

Analytical and Nutritional Aspects of Folate in Cereals

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Abstract

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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 C₁₈ column.

Optimising the sample purification prior to quantification can also improve the HPLC method. Reversed-phase sorbents, *e.g.* phenyl-encapped and cyclohexyl-encapped, 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-CH₃-H₄folate, HPLC, SPE purification, enzyme treatment, folate absorption, fortification, gluten-free products, wheat breakfast rolls

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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 celledelning 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 ryggmärgsbräck. 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 diskuteras man därför en allmän folatberikning av vetemjöl och rågsikt. I dagsläget är endast barmat 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 B₁₂-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 C₁₈-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.

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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

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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 ₄ folate	10-formyl-tetrahydrofolate
5,10-CH ⁺ -H ₄ folate	5,10-methylene-tetrahydrofolate
5,10-CH ₂ -H ₄ folate	5,10-methenyl-tetrahydrofolate
5-CH ₃ -H ₄ folate	5-methyl-tetrahydrofolate
5-HCO-H ₄ folate	5-formyl-tetrahydrofolate
ACN	acetonitrile
As	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 ₂ folate	dihydrofolate
H ₄ folate	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 than 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 (H₂folate) or fully reduced (H₄folate). Reduced folate can exist with or without one-carbon substituents. Substituents can exist on either the N⁵ or N¹⁰ positions (predominantly as methyl, formyl or formino groups), or as methylene or methenyl units bridging between N⁵ and N¹⁰. 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 H₄folate and its 5-HCO- and 5-CH₃-derivatives are the [6S, αS] diastereoisomers, whereas the natural diastereoisomers of 10-HCO-, 5, 10-CH₂- and 5,10-CH⁺-H₄folate 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 H₄folate 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 H₄folate or H₂folate (Hawkes & Villota, 1989; Ball, 1998). The order of stability of H₄folate forms in aqueous solutions is 5-HCO-H₄folate > 5-CH₃-H₄folate > 10-HCO-H₄folate > H₄folate since substitution at N⁵ and N¹⁰ 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 C⁹-N¹⁰ bond, the pteridine ring system or both (Gregory, 1996). It is known that oxidative cleavage of H₄folate, H₂folate and folic acid yield nutritionally inactive products (*p*-aminobenzoylglutamate and a pterin) (Maruyama, Shiota & Krumdieck, 1978; Tannenbaum, Young & Archer, 1985; Gregory, 1996).

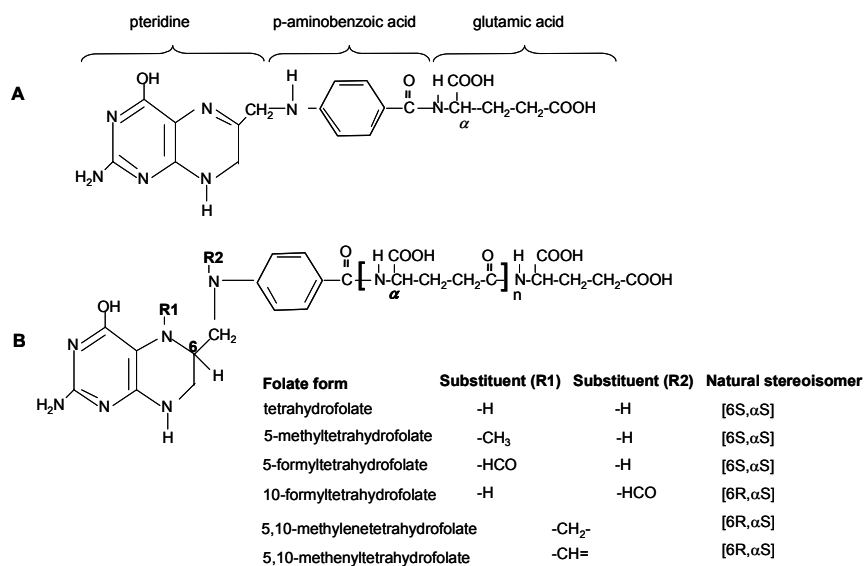


Figure 1. The folate molecules: A, Folic acid, B, Polyglutamyl tetrahydrofolate

Folate undergoes interconversion in ionic form as a function of pH. In acidic pH 10-HCO-H₄folate tends to form 5, 10-CH⁺-H₄folate. 5-HCO-H₄folate also forms 5,10-CH⁺-H₄folate in acidic media (pH 1-2) but at a much slower rate (Pfeiffer, Rogers & Gregory, 1997a). When a solution of 5,10-CH⁺-H₄folate is neutralized, hydrolysis occurs to yield 10-HCO-H₄folate, and a small amount of 5-HCO-H₄folate (Gregory, 1989). 10-HCO-H₄folate is completely oxidised to 10-HCO-H₂folate and 10-HCO-folic acid during food preparation (Konings, 1999). 5,10-CH₂-H₄folate is readily dissociated to H₄folate at acidic, near neutral pH (Horne, 2001). 5-CH₃-H₄folate has been reported to have an oxidation rate of about one tenth of that of H₄folate (Hawkes & Villota, 1989). 5-CH₃-H₄folate is oxidised to 5-CH₃-H₂folate 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 H₄folate 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-CH₃-H₄folate 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-CH₃-H₄folate monoglutamate and folate in human plasma exists almost exclusively in this form (Pfeiffer *et al.*, 2004). Folic acid is reduced to H₂folate and then converted to 5-CH₃-H₄folate 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 μ g/dose of supplemental folic acid.

After absorption, 5-CH₃-H₄folate 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-CH₃-H₄folate and 10-HCO-H₄folate (Combs, 1992; Herbert & Das, 1994). Folate undergoes substantial enterohepatic recirculation, with as much as 100 μ g 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-CH₃-H₄folate. 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

