är nödvändigt vid all typ av celldelning t.ex. vid fosterutveckling. Folater är mycket viktigt att få i sig före och under de första graviditetsveckorna då vitaminet skyddar mot fosterskador på neuralröret, vilket kan leda till missfall eller missbildningar i form av ryggmärgsbråck. Det finns även studier som visar att ett högt intag av folater under graviditeten skyddar mot Downs syndrom. Folater krävs också i aminosyrametabolismen, för omvandlingen av homocystein till metionin. Höga halter av homocystein i blodet anses öka risken för att drabbas av hjärt-kärlsjukdom samt öka risken för att utveckla vissa demenssjukdomar som t.ex. Alzheimers sjukdom. Rika folatkällor är inälvsmat, bladgrönsaker, spannmålsprodukter, vissa frukter och bär och mejeriprodukter. I vår svenska kost står spannmålsprodukter för 22% av folatintaget.

Det rekommenderade intaget av folater är 400 µg för kvinnor i barnafödande ålder, 500 µg för gravida och ammande och 300 µg per dag för övriga vuxna enligt den senaste upplagan (2004) av ”Nordiska näringsrekommendationer”. Enligt en kostundersökning från 1998, ”Riksmaten”, ligger svenskarnas folatintag på 215-230 µg per dag. Från myndighetshåll diskuterar man därför en allmän folatberikning av vetemjöl och rågsikt. I dagsläget är endast barnmat i form av välling och gröt obligatoriskt berikad med folsyra. Folsyra är den syntetiska vitaminformen av naturligt förekommande folat som används vid berikning och som vitamintillskott. I USA, där folsyraberikning varit obligatorisk i cerealieprodukter sedan 1998, har man sett en signifikant ökning av folatintaget samt en sänkning av homocysteinhalterna. Antalet födda barn med missbildningar på neuralröret har dessutom minskat med 19%. I dagsläget har 38 länder introducerat obligatorisk folsyraberikning, men inget EU-land är i bland dessa. I Europa befarar man att neurologiska symptom som en följd av vitamin B_{12}-brist ska döljas av höga halter av folsyra.

Folater finns i många olika kemiska former i livsmedel och skillnader mellan deras stabilitet har gjort vitaminet svårt att analysera. Den enda analysmetod som idag kan skilja mellan folatformer är HPLC. Flera HPLC-metoder har utvecklats de senaste åren, men provupparbetningen och känsligheten vid kvantifieringen måste förbättras för att tillförlitliga folathalter ska kunna anges för olika livsmedel. Nyligen har nya HPLC-kolonner utvecklats för att passa till polära föreningar, som folater. I detta arbete har tio stycken HPLC-kolonner studerats med avseende på hur väl de separerar de fem dominerande folatformerna. Dessutom studerades utseendet på de resulterande topparna i HPLC-kromatogrammet. Den bästa separationen, med ett relativt kort analysprogram, sågs på en Aquasil C_{18}-kolonn och denna kolonn användes sedan för att bestämma folathalten i några glutenfria livsmedel. Folater finns i relativt låga koncentrationer i vissa livsmedel och dessa provextrakt behöver renas från störande föreningar för att folathalten ska kunna bestämmas. Flera fastfas (SPE) extraktionskolonner har studerats för olika livsmedelsmatriser och två stycken olika ”reversed-phase” kolonner har visat sig

vara mycket bra. Utöver detta har även enzymbehandlingen och stabiliseringen av folater under provupparbetning optimerats i syfte att uppnå säkrare analysresultat.

Nyligen genomförda populationsstudier har uppskattat antalet personer som lider av glutenintolerans till 1 på 200. Personer med glutenintolerans har en specialkost bestående av glutenfria livsmedel. I två svenska studier har det visats att personer med glutenintolerans får i sig mindre folat än den genomsnittlige svensken. I en av dessa studier spekulerades det i att orsaken till det låga folatintaget var den låga folathalten hos glutenfritt bröd. Några olika glutenfria produkter har därför analyserats med avseende på folathalten. Resultaten visar att folathalten i glutenfria produkter är betydligt lägre än i motsvarande produkter med gluten. Vi rekommenderar därför att man även berikar dessa livsmedel om berikning av folsyra införs i Sverige.

Förutom att känna till folathalten i olika livsmedel, behöver vi också veta hur mycket av det intagna vitaminet som kroppen kan tillgodogöra sig. I två olika humanstudier har detta undersökts inom ramen för detta forskningsprojekt. I en korttidsstudie med nio ileostomister har folatupptaget från olika folatkällor studerats. Absorptionen av folsyra i folsyraberikat bröd har jämförts med i livsmedel naturligt förekommande folatformer dels som farmaceutiskt preparat dels som en naturlig del av jästflingor. Absorptionen av folsyra i berikat bröd uppmättes till ca 80%. Upptaget av folsyra i brödet var dock något lägre än den från samma mängd folsyra i form av ett kosttillskott och de naturliga folaterna i jästflingor. Jästsvampar med hög folatproduktion skulle kunna användas för att ta fram folatrika livsmedel. I en långtidsinterventionsstudie över tre månader bekräftades resultaten från korttidsstudien, d.v.s. att folsyra från berikat bröd absorberas väl i kroppen. I interventionsstudien studerades effekten av daglig folsyrakonsumtion i form av berikat bröd hos 29 kvinnor. Kvinnorna åt en folsyraberikad fralla varje dag, men ändrade förövrigt inte sin kost. Folatstatusen hos kvinnorna bestämdes genom att koncentrationen av folat och homocystein i blodet uppmättes. Vi upptäckte att ett dagligt folsyraintag på 170 μg var tillräckligt för att höja folatstatusen hos kvinnorna genom att deras homocysteinkoncentrationer i blodet sänktes signifikant ($p < 0.01$) med 20% samtidigt som koncentrationen av folat ökade signifikant ($p < 0.001$) med 30% i de röda blodkropparna. Vi anser därför att bröd är ett lämpligt livsmedel för folsyraberikning då vi har visat att den tillsatta folsyran tas upp i kroppen och har positiv effekt på folatstatusen. I USA berikar man alla cerealieprodukter med 140 μg/100 g produkt. Kvinnor i Sverige äter dagligen ca 100-150 g av livsmedel baserade på vetemjöl och rågsikt. Om dessa livsmedel berikades med folsyra i samma halt som i USA, skulle svenska kvinnor få i sig mellan 140-210 μg mer folsyra per dag och tillsammans med sitt naturliga folatintag från övriga kosten nå det önskvärda folatintaget på 400 μg. Vi fann att vid bakning av frallor uppgick förlusterna av folsyra till 12-25%. Därför måste mängden folsyra anpassas så att förväntade processförluster kompenseras vid berikning i mjöl.

Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Johansson, M., Jastrebova, J., Grahn, A. and Jägerstad, M. Separation of dietary folates by reversed-phase high performance liquid chromatography: comparison of conventional and alternative silica based stationary phases. *Submitted*

II. Nilsson, C., Johansson M., Yazynina, E., Strålsjö, L. and Jastrebova, J. (2004) Solid-phase extraction for HPLC analysis of dietary folates. *European Food Research and Technology 219: 199-204*

III. Yazynina, E., Jastrebova, J., Johansson, M. and Jägerstad M. Folates in gluten-free cereals and starch products. *Manuscript to be submitted*

IV. Witthöft, C. M., Arkbåge, K., Johansson, M., Lundin, E., Berglund, G., Zhang, J-X., Lennernäs, H. and Dainty J. Folate absorption from folate-fortified and processed foods using a human ileostomy model. *Submitted*

V. Johansson, M., Witthöft, C. M., Bruce, Å. and Jägerstad M. (2002) Study of wheat breakfast rolls fortified with folic acid. The effect on folate status in women during a 3-month intervention. *European Journal of Nutrition 41: 279-286*

Madelene Johansson´s contribution to the papers

I. Participation in planning of the experimental work, performance of the analytical work, participation in preparation of evaluating results and writing of the manuscript.

II. Performance of parts of the analytical work, participation in writing the manuscript.

III. Development of experimental methods used (extraction procedures, enzymatic treatment, purification and quantification), preparation of the manuscript.

IV. Participation in planning and preparation of bread and yeast test foods, performance of parts of the human and analytical work, participation in writing the manuscript.

V. Participation in planning and designing human experiment, organisation of information meetings for subjects and involved staff, coordination of sampling days and analyses of clinical samples, production, distribution and analyses of breakfast rolls, evaluation of results, preparation of the manuscript.

List of abbreviations

10-HCO-folic acid	10-formylfolic acid
10-HCO-H_4folate	10-formyl-tetrahydrofolate
5,10-CH^+-H_4folate	5,10-methylene-tetrahydrofolate
5,10-CH_2-H_4folate	5,10-methenyl-tetrahydrofolate
5-CH_3-H_4folate	5-methyl-tetrahydrofolate
5-HCO-H_4folate	5-formyl-tetrahydrofolate
ACN	acetonitrile
A*s*	peak asymmetry
AUC	area under the (plasma concentration) curve
CH	cyclohexyl
Ches	2-(N-cyclohexylamino)-ethanesulfonic acid
CP	chicken pancreas
DMP	2,3-dimercapto-1-propanol
DTT	dithiothreitol
EC	endcapped
Ex	excitation wavelength
Em	emission wavelength
FBP	folate binding-protein
FLD	fluorescence detector
FFQ	frequency questionnaire
GC	gas chromatography
GI	gastrointestinal
H_2folate	dihydrofolate
H_4folate	tetrahydrofolate
Hepes	N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid
HK	hog kidney
HPLC	high performance liquid chromatography
i.m	intramuscular
LC	liquid chromatography
LOQ	limit of quantification
MA	microbiological assay
MCE	2-mercaptoethanol
MS	mass spectrometry
MTHFR	methylene tetrahydrofolate reductase
NTD	neural tube defect
PBA	protein-binding assay
PH	phenyl
PteGlu	pteroylmono-γ-L-glutamic acid, folic acid
$PteGlu_3$	pteroyltri-γ-L-glutamic acid
RP	rat plasma
RS	rat serum
RPBA	radio protein-binding assay
r^2	coefficient of correlation
RT	retention time
SAX	strong anion exchange
SD	standard deviation
SPE	solid phase extraction
TBAP	tetrabutylammonium phosphate
tHcy	total homocysteine
UV	ultraviolet
$w_{0.5}$	peak width at half-height

Introduction

Folate is a water-soluble B-vitamin essential for a wide range of biochemical pathways which acts as a carbon donor and acceptor. In particular, folate plays an essential role in cell replication and pregnancy because it is required for synthesis of purines and pyrimidines, the building blocks of DNA. Marked protection against neural-tube defects has been shown in women with good folate status before conception (MRC Vitamin Study Research Group, 1991; Honein *et al.*, 2001; Liu *et al.*, 2004). The remethylation of homocysteine to methionine is dependent on an adequate supply of folate. Thus, low folate status results in elevated homocysteine concentrations which might be a risk factor or marker of cardiovascular disease (Brattström & Wilcken, 2000; Mangoni & Jackson, 2002).

Folate exists in many different chemical forms in foods and differences in its stability have led to difficulties in characterising the vitamin and establishing accurate data on food folate content. The most frequently used folate quantification methods are microbiological assays (MA), protein-binding assays (PBA) and high performance liquid chromatography (HPLC). However, there is today no method with a status as reference method for the measurement of natural folate in foods since all methods are complicated by difficulties in sample preparation, including extraction, deconjugation and purification (Martin, 1995; Vahteristo, 1998). Currently, the only method to differentiate between folate forms is HPLC. Differentiation between folate forms is important since the individual folate derivatives show different stability (O'Broin *et al.*, 1975) and bioavailability (Gregory *et al.*, 1992). Despite much progress in development of improved HPLC methods for folate, there is still a need to improve detection limits and selectivity when analysing complex matrices such as cereals with extremely low level of folate (Ruggeri *et al.*, 1999; Kariluoto, Vahteristo & Piironen, 2001).

Cereals are important sources of vitamin E and B. When white flour is produced, the vitamins in the whole-grain are fractionated into the bran and germ fractions so that white flour contains less then half the level of vitamins present in the whole-grain (Håkansson *et al.*, 1987). In Sweden, flour is fortified with thiamine, riboflavin, niacin and pyridoxine. Folate, however, is not compensated for, although this vitamin is lost during processing of flour. In the US and Canada, folic acid fortification has been mandatory for cereal products since 1998 and this has resulted in a significant increase of the mean folate intake (Quinlivan & Gregory, 2003; Liu *et al.*, 2004). In Europe, however, mandatory action has not been taken.

This thesis, therefore, focuses on cereals and cereal products as dietary sources of native folate and vehicle for folic acid fortification. The thesis consists of three parts: The first reviews the literature on folate concerning chemistry, occurrence, bioavailability and analyses. In the second part the development and validation of an HPLC method for quantification of food folate is presented and applied on starch and bread products. Using two different human models, folate absorption from folic acid-fortified bread is investigated. The third and last part consists of five papers, which together form the basis of this thesis.

Folate review

Nomenclature, chemical structure, stability and solubility

The generic term "folate" should be used for the class of compounds with chemical structure and nutritional activity similar to that of pteroyl-L-glutamic acid (folic acid, PteGlu) according to recommendations from IUPAC-IUB Joint Commission on Biochemical Nomenclature 1986 (Blakley, 1988). More than 100 analogues based on folic acid exist in nature (Gregory, 1989) but few analogues are stable enough to be isolated for full chemical analysis.

Folate consists of a pteridine ring attached to a para-aminobenzoate, which in turn is linked to L-glutamate (Figure 1). In folic acid, the pteridine moiety is fully oxidized. In naturally occurring folate the pteridine ring is partially reduced at the 7, 8-position (H_2folate) or fully reduced (H_4folate). Reduced folate can exist with or without one-carbon substituents. Substituents can exist on either the N^5 or N^{10} positions (predominantly as methyl, formyl or formino groups), or as methylene or methenyl units bridging between N^5 and N^{10}. All fully reduced folate has two chiral centres: the α-C atom in the glutamic acid moiety and the C atom in position 6 of the pteroyl moiety (Figure 1). Four possibilities of diastereoisomers exist which are [6S, αS], [6S, αR], [6R, αS], [6R, αR]. The naturally occurring diastereoisomer of H_4folate and its 5-HCO- and 5-CH_3-derivatives are the [6S, αS] diastereoisomers, whereas the natural diastereoisomers of 10-HCO-, 5, 10-CH_2- and 5,10-CH^+-H_4folate are [6R, αS] diastereoisomers. It has been agreed that all natural diastereoisomers of reduced folate should be defined as L-diastereoisomers and all unnatural ones as D-diastereoisomers (Groehn & Moser, 1999). Most naturally occurring folate has a side chain of 3 to 11 glutamate residues with γ-peptide linkage (Gregory, 1996; Scott, Rébeille & Fletcher, 2000). It is generally assumed that approximately 80% of the food folate exists in polyglutamyl form. Folic acid is not present in biological systems but it is the form used in pharmaceutical and fortified food products (Gregory, 1996).

Large differences in stability exist among the various H_4folate as a result of the influence of the one-carbon substituents on susceptibility to oxidative degradation, their thermal stability and the pH dependency. Folic acid exhibits greater oxidative stability than the H_4folate or H_2folate (Hawkes & Villota, 1989; Ball, 1998). The order of stability of H_4folate forms in aqueous solutions is 5-HCO-H_4folate > 5-CH_3-H_4folate > 10-HCO-H_4folate > H_4folate since substitution at N^5 and N^{10} position increases oxidative stability (Gregory, 1989; Mullin & Duch, 1992; Eitenmiller & Landén, 1999). Stability of each folate is dictated only by the chemical nature of the pteridine ring system with no influence from the polyglutamyl chain length. Folate degradation generally involves changes at the C^9-N^{10} bond, the pteridine ring system or both (Gregory, 1996). It is known that oxidative cleavage of H_4folate, H_2folate and folic acid yield nutritionally inactive products (*p*-aminobenzoylglutamate and a pterin) (Maruyama, Shiota & Krumdieck, 1978; Tannenbaum, Young & Archer, 1985; Gregory, 1996).

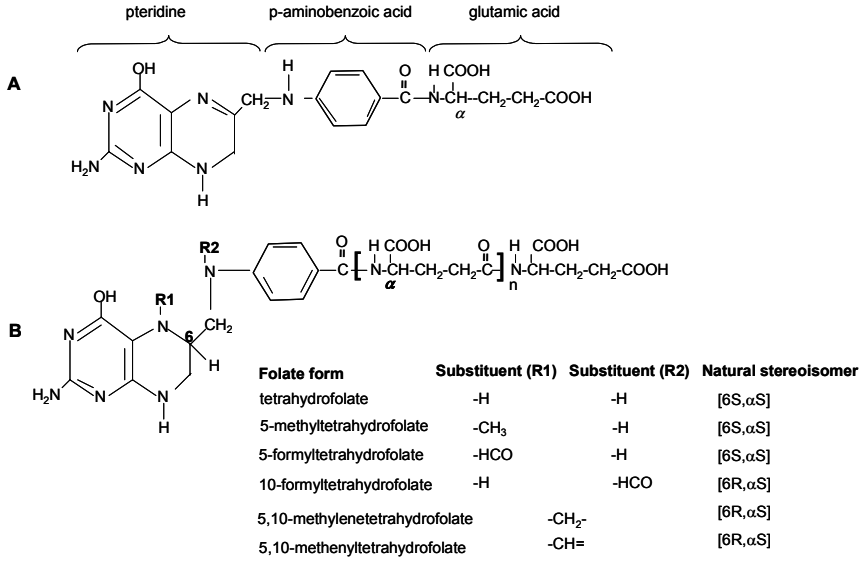

Figure 1. The folate molecules: A, Folic acid, B, Polyglutamyl tetrahydrofolate

Folate undergoes interconversion in ionic form as a function of pH. In acidic pH 10-HCO-H_4folate tends to form 5, 10-CH^+-H_4folate. 5-HCO-H_4folate also forms 5,10-CH^+-H_4folate in acidic media (pH 1-2) but at a much slower rate (Pfeiffer, Rogers & Gregory, 1997a). When a solution of 5,10-CH^+-H_4folate is neutralized, hydrolysis occurs to yield 10-HCO-H_4folate, and a small amount of 5-HCO-H_4folate (Gregory, 1989). 10-HCO-H_4folate is completely oxidised to 10-HCO-H_2folate and 10-HCO-folic acid during food preparation (Konings, 1999). 5,10-CH_2-H_4folate is readily dissociated to H_4folate at acidic, near neutral pH (Horne, 2001). 5-CH_3-H_4folate has been reported to have an oxidation rate of about one tenth of that of H_4folate (Hawkes & Villota, 1989). 5-CH_3-H_4folate is oxidised to 5-CH_3-H_2folate which is further decomposed slowly to unidentified products (Maruyama, Shiota & Krumdieck, 1978).

The rate of folate degradation depends on the folate form and the food matrix, particularly with respect to pH, buffer composition, catalytic trace elements and antioxidants (Gregory, 1989). Oxidation of H_4folate may be retarded or prevented by sufficient amounts of antioxidants *e.g.* ascorbic acid and thiols (Blakley, 1960).

Folate exhibits minimum solubility in mildly acidic solvents (pH 2 to 4), where monocationic and neutral forms dominate. Solubility increases above pH 4 where anionic species increase in concentration. Folate is soluble at very low pH since cationic species are the dominant form (Gregory, 1989). At neutral to alkaline pH levels, polyglutamyl folate is more soluble than folic acid due to the presence of additional ionisable α-carboxyl groups. Long chain polyglutamyl folate is more

hydrophobic than short-chain polyglutamyl folate at low pH levels where the α-carboxyl groups are predominantly protonated (Gregory, 1989).

Folate nutrition

Absorption

In the human, the entire small intestine is capable of absorbing folate monoglutamates but absorption mainly occurs in the jejunum (Gregory, 1996; Ball, 1998). In order to absorb dietary folate (which exists predominantly as polyglutamates), the polyglutamate chain is hydrolysed by the brush-border γ-glutamyl hydrolase (also called folylpolyglutamate carboxypeptidase or folate conjugase) (Chandler, Wang & Charles, 1986; Mullin & Duch, 1992). After cleavage to monoglutamates, the folate is absorbed by a pH dependent carrier-mediated process (pH 6.3). In addition the folate monoglutamates being small water-soluble molecules can diffuse across the membrane. If the folate intake is increased by fortification or supplementation, the diffusion process becomes more important (Ball, 1998; Scott, Rébeille & Fletcher, 2000). If the dietary folate is not already present in the 5-CH_3-H_4folate forms, which is true for most food folate, it will be converted to this form during the transit through the intestinal mucosal cells (Lucock, 2000). Thus, usually the only form entering the human circulation from the intestinal cells is 5-CH_3-H_4folate monoglutamate and folate in human plasma exists almost exclusively in this form (Pfeiffer *et al.*, 2004). Folic acid is reduced to H_2folate and then converted to 5-CH_3-H_4folate during the transit through the gut mucosa (Scott, 1999). However, this reduction process is readily saturated, and Kelly et al. (1997) suggested that significant amounts of folic acid are found in plasma of humans ingesting ~250 µg/dose of supplemental folic acid.

After absorption, 5-CH_3-H_4folate is released into the portal vein. Approximately 10-20% of the absorbed folate is retained by the liver while the rest circulates to other tissues (Gregory, 1995). In humans, the total body content of folate is 5-10 mg, of which half resides in the liver in the form of tetra- penta- hexa- and heptaglutamates of 5-CH_3-H_4folate and 10-HCO-H_4folate (Combs, 1992; Herbert & Das, 1994). Folate undergoes substantial enterohepatic recirculation, with as much as 100 µg folate undergoing biliary excretion and reabsorption each day (Herbert & Das, 1994).

Bioavailability

Bioavailability is defined in many different ways, most of which focus on the efficiency of intestinal absorption. Factors affecting folate bioavailability include species of folate (Gregory *et al.*, 1992; Bostom *et al.*, 2000), length of polyglutamate chain (Konings *et al.*, 2002), concentration of folate (Malinow *et al.*, 1998), food matrix such as dietary fibres (Keagy, Shane & Oace, 1988; Finglas *et al.*, 2002), inhibition of deconjugation by other dietary constituents such as organic acids (Wei & Gregory, 1998), other dietary constituents affecting folate stability during digestion *e.g.* folate-binding proteins (Jones & Nixon, 2002; Arkbåge *et al.*, 2003; Verwei *et al.*, 2003). The bioavailability of folate is also

affected by the nutrient status of the body (Herbert & Das, 1994), host-related factors such as pregnancy (Gregory, 2001), genetic factors (Jacques *et al.*, 1996) and gastro-intestinal diseases (Halsted, 1990). The bioavailability of folate is therefore affected by the net result of release of folate from the food matrix, uptake by the brush border, deconjugation if folate is present as polyglutamates, active transport or diffusion, and finally conversion to 5-CH_3-H_4folate. The bioavailability of folate from a mixed diet is estimated to be about 50% (Gregory, 1995).

Studies of folate bioavailability were initially performed using bioassays. The response to various folate and different folate-containing foods was investigated. However, large differences that exist in the process of digestion and absorption of folate between animals and humans and the relevance of bioassays to folate bioavailability in human nutrition can be questioned (Scott, Rébeille & Fletcher, 2000; Gregory, 2001).

In humans, short-term protocols can be used. The change in area under the plasma response curve (AUC) or urinary folate excretion is then measured, usually after a single folate dose in the form of a pharmaceutical preparation, or a food (Pfeiffer *et al.*, 1997b; Prinz-Langenohl *et al.*, 1999). However, it has been suggested that the AUC method is relatively insensitive, which makes it suitable only for foods with folate concentrations >300 µg/dose, depending on liver stores otherwise no detectable rise in the plasma folate is seen (Gregory, 2001). This is because the absorbed folate is firstly removed into liver tissues. To avoid this, volunteers can be given large oral doses of milligrams of folic acid for days or weeks before the study to saturate the tissues (Gregory, 2001). However, a physiological condition is then not observed. In long-term protocols the impact of repeated folate ingestion on folate status is evaluated by *e.g*. feeding fortified foods, natural food folate or folic acid supplements (Cuskelly, McNulty & Scott, 1996, 1999). Folate concentrations in erythrocytes or fastened serum were quantified. Erythrocytes are used as a measure of folate status since they are not affected by recent dietary intake (Lucock, 2000). Erythrocyte folate is considered to be the best indicator of long-term status since the lifespan of the erythrocyte is 120 days, and folate is retained in the erythrocyte for the duration of its life. Folate is incorporated into the developing erythroblast during erythropoiesis in the marrow and less than 1% of circulating erythrocytes are replaced daily (Gregory, 2001).

Stable isotope protocols to study folate absorption have also been used (Pfeiffer *et al.*, 1997b; Finglas *et al.*, 2002). The bioavailability from isotopically labelled folate in "fortified" foods or pharmaceutical preparations is investigated using LC-MS or GC-MS techniques. The advantage of using stable isotopes is that isotopically labelled folate from the dose can be differentiated from endogenous folate in body fluids.

Wigertz (1997) applied a new model to assess folate bioavailability by using healthy volunteers with ileostomy. Konings *et al.* (2002) improved the model and compared folate absorption based on stomal effluent data with data from plasma AUC. They reported the bioavailability of folate in spinach to be 80% of that of an oral dose of folic acid. Witthöft *et al.* (2003) use an intramuscular injection of

(6S)-5-CH_3-H_4folate as a reference dose when calculating the folate bioavailability from strawberries and broccoli to 65 and 50% respectively. A major advantage of using ileostomists in folate bioavailability studies is that any interference from folate synthesis by microbes in the large intestine can be avoided. Thereby direct folate absorption can be calculated by comparing stomal folate excretion with relative folate absorption from plasma AUC.

Metabolic function

Mammals can synthesise the pteridine ring in the folate molecule but can not conjugate it to the *p*-aminobenzoate and glutamate chain. Therefore mammals need to obtain folate from the diet. Small amounts of folate may also be synthesised by the intestinal flora (Finglas *et al.*, 2003). The major folate form in the plasma, 5-CH_3-H_4folate monoglutamate, is transported into the cell, where it is converted to the polyglutamate form by polyglutamate synthetase (Figure 2). This enzyme uses H_4folate as substrate and therefore the 5-CH_3-H_4folate must be converted to H_4folate prior to polyglutamation (Lucock, 2000). The function of the polyglutamyl chain is two-fold: it is required for the retention of the vitamin in the cell, since the long chain prevents folate from passing through cell membranes, and it enhances the binding of the folate cofactor to folate requiring enzymes (Brody, 1999). The synthetic folic acid existing in supplements and fortified foods will be reduced to H_2folate in the cell and then further to H_4folate by the enzyme dihydrofolate reductase. The resulting H_4folate can take part in all folate.requiring reactions (Scott, 1999).

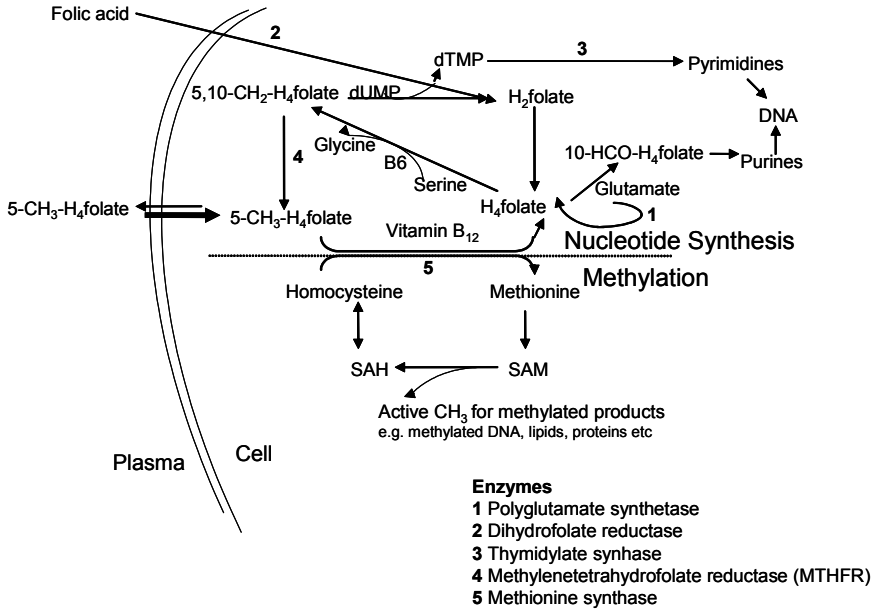

Figure 2. Folate functions in the cell

Folate is cofactor in one-carbon reactions within the cell. These one-carbon transfers occur in two important cycles in both plant and mammalian cells: the nucleotide synthesis cycle and the methylation cycle (Figure 2). This single carbon metabolism is principally mediated by the interconversion of the H_4folate. Folate is essential in the nucleotide synthesis cycle because two of the carbons in the purine ring directly derive from 10-HCO-H_4folate. Thymidylate synthase converts uracil to the pyrimidine base thymine by using the one-carbon unit attached to 5,10-CH_2-H_4folate. (Reviewed by (Scott, 1999).

Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-CH_2-H_4folate to 5-CH_3-H_4folate, which donates a methyl group to homocysteine to generate methionine in the so-called methylation cycle (Figure 2). This reaction is catalysed by methionine synthase and requires vitamin B_{12} as a cofactor. Methionine can then be utilized to synthesise S-adenosylmethionine, a primary methylating agent which is required for a multitude of methylation reactions including DNA and protein methylation, phospholipid synthesis and neurotransmitter synthesis (Rozen, 2004). When vitamin B_{12} is deficient an accumulation of homocysteine and 5-CH_3-H_4folate occurs, blocking the recycling of H_4folate; this event is referred to as the methyl-folate trap.

Status, deficiency and health

Since folate play an integral role in the *in novo* synthesis of DNA, a good folate status is essential to maintain a normal rate of cell division. Two of the first symptoms of folate deficiency are macrocytic anaemia and GI mucosa deterioration as a result of decreased replacement of red blood and mucosa cells. Inadequate intake of folate is possibly the main cause, but folate deficiency may also result from impaired absorption for example caused by coeliac disease. People suffering from coeliac disease react; their small intestine is inflamed, if gluten is digested (Feighery, 1999; Murray, 1999). Gluten intake cause atrophy of the intestinal villi and seriously reduces the absorptive surface of the intestinal tract. Disaccharides, *e.g.* lactase, and the carrier molecules normally found on the villi disappear leading to malabsorption of several important nutrients including iron, folate, calcium and fat-soluble vitamins (Whitney, Cataldo & Rolfes, 1994). Deficiency can occur wherever cell multiplication must increase: in pregnancies especially involving twins, during cancer, in skin-destroying diseases, in burns, blood loss and GI tract damage (Whitney, Cataldo & Rolfes, 1994).

Increased folate intake during the first weeks of pregnancy has been shown to decrease the risk of birth defects such as neural tube defects (NTD) (Czeizel & Dudas, 1992; Berry *et al.*, 1999; Honein *et al.*, 2001; Liu *et al.*, 2004). Worldwide, between 300,000-400,000 infants are born each year with NTD (Whitney, Cataldo & Rolfes, 1994). In Sweden, the NTD frequency is 9-11 per 10,000 pregnancies, when elective abortions are included. This estimate is slightly higher than in Norway: 8 per 10,000 pregnancies and Finland: 7.5 per 10,000 pregnancies, but lower than in South America where the NTD frequency is 21 per 10,000 pregnancies (Personal communication G. Annerén). Prevalence of NTD babies born with *spina bifida* and anencephaly, the two most common forms of NTDs, may vary as a function of ethnical and racial background. In the US, higher rates

are seen among hispanics and whites than among blacks (Botto *et al.*, 1999). However, in northern China the rate of NTD is 48 per 10,000 pregnancies, whereas in the south it is 10 per 10,000. Because the ethnical background in both areas is the same other factors may have a role in the risk of having a foetus or infant with NTD (Berry *et al.*, 1999). As a result of the findings of the relationship between folate intake and decreased NTD risk, the Food and Drug Administration in the US started mandatory folic acid fortification in cereal grain products in 1998, in an effort to help women increase their consumption of folic acid. Increased consumption of folic acid might also protect against other adverse pregnancy outcomes including cleft lip, Down´s syndrome, low birth weight and miscarriage (Lucock, 2000; George *et al.*, 2002; Caudill, 2004).

Plasma total homocysteine (tHcy) concentrations are used as an indicator of folate status since the remethylation of homocysteine to methionine by methylenetetrahydrofolate reductase (MTHFR) is dependent on an adequate supply of folate. Thus, low folate status results in elevated tHcy concentrations causing mild hyperhomocysteinaemia (Kang, Wong & Norusis, 1987) which might be a risk factor, for cardiovascular disease (Brattström & Wilcken, 2000; Mangoni & Jackson, 2002). Over 100 studies have investigated the relationship between moderately elevated homocysteine concentrations and cardiovascular disease and Wald, Law & Morris (2002) in a meta-analysis including 16 prospective studies reported a 23% increase in heart disease risk for every 5 μmol/L increase of plasma homocysteine. Interestingly, several studies found no or only a weak association whereas others found a stronger relationship. Currently, several large-scale clinical trials in the United States, Europe, Canada and Australia are being carried out to examine how lowering plasma homocysteine levels affects cardiovascular disease. However, supplementation with folic acid, even at low doses, leads to a reduction in plasma homocysteine concentrations in healthy subjects (Schorah *et al.*, 1998; Brouwer *et al.*, 1999).

Several inherited enzyme polymorphisms involved in the folate metabolism in the body have been discovered during the past 10 years (Finglas *et al.*, 2003). One of the most studied enzymes is 5,10-methylenetetrahydrofolate reductase (MTHRF) (Figure 2), and it is now known as a common polymorphism affecting about 10% of the population (Lucock, 2000). This polymorphism is a thermo-labile enzyme variant, which causes reduction of the enzyme activity to less than 20% (Rozen, 2004). The mutation is found when a substitution of C to T at nucleotide 677 has occurred. This mutation causes elevated serum homocysteine concentrations and sometimes low serum folate concentrations for people homozygous for the thermo-labile variant (Molloy *et al.*, 1997; Caudill, 2004).

Folate insufficiency has also been linked to several cancers including colon, colorectal, breast, pancreas, brain and lung cancers (Choi & Mason, 2000). The relationship between folate intake and cancer is most clearly defined for colorectal cancer (Bailey, Rampersaud & Kauwell, 2003). Low blood levels of folate and elevated homocysteine levels have also been associated with Alzheimer's disease (Lucock, 2000; Seshadri *et al.*, 2002).

Estimated and recommended intake

In Sweden, the recommended daily intake of dietary folate is 300 µg for adults but 400 µg for fertile women (Becker *et al.*, 2004). Pregnant women are recommended a daily intake of 500 µg from the start of the pregnancy. Also lactating women are also recommended a daily intake of 500 µg. Both FAO/WHO (2002) and the US authorities (Yates, Schlicker & Suitor, 1998) recommend all adults a daily intake of 400 µg, pregnant women 600 µg and lactating women 500 µg. In order to prevent neural tube defects, Swedish women who wish to become pregnant are advised to increase their dietary folate intake by eating more vegetables and fruits (Socialstyrelsens meddelande blad nr 1/2001, 2001). They can also increase their intake by taking folic acid supplements. Folic acid can mask the diagnosis of neurological damage caused by vitamin B_{12} deficiency (Scott, Rébeille & Fletcher, 2000). This is why the Nordic nutritional recommendations have set the tolerable upper intake level of folic acid at 1 mg/day (Becker *et al.*, 2004) which is similar to that set by the FAO/WHO as well as the US Institute of Medicine (Yates, Schlicker & Suitor, 1998; FAO/WHO, 2002).

According to the dietary survey "Riksmaten" performed in 1997-98 Swedish women have an overall daily folate intake of 217 µg while Swedish men have an intake of 232 µg/day (Becker, 1999). These figures were almost the same as those in the previous Swedish dietary survey "Hulk" from 1989. In a review by de Bree *et al.* (1997) it is stated that the Swedish females together with British and Irish females, have the lowest mean folate intake in Europe.

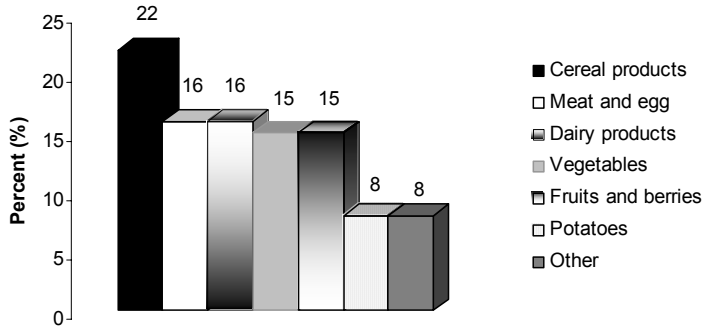

Figure 3. The contribution of different foods to the folate intake of the Swedish population.

Folate is present in a wide range of foods. Especially leafy green vegetables, legumes, yeast and liver are rich sources of folate (Witthöft *et al.*, 1999). However, cereal products, vegetables, dairy products, fruits and potatoes appear to be the most significant sources of folate in the Swedish diet (Becker, 2000). The food folate contribution (%) to the Swedish population is presented in Figure 3. Since only a few percent of the women reach a folate intake of 400 µg/day, Swedish authorities are discussing how to increase the folate intake. One strategy

to increase the population's folate intake is to recommend them to eat folate-rich foods such as spinach and orange juice. However, the compliance to a diet with high amounts of fruit and vegetables appears to be low in Sweden, as in many countries (Becker, 1999, 2000). Another is therefore to increase the population´s folate intake through their normal diet *e.g.* by producing foods that contain higher content of natural folate by adding folate-rich cereal fractions, such as bran and germs, or by fermentation. Today non-fortified cereal products contribute considerably to the folate intake in the Swedish diet (Becker, 2000) and can therefore be an important dietary source when trying to increase folate consumption. A third option could be to introduce mandatory folic acid fortification in *e.g*. wheat flour and sifted rye flour.

Folate in cereals and cereal products

The Swedish survey "Riksmaten" demonstrated that cereals and cereal products are the predominant sources of folate in the Swedish population, providing approximately 22% of the daily intake (Figure 3). This proportion is very similar in several European countries (de Bree *et al.*, 1997). In Table 1, folate content in common cereals and cereal products from the Swedish Food Composition Table (2002) are shown (Livsmedelsverket, 2002). The figures should not be interpreted as absolute values since variations in varieties, condition of growth and maturity can influence the nutritional value as stated in the Food table. Furthermore, the values have been obtained using different quantification methods.

According to the Swedish Food Composition Table wheat flour contains 20 µg total folate/100 g, while white bread such as the Swedish teacake contains 33 µg/100 g. The increase is attributed to the yeast added to bread. Keagy, Stokstad & Fellers (1975) estimate that as much as 65% of folate in wheat bread derives from yeast.

Osseyi, Wehling & Albrecht (2001) showed the distribution of endogenous folate during the bread baking procedure. In commercial bread flour (48 µg folate/100 g dry basis), containing wheat flour and malted barley, the distribution of folate derivatives were: 60% 5-HCO-H_4folate, 17% 10-HCO-folate, 17% H_4folate and 6% 5-CH_3-H_4folate. In the final bread the total folate content had increased to 56 µg/100 g and the distribution of folate forms had changed. The content of 5-CH_3-H_4folate had increased 8-fold while the 5-HCO-H_4folate content had decreased by half, the other forms being unaffected. However, Kariluoto *et al* (2004) showed a different distribution of folate forms when baking sour-dough rye bread. Practically no H_4folate was detected and a decrease of 5-CH_3-H_4folate was observed. Several studies have reported that the losses in reduced folate during the baking stage at 220-240 ºC is 20-32% (Keagy, Stokstad & Fellers, 1975; Osseyi, Wehling & Albrecht, 2001; Kariluoto *et al.*, 2004). Folic acid added to the flour before the baking procedure has however, shown smaller losses (8-20%) during the baking stage (Keagy, Stokstad & Fellers, 1975; Osseyi, Wehling & Albrecht, 2001).

Table 1. *Total folate content in cereals and related products*[a]

Cereals and related products	**Folate concentration in µg/100 g edible part**
Rich sources (>100 µg/100 g)	
Yeast bakers compressed[b,c]	1000
Wheat germs	330
Wheat bran	260
Mora Brungräddat crispbread, rye	104
Medium sources (10-100 µg/100 g)	
Falu råg-rut crispbread, rye	83
Oats rolled with roasted wheat bran	82
Croissant	75
Oats rolled and oat flour	56
Rye flour whole	56
Brown rice	53
Oat bran	52
Gluten-free crispbread	48
Wheat flour wholemeal	44
Buckwheat whole or crushed grains	40
Wheat crushed grain or flakes	40
Rye bread	35
White bread, Swedish teacake	33
Sweet wheat bread	30
Gluten-free dark brown bread	28
Gluten-free light brown bread	26
Oat crunches	22
Couscous	20
Wheat flour	20
Corn flakes	20
White bread, Pita bread	18
Rice precooked	13
Pasta	12
Poor sources (<10 µg/100 g)	
Wafers plain	9
Oat meal porridge	8
Taco shells	5
Brown rice boiled	4
Rice precooked, boiled	4
Rice flour	4
Pasta, boiled	4
Corn starch	0
Beer	0

[a](Livsmedelsverket, 2002), [b]Fresh weight, [c]An important folate source in some cereal products.

In as early as 1975 Keagy, Stokstad and Fellers reported that the folate concentration in bread is independent of whether straight-dough or sponge-dough methods are used, which was confirmed by Kariluoto *et al.* (2004). The folate content can differ 10-fold in different parts of the kernel and the highest concentration is known to be in the bran fraction. This is seen in Table 1 when comparing folate content in wheat bran and flour.

During the last ten years wheat aleurone flour has been commercially available in Australia. It has the potential to make an important contribution to the folate intake, with a folate content between 400 and 600 µg/100 g (Fenech *et al.*, 1999).

Various thermal processing conditions have different effects on folate stability. For example, popping whole-wheat grains appears to cause much greater folate loss than autoclaving or steam flaking (Håkansson *et al.*, 1987). Storage studies of flour indicated losses of native folate up to 40% at 49 ºC and 15% at 29 ºC after 12 weeks storage (Keagy, Stokstad & Fellers, 1975). However, added folic acid seems to be stable in stored flour. At 28 ºC and 38 ºC only small losses of folic acid were seen during storage for 52 weeks (Keagy, Stokstad & Fellers, 1975).

Kariluoto *et al*. (2004) investigated variation in folate concentrations between different rye varieties; grown in the same year and on the same location and they found a variation of 10%. Three studies have been performed to analyse the folate content in different wheat varieties; in two studies it was shown that soft wheat contains higher levels of folate compared to hard varieties (Mullin & Jui, 1986; Arcot *et al.*, 2002). In the third study, flour from hard wheat contained the highest folate concentration (Keagy *et al.*, 1980). Hard varieties are usually used for bread making.

Boiled pasta is considered a poor folate contributor (Table 1). However, Ranhotra *et al.* (1985) investigated the retention of folate in several pasta products and found a retention of 54-100% after cooking. Both boiled brown rice and precooked rice contain 4 µg folate/100 g, while differences in the unboiled are considerable. Folate may be leaking into the cooking water and may be destroyed by heat (Ball, 1998).

According to the Swedish Food Composition Table (Table 1) beer contains no folate. However, in a recent study within the EU project: Folate: From Food to Functionality and Optimal Health (QLRT-1999-00576) a survey on a total of 120 beer varieties was carried out and on average the beers contained 80 µg/L ranging between 30-180 µg/L. The folate content was related to the alcohol since higher alcohol content requires more cereal. Beers produced from wheat contained higher content of folate compared to that produced from barley. During germination/malting, of the grain, folate is synthesised because it is needed for growth and cell differentiation. The folate content can increase 2-4 fold using optimised temperature during malting (Liukkonen *et al.*, 2003; Jägerstad *et al,*in press).

Fortification

Mandatory folic acid fortification has been introduced in several countries, namely in South America, Canada and the United States (Honein *et al.*, 2001; Darnton-Hill & Nalubola, 2002). In the United States, the added amount of 140 μg per 100 g grain product was estimated to increase folate intake by 100 μg folic acid/day for women of childbearing age (Mills & England, 2001). In the US population, a substantial improvement in folate status and decrease in homocysteine levels have been demonstrated (Jacques *et al.*, 1999). Furthermore, the frequency of NTD birth prevalence has been reduced by 19% (Honein *et al.*, 2001). In Newfoundland, Canada, the NTD frequency decreased by 78% after fortification was introduced; from an average of 4.36 per 1000 NTD birth in 1991-1997 to 0.96 per 1000 NTD births during 1998-2001 (Liu *et al.*, 2004). However, in the US it is now suggested that folic acid consumption from fortified products is > 200 μg/day. This folic acid intake is approximately twice the amount that was aimed for (Choumenkovitch *et al.*, 2002; Quinlivan & Gregory, 2003). This may be due to a wide-scale overfortification since Rader, Weaver and Angyal (2000) observed that fortified products typically contain 150-175% of their predicted folate content. Lewis *et al.*, (1999) estimated that 15-25% of children aged 1-8 years and 0.5-5% of the adults has an intake above 1 mg as dietary folate equivalent (DFE) per day (upper tolerable level).(1 DFE=1 μg food folate=0.6 μg folic acid consumed with food=0.5 μg folic acid taken on empty stomach (Yates *et al.* 1998)). Folic acid is retained and metabolised independently of vitamin B_{12} in the cell and a high intake of folic acid can prevent folate deficiency and thereby delay diagnosis of an underlying vitamin B_{12} deficiency. Folic acid has historically been used as a food fortificant because it is inexpensive to synthesise and relatively stable. Intake of natural 5-CH_3-H_4folate is not masking B_{12} deficiency, but it is much more expensive to synthesise and less stable than folic acid. Furthermore it was just recently that only the 6S-form could be synthesised; previously always, a 50:50 mixture of 6S and 6R was produced (Wright, Finglas & Southon, 2001). Ericson, Källén & Åberg (2001) interpreted results from an epidemiological study that folic acid supplementation increased the frequency of giving birth to twins. Twins are more likely to be premature and to have low birth weight. An increase of twin pregnancies in the US, after folic acid fortification was introduced, has however, not been confirmed (Shaw *et al.*, 2003; Waller, Tita & Annegers, 2003).

Nutritional aspects of folate in cereals

Considering the importance of cereals regarding the daily intake of folate, studies on bioavailability of folate or folic acid in cereals are still few. There are, however, some studies investigating the absorption of folic acid in fortified cereal grain products (Colman, Green & Metz, 1975; Margo *et al.*, 1975; Pfeiffer *et al.*, 1997b; Malinow *et al.*, 1998; Schorah *et al.*, 1998; Finglas *et al.*, 2002) and some on the bioavailability of natural cereal folate (Fenech *et al.*, 1999; Vahteristo *et al.*, 2002). Colman, Green & Metz (1975), Margo *et al.* (1975) and Finglas *et al.* (2002) estimate that the bioavailability of folic acid as fortificant in cereal products is incomplete due matrix effects, but this hypothesis is not supported by Pfeiffer *et al.* (1997b). Fenech *et al.* (1999) show a good short-term (7 h)

bioavailability of natural folate from novel wheat aleurone flour in humans. Vahteristo *et al.* (2002) estimate the bioavailability of natural folate from different rye products and orange juice to be similar to that of folic acid from fortified wheat bread.

Malabsorption caused by diseases affecting the jejunal mucosa can also affect folate absorption. Recent population studies have estimated that coeliac disease may affect 1 individual in 200 (Feighery, 1999; Murray, 1999; Gallagher, Gormley & Arendt, 2004) but remarkably few studies have questioned whether the composition of a strict gluten-free diet is nutritionally balanced. Thompson (2000) and Hallert *et al.* (2002) conclude that coeliac patients might be vulnerable to inadequate intake of folate and Grehn *et al.* (2001) estimate that both women and men with coeliac disease have a low daily folate intake, 186 µg/day and 172 µg/day respectively. This was confirmed by Hallert *et al.* (2002) and they suggested the reason for lower folate intake among coeliac patients to be that gluten-free bread contains significantly less folate compared with bread consumed by the general population. Also, in the US, gluten-free cereal products are generally not enriched with folic acid (Thompson, 2000).

Folate analysis

The existence of folate in many different chemical forms in foods and differences in their stability has led to considerable difficulties in characterizing the vitamin and establishing accurate data for food folate. Currently, the three most frequently used folate quantification methods are microbiological assay, competitive protein-binding procedures and high performance liquid chromatography (HPLC). None of these methods has official status as reference method for the measurement of natural folate in foods since they all are subject to error, as well as being complicated by sample preparation, including extraction, deconjugation and purification (Martin, 1995; Vahteristo, 1998).

Extraction of folate from food matrix

Folate is usually extracted from the food matrix using heat treatment in buffer with added antioxidants. The buffers commonly have a pH close to neutral or mildly acidic/alkaline to stabilise the folate analogues. Each vitamer has its own unique pH stability profile, and it is therefore sometimes difficult to optimise extraction conditions for several folate forms in a single extraction procedure (Vahteristo & Finglas, 2000). Several folate derivatives can interconvert during extraction. Homogenisation and heat treatment (5 to 60 min at 100 °C or 120 °C) disrupt the cellular structure and the folate is released to the buffer (Ball, 1998). Freeze drying prior to extraction is sometimes performed (Gregory, 1996). Ascorbic acid is the most commonly used antioxidant but 2-mercaptoethanol, dithiothreitol and 2,3-dimercapto-1-propanol, can be used in addition as well (Blakley, 1969; Vahteristo & Finglas, 2000). Exclusion of oxygen by adding nitrogen gas to the extract prior homogenisation or heating as well as cooling and shelter from light improves the folate stability (Gounelle, Ladjimi & Prognon, 1989).

Enzyme treatment

Food folate is mostly present as poly-γ-glutamates. The glutamyl residues can be hydrolysed with γ-glutamylhydrolase (conjugase) (EC 3.4.22.12). Chicken pancreas and hog kidney are the two most common sources of conjugase enzyme, although human plasma and rat plasma, or serum also has been used. Conjugases from different sources differ in their mode of action, end product and pH optima. The end product of chicken pancreas conjugase treatment is mainly diglutamates while hog kidney and plasma conjugases produce monoglutamates. Since *Lactobacillus rhamnosus* responds equally to mono- and diglutamates, chicken pancreas is usually used as conjugase source during microbiological assay since it is readily available and highly active. For most HPLC methods, which separate individual folate monoglutamates, a conjugase producing folate monoglutamates as end product must be applied. The type and activity of the conjugase and conditions during deconjugation process, pH, time and temperature and the nature of the sample can all affect this step which crucially affects outcome of analysis. The efficiency of the conjugase can also be affected by components in the food extract (Reviewed by Scott, Rébeillé and Fletcher (2000)).

De Souza & Eitenmiller (1990) used different enzyme treatments including conjugase, α-amylase and protease and they observed significant increases in measurable folate by using the trienzyme combination prior to microbiological and protein-binding assays. Rader, Weaver & Angyal (1998) determine total folate, by MA, in 56 cereal-based enriched foods after trienzyme treatment. In 30% of the products the concentrations of folate were significantly higher after trienzyme treatment than with only conjugase treatment. Several researchers have confirmed these findings in different food sources (Aiso & Tamura, 1998; DeVries *et al.*, 2001; Johnston, Lofgren & Tamura, 2002; Yon & Hyun, 2003) while others have not seen these effects (Shrestha, Arcot & Paterson, 2000; Ndaw *et al.*, 2001). However, today there is no standardisation about which foods require trienzyme treatment and how this should be performed *i.e.,* in what order the enzymes should be added, at which pH, which enzyme source should be used, at what temperature and for how long the treatment should be carried out. One disadvantage of the enzymatic extraction method is the formation of a large number of UV-absorbing compounds during the enzymatic hydrolysis. These can interfere with the quantification of the folate peaks. However, Pfeiffer, Rogers & Gregory (1997a) used trienzyme treatment successfully prior to HPLC analysis in combination with a purification method based on affinity chromatography.

It is important during sample pre-treatment to consider that protease and conjugase preparations contain endogenous folate, which results in high background values if not properly purified before use. When calculating the folate content in food, corrections must be made for the folate content in the enzyme suspensions added to the extract during sample preparation (Scott, Rébeille & Fletcher, 2000).

Quantification

Microbiological assay (MA)

Traditionally, the determination of total folate content in foods is carried out using a microbiological assay with *Lactobacillus rhamnosus* ATCC No. 7469 (previously *L. casei*). Today the only officially recognised AOAC method (AOAC, 2000) for folate analysis is the microbiological method to quantify folic acid in vitamin preparations (944.12) and infant formula (992.05). Microbiological growth assays are based on the nutritional requirements of the microorganisms for folate. With the appropriate pH in the growth medium, *L. rhamnosus* is reported to respond equally to all folate mono-, di- and tri glutamates and not to respond to pteroic acid, a common folate degradation product. However, Newman & Tsai (1986) demonstrated small differences in growth response between folic acid, 5-CH_3-H_4folate and 5-HCO-H_4folate. Goli & Vanderslice (1989) reported significant growth response differences to various folate forms. The relative growth response to H_2folate, H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate and 10-HCO-H_4folate were 29%, 15%, 83%, 115% and 95% respectively compared to folic acid, under the conditions of their study. Since standard curves are in most cases established with folic acid, unequal growth response to naturally occurring folate could significantly affect accuracy. Today some laboratories use 5-HCO-H_4folate as a standard since a sudden loss of response to folic acid by the assay organism has been observed (Tamura, 1990). Stimulation and inhibition of *L. rhamnosus* growth by non-folate compounds found in food matrices have been observed by several workers as cited by Finglas, Faulks & Morgan (1988a). Microbiological procedures for folate are considered time consuming and laborious even though more and more laboratories are using microtitre plate format assays (Newman & Tsai, 1986; Finglas, Faulks & Morgan, 1988a; Horne, 1997; Molloy & Scott, 1997; Kariluoto, Vahteristo & Piironen, 2001). However, despite this, MA is still considered to be the best method for total folate quantification in foods. A joint collaboration study with 13 participating laboratories was carried out to measure total folate by MA with trienzyme treatment in a wide range of cereal products. It was recommended that this method should be adopted as a First Approval Method of Analysis of AACC (DeVries *et al.*, 2001).

Protein-binding assays (PBA)

The competitive radio protein-binding assay (RPBA) is based on competition between endogenous folate in the sample with radiolabelled folate in the kit for the binding site of a folate binding protein. RPBAs have several advantages over the MA, simple to perform and having a high sample throughput. Furthermore, RPBAs are more suitable when analysing serum or erythrocyte folate since they are less subject to variation if antibiotics and other folate antagonists are present (Finglas *et al.*, 1988b; Hawkes & Villota, 1989). RPBA methods are therefore widely used for clinical samples, but the variation of ligand-binding affinity for different folate forms has limited their use for food analysis (Gregory, 1996). In particular, the affinity of the folate binding protein to 5-HCO-H_4folate is very low (Wigertz & Jägerstad, 1995; Arcot, Shrestha & Gusanov, 2002; Strålsjö *et al.*, 2002) while the affinity for H_4folate is much stronger than for 5-CH_3-H_4folate

(Strålsjö *et al.*, 2002). Despite this, several protein-binding methods have been used to quantify folate in food (Wigertz & Jägerstad, 1995; Arcot, Shrestha & Gusanov, 2002; Strålsjö *et al.*, 2002). Arcot, Shresta and Gusanov (2002) quantified folic acid in breakfast cereals using both an enzyme protein binding assay and MA. They reported that the result were similar using both quantification methods. Strålsjö *et al.* (2002) demonstrated an optimised RPBA method to be suitable for the quantification of folate in berries and milk, containing mainly 5-CH_3-H_4folate, when 5-CH_3-H_4folate was used for calibration. However matrix effects were found to be considerable for some berry samples *e.g.* black currant and blueberries (Strålsjo *et al.*, 2003). For these low folate containg matrices the false RPBA response was estimated to approximately 50%. However the authors solved this problem by purifying the samples with SPE prior to RPBA quantification.

HPLC and LC-MS methods

Neither the MA nor RPBA allow differentiation between folate forms. Currently, the only method to differentiate between folate forms is high performance liquid chromatography (HPLC). Several HPLC methods have been developed for quantification of folate, based on both ion-exchange and reversed-phase chromatography. In reversed-phase separations, suppression or enhancement of the ionisation of functional groups by pH can effectively be used to regulate retention on the column. The pH, polarity and ionic strength of the solvents are used to optimise separation. Separation with ion pairing is accomplished at near neutral pH with cationic surfactants usually involving methanol and tetrabutylammoniumphosphate (TBAP) (Holt, Wehling & Zeece, 1988). However, lately reversed-phase chromatography has become the method of choice due to its compatibility with detection techniques such as mass spectrometry (Stokes & Webb, 1999; Garbis *et al.*, 2001; Nelson, Dalluge & Margolis, 2001; Pawlosky & Flanagan, 2001; Hart *et al.*, 2002; Freisleben, Schieberle & Rychlik, 2003a; Freisleben, Schieberle & Rychlik, 2003b; Kok *et al.*, 2004). Determination of naturally occurring folate can be accomplished as deconjugated food folate monoglutamates or as intact folate polyglutamates on reversed-phase HPLC (Eitenmiller & Landén, 1999; Bagley & Selhub, 2000; Vahteristo & Finglas, 2000). However, there is a lack of commercially available reduced folate polyglutamate standards. Reversed-phased separation of folate is generally performed on octadecyl (C_{18}) columns (Wigertz & Jägerstad, 1995; Vahteristo *et al.*, 1996; Pfeiffer, Rogers & Gregory, 1997a; Konings, 1999; Ruggeri *et al.*, 1999; Kariluoto, Vahteristo & Piironen, 2001; Ndaw *et al.*, 2001; Osseyi, Wehling & Albrecht, 2001; Pawlosky & Flanagan, 2001; Hart *et al.*, 2002; Doherty & Beecher, 2003; Freisleben, Schieberle & Rychlik, 2003a; Kok *et al.*, 2004) but some studies have been performed on C_8 columns (Jastrebova *et al.*, 2003; Pfeiffer *et al.*, 2004) and on phenyl-bonded silica-based stationary phases (Gregory, Sartain & Day, 1984; Lucock *et al.*, 1995; Bagley & Selhub, 2000; Nelson, Dalluge & Margolis, 2001). Garbis *et al.* (2001) used HILIC column to measure folate in plasma. The most commonly used mobile phase is a binary mixture of phosphate or acetate buffer at low pH (2-3.5) and acetonitrile or methanol. Due to the different polarities of the different folate derivatives, gradient conditions are

usually used. Gradient elution provides narrower peaks than isocratic elution, which is an advantage when resolving the analytes from each other and from matrix interferences.

A number of different detection systems are used including UV, fluorometric, electrochemical and mass spectrometry. Due to its high sensitivity, selectivity and lower cost compared to a mass spectrometry, fluorescence detection is most commonly used. H_2folate and H_4folate other than 5, 10-CH^+-H_4folate fluoresce maximally at excitation 300-320 nm and emission 360-425 nm (Eitenmiller & Landén, 1999). For LC quantification by fluorescence, native fluorescence of H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate is sufficient at pH 2.3 to allow detection at pmol levels (Eitenmiller & Landén, 1999). Fluorescence intensity of H_2folate and 10-HCO-H_4folate is too low to allow quantification, and folic acid does not fluoresce (Gregory, Sartain & Day, 1984). Native fluorescence of 5-CH_3-H_4folate is about ten times stronger than that of 5-HCO-H_4folate and twice as strong as that of H_4folate (Gounelle, Ladjimi & Prognon, 1989).

Recently the first methods with mass spectrometry as detection technique for quantification of food folate were developed (Freisleben, Schieberle & Rychlik, 2003b; Pawlosky, Flanagan & Doherty, 2003). An advantage of LC/MS is that it is not limited by the low fluorescence activity of folic acid and 5-HCO-H_4folate. Use of LC/MS quantification of folate in food is today, however, limited due to a low number of commercially available internal standards and so far, low sensitivity for some of the folate compounds.

Quantification is usually based on external standard methods when using UV and fluorescence detectors, whereas internal standard methods with stable isotope label are often used in LC-MS (Pawlosky & Flanagan, 2001; Freisleben, Schieberle & Rychlik, 2003b; Pawlosky, Flanagan & Doherty, 2003). These stable isotope dilution methods are considered most accurate because they allow correction for vitamin losses during extraction and purification and can also compensate for variations in ionization efficiency due to matrix interferences (Rychlik, 2004).

Only some researchers have used HPLC for folate quantification in cereal based foods (Pfeiffer, Rogers & Gregory, 1997a; Stokes & Webb, 1999; Konings *et al.*, 2001; Osseyi, Wehling & Albrecht, 2001; Freisleben, Schieberle & Rychlik, 2003a; Pawlosky, Flanagan & Doherty, 2003; Kariluoto *et al.*, 2004). Stokes & Webb (1999) used a single quadruple as detector, Pawlosky, Flanagan & Doherty (2003) used an ion-trap and Freisleben, Schieberle & Rychlik (2003a) used tandem MS for detection, while others use fluorescence detection and UV absorbance. As already mentioned in the Section *Folate in cereals and cereal products*, Osseyi, Wehling & Albrecht (2001) and Kariluoto *et al.* (2004) investigated the effect of baking on individual folate forms. Osseyi, Wehling & Albrecht (2001) used ion pairing chromatography for separation of H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, 10-HCO-folic acid and folic acid. The mobile phase was composed of 24% methanol in aqueous phosphate buffer pH 6.8 and TBAP as ion-pairing agent and isocratic conditions was used. Kariluoto *et al.* (2004) detected the same folate forms but used reversed-phase chromatography with a binary mixture of 30 mM phosphate buffer pH 2.2 and acetonitrile as

mobile phase and used a gradient. Similar mobile phase and gradient program were used by Konings *et al.* (2001) and Pfeiffer, Rogers & Gregory (1997a) when they quantified individual folate forms and calculated the total folate content in several cereal based foods. Stokes & Webb (1999) developed the first LC-MS method for folate quantification in foods. They tried to quantify 5-HCO-H_4folate and folic acid in breakfast cereal but they had serious problems due to interfering compounds from the matrix. Pawlosky, Flanagan & Doherty (2003) compared fluorescence detection with ion trap mass spectrometry to analyse 5-CH_3-H_4folate and folic acid in whole meal flour and infant formula and stated that fluorescence detection may be used to accurately quantify folate in foods. Freisleben, Schieberle & Rychlik (2003a) on the other hand, demonstrated that when fluorescence detection is used for quantifying folate in wheat bread, 5-HCO-H_4folate is not detected, but when tandem MS is used this form can be measured.

Sample purification

Folate is present in relatively low concentrations in foods, and procedures to remove interfering compounds from the food extract are required for accurate quantification by HPLC. In recent years, purification methods of choice to improve detection limits and selectivity in the HPLC have involved the use of solid-phase extraction (SPE) utilising strong anion exchange sorbent bonded to silica (Jacoby & Henry, 1992; Wigertz & Jägerstad, 1995; Vahteristo *et al.*, 1996; Osseyi, Wehling & Albrecht, 2001; Jastrebova *et al.*, 2003; Strålsjö *et al.*, 2003). A few applications with sorbents bonded with phenyl and C_{18} (Lucock, Hartley & Smithells, 1989; White, 1990; Stokes & Webb, 1999; Pawlosky & Flanagan, 2001) have also been published. Affinity chromatography using immobilized folate-binding protein has over the past decade attracted interest, as a highly selectively purification method for folate, especially for complex food matrices such as cereals since the sample extract can be concentrated (Pfeiffer, Rogers & Gregory, 1997a; Konings, 1999; Bagley & Selhub, 2000; Kariluoto, Vahteristo & Piironen, 2001; Freisleben, Schieberle & Rychlik, 2003a; Nelson *et al.*, 2003). Affinity chromatography has proved to enable sensitive quantifications with detection limits 10-fold lower than after solid-phase extraction (Freisleben, Schieberle & Rychlik, 2003a). However, drawbacks of this purification method are that affinity columns are not commercially available. Besides, this method is time consuming and expensive. In addition, the affinity chromatography columns show separation of R- and S-diastereomers due to steroeselectivity of folate binding protein. This can complicate the use of synthesised folate standards which are equimolar mixtures of R- and S-diastereomers, as for instance, stable isotope-labelled internal standards in LC-MS methods (Freisleben, Schieberle & Rychlik, 2003a).

Objectives

According to the state of the art, briefly summarised in the previous section, there is little reliable information about concentrations of individual folates in foods. The main reasons for this are the existence of numerous native folate forms of which the most are unstable and occur in low concentrations. HPLC is the method of choice for separation and characterisation and several HPLC methods have been developed. Some approaches are potentially suitable for food applications although methods for extraction, enzymatic pre-treatment and sample purification must be optimised to permit application to a broad range of food. This is particularly the case for folates in cereals and cereal products - a major contributor to dietary folate intake - where matrix effects are known to interfere with the HPLC quantification.

According to dietary surveys, only a few percent of the Swedish women reach a daily intake of the recently recommended target of 400 µg per day. In Sweden, mandatory fortification with folic acid is not carried out, except for infant formulas and baby foods such as porridge and gruels. Because not all women achieve the recommended folate intake and because some pregnancies are unplanned, the authorities are discussing whether Sweden should introduce mandatory folic acid fortification of wheat and sifted rye flour. The issue to what concentration of folic acid the flour and perhaps also gluten-free products should be fortified is important. Another critical issue is the evaluation of dietary sources concerning folate bioavailability. According to the literature, folate bioavailability from a mixed diet is estimated to be about 50% (Gregory, 1995).

The main objectives of the present work were:

- To optimise chromatographic separation of dietary folate through a comparison of different silica-based columns (Paper I).
- To optimise the sample purification procedures before HPLC quantification (Paper II).
- To optimise sample pre-treatment (extraction and enzymatic treatments) prior to purification and quantification (Paper III).
- To determine folate content in cereals and gluten-free products (Paper III & IV).
- To study the stability of folic acid as fortificant in breakfast roll during baking and storage (Paper V).
- To study folate absorption in humans
  - -using a short-term ileostomy model to compare the absorption of folate and folic acid from different food sources and pharmaceutical preparations (Paper IV).
  - -using an intervention model to investigate the effect of consumption of folic acid-fortified bread on folate status (Paper V).

Table 2. *Overview of the experimental design*

Papers	***I***	***II***	***III***	***IV***	***V***
	HPLC optimisation	*SPE-optimisation*	*Pre-treatment optimisation*	*Ileostomy study*	*Intervention study*
Samples	Folate standards	Folic acid fortified breakfast rolls [a]	Gluten-free products	Folic acid fortified wheat bread Yeast flakes/desert creme[b] Plasma[c], Stomal effluent[c]	Breakfast rolls[e]
Food sample pre-treatment		Trienzyme (amylase, protease, HK)	Monoenzyme (RS) Dienzyme (amylase, RS) Trienzyme (amylase, protease, RS)	Trienzyme (amylase, protease, HK)	Trienzyme (amylase, protease, HK)
Purification		SPE- several sorbents	SPE-Phenyl (EC)	SPE-SAX Affinity chromatography	SPE-SAX
Quantification	HPLC-different columns	HPLC-Zorbax C_8	HPLC-Aquasil C_{18}	HPLC-Zorbax C_8 MA[d]	HPLC-Zorbax C_8 MA[d]

HK=hog kidney conjugase, SPE=solid phase extraction, EC=endcapped, SAX=strong anion exchange, RS=rat serum conjugase MA=microbiological assay. [a] further samples; strawberries, rose hips, baker´s yeast, orange juice (for details on sample preparation see Paper II), [b] further food samples: milk, fermented milk, standardized lunch & snack (for details on sample pre-treatment see Paper IV), [c]for details on sample pre-treatment see Paper IV, [d]performed at the National Food Administration with modification from AOAC Official method 992.05, [e]subjects blood samples were quantified for serum folate, erythrocyte folate and serum homocysteine at the University Hospital, Uppsala.

Material and methods

The following section gives a brief description of material and methods used. More detailed information is found in Papers I-V. An overview of the experimental design is seen in Table 2.

Materials

All chemicals were of HPLC quality or p.a. grade and purchased from E. Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, USA). Preparation of hog kidney conjugase suspension was prepared from (10 g) hog kidney acetone powder (cat. No. K7250, Sigma Chemical Co., St. Louis, USA) and dialysed overnight according to Jastrebova *et al.* (2003). Thermostable α-amylase (E-BLAAM) was purchased from Megazyme International, Ireland. Protease (Cat. No. P5147) from Sigma Chemical Co., St. Louis, USA and rat serum was obtained from Scanbur, Sollentuna, Sweden, and both were dialysed for 2 h before use. Lyophilised chicken pancreas was purchased from Difco, Detroit, USA). Folate standards (6S)-H_4folate, (6S)-5-CH_3-H_4folate, (6S)-5-HCO-H_4folate, folic acid were kindly provided by MERCK Eprova AG (Schaffhausen, Switzerland). Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-HCO-folic acid was purchased from Dr Schircks laboratories (Jona, Switzerland). De-ionised water was of Milli-Q grade.

Bovine folate-binding proteins for affinity chromatography (Cat. No. F0524, Sigma Chemical Co., St. Louis, USA) was purchased from Scripps Laboratories (San Diego, USA), and agarose affi gel 10 and poly prep chromatography columns from Bio-Rad Laboratories (Richmond, USA). Different silica-based sorbents of the trademark Isolute from International Sorbent Technology (Mid-Glamorgan, UK) SAX (strong anion exchange), PH EC (phenyl-endcapped), PH, CH EC (cyclohexyl-endcapped) have been used for sample purification by solid-phase extraction.

Table 3. *Reversed-phase HPLC columns investigated for folate analysis*

Conventional alkyl (C8-C18) bonded phase	*Novel alkyl bonded phases*	*Polar bonded phase*	*Conventional and novel phenyl bonded phases*
Zorbax SB C_8 (ZoC8)	HyPurityAdvance (HyPA)	Zorbax SB CN (ZoCN)	Luna Phenyl-Hexyl (LuPH)
Synergi Max (SyC12)	Zorbax SB Aq (ZoAq)		Fluophase PFP (FlouP)
Genesis C_{18} (GeC18)	Aquasil C_{18} (AqC18)		Synergi Polar (SyPo)

Several analytical HPLC columns have been investigated (Table 3). All columns were 150 x 4.6 mm and based on ultra-pure silica. More information on the columns is available in Paper I.

Several gluten-free products (Table 4) were selected for folate quantification and optimisation of sample pre-treatment (Paper III). The folic acid-enriched wheat bread used as test food in the ileostomy model (Paper IV) was intended to contain 400 µg folic acid/100 g and was baked at the pilot plant at Cerealia, Järna, Sweden. Yeast flakes were mixed with a commercial dessert crème to hide the taste before the yeast flakes was given to the ileostomy volunteers (Paper IV). Gelatine capsules with either (6S)-5-CH_3-H_4folate (192 µg) or folic acid (199 µg) were produced by Merck Eprova AG, Schaffhausen, Switerland. The test foods in the intervention study (Paper V) were folic acid-enriched wheat breakfast rolls. They were baked at Skogaholms bakery, Stockholm, Sweden as two types of breakfast rolls, one with an intended folic acid content of 400 µg (roll H) and one with an intended folic acid content of 200 µg (roll L). These rolls were also used for optimisation of the sample purification procedure (Paper II).

During the short-term folate absorption study venous blood samples, urine and stomal effluent were collected for 10 h post-dose (Paper IV). During the intervention study fasting venous blood samples were collected on day 0, 30, 60 and 90 for serum folate, serum homocysteine and erythrocyte folate analysis (Paper V).

Table 4. *Samples used*

Gluten-free products[a]	Test foods	Pharmaceuticals[2]	Clinical samples
Corn starch	Wheat bread[b] – fortified with 400 µg folic acid/100 g (paper IV)	Folic acid-gelatine capsule (paper IV)	Plasma (paper IV)
Potato starch			Urine (paper IV)
Rice flour		(6S)- 5-CH_3-H_4folate-gelatine capsule (paper IV)	Stomal effluent (paper IV)
Flour mix (wheat starch)	Yeast flakes[b] in dessert crème- yeast flakes were mixed with lemon creme (paper IV)		
Coarse flour mix (rice flour, corn starch, potato starch)			Blood (paper V)
Coarse crispbread	Wheat breakfast rolls-fortified with 200 µg or 400 µg folic acid/roll (paper V)		
Crispbread (lactose free)			
Lunch rolls			

[a]details on the products are given in Paper III, Table 1, [b]details are found in Paper IV Table 1,

Methods

Food sample pre-treatment

An overview of the sample pre-treatment is shown in a flow chart (Figure 4). The flow chart describes the extraction and enzyme procedures prior to purification and quantification in different foods. Tested conditions included the use of different buffer systems (0.1 M phosphate buffer, pH 6.1 (Vahteristo *et al.*, 1996), Hepes/Ches pH 7.85 (Wilson & Horne, 1984), acetate buffer pH 5.0 (Gregory *et al.* 1984)), temperature of extraction (75-100 ºC), duration of heat treatment (10-60 min), protease treatment (1-3 h), conjugase sources (CP, HK, RP and RS). To increase homogeneity of the samples and improve the extraction procedure, some samples were freeze-dried and milled. Different antioxidants (2-mercaptoethanol (MCE), 2.3-dimercapo-1-propanol (DMP) and dithiothreitol (DTT)) in combination with sodium ascorbate have been investigated with different buffer systems to achieve the maximal stabilising effect and maybe be able to replace the toxic MCE. To prevent folate oxidation samples were protected by nitrogen and subdued light and cooled on ice throughout sample preparation. Three different enzyme treatment procedures were used: monoenzyme treatment (by either dialysed rat serum (RS) or chicken pancreas (CP) suspension), dienzyme treatment (with α-amylase and RS) or trienzyme treatment (α-amylase, protease and RS or dialysed hog kidney (HK) suspension).

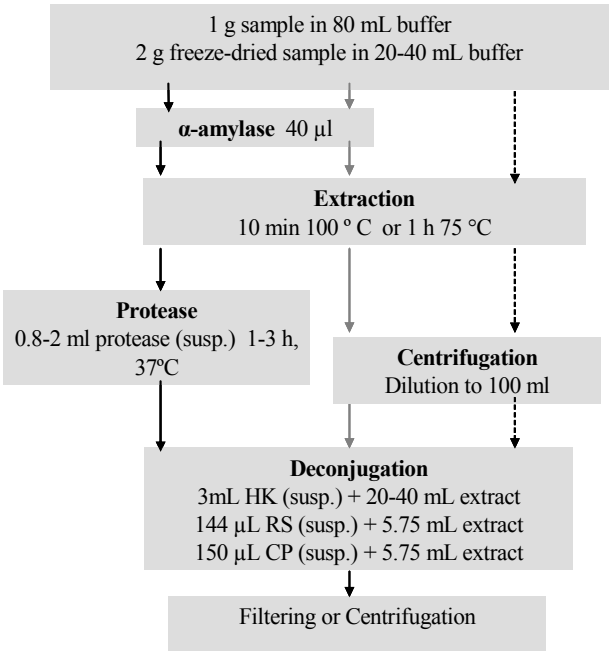

Figure 4. A flow chart describing an overview on food sample preparation prior to analysis. Monoenzyme treatment ⇢ dienzyme treatment → and trienzyme treatment →

Purification

To purify the food sample extracts prior to HPLC quantification, different solid phase extraction (SPE) sorbents have been tested and used. SAX cartridges were used according to Jastrebova *et al.* (2003) for food samples (Papers II, III, IV & V) and human samples (Paper IV). Aliquots of extract were applied to the preconditioned cartridges and eluted with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% AA and 0.1% MCE (Vahteristo *et.al.* 1996). PH, PH EC and CH EC were conditioned using 0.03 M H_3PO_4 containing 1% AA and extracts were eluted with 0.1 M sodium acetate containing 10% acetonitrile (ACN), 1% AA and 0.1% MCE (pH 4.5). To study the ability to concentrate the sample on PH and CH cartridges, elution buffers with acetonitrile content up to 15% were also investigated. PH EC cartridges were used to purify the gluten-free sample extracts with the above-described procedure (Paper III). The combination of SPE procedures on PH EC and SAX cartridges were also used in order to eliminate interferences (Paper III) Affinity chromatography using immobilized folate-binding protein was used to purify urine samples (Konings, 1999; Kariluoto, Vahteristo & Piironen, 2001) (Paper IV).

Quantification

HPLC quantification

The main in-house HPLC method was carried out using an Agilent 1100 HPLC system (Papers II, IV & V). A Zorbax SB C_8, 150 x 4.6 mm, 5 µm (Agilent Technologies, USA) analytical column was used to separate the folate. The chromatographic conditions were as follows: column temperature, 23 ºC; autosampler temperature, 8 ºC; flow rate 0.4 ml/min; volume injected, 20 µl; fluorescence detection, 290 nm excitation and 360 nm emission; UV detection, 290 nm. The mobile phase was a binary mixture of phosphate buffer pH 2.3 or acetic acid pH 3.1 and ACN under linear gradient elution condition. The gradient started at 6% ACN with a lag of 5 min, and then increased to 25% within 20 min.

To analyse the folate content in gluten-free products (Paper III) the analytical column Aquasil C_{18}, 150 x 4.6 mm; 3µm (Thermo Electron Corporation, USA) was used instead, with same conditions as described above.

To optimise the selectivity and sensitivity during folate quantification ten different silica-based analytical columns (Table 3) were investigated (Paper I). Retention time (RT), separation, elution order, peak height, peak width ($w_{0.5}$) and asymmetry (*As*) were studied. Two different mobile phase buffers were used: 0.03 M phosphate buffer, pH 2.3 for optimal fluorescence response of H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate and 0.03 M acetate, pH 3.1 since this buffer is volatile and therefore compatible with a mass spectrometric detector.

During HPLC quantification, retention times were used for peak identification. The ratio of sample peaks from fluorescence and DAD detectors to ratio of standard peaks as well as fluorescence and diode array spectra was used for verification of peaks. Quantification was based on an external standard method with multilevel calibration curve (n>6) for all folate forms.

Quantification with microbiological assay and protein binding assay

The total folate concentration in the breakfast rolls used in the intervention study (Paper V) was analysed using the microbiological assay at the Swedish Food Administration, with minor modifications according to AOAC (2000). The blood samples in the intervention study were analysed for total serum homocysteine at the Clinical Chemistry and Pharmacology Centre for Laboratory Medicine, Uppsala University Hospital using a fluorescence polarization immunoassay (Imx System, Homocysteine, Abbot Laboratories, USA). Serum and erythrocyte folate were quantified using a protein-binding assay (Abbot Axsym System Folate, Abbot Laboratories, USA).

Models to evaluate folate absorption

Two studies have been performed to investigate folate absorption in humans. A study on short-term kinetics of folate and folic acid absorption from yeast and fortified wheat bread was carried out using ileostomy volunteers (Paper IV). Effects of consumption of folic acid-fortified bread on folate status in women were studied during a three-month intervention (Paper V).

The human ileostomy model

A human ileostomy model (Figure 5) described in detail by Witthöft *et al.* (2003) was used to study relative and apparent folate absorption of folic acid-fortified bread and natural polyglutamates in yeast flakes in a dessert crème (Paper IV). In our study, the volunteers' folate status was standardised by liver pre-saturation to minimise the hepatic first-pass uptake affecting the plasma response.

Randomised test days:

Day N: no folate
Day I: i.m. injection of (6S)-5-CH_3-H_4folate
Day O: oral dose of (6S)-5-CH_3-H_4folate capsule
Day C: oral dose of folic acid capsule
Day B: folic acid fortified bread
Day Y: yeast flakes 5-CH_3-H_4folate polyglutamates
Day P: pasteurised milk + (6S)-5-CH_3-H_4folate + FBP
Day F: fermented milk + (6S)-5-CH_3-H_4folate + FBP
Day U: fermented milk + (6S)-5-CH_3-H_4folate

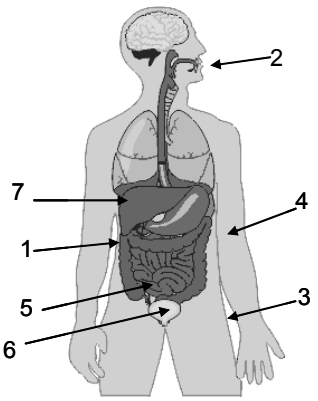

Figure 5. The human ileostomy model.
Left: Information on test days. Right: Details of the human model, 1) colon removed and a stoma (5) is made from the ileum and brought out through the abdomen to allow for defecation, 2) application of test foods or pharmaceutical folate, 3) i.m. injection of (6S)-5-CH_3-H_4folate, 4) collection of blood, 5) collection of stomal effluent, 6) urine collection, 7) liver.Picture from (http://dimkin.df.ru/clipart/clipart_show.html?cat=100000027&start=10).

The folate absorption was determined by the area-under-the-plasma curve (AUC) technique, previously used by several researchers (Prinz-Langenohl *et al.*, 1999; Konings *et al.*, 2002; Witthöft *et al.*, 2003). All volunteers accomplished randomly all study days and the resulting AUCs were compared within individuals since high inter-individual variation of plasma response was expected due to differences in body weight and volume of folate distribution (Loew *et al.*, 1987). The folate absorption was compared from the AUC/dose ratio after consumption of a test food dose as compared to a pharmaceutical folate/folic acid dose. Mean apparent absorption was estimated by kinetic modelling of plasma concentration curves. Folate excretion into post-dose ileostomal effluent reflected non-absorbed folate. Folate excretion into urine was used to evaluate if the ingested doses were within physiological range.

The intervention study

The women (n=29) were randomly assigned into two groups receiving wheat breakfast rolls fortified with either 400 µg (roll H) or 200 µg (roll L) folic acid (Paper V). The women consumed one roll daily during 12 weeks without changing their usual dietary habits. Fasting venous blood samples were collected at day 0, 30, 60, and 90. As indicators for folate status, serum folate, erythrocyte folate and serum homocysteine were used. Dietary habits were assessed twice during the intervention, using a self-administrated food-frequency questionnaire (FFQ).

Statistical analysis

Average concentrations and standard deviations (SD) were calculated for all samples using Excel from Microsoft. The correlation between different SPE procedures, when measuring food folate concentrations, was estimated using linear regression (Paper II). Statistical analyses in the human ileostomy study (Paper IV) were made using the Minitab programme (release 14). The treatment means of different variables were calculated and compared using one-way ANOVA and Tukey's pairwise comparison. To compare the characteristics of the two groups of women in the intervention study, two-sample t, Minitab, version 13 was used (Paper V). To compare whether the daily average folate intake changed during the intervention, based on the FFQ, a paired t-test was used (Minitab 13). Comparison of changes in folate status parameters during the intervention was carried out using the procedure "Mixed", SAS (Statistical Analysis System INC, Cary, USA). Changes in folate concentrations in the rolls during storage were analysed using general linear modelling, SAS. For all statistical analyses, differences were considered significant at $p < 0.05$.

Results and discussion

Method development and folate analysis

Optimisation of HPLC separation (Paper I)

There is still a need for higher selectivity and better detection limits when analysing foods with extremely low levels of folate by HPLC (Ruggeri *et al.*, 1999; Kariluoto, Vahteristo & Piironen, 2001). Recently, a number of alternative stationary phases based on ultra-pure silica, specially developed for the analysis of polar compounds, have become available. These alternative phases with additional polar groups offer higher stability under aqueous conditions and different selectivity compared with conventional C_{18} phases (McCalley, 1999; Vervoort *et al.*, 2000; Layne, 2002). In the present study, ten silica-based stationary phases, such as conventional alkyl-bonded (C_8, C_{12}, C_{18}), phenyl-bonded, polar-bonded (cyano) as well as alternative polar-embedded and polar-endcapped phases, all with unique selectivity for polar compounds, were compared for the separation of five different dietary folate analogues. Moreover, different gradient program were tested. The separation of the early eluting derivatives (H_4folate and 5-CH_3-H_4folate) were excellent on all phases except HyPA, but separation between the late eluting forms 10-HCO-folic acid, 5-HCO-H_4folate and folic acid were only achieved on AqC18 when using linear acetonitrile gradient from 6 to 25% within 20 min as used for Zorbax C_8 in Paper IV & V (Figure 6). However, when the gradient program was changed to a linear acetonitrile gradient from 5 to 25% within 37 min and the flow was reduced to 0.2 ml/min (instead of 0.4 ml/min); separation of the late eluting folate forms could also be achieved on SyC12 (Figure 5, Paper I). However, longer run times reduce sample throughput.

Good peak shape expressed as peak height, $w_{0.5}$ and A_S are shown in Table 5 for H_4folate, 5-CH_3-H_4folate, 10-HCO-folic acid and folic acid in phosphate or acetate buffered mobile phase. However, for 5-HCO-H_4folate, a good peak shape was difficult to achieve. On all phases the peaks of 5-HCO-H_4folate were broad ($w_{0.5}$ > 0.3 min) but symmetrical (As = 0.8-1.2) in both mobile phases. Due to the rather broad peaks no column could therefore, be listed as good for 5-HCO-H_4folate in Table 5. In conclusion, the use of acetate buffer generally resulted in impaired peak shape and only ZoAq and ZoC8 could be selected for use in LC/MS based on peak shape. Since all folate forms could be separated using linear acetonitrile gradient from 6 to 25% within 20 min on AqC18 (Figure 6) this column was used for folate analysis in gluten-free products (Paper III). However, when evaluating the limit of quantification (LOQ) it was discovered that the LOQ for 5-HCO-H_4folate was lower on Aquasil C_{18} (8 ng/ml) compared to Zorbax C_8 (2 ng/ml), unchanged for H_4folate (0.5 ng/ml), but improved for 5-CH_3-H_4folate (0.2 ng/ml on Aquasil C_{18} and 0.3 ng/ml on Zorbax C_8).

Table 5. *Columns where different folate derivatives exhibited good peak shape. Peak shape was defined after investigating the peak height, width (w0.5 <0.22) and symmetry (AS =0.9-1.6)*[a].

	Phosphate buffer	Acetate buffer
H_4folate	All alkyl bonded phases, FlouP, SyPo	ZoC8, ZoAq, GeC18
5-CH_3-H_4folate	All columns except ZoCN	ZoC8, ZoAq, GeC18
10-HCO-folic acid	All phases except ZoCN and HyPA	All phases except HyPA[b]
Folic acid	All phases except ZoCN and HyPA	All phases except HyPA[b] and GeC18

[a]Further details are given in Paper I, Figure 3 and 4, [b] No peaks of these compounds were found

Optimisation of sample purification (Paper II)

Folate is present in low concentrations in foods, and procedures to remove interfering compounds from the food extract are required for accurate quantification by HPLC. Affinity chromatography has proved to be a very specific clean up procedure that enables sensitive quantification. However, a drawback of this purification method is that the affinity chromatography columns shows separation of R- and S-diasteriomers which can complicate the use of synthesised equimolar mixtures of folate standards, as for instance, stable isotope internal standards in LC-MS methods (Freisleben, Schieberle & Rychlik, 2003a). Solid-phase extraction (SPE) is the most common method for purification of food extracts prior to LC-analysis of folate. Strong anion-exchange (SAX) materials based on trimethylaminopropyl silica are widely used SPE sorbents for purification of folate-containing food extracts. SAX sorbents commonly provides high recovery for different folate forms, but pre-concentration of samples is usually not possible due to the limited capacity of SAX cartridges and the necessity of using large buffer volumes to elute folate quantitatively (Rebello, 1987; Vahteristo *et al.*, 1996; Breithaupt, 2001; Jastrebova *et al.*, 2003). Furthermore, the selectivity of this purification method is not sufficient for *e.g.* 5-HCO-H_4folate since undesirable compounds from the food matrix are co-eluting during the SPE procedure and interfering with chromatographic peaks of interest during HPLC analysis. The use of other SPE sorbents may provide better selectivity and higher pre-concentrations of samples compared with SAX sorbents (Rebello, 1987; Lucock, Hartley & Smithells, 1989; van Tellingen *et al.*, 1989; Nelson, Dalluge & Margolis, 2001). However, this approach is poorly studied for food matrices; only a few applications with phenyl and C_{18} bonded sorbents have been published regarding SPE purification of food extracts prior to HPLC analyses of folate (White, 1990; Stokes & Webb, 1999; Pawlosky & Flanagan, 2001).

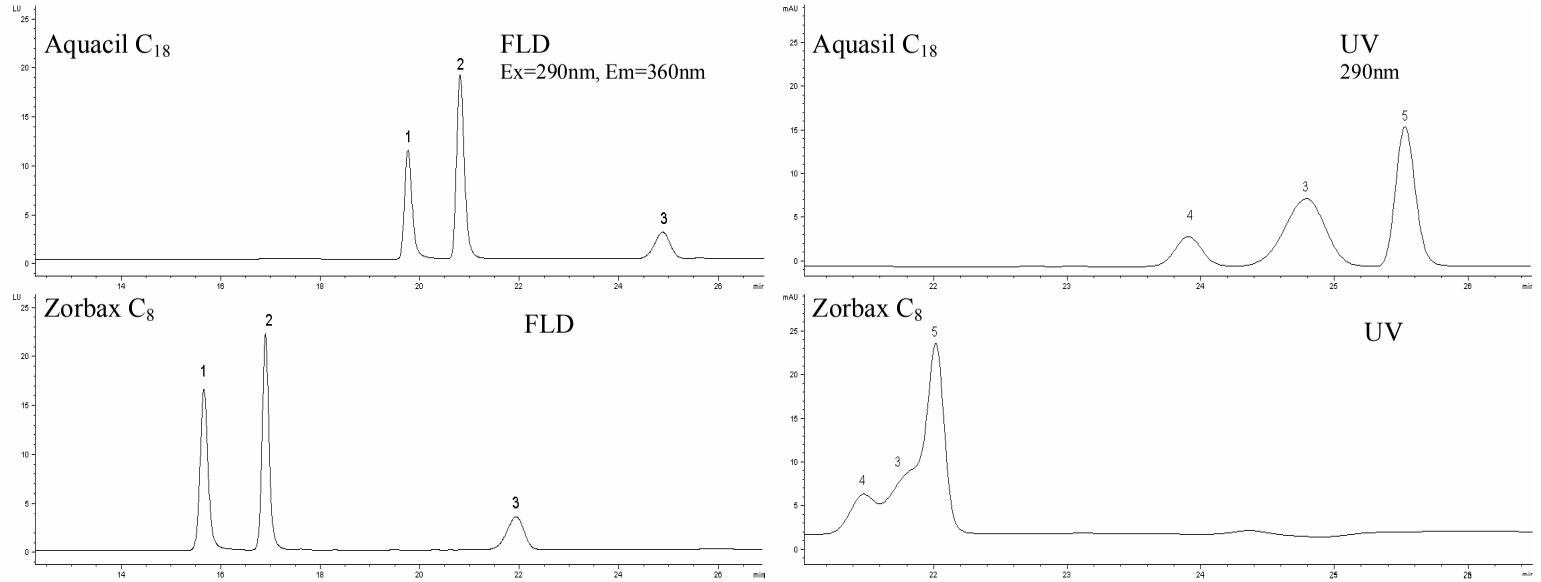

Figure 6. Separation of individual folate forms on two columns, AqC18 and ZoC8. Standard mixture containing 100 ng/ml of H_4folate, 5-CH_3-H_4folate, and 1000 ng/ml of 5-HCO-H_4folate, 10-HC0-folic acid, folic acidl. Peaks: 1=H_4folate, 2=5-CH_3-H_4folate, 3=5-HCO-H_4folate, 4=10-HC0-folic acid, 5=folic acid. Mobile phase: acetonitrile-phosphate buffer (pH 2.3). Linear acetonitrile gradient from 6 to 25% within 20 min with a 5-min lag phase and flow rate 0.4 ml/min.

Therefore, SPE of folate using reversed-phase and anion-exchange sorbents was compared in terms of their recoveries and selectivity and the developed purification procedure was applied to the analysis of folate in a number of food extracts.

Three developed clean-up methods using PH EC, CH EC sorbents or the combination of SAX and PH EC sorbents were applied to the determination of folate in different food extracts and the procedures were verified by correlation with the most common purification method with SAX sorbent. On anion-exchange sorbents folate analogues are retained due to ionic interaction and require ionisation of folate compounds in contrast to the retention on non-polar reversed-phased sorbents where folate analogues are retained in their neutral form due to hydrophobic interactions. Therefore 0.03 M H_3PO_4 was chosen as conditioning and washing buffer for the SPE procedure on reversed-phase PH, PH EC and CH EC cartridges, since this buffer suppressees the ionisation of the carboxyl groups on the folate molecule during application and washing steps. Good recoveries (93-100%) were obtained on PH EC, CH EC cartridges for H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate after some improvements of the method. The need for AA in the conditioning buffer to stabilise H_4folate was discovered since the recovery of H_4folate was improved from 79% (without AA) to 95% when 1% AA was added to 0.03 M H_3PO_4 (the conditioning buffer).

The folate was eluted with 0.1 M sodium acetate containing 10% (v/v) acetonitrile 1% AA, and 0.1% (v/v) acetonitrile and 0.1% (v/v) MCE (pH 4.5). This elution buffer ionise the carboxyl groups and provides higher solubility of folate analogues, therefore, complete desorption of folate during the elution step was observed. Different acetonitrile concentrations (10-15%) in the elution buffer were investigated to improve the elution rate and be able to pre-concentrate the sample. The effectiveness of the elution of folate was improved with higher acetonitrile content. However, the recovery of H_4folate was decreased below 80% due to degradation. Therefore the elution buffer containing 12% acetonitrile was chosen. The stability of the eluates in the autosampler (8 ºC) for 5-30 h was also investigated. The use of MCE together with AA was found to be essential in order to achieve good stability of H_4folate during storage in the autosampler. If 0.1% MCE and 1% AA were added to the elution buffer containing 12% acetonitrile, the loss of the initial H_4folate concentration was only 2% after 30 h. The capacity of SPE cartridges vary widely for different food matrices because the interfering components present at high levels in food samples may saturate the loading capacity of the sorbents, which results in drastic reduction of the breakthrough volumes compared to standard solutions (Snyder, Kirkland & Glajch, 1987). Therefore a capacity test with different foods containing 5-CH_3-H_4folate was performed. It was found that sample volumes up to 5 ml (containing 0.1-0.3 g matrix/ml extract) could be loaded onto both PH EC and CH EC without any noticeable losses of 5-CH_3-H_4folate.

To test the SPE procedure, several food samples including breakfast rolls were purified using PH EC. The SPE procedure with SAX cartridges was used as a reference method. A good correlation in 5-CH_3-H_4folate concentration between these two different purification procedures was found with correlation coefficients

(r^2) of 0.999. For complex matrices, a combination of two SPE procedures on sorbents exhibiting different retention mechanisms is of interest since it is possible to eliminate interferences more effectively. This procedure was therefore also investigated. The food samples were first purified on SAX cartridges and then the eluate was loaded onto PH EC cartridges. A good correlation in 5-CH_3-H_4folate concentration between the combined procedure and the SAX procedure was found with correlation coefficients (r^2) of 0.999. It was also discovered that the combined procedure provided the cleanest chromatograms by minimising the interferences from the matrix for all food samples.

Optimisation of food sample pre-treatment (Paper III)

Different methods of sample pre-treatment prior to purification and quantification have been evaluated (Figure 4). Folate was extracted from the food matrix using 0.1 M phosphate buffer at pH 6.1 with added antioxidants. This buffer stabilised the folate both with MCE and DMP in combination with sodium ascorbate. As seen in Table 6, DMP protects the H_4folate during heat treatment in all buffers. However stability during storage in the freezer as well as freeze-thaw tests have still not been fully evaluated and MCE has therefore been used throughout this project.

Table 6. *Losses (in %) of H_4folate (200 ng) in different buffers in a boiling water bath for 1 h. Sodium ascorbate (2%) and 0.1% of the antioxidant was added to each sample*.

Antioxidants	Acetate pH 5.0	Phosphate pH 6.1	Hepes/Ches pH 7.85
2-mercaptoethanol (MCE)	- 6%	-2%	-1%
2.3-dimercapto-1-propanol (DMP)	No loss	No loss	No loss
Dithiothreitol (DTT)	No loss	No loss	- 4%

Another advantage of using phosphate buffer pH 6.1 as extraction buffer was that α-amylase, protease and RS all have activity at this pH simplifying further pre-treatment procedure. The crucial question of whether enzyme treatment using α-amylase and protease is necessary could not be answered definitively by our studies. The gluten-free products were all rich in starch, but an increase in folate content due to liberation from the matrix after α-amylase treatment was not observed for any of these samples (Paper III). However, for some cereal samples α-amylase treatment is essential to simplify further sample pre-treatment procedures such as pH measurements, centrifugation and filtration since the sample extract otherwise forms a gel.

The enzyme activity from the hog kidney powder varied between batches and the activity was lower than in a preparation from fresh kidney: furthermore, the activity was decreased during storage at -20 ºC. However the conjugase suspension from the fresh hog kidney was much more time-consuming to prepare. Both RP and RS preparations have been evaluated. The activity of RP and RS was compared by studying the conversion of $PteGlu_3$ to folic acid and was found to be similar for both preparations. However, the use of RS instead of RP as conjugase

source was found to be preferable because rat plasma contained heparin which damaged the HPLC column. RS preparation is easily to prepare since it only needs to be dialysed for 2 h before use.

Analysis of native folate and folic acid in food samples

Gluten-free products were found to be a poor to moderate folate source based on the samples analysed within this project (Paper III). Corn, and potato starch and gluten-free flour mix samples contained almost no folate while rice flour contained a small amount (6.3 µg/100 g). The crispbread samples contained 31 and 36 µg/100 g total folate depending on ingredients and the lunch roll used in this study contained 15 µg folate/100 g. Based on our results for the lunch rolls and crispbread it was obvious that the yeast is the main folate contributor in cereal products. Several researchers (Pfeiffer, Rogers & Gregory, 1997a; Kariluoto, Vahteristo & Piironen, 2001; Osseyi, Wehling & Albrecht, 2001) report a large proportion of 5-HCO-H_4folate in cereal products when analysing the individual folate forms; however, no 5-HCO-H_4folate was detected in the analysed gluten-free products. This might be due to the relatively high LOQ (8 ng/ml) achieved on the Aquasil C_{18} column. However, to eliminate interferences disturbing the formyl folates in the chromatograms, a combination of PH EC and SAX was used. The result from this procedure was that no formyl folates could be found in gluten-free products.

The distribution between folate monoglutamates and polyglutamates was also estimated, as a ratio after treatment with either CP solution or RS solution. Interestingly, the distribution between different kinds of products differed. The flour samples contained only folate in monoglutamate form and in crispbread samples almost all folate (-90%) was in polyglutamate form. The three starch products showed no common pattern, rice flour contained 11% polyglutamates,

Table 7. *Comparison of folate content in gluten-free products (µg/100 g) between our obtained results with HPLC and the listed values in the Swedish Food Table*

Products	Present study			Swedish
	H_4-folate	5-CH_3-H_4folate	Sum of folate[a]	Food Table[a]
Corn starch	n.d	0.4	0.4	0
Potato starch	n.d	0.4	0.4	0
Rice flour	1.4	5.0	6.3	4
Flour mix	n.d	0.5	0.5	n.a
Coarse flour mix	n.d	1.0	1.0	n.a
Coarse crispbread	4.1	28.1	30.9	38-50
Crispbread (lactose free)	5.1	31.9	35.9	38-50
Lunch rolls	3.4	12.1	15.1	26[b]

n.d=not detected, n.a =not analysed [a]expressed as folic acid, [b]white bread

corn starch 48% and potato starch 76%. The recovery of H_4folate was rather low in this study (48-56%). However, despite the low recovery for H_4folate and the fact that no 5-HCO-H_4folate was detected, the results in our study were of similar

magnitudes to those in the Swedish Food Table, analysed with the microbiological assay (Table 7).

The portions (170 g) of lemon crème with yeast flakes used in the ileostomy study (Paper IV) contained 68-75 µg 5-CH_3-H_4folate polyglutamates. The folic acid-fortified bread used in the same study, contained 12 µg endogenous 5-CH_3-H_4folate and 217 µg folic acid per portion (50 g). The native folate content in the folic acid-fortified breakfast rolls used in the intervention study (Paper V) was 2.5 µg H_4folate, 10 µg 5-CH_3-H_4folate for both of the rolls (51.5 ± 0.7 g/roll). No detectable amounts of 5-HCO-H_4folate in the rolls were found. Roll L contained 166 µg folic acid and roll H 355 µg folic acid. The folate content analysed both with MA and HPLC is seen in Table 8.

Table 8. *Comparison of folate content (µg/roll) in breakfast rolls used in the intervention study (Paper V) analysed using the in-house HPLC method with Zorbax C_8 column and a microbiological assay.*

	Folate concentration (µg/roll)[a]	
	Roll L (51g)	Roll H (51g)
HPLC-Zorbax C_8		
Sum of folate	177 ± 17	367 ± 34
MA		
Total folate	198 ± 80	365 ± 145

[a]expressed as folic acid

The stability of folic acid during the baking procedure has been investigated (Paper V). The loss of the added folic acid was about 20% when baking the 50g rolls, but only 12% when the 70g rolls were produced (during test baking). This was probably a result of the relatively smaller surface in the heavier rolls.

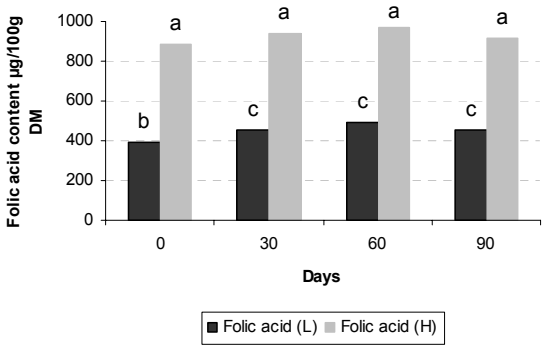

Figure 7. Content of folic acid in the two different breakfast rolls (L and H) during storage at -20ºC. Different letters show statistically significant ($p<0.05$) differences in folate content between days.

During storage in the freezer the content of natural folate did not change. No significant ($p > 0.197$, roll H; $p > 0.777$, roll L day 30-90) losses of folic acid could be observed (Figure 7) during storage at -20 º C.

Nutritional aspects

Absorption from pharmaceutical preparations of folic acid and (6S)-5-CH_3-H_4folate (Paper IV)

Traditionally folic acid has been used as a supplement since it is more stable than other folate forms and inexpensive to synthesise. However, folic acid can delay diagnosis of an underlying vitamin B_{12} deficiency. Intake of 5-CH_3-H_4folate does not mask B_{12} deficiency, but it is more expensive to synthesise and less stable than folic acid. Previously always an equimolar mixture of 6S and 6R was produced (Wright, Finglas & Southon, 2001) but today the biological active 6S-form can be synthesised alone. The absorption differences between these two folate forms given as pharmaceutical folic acid and (6S)-5-CH_3-H_4folate preparations were also studied in the ileostomy model for the first time. Interestingly, no significant ($p= 0.541$) effect from the ingested folate form on absorption could be demonstrated when comparing the pharmaceutical capsules of (6S)-5-CH_3-H_4folate (day O) and folic acid (day C). When folate absorption was expressed by means of dose-normalized AUCs (AUC/dose ratio) three volunteers showed higher mean folate absorption after ingestion of 5-CH_3-H_4folate, three after folic acid ingestion and three volunteers had similar ratios (Figure 8). Also, mean total folate excretion was similar after ingestion of both folate forms. In an intervention study by Lamers *et al.* (2004) no differences between these two folate forms could be observed. This intervention was performed to investigate the effect on plasma folate concentrations after ingestion of these supplements. One group of women (n=36) received 400 μg folic acid and another group (n=36) 416 μg (6S)-5-CH_3-H_4folate during 24 weeks. The plasma folate concentration increased by 151% for women receiving folic acid and 164% for women receiving (6S)-5-CH_3-H_4folate, but the two groups did not differ significantly.

Absorption of folic acid from fortified bread (Paper IV)

The absorption of folic acid from fortified bread was interesting to study since although mandatory folic acid fortification is already carried out in *e.g.* the US and Canada, information on the fortificant absorption is only sparsely available (Pfeiffer *et al.*, 1997b; Schorah *et al.*, 1998; Vahteristo *et al.*, 2002). Mean apparent absorption of folic acid from fortified bread was estimated in our study to approximately 80%, by kinetic modelling of plasma concentration curves. However, the mean folate absorption (expressed as AUC/dose ratio) was significantly ($p=0.005$) lower from folic acid as a fortificant in the bread compared to folic acid from capsules (Figure 8). Finglas *et al.* (2002) observe the samilar effect when comparing absorption from folic acid capsules with folic acid-fortified white bread and suggested that the cereal matrix has some inhibitory effect on absorption of the fortificant folic acid added to the food. However, in our study

only low quantities (traces-17 μg/10 h) of folic acid were found in the stomal effluent demonstrating that the absorption was not severely hampered.

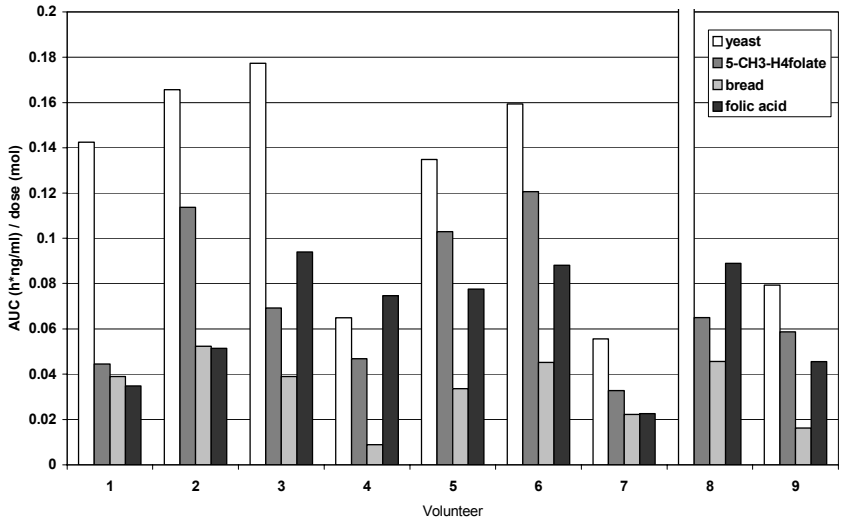

Figure 8. Plasma AUC dose/ratios in 9 volunteers after ingestion of different folate forms. The folate sources were the natural 5-CH_3-H_4folate polyglutamates from yeast flakes (Y), (6S)-5-CH_3-H_4folate in a capsule (O), folic acid fortificant in bread (B) and folic acid in a capsule (C). Mean of Y > B ($p=0.001$), mean of C > B ($p=0.005$), no significant differences between C and O ($p=0.541$).

Absorption of natural 5-CH_3-H_4folate polyglutamates from yeast (Paper IV)

Yeast folate offers the possibility to study absorption of natural polyglutamate. In bread the major part (> 60%) of native folate originates from yeast. Folate in yeast exists only in the polyglutamyl form comprising of 63% 5-CH_3-$PteGlu_7$, 26% 5-CH_3-$PteGlu_8$ and 11% 5-CH_3-$PteGlu_6$ (Ndaw *et al.*, 2001). Various researchers have studied the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid (Keagy, Shane & Oace, 1988; Konings *et al.*, 2002; Melse-Boonstra *et al.*, 2004). From these studies the bioavailability of polyglutamyl folic acid relative to monoglutamyl folic acid has been found to vary from 55 to 100% depending on the study design. However, due to the lack of pharmaceuticals containing reduced folate polyglutamates, absorption studies of natural folate polyglutamates have not yet been performed.

Mean folate absorption (expressed as AUC/dose ratio) was significantly ($p = 0.001$) increased after ingestion of yeast flakes (day Y) compared to folic acid fortified bread (day B) as seen in Figure 8. This is interesting since in the US the estimated average requirement (EAR) is calculated in dietary folate equivalents (DFE). The DFE calculation is based on the assumptions that added folic acid to 85% available and natural folate only to 50%. Fenech *et al.* (1999) studied the bioavailability of natural folate from aleurone flour and observe that natural folate was absorbed in a similar magnitude to that from a folic acid tablet. This was also

observed in our study where the absorption of folate from yeast flakes did not significantly differ ($p = 0.970$) from folic acid capsules. In our study the mean apparent absorption from natural folate polyglutamates in yeast flakes was estimated to 85%. This finding demonstrates that high folate-producing yeast strains could be used as "biofortificants" to increase the folate content in different foods as yeast folate polyglutamates seems highly absorbable.

Only very small quantities of 5-CH_3-H_4folate (3.4 μg/10 h) were found in volunteers' stomal effluent when no folate dose (day N) was given, which indicates that neither bile folate nor the low-folate low-fat lunch and snack influenced post-dose plasma folate concentrations particularly. Only small quantities of intact 5-CH_3-H_4folate were found (1-20 μg/ 10h), in the urine during study days indicating that all doses were at physiological range.

In summary, this human ileostomy model was used to compare folate absorption from different sources such as pharmaceutical preparations, fortified food and a natural dietary folate source. Yeast folate polyglutamates and a folic acid supplement seemed highly absorbable in comparison with folic acid fortified bread. The volunteers were randomly assigned to study days and resulting AUCs are compared within individuals to compensate for the high-intervidual variability. The ileostomy model could be further improved by additional collection of stomal effluent samples (up to 12 or 14 h post-dose) since ileostomal folate excretion could be underestimated. This model would be strengthened using stable-isotope techniques. The ileostomy model was used for short term studies of folate absorption, however, to investigate effects of folic acid ingestion on folate status, long term studies are needed.

Effect of folic acid fortified bread on folate status in healthy fertile women - intervention study (Paper V)

The 3-month intervention study confirms the results from the ileostomy study regarding folic acid absorption from fortified bread. Here, serum folate, erythrocyte folate and homocysteine levels were used as biomarkers for the folate status in subjects. Estimates of native folate intake based on food frequency questionnaires (FFQ) indicated a mean intake of 214 μg native folate/day which were neither significantly different between the two groups nor between day 0 and 90 of the intervention period. Additionally, the two groups of the women received 166 μg folic acid/day and 366 μg folic acid per day, respectively, from fortified bread. A significant ($p < 0.01$) increase of 22-25% in serum folate was observed as early as day 30 in both groups of women, which persisted during the whole intervention period. Erythrocyte folate concentrations did not increase significantly after four weeks, but were significantly ($p < 0.05$) increased at day 90 compared with initial concentration for both groups of women (Figure 9A). Women receiving 166 μg folic acid/day increased their erythrocyte folate concentration with 20% and the women receiving 355 μg folic acid/day with 26% after 90 days of intervention. Women receiving 355 μg folate daily showed significantly higher ($p < 0.001$) mean erythrocyte folate concentrations than women consuming half the amount.

Cuskelly, McNulty & Scott (1996) compare changes in red cell folate concentrations in women after a three-month intervention with either a folic acid supplement, or folic acid-fortified foods or a diet containing of 400 µg natural folate/day. A fourth group received only dietary advice. Interestingly, only the groups consuming either folic acid supplements or food fortified with folic acid showed significant increases in red cell folate concentrations whereas the other groups did not improve in their folate status parameters. However, Vahteristo *et al.* (2002) show already a significantly increase of red cell folate concentrations of 17% after consumption of 184 µg dietary folate/day from a test food containing rye containing products and orange juice after 27 days. This increase in red cell folate concentrations was comparable with that after consumption of folic acid-fortified white bread.

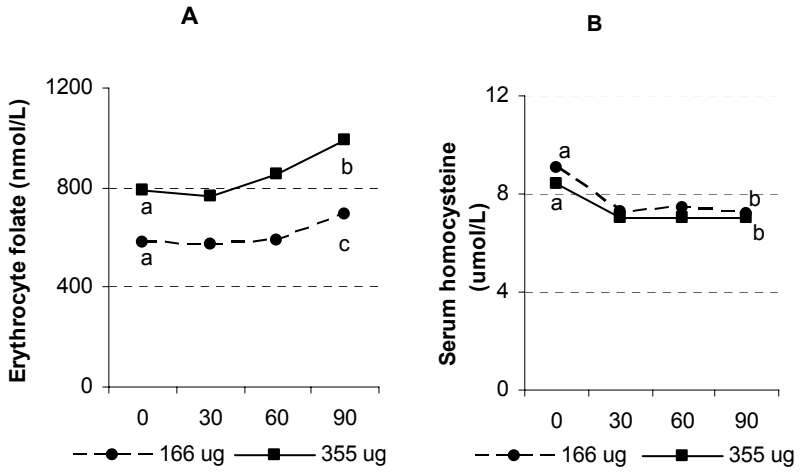

Figure 9. Mean erythrocyte folate concentration (A) and mean serum homocysteine concentration (B) in women receiving 166 µg folic acid/day (n=14) and 355 µg folic acid/day (n=15). Different letters between days and groups show statistically significant differences in concentrations ($p < 0.05$).

A significant decrease in serum homocysteine was not observed by Vahteristo *et al.*, neither after the consumption of folic acid-fortified bread nor the rye and orange juice diet. However in our study, both fortification levels resulted in a significant ($p < 0.05$) decrease of 16-20% in serum homocysteine concentrations already after the first four weeks of intervention (Figure 9B). Mean initial homocysteine concentrations in our subjects of 9.1 ± 2.0 µmol/L (group L) and 8.4 ± 1.8 µmol/L (group H) were similar to those in Vahteristo´s study where the subjects had mean initial homocysteine concentrations of 8.6 ± 3.0 µmol/L (rye and orange juice group) and 7.9 µmol/L (wheat bread group), respectively. Results from our study indicate that an intake of 166 µg folic acid/day seems sufficient to decrease serum homocysteine in healthy women. Schorah *et al.* (1998) reported a significant decrease in mean plasma homocysteine concentrations among 33 subjects after an eight-week intervention with 200 µg folic acid from fortified

breakfast cereals. The subjects in that study had similar initial serum homocysteine concentrations to ours. In contrast to us, Schorah and co-workers observed no significant decrease in homocysteine concentration after four weeks. These findings, however, confirm those of Vahteristo and co-workers which did not observe a significant decrease; their intervention period was only 27 days. In an ongoing meta-analysis of 9 human studies, within an EU project: Folate: From Food to Functionality and Optimal Health (QLRT-1999-00576) the decreases in homocysteine concentrations were studied as an effect of increased folate/folic acid intake (Personal communication Dr. Kees de Meer). After intake of either natural folate or folic acid-fortified foods, the 276 subjects had a mean extra folate intake of 200 μg/day. A significant decrease (16%) in mean plasma homocysteine after 28 days could only be observed in subjects with an initial plasma homocysteine concentration above 12 μM. The pronounced decrease in serum homocysteine in our study already after 4 weeks is therefore difficult to explain: however, after 8 weeks one can expect a significant decrease in the population serum homocysteine levels if folic acid is regularly consumed.

In conclusion, wheat bread is a suitable vehicle for folic acid fortification. Based on the results from both our human studies, we can state that the added folic acid is well absorbed.

General discussion and concluding remarks

In the latest edition of the Nordic Nutritional Recommendations (2004) the recommended daily folate intake for fertile women was increased from 300 µg to 400 µg. For pregnant and lactating women the recommendation was increased from 400 µg to 500 µg (Becker *et al.*, 2004). However, the mean folate intake for Swedish women is only 217 µg/day (Becker, 1997). In our intervention study with folic acid-fortified bread, we showed that an additional amount of 166 µg folic acid fortificant/day is sufficient to increase the folate status in women. If Swedish women increase their daily folate intake by approximately 150 µg they should have an average intake close to 400 µg, as recommended. Women in Sweden consume foods containing white wheat and sifted flour amounting to about 100-150 g/day (Becker, 1999). White wheat and sifted flour are the two vehicles that are discussed for folic acid fortification in Sweden. Thus, a folic acid fortification level of flour corresponding to 140 µg per 100 g, as is mandatory in the US today, would supply the Swedish women with additional folic acid between 140-210 µg per day.

In two Swedish studies with coeliac patients, it was demonstrated that the patients have an even lower folate intake compared to the average population (Grehn *et al.*, 2001; Hallert *et al.*, 2002). Hallert et al. (2002) suggested the reason for lower folate intake among coeliac patients to be that gluten-free breads contain significantly less folate compared with bread consumed by the general population. This was confirmed by our data on folate content in several common gluten-free products. The starch from corn and potato, and gluten-free flour mix contained almost no folate, whereas rice flour contained only small amounts (6 µg/100 g). The crispbread samples contained 31 and 36 µg/100 g depending on ingredients, and the lunch roll contained 15 µg folate/100 g. Further studies to confirm findings for more products are needed but we suggest that gluten-free products should also be considered for folic acid fortification. The steadily growing prevalence of coeliac disease worldwide recognised during the last decade (from 1/1000 to 1/200 individuals) emphasises this need.

Since mandatory folic acid fortification was introduced, especially in the US, several issues have been discussed. The main aim of the mandatory fortification was to decrease the frequency of neural-tube defects in foetus, which is related to folate deficiency in mothers. NTD birth prevalence has been reduced in the US by 19% since 1998 (Honein *et al.*, 2001) and in some parts of Canada, by even 78% after fortification was introduced (Liu *et al.*, 2004). However, it is now estimated that the average folic acid consumption from fortified products is twice the intended amount of 70-130 µg (Choumenkovitch *et al.*, 2002; Quinlivan & Gregory, 2003). This discrepancy is probably due to a widespread over-fortification. Rader, Weaver & Angyal (2000) observed that fortified products typically contain 150-175% of their predicted folate content. This over-fortification lead Lewis *et al.* (1999) to estimate that 15-25% of children 1-8 years and 0.5-5% of adults have an intake above 1 mg expressed as dietary folate equivalent (DFE) per day, the defined upper tolerable level. It is therefore important to quantify the correct amounts of folate in each fortified products. Our

results on the stability of folic acid during the baking procedure may therefore give important information for the food industry. We were able to show that the loss of the added folic acid was higher when baking the small rolls compared to larger rolls probably as a result of the relatively smaller surface area of the larger rolls. Losses were much lower than the overage of 50-75% as seen in fortified products in the US. We also demonstrated that no losses in folic acid occurred when the rolls were stored at -20 ºC for three months. However, prior to introduction of folate fortification systematic stability studies are needed to avoid intake above the upper tolerable level.

Linked to this, another problem of a too high intake of folic acid (> 200 μg in a single dose) is the appearance of unmetabolised folic acid in the plasma due to overloading the metabolic capacity for folic acid conversion to 5-CH_3-H_4folate before entering the portal blood (Kelly *et al.*, 1997). Folic acid is retained and metabolised independently of vitamin B_{12} in the cell and a high intake of folic acid may prevent the folate deficiency by possibly delay diagnosis of the accompanying underlying vitamin B_{12} deficiency, even to a point where irreversible neurological damage has occurred (Quinlivan & Gregory, 2003). So far, only one study suggests that no harm has occurred in the US population since folic acid fortification started (Mills *et al.*, 2003). Yet there is certainly a need in countries where folic acid fortification is mandatory to monitor populations associated with low vitamin B_{12} status *e.g.* the elderly. Previously, the issue of an increased frequency of giving birth to twins after folic acid supplementation was raised (Ericson, Källén & Åberg, 2001). However, today this is not seen as a problem since an increase of twin pregnancies in the US has not been confirmed after folic acid fortification was introduced (Shaw *et al.*, 2003; Waller, Tita & Annegers, 2003).

A diet high in folate-rich foods such as vegetables and fruits has been shown to improve the folate status, as shown by decreasing plasma homocysteine concentrations (Brouwer, van Dusseldorp, West *et al.*, 1999). However, today vegetables and fruits comprise 30% of the total folate intake in a Swedish diet, and Swedish women would have to increase their intake of fruit and vegetable 6-fold to reach recommendations. One alternative is therefore to increase the population's folate intake by producing food with a higher content of natural folate by adding folate-rich cereal fractions, such as bran and germs, or by fermentation. Another strategy to increase the population's folate intake, but without introduction of folic acid fortification, is to fortify with an alternative fortificant, (6S)-5-CH_3-H_4folate. An intake of (6S)-5-CH_3-H_4folate does not mask B_{12} deficiency (Lamers, *et al.* 2004) and in our ileostomy study, we observed no significant differences in folate absorption between folic acid-fortified bread and (6S)-5-CH_3-H_4folate-fortified fermented milk. Also, no differences in folate absorption between supplements containing folic acid or (6S)-5-CH_3-H_4folate were seen. Furthermore, folate absorption from natural 5-CH_3-H_4folate polyglutamates from yeast was significantly higher than for both fortified foods.

Folate was discovered as early as 1930 but still today the only officially recognised AOAC methods for folate analysis are the microbiological method to quantify folic acid in vitamin preparations and infant formula. So far no HPLC

method, which can differentiate between folate forms, has achieved official status. There is for instance, still a need to achieve higher selectivity in order to reduce matrix effects from various foods when analysing foods containing low levels of folate by HPLC. One solution can be the use of reversed-phase stationary phases with unique selectivity recently appearing on the market. In the present study, ten silica-based stationary phases, all with unique selectivity for polar compounds, were compared for the separation of five different dietary folate analogues. Alkyl-bonded stationary phases were found to be best for the separation of folate forms in terms of selectivity and peak shape. Polar-endcapped Aquasil C_{18} was therefore used to quantify the folate concentration in different gluten-free products.

Another important issue is to achieve reliable analytical methods with optimal conditions during sample preparation of food for folate analysis. For instance, the stability of individual folate forms is not fully evaluated nor is the interconversion at different pH and temperatures. In our work we have focused on the stability of H_4folate, which is known to be a most unstable folate form. We were able to demonstrate that the amount of acetonitrile used in elution buffer during the SPE procedure affects the H_4folate stability. The importance of adding ascorbic acid as reducing agent to the conditioning buffer was also confirmed. Furthermore, H_4folate can be stable for 30 h at 8 ºC (the conditions in an autosampler) if both 2-mercaptoethanol and ascorbic acid are added to the sample extract.

As solid-phase extraction (SPE) with strong anion exchange (SAX) is today the most common method for food extract purification prior to folate analysis by HPLC we have investigated new SPE sorbents to provide better selectivity. Good recoveries were obtained on phenyl-endcapped and cyclohexyl-endcapped cartridges. As mentioned earlier it was also found that the combined procedure involving both SAX and endcapped-phenyl cartridges provided the cleanest chromatograms by minimising the interferences from the matrix. This can therefore be a solution in the future for complex food matrices.

The question of whether trienzyme treatment with α-amylase and protease is necessary for folate analysis in cereals could not be answered definitively by our studies. For some cereal samples, *e.g.* breakfast rolls, α-amylase treatment is essential to simplify further sample pre-treatment procedures such as pH measurements and filtration. The gluten-free products were all rich in starch, but an increase in folate content after α-amylase treatment due to liberation from the matrix was not observed and we conclude that for this type of products trienzyme treatment is not necessary.

The aim of the presented analytical work was to contribute towards validated methodology for accurate and precise quantification of food folate. Some further improvements are needed concerning selectivity and sensitivity. Both LC-MS and LC-MS-MS methods offer a promising approach to verify the identity of LC separated peaks. Until these detection methods can be used routinely, HPLC offers good possibilities to characterise and quantify folate in foods. Optimisation regarding procedures for folate stabilisation, sample pre-treatment and purification, as presented in this thesis, can easily be applied in the future using mass spectrometry detection methods.

References

Aiso, K. & Tamura, T. 1998. Trienzyme treatment for food folate analysis: Optimal pH and incubation time for alfa-amylase and protease treatments. *Journal of Nutrition Science and Vitaminology,* 44**,** 361-370.

AOAC. 2000. Official methods of analysis. In *AOAC official method 992.05 folic acid (pteroylglutamic acid) in infant formula* Vol. 2 (Eds, Horwitz, W.) AOAC International, Gaithersburg, MD., 50.024-050.026.

Arcot, J., Shrestha, A. K. & Gusanov, U. 2002. Enzyme protein binding assay for determining folic acid in fortified cereal foods and stability of folic acid under different extraction conditions. *Food Control,* 13**,** 245-252.

Arcot, J., Wootton, M., Alury, S., Chan, H. Y. & Shrestha, A. K. 2002. Folate levels in twelve Australian wheats and changes during processing into bread. *Food Australia,* 54**,** 18-20.

Arkbåge, K., Verwei, M., Havenaar, R. & Witthöft, C. 2003. Bioaccessibility of folic acid and (6S)-5-methyltetrahydrofolate decreases after the addition of folate-binding protein to yogurt as studied in a dynamic in vitro gastrointestinal model. *Journal of Nutrition,* 133**,** 3678-3683.

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

Bailey, L. B., Rampersaud, G. C. & Kauwell, G. P. A. 2003. Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: Evolving science. *Journal of Nutrition,* 133**,** 1961S-1968.

Ball, G. F. M. 1998. *Bioavailability and analysis of vitamins in foods,* Chapman & Hall, London, United Kingdom.

Becker, W. 1997. Bioavailability '97-från absorption till utnyttjande. *Scandinavian Journal of Nutrition,* 41**,** 139-140.

Becker, W. 1999. Svenskarna äter nyttigare-allt fler väljer grönt. *Vår Föda***,** 24-27.

Becker, W. 2000. Vilka är källorna till våra näringsämnen? *Vår Föda***,** 16-20.

Becker, W., Lyhne, N., Pedersen, A. N., Aro, A., Fogelholm, M., Phorsdottir, I., Alexander, J., Anderssen, S. A., Meltzer, H. M. & Pedersen, J. I. 2004. Nordic nutrition recommendations 2004-integrating nutrition and physical activity. *Scandinavian Journal of Nutrition,* 48**,** 178-187.

Berry, R. J., Li, Z., Erickson, D., Li, S., Moore, C. A., Wang, H., Mulinare, J., Zhao, P., Wong, L. C., Gindler, J., Hong, S-X. & Correa, A. 1999. Prevention of neural-tube defects with folic acid in China. *The New England Journal of Medicine,* 341**,** 1485-1490.

Blakley, R. L. 1960. Spectrophotometric studies on combination of formaldehyde with tetrahydropteroylglutamic acid and other hydropteridines. *Journal of Biochemistry,* 74**,** 71-82.

Blakley, R. L. 1969. *The biochemistry of folic acid and related pteridines,* North Holland Publishing Company, Amsterdam, London.

Blakley, R. L. 1988. IUPAC-IUB joint commission on biochemical nomenclature (JCBN) nomenclature and symbols for folic acid and related compounds recommendations 1986. *The Journal of Biological Chemistry,* 263**,** 605-607.

Bostom, A. G., Shemin, D., Bagley, P., Massy, Z. A., Zanabli, A., Christopher, K., Spiegel, P., Jacques, P. F., Dworkin, L. & Selhub, J. 2000. Controlled comparison of L-5-methyltetrahydrofolate versus folic acid for the treatment of hyperhomocysteinemia in hemodialysis patients. *Circulation,* 101**,** 2829-2832.

Botto, L. D., Moore, C. A., Khoury, M. J. & Erickson, J. D. 1999. Neural tube defects. *The New England Journal of Medicine,* 341**,** 1509-1519.

Brattström, L. & Wilcken, D. E. L. 2000. Homocysteine and cardiovascular disease: Cause or effect? *American Journal of Clinical Nutrition,* 72**,** 1-9.

Breithaupt, D. E. 2001. Determination of folic acid by ion-pair RP-HPLC in vitamin-fortified fruit juices after solid-phase extraction. *Food Chemistry,* 74**,** 521-525.

Brody, T. 1999. Folate. In *Nutritional biochemistry* (Eds, Brody, T.) Academic Press, San Diego, 357-376.
Brouwer, I. A., van Dusseldorp, M., Thomas, C. M. G., Duran, M., Hautvast, J. G. A. J., Eskes, T. K. A. B. & Steegers-Theunissen, R. P. M. 1999. Low-dose folic acid supplementation decreases plasma homocysteine concentrations: A randomized trial. *American Journal of Clinical Nutrition,* 69**,** 99-104.
Caudill, M. 2004. The role of folate in reducing chronic and developmental disease risk: An overview. *Journal of Food Science,* 69**,** S55-S59.
Chandler, C. J., Wang, T. T. Y. & Charles, H. H. 1986. Pteroylpolyglutamate hydrolase for human jejunal brush borders purification and characterization. *The Journal of Biological Chemistry,* 261**,** 928-933.
Choi, S.-W. & Mason, J. B. 2000. Folate and carcinogenesis: An integrated scheme. *Journal of Nutrition,* 130**,** 129-132.
Choumenkovitch, S. F., Selhub, J., Wilson, P. W. F., Rader, J. I., Rosenberg, I. H. & Jacques, P. F. 2002. Folic acid intake from fortification in United States exceeds predictions. *Journal of Nutrition,* 132**,** 2792-2798.
Colman, N., Green, R. & Metz, J. 1975. Prevention of folate deficiency by food fortification. II. Absorption of folic acid from fortified staple foods. *American Journal of Clinical Nutrition,* 28**,** 459-464.
Combs, G. F. 1992. Folate. In *The vitamins fundamental aspects in nutrition and health* (Eds, Combs, G. F.) Academic Press, San Diego, 377-401.
Cuskelly, G. J., McNulty, H. & Scott, J. M. 1996. Effect of increasing dietary folate on red-cell folate: Implications for prevention of neural tube defects. *The Lancet,* 347**,** 657-659.
Cuskelly, G. J., McNulty, H. & Scott, J. M. 1999. Fortification with low amounts of folic acid makes a significant difference in folate status in young women: Implications for the prevention of neural tube defects. *American Journal of Clinical Nutrition,* 70**,** 234-239.
Czeizel, A. E. & Dudas, I. 1992. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *The New England Journal of Medicine,* 327**,** 1832-1835.
Darnton-Hill, I. & Nalubola, R. 2002. Fortification strategies to meet micronutrient needs: Successes and failures. *Proceedings of the Nutrition Society,* 61**,** 231-241.
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**,** 643-660.
De Souza, S. & Eitenmiller, R. 1990. Effects of different enzyme treatments on extraction of total folate in various foods prior to microbiological assay and radioassay. *Journal of Micronutrient Analysis,* 7**,** 37-57.
DeVries, J. W., Keagy, P. M., Hudson, C. A. & Rader, J. I. 2001. AACC collaborative study of a method for determining total folate in cereal products-microbiological assay using trienzyme extraction (AACC method 86-47). *Cereal Foods World,* 46**,** 216-219.
Doherty, R. F. & Beecher, G. R. 2003. A method for the analysis of natural and synthetic folate in foods. *Journal of Agricultural and Food Chemistry,* 51**,** 354-361.
Eitenmiller, R. R. & Landén, W. O. 1999. Folate. In *Vitamin analysis for the health and food sciences* (Eds, Eitenmiller, R. & Landen, W. O.) CRC Press, Boca Raton, Florida, 411-466.
Ericson, A., Källén, B. & Åberg, A. 2001. Use of multivitamins and folic acid in early pregnancy and multiple births in Sweden. *Twin Research,* 4**,** 63-66.
FAO/WHO. 2002. Human vitamin and mineral requirements. Rome, 53-63.
Feighery, C. 1999. Fortnightly review: Coeliac disease. *BMJ,* 319**,** 236-239.
Fenech, M., Noakes, M., Clifton, P. & Topping, D. 1999. Aleurone flour is a rich source of bioavailable folate in humans. *Journal of Nutrition,* 129**,** 1114-1119.
Finglas, P. M., Faulks, R. M. & Morgan, M. R. A. 1988a. The development and characterisation of a protein- binding assay for the determination of folate-potential use in food analysis. *Journal of Micronutrient Analysis,* 4**,** 295-308.
Finglas, P. M., Kwiatkowska, C., Faulks, R. M. & Morgan, M. R. A. 1988b. Comparison of a non-isotopic, microtitration plate folate-binding protein assay and a microbiological

method for the determination of folates in raw and cooked vegetables. *Journal of Micronutrient Analysis,* 4**,** 309-322.
Finglas, P. M., Witthöft, C. M., Vahteristo, L., Wright, A. J. A., Southon, S., Mellon, F. A., Ridge, B. & Maunder, P. 2002. Use of an oral/intravenous dual-label stable-isotope protocol to determine folic acid bioavailability from fortified cereal grain foods in women. *Journal of Nutrition,* 132**,** 936-939.
Finglas, P. M., Wright, A. J. A., Wolfe, C. A., Hart, D. J., Wright, D. M. & Dainty, J. R. 2003. Is there more to folates than neural-tube defects? *Proceedings of the Nutrition Society,* 62**,** 591-598.
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**,** 149-156.
Gallagher, E., Gormley, T. R. & Arendt, E. K. 2004. Recent advances in the formulation of gluten-free cereal-based products. *Trends in Food Science & Technology,* 15**,** 143-152.
Garbis, S. D., Melse-Boonstra, A., West, C. E. & van Breemen, R. B. 2001. Determination of folates in human plasma using hydrophilic interaction chromatography-tandem mass spectrometry. *Analytical Chemistry,* 73**,** 5358-5364.
George, L., Mills, J. L., Johansson, A. L. V., Nordmark, A., Olander, B., Granath, F. & Cnattingius, S. 2002. Plasma folate levels and risk of spontaneous abortion. *JAMA,* 288**,** 1867-1873.
Goli, D. P. & Vanderslice, J. T. 1989. Microbiological assay of folacin using a CO_2 analyzer system. *Journal of Micronutrient Analysis,* 6**,** 19-23.
Gounelle, J.-C., Ladjimi, H. & Prognon, P. 1989. A rapid and specific extraction procedure for folates determination in rat liver and analysis by high-performance liquid chromatography with fluorometric detection. *Analytical Biochemistry,* 176**,** 406-411.
Gregory, J., Bhandari, S., Bailey, L., Toth, J., Baumgartner, T. & Cerda, J. 1992. Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *American Journal of Clinical Nutrition,* 55**,** 1147-1153.
Gregory, J. F. 1989. Chemical and nutritional aspects of folate research: Analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates. *Advances in Food and Nutrition Research,* 33**,** 1-101.
Gregory, J. F. 1995. The bioavailability of folate. In *Folate in health and disease* (Eds, Bailey, L. B.) Marcel Deeker, New York, 195-235.
Gregory, J. F. 1996. Vitamins. In *Food chemistry* (Eds, Fennema, O. R.) Marcel Dekker Inc, New York, 531-610.
Gregory, J. F. 2001. Case study: Folate bioavailability. *Journal of Nutrition,* 131**,** 1376S-1382S.
Gregory, J. F., Sartain, D. B. & Day, B. P. F. 1984. Fluorometric determination of folacin in biological materials using high performance liquid chromatography. *Journal of Nutrition,* 114**,** 341-353.
Grehn, S., Fridell, K., Lillecreutz, M. & Hallert, C. 2001. Dietary habits of Swedish adult coeliac patients treated by a gluten-free diet for 10 years. *Scandinavian Journal of Nutrition,* 45**,** 178-182.
Groehn, V. & Moser, R. 1999. Synthesis of optically pure diastereoisomers of reduced folates. *Pteridines,* 10**,** 95-100.
Hallert, C., Grant, C., Grehn, S., Gränno, C., Hultén, S., Midhagen, G., Ström, M., Svensson, H. & Valdimarsson, T. 2002. Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Alimentary Pharmacology & Therapeutics,* 16**,** 1333-1339.
Halsted, C. H. 1990. Intestinal absorption of dietary folates. In *Folic acid metabolism in health and disease* Vol. 13 (Eds, Picciano, M. F., Stokstad, E. L. R. & Gregory III, J. F.) Wiley-Liss, New York, 23-46.

Hart, D. J., Finglas, P. M., Wolfe, C. A., Mellon, F., Wright, A. J. A. & Southon, S. 2002. Determination of 5-methyltetrahydrofolate (^{13}C-labeled and unlabeled) in human plasma and urine by combined liquid chromatography mass spectrometry. *Analytical Biochemistry,* 305**,** 206-213.

Hawkes, J. G. & Villota, R. 1989. Folates in foods: Reactivity, stability during processing, and nutritional implications. *Critical Reviews in Food Science and Nutrition,* 28**,** 439-526.

Herbert, V. & Das, K. C. 1994. Folic acid and vitamin B_{12}. In *Modern nutrition in health and disease* Vol. 1 (Eds, Shils, M., Olson, J. M. & Shike, M.) Lea & Febiger, Philadelphia, 402-425.

Holt, D. L., Wehling, R. L. & Zeece, M. G. 1988. Determination of native folates in milk and other dairy-products by high-performance liquid-chromatography. *Journal of Chromatography,* 449**,** 271-279.

Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D. & Wong, L.-Y. C. 2001. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA,* 285**,** 2981-2986.

Horne, D. W. 1997. Microbiological assay of folates in 96-well microtiter plates. *Methods in Enzymology,* 281**,** 38-43.

Horne, D. W. 2001. High-performance liquid chromatographic measurement of 5,10-methylenetetrahydrofolate in liver. *Analytical Biochemistry,* 297**,** 154-159.

http://dimkin.df.ru/clipart/clipart_show.html?cat=100000027&start=10, -. N. 13-Nov 2004

Håkansson, B., Jägerstad, M., Öste, R., Åkesson, B. & Jonsson, L. 1987. The effects of various thermal processes on protein quality, vitamins and selenium content in whole-grain wheat and white flour. *Journal of Cereal Science,* 6**,** 269-282.

Jacoby, B. T. & Henry, F. T. 1992. Liquid chromatographic determination of folic acid in infant formula and adult medical nutritionals. *Journal of AOAC International,* 75**,** 891-898.

Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J. & Rozen, R. 1996. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation,* 93**,** 7-9.

Jacques, P. F., Selhub, J., Bostom, A. G., Wilson, P. W. F. & Rosenberg, I. H. 1999. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *The New England Journal of Medicine,* 340**,** 1449-1454.

Jägerstad, M., Piironen, V., Walker, C., Ros, G., Carnovale, E, Holasova**,** M, Nau,H. Increasing natural food folates through bioprocessing and biotechnology. *Trends in Food science and Technology, in press*

Jastrebova, J., Witthöft, C., Grahn, A., Svensson, U. & Jägerstad, M. 2003. HPLC determination of folates in raw and processed beetroots. *Food Chemistry,* 80**,** 579-588.

Johnston, K. E., Lofgren, P. A. & Tamura, T. 2002. Folate concentrations of fast foods measured by trienzyme extraction method. *Food Research International,* 35**,** 565-569.

Jones, M. L. & Nixon, P. F. 2002. Tetrahydrofolates are greatly stabilized by binding to bovine milk folate-binding protein. *Journal of Nutrition,* 132**,** 2690-2694.

Kang, S.-S., Wong, P. W. K. & Norusis, M. 1987. Homocysteinemia due to folate deficiency. *Metabolism,* 36**,** 458-462.

Kariluoto, S., Vahteristo, L., Salovaara, H., Katina, K., Liukkonen, K. H. & Piironen, V. 2004. Effect of baking method and fermentation on folate content of rye and wheat breads. *Cereal Chemistry,* 81**,** 134-139.

Kariluoto, S. M., Vahteristo, L. & Piironen, V. 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.

Keagy, P., Shane, B. & Oace, S. 1988. Folate bioavailability in humans: Effects of wheat bran and beans. *American Journal of Clinical Nutrition,* 47**,** 80-88.

Keagy, P. M., Borenstein, B., Ranum, P., Connor, M. A., Lorenz, K., Hobbs, W. E., Hill, G., Bachman, A. L., Boyd, W. A. & Kulp, K. 1980. Natural levels of nutrients in commercially milled wheat flours. II. Vitamin analysis. *Cereal Chemistry,* 57**,** 59-65.

Keagy, P. M., Stokstad, E. L. R. & Fellers, D. A. 1975. Folacin stability during bread processing and family flour storage. *Cereal Chemistry,* 52**,** 348-356.
Kelly, P., McPartlin, J., Goggins, M., Weir, D. G. & Scott, J. M. 1997. Unmetabolized folic acid in serum: Acute studies in subjects consuming fortified food and supplements. *American Journal of Clinical Nutrition,* 65**,** 1790-1795.
Kok, R. M., Smith, D. E. C., Dainty, J. R., van den Akker, J. T., Finglas, P. M., Smulders, Y. M., Jakobs, C. & de Meer, K. 2004. 5-methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: Applications to folate absorption and metabolism. *Analytical Biochemistry,* 326**,** 129-138.
Konings, E. J., Roomans, H. H., Dorant, E., Goldbohm, R. A., Saris, W. H. & 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**,** 765-776.
Konings, E. J. M. 1999. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. *Journal of AOAC International,* 82**,** 119-125.
Konings, E. J. M., Troost, F. J., Castenmiller, J. J. M., Roomans, H. H. S., van den Brandt, P. A. & Saris, W. H. M. 2002. Intestinal absorption of different types of folate in healthy subjects with an ileostomy. *British Journal of Nutrition,* 88**,** 235-242.
Lamers, Y., Prinz-Langenohl, R., Moser, R. & Pietrzik, K. 2004. Supplementation with (6S)-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. *American Journal of Clinical Nutrition,* 79**,** 473-478.
Layne, J. 2002. Characterization and comparison of the chromatographic performance of conventional, polar-embedded, and polar-endcapped reversed-phase liquid chromatography stationary phases. *Journal of Chromatography A,* 957**,** 149-164.
Lewis, C. J., Crane, N. T., Wilson, D. B. & Yetley, E. A. 1999. Estimated folate intakes: Data updated to reflect food fortification, increased bioavailability, and dietary supplement use. *American Journal of Clinical Nutrition,* 70**,** 198-207.
Liu, S., West, R., Randell, E., Longerich, L., O´Connor, K., Scott, H., Crowley, M., Lam, A., Prabhakaran, V. & McCourt, C. 2004. A comprehensive evaluation of food fortification with folic acid for the primary prevention of neural tube defects. *BMC Pregnancy Childbirth,* 4**,** 20.
Liukkonen, K-H., Katina, K., Wilhelmsson, A., Myllymäki, O., Lampi, A-M., Kariluoto, S., Piironen, V., Heinonen, S. M., Nurmi, T., Adlercreutz, H., Peltoketo, A., Pihlava, J-M., Hietaniemi, V. & Poutanen, K. 2003. Process-induced changes on bioactive compounds in whole grain rye. *Proceedings of the Nutrition Society,* 62**,** 117-122.
Livsmedelsverket. 2002. Food composition table-energy and nutrients. Uppsala, Sweden,
Loew, D., Eberhardt, A., Heseker, H. & Kübler, W. 1987. Zur Plasmakinetik und Elimination der Folsäure. *Klinische Wochenschrift,* 65**,** 520-524.
Lucock, M. 2000. Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. *Molecular Genetics and Metabolism,* 71**,** 121-138.
Lucock, M. D., Green, M., Priestnall, M., Daskalakis, I., Levene, M. I. & Hartley, R. 1995. Optimisation of chromatographic conditions for the determination of folates in foods and biological tissues for nutritional and clinical work. *Food Chemistry,* 53**,** 329-338.
Lucock, M. D., Hartley, R. & Smithells, R. W. 1989. A rapid and specific HPLC-electrochemical method for the determination of endogenous 5-methyltetrahydrofolic acid in plasma using solid phase sample preparation with internal standardization. *Biomedical Chromatography,* 3**,** 58-63.
Malinow, M. R., Duell, P. B., Hess, D. L., Anderson, P. H., Kruger, W. D., Phillipson, B. E., Gluckman, R. A., Block, P. C. & Upson, B. M. 1998. Reduction of plasma homocyst(e)ine levels by breakfast cereal fortified with folic acid in patients with coronary heart disease. *The New England Journal of Medicine,* 338**,** 1009-1015.
Mangoni, A. A. & Jackson, S. H. D. 2002. Homocysteine and cardiovascular disease: Current evidence and future prospects. *The American Journal of Medicine,* 112**,** 556-565.
Margo, G., Barker, M., Fernandes-Costa, F., Colman, N., Green, R. & Metz, J. 1975. Prevention of folate deficiency by food fortification. VII. The use of bread as a vehicle for folate supplementation. *American Journal of Clinical Nutrition,* 28**,** 761-763.

Martin, C. A. 1995. Folate analysis in foods - how accurate is it? *BNF Nutrition Bulletin,* 20**,** 8-15.

Maruyama, T., Shiota, T. & Krumdieck, C. L. 1978. The oxidative cleavage of folates. *Analytical Biochemistry,* 84**,** 277-295.

McCalley, D. V. 1999. Comparison of the performance of conventional C_{18} phases with others of alternative functionality for the analysis of basic compounds by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A,* 844**,** 23-38.

Melse-Boonstra, A., West, C. E., Katan, M. B., Kok, F. J. & Verhoef, P. 2004. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. *American Journal of Clinical Nutrition,* 79**,** 424-429.

Mills, J. L. & England, L. 2001. Food fortification to prevent neural tube defects. Is it working? *JAMA,* 285**,** 3022-3023.

Mills, J. L., Von Kohorn, I., Conley, M. R., Zeller, J. A., Cox, C., Williamson, R. E. & Dufour, D. R. 2003. Low vitamin B-12 concentrations in patients without anemia: The effect of folic acid fortification of grain. *American Journal of Clinical Nutrition,* 77**,** 1474-1477.

Molloy, A. M., Daly, S., Mills, J. L., Kirke, P. N., Whitehead, A. S., Ramsbottom, D., Conley, M. R., Weir, D. G. & Scott, J. M. 1997. Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: Implications for folate intake recommendations. *The Lancet,* 349**,** 1591-1593.

Molloy, A. M. & Scott, J. M. 1997. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. *Methods in Enzymology,* 281**,** 43-53.

MRC Vitamin Study Research Group. 1991. Prevention of neural tube defects: Results of the medical research council vitamin study. *The Lancet,* 338**,** 131-137.

Mullin, R. J. & Duch, D. S. 1992. Folic acid. In *Modern chromatographic analysis of vitamins* Vol. 60 (Eds, De Leenheer, A. P. L., W.E. Nelis, H.J.) Marcel Deeker, New York, 261-283.

Mullin, W. J. & Jui, P. Y. 1986. Folate content of bran from different wheat classes. *Cereal Chemistry,* 63**,** 516-518.

Murray, J. A. 1999. The widening spectrum of celiac disease. *American Journal of Clinical Nutriton,* 69**,** 354-365.

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-methylterahydrofolates. *Journal of Chromatography,* 928**,** 77-90.

Nelson, B. C., Dalluge, J. J. & Margolis, S. A. 2001. Preliminary application of liquid chromatography-electrospray-ionization mass spectrometry to the detection of 5-methyltetrahydrofolic acid monoglutamate in human plasma. *Journal of Chromatography B: Biomedical Sciences and Applications,* 765**,** 141-150.

Nelson, B. C., Pfeiffer, C. M., Margolis, S. A. & Nelson, C. P. 2003. Affinity extraction combined with stable isotope dilution LC/MS for the determination of 5-methyltetrahydrofolate in human plasma. *Analytical Biochemistry,* 313**,** 117-127.

Newman, E. M. & Tsai, J. F. 1986. Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. *Analytical Biochemistry,* 154**,** 509-515.

O'Broin, J., Temperley, I., Brown, J. & Scott, J. 1975. Nutritional stability of various naturally occurring monoglutamate derivatives of folic acid. *American Journal of Clinical Nutrition,* 28**,** 438-444.

Osseyi, E. S., Wehling, R. L. & Albrecht, J. A. 2001. HPLC determination of stability and distribution of added folic acid and some endogenous folates during breadmaking. *Cereal Chemistry,* 78**,** 375-378.

Pawlosky, R. J. & Flanagan, V. P. 2001. A quantitative stable-isotope LC-MS method for the determination of folic acid in fortified foods. *Journal of Agricultural and Food Chemistry,* 49**,** 1282-1286.

Pawlosky, R. J., Flanagan, V. P. & Doherty, R. F. 2003. A mass spectrometric validated high-performance liquid chromatography procedure for the determination of folates in foods. *Journal of Agricultural and Food Chemistry,* 51**,** 3726-3730.

Pfeiffer, C. M., Fazili, Z., McCoy, L., Zhang, M. & Gunter, E. W. 2004. Determination of folate vitamers in human serum by stable-isotope-dilution tandem mass spectrometry and comparison with radioassay and microbiologic assay. *Clinical Chemistry,* 50**,** 423-432.

Pfeiffer, C. M., Rogers, L. M., Bailey, L. B. & Gregory, J. F. 1997b. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined using a dual-label stable-isotope protocol. *American Journal of Clinical Nutrition,* 66**,** 1388-1397.

Pfeiffer, C. M., Rogers, L. M. & Gregory, J. F. 1997a. Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. *Journal of Agricultural and Food Chemistry,* 45**,** 407-413.

Prinz-Langenohl, R., Brönstrup, A., Thorand, B., Hages, M. & Pietrzik, K. 1999. Availability of food folate in humans. *Journal of Nutrition,* 129**,** 913-916.

Quinlivan, E. P. & Gregory, J. F. 2003. Effect of food fortification on folic acid intake in the United States. *American Journal of Clinical Nutrition,* 77**,** 221-225.

Rader, J. I., Weaver, C. M. & Angyal, G. 1998. Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products. *Food Chemistry,* 62**,** 451-465.

Rader, J. I., Weaver, C. M. & Angyal, G. 2000. Total folate in enriched cereal-grain products in the United States following fortification. *Food Chemistry,* 70**,** 275-289.

Ranhotra, G. S., Gelroth, J. A., Novak, F. A. & Matthews, R. H. 1985. Retention of selected B vitamins in cooked pasta products. *Cereal Chemistry,* 62**,** 476-477.

Rebello, T. 1987. Trace enrichment of biological folates on solid-phase adsorption cartridges and analysis by high-pressure liquid-chromatography. *Analytical Biochemistry,* 166**,** 55-64.

Rozen, R. 2004. Folate and genetics. *Journal of Food Science,* 69**,** S65-S67.

Ruggeri, S., Vahteristo, L., Aguzzi, A., Finglas, P. M. & Carnovale, E. 1999. Determination of folate vitamers in food and in Italian reference diet by high-performance liquid chromatography. *Journal of Chromatography A,* 855**,** 237-245.

Rychlik, M. 2004. Revised folate content of foods determined by stable isotope dilution assays. *Journal of Food Composition and Analysis,* 17**,** 475-483.

Schorah, C. J., Devitt, H., Lucock, M. & Dowell, A. C. 1998. The responsiveness of plasma homocysteine to small increases in dietary folic acid: A primary care study. *European Journal of Clinical Nutrition,* 52**,** 407-411.

Scott, J., Rébeille, F. & Fletcher, J. 2000. Folic acid and folates: The feasibility for nutritional enhancement in plant foods. *Journal of the Science of Food and Agriculture,* 80**,** 795-824.

Scott, J. M. 1999. Folate and vitamin B_{12}. *Proceedings of the Nutrition Society,* 58**,** 441-448.

Seshadri, S., Beiser, A., Selhub, J., Jacques, P. F., Rosenberg, I. H., D'Agostino, R. B., Wilson, P. W. F. & Wolf, P. A. 2002. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *The New England Journal of Medicine,* 346**,** 476-483.

Shaw, G. M., Carmichael, S. L., Nelson, V., Selvin, S. & D.M, S. 2003. Food fortification with folic acid and twinning among Califonia infants. *American Journal of Medical Genetics,* 119A**,** 137-140.

Shrestha, A. K., Arcot, J. & Paterson, J. 2000. Folate assay of food by traditional and tri-enzyme treatments using cryoprotected *Lactobacillus casei*. *Food Chemistry,* 71**,** 545-552.

Snyder, L. R., Kirkland, J. J. & Glajch, J. L. (Eds.) 1987. *Practical HPLC method development,* John Wiley & Sons, New York.

Socialstyrelsens meddelande blad nr 1/2001. 2001. Folsyra i samband med graviditet. Stockholm,

Stokes, P. & Webb, K. 1999. Analysis of some folate monoglutamates by high-performance liquid chromatography-mass spectrometry. I. *Journal of Chromatography A,* 864**,** 59-67.

Strålsjö, L., Åhlin, H., Witthöft, C. M. & Jastrebova, J. 2003. Folate determination in Swedish berries by radioprotein-binding assay (RPBA) and high performance liquid chromatography (HPLC). *European Food Research and Technology,* 216**,** 264-269.

Strålsjö, L., Arkbåge, K., Witthöft, C. & Jägerstad, M. 2002. Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk. *Food Chemistry,* 79**,** 525-534.

Tamura, T. 1990. Microbiological assay of folates. In *Folic acid metabolism in health and disease* Vol. 13 (Eds, Picciano, M. F., Stokstad, E. L. & Gregory III, J. F.) Wiley-Liss, New York, 121-138.

Tannenbaum, S. R., Young, V. R. & Archer, M. C. 1985. Vitamins and minerals. In *Food chemistry* (Eds, Fennema, O. R.) Marcel Dekker, New York, 477-544.

Thompson, T. 2000. Folate, iron, and dietary fiber contents of the gluten-free diet. *Journal of the American Dietetic Association,* 100**,** 1389-1396.

Vahteristo, L. 1998. Food folates and their analysis: Determination of folate derivatives and their stability by high-performance liquid chromatography, PhD Thesis. *Department of Applied Chemistry and Microbiology University of Helsinki*, Helsinki.

Vahteristo, L. & Finglas, P. M. 2000. Chromatographic determination of folates. In *Modern chromatographic analysis of vitamins* (Eds, De Leenheer, A. P., Lambert, W. E. & Van Bocxlaer, J. F.) Marcel Dekker, Inc., New York.

Vahteristo, L., Kariluoto, S. M., Bärlund, S., Kärkkäinin, M., Lamberg-Allardt, C., Salovaara, H. & Piironen, V. 2002. Functionality of endogenous folates from rye and orange juice using human *in vivo* model. *European Journal of Nutrition,* 41**,** 271-278.

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**,** 477-482.

Wald, D. S., Law, M. & Morris, J. K. 2002. Homocysteine and cardiovascular disease: Evidence on causality from a meta-analysis. *British Medical Journal,* 325**,** 1202-1206.

Waller, D. K., Tita, A. T. N. & Annegers, J. F. 2003. Rates of twinning before and after fortification of foods in the US with folic acid, Texas 1996 to 1998. *Paediatric and Perinatal Epidemiology,* 17**,** 378-383.

van Tellingen, O., van der Woude, H. R., Beijnen, J. H., van Beers, C. J. T. & Nooyen, W. J. 1989. Stable and sensitive method for the simultaneous determination of N^5-methyltetrahydrofolate, leucovorin, methotrexate and 7-hydroxymetrexate in biological fluids. *Journal of Chromatography,* 488**,** 379-388.

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.

Verwei, M., Arkbåge, K., Havenaar, R., van den Berg, H., Witthöft, C. & Schaafsma, G. 2003. Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. *Journal of Nutrition,* 133**,** 2377-2383.

Vervoort, R. J. M., Debets, A. J. J., Claessens, H. A., Cramers, C. A. & de Jong, G. J. 2000. Optimisation and characterisation of silica-based reversed- phase liquid chromatographic systems for the analysis of basic pharmaceuticals. *Journal of Chromatography A,* 897**,** 1-22.

White, D. R. J. 1990. Determination of 5-methyltetrahydrofolate in citrus juice by reversed-phase high-performance liquid chromatography with electrochemical detection. *Journal of Agricultural and Food Chemistry,* 38**,** 1515-1518.

Whitney, E. N., Cataldo, C. B. & Rolfes, S. R. 1994. *Understanding normal & clinical nutrition,* West publishing Company, Minneapolis.

Wigertz, K. 1997. Milk folates. Characterisation and availability, PhD Thesis. *Department of Applied Nutrition and Food Chemistry, Lund University*, Lund, Sweden.

Wigertz, K. & Jägerstad, M. 1995. Comparison of a HPLC and radioprotein-binding assay for the determination of folates in milk and blood samples. *Food Chemistry,* 54**,** 429-436.

Wilson, S. D. & Horne, D. W. 1984. High-performance liquid chromatographic determination of the distribution of naturally occurring folic acid derivatives in rat liver. *Analytical Biochemistry,* 142**,** 529-535.

Witthöft, C., Strålsjö, L., Berglund, G. & Lundin, E. 2003. A human model to determine folate bioavailability from food: A pilot study for evaluation. *Scandinavian Journal of Nutrition,* 47**,** 6-18.
Witthöft, C. M., Forssén, K., Johannesson, L. & Jägerstad, M. 1999. Folates-food sources, analysis, retention and bioavailability. *Scandinavian Journal of Nutrition,* 43**,** 138-146.
Yates, A. A., Schlicker, S. A. & Suitor, C. W. 1998. Dietary reference intakes: The new basis for recommendations for calcium and related nutrients, B vitamins, and choline. *Journal of the American Dietetic Association,* 98**,** 699-706.
Yon, M. & Hyun, T. H. 2003. Folate content of foods commonly consumed in Korea measured after trienzyme extraction. *Nutrition Research,* 23**,** 735-746.

Acknowledgement/Tack

Först och främst vill jag rikta ett stort och varmt tack till alla som jobbat på LMV och LMK i synnerhet, under min doktorandtid. Många personer har hjälp mig under de här åren som forskarstuderande och jag vill framförallt tacka:

Min huvudhandledare Margaretha Jägerstad för att du alltid har tid för mig trots ditt fullttecknade schema. Tack för att du alltid försöker se en rationell lösning på alla problem och för att jag alltid har fått ditt stöd. Ett stort tack för alla utmärkta ideer och förbättringar i mina artikar och i avhandlingen.

Cornelia Witthöft, min biträdande handledare, först och främst ett stort tack för din stora entusiasm för folaternas värld. Den har smittat av sig! Ett jättetack för kul och intressant samarbete i samband med humanstudierna och för ALLA kommentarer på kappan.

Jelena Jastrebova, min biträdande handledare, ett stort tack för att du delat med dig av ditt stora analytiska kunnande. Det har varit lärorikt. Tack för allt arbete du lagt ner på våra gemensamma publikationer och för kommentarer på avhandlingen.

Lena och Karin, mina folatsystrar. Jag har saknat er det här sista halvåret. Tack för alla gemensamma stunder vi har haft, både med skratt och gråt. Tack för att ni alltid har haft tid att diskutera folatrelaterade frågor (och allt annat förstås!) och även haft kommentarer på den här boken.

To all my co-authors for excellent help, without you this thesis would not have become reality. Special thanks to Charlotte and Elena for all the hard work.

Till alla mina rumskamrater under dessa år. Ett speciellt tack till Ninni, Barbara, Irina och Johan för trevliga pratstunder.

Alla doktorander, gamla som nya, för alla roliga påhitt som; julpyssel, chokladprovning, restaurangbesök, studieresor och planeringar inför disputationsspex. Ett stort tack till er som även blivit mina vänner utanför jobbet.

Till lunchgänget med Gertrud och Lotta W i spetsen. Det har varit ett trevligt avbrott i arbetet.

Lars och Maria för alla peppningar och trevliga pratstunder.

Till Carina, Eva, Maggan och Einar för att ni fixar allt det praktiska. Ett speciellt tack till Eva för all hjälp i samband med min USA-resa.

I also wish to thank Dr. van Breemen at University of Illinois at Chicago, whom I could visit in the autumn 2002. The training in your lab was a great experience for me. Thanks to the van Breemen family for inviting me to celebrate Thanksgiving with you. Thanks to everyone in the lab for helping me with a lot of things

including furniture and kitchen utensils for my studio. Thanks to Spiros for all the interesting chats and special thanks to Mihae who made my visit such a pleasant memory.

En stor kram till alla mina vänner och släktingar som jag har varit alldeles för dålig på att hålla kontakt med den senaste tiden.

Till farmor, du är en förebild och till mormor för att du alltid har visat ett stort intresse för vad jag gör.

Till Weistrands, ett jättetack för allt ni gör för oss, fixar duschen, lampor, putsar fönster, flyttstädar och lagar en massa god mat. Jag är er evigt tacksam.

Mamma och pappa för att ni alltid finns där och hjälper mig när jag behöver det. Pappa för att du har lärt mig att aldrig ge upp och mamma för att du är min bästa kompis. Min systeryster Caroline för att vi alltid står enade mot andra och för att du alltid reser till så kul ställen där jag kan besöka dig.

Min lilla familj, Ola och Nils. Vad vore jag utan er? Ola, för att du alltid stöttar och uppmuntrar mig och Nils för att du gör mitt liv så mycket roligare.