Folate Bioavailability

In vitro Experiments and Human Trials

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2009

Acta Universitatis agriculturae Sueciae
2009: 63

ISSN 1652-6880
ISBN 978-91-576-7410-4

Print: SLU Service/Repro, Uppsala 2009

Folate Bioavailability. *In vitro* Experiments and Human Trials

Abstract

An inadequate folate status is associated with increased risk of anaemia and neural tube defects. In many countries a folate intake below recommendations has been reported for women in childbearing age. However, data on folate intake and status are not always associated, since factors other than intake, *e.g.* bioavailability, affect folate status.

This thesis studied the bioavailability of folate using *in vivo* and *in vitro* models. The effect of two pieces of Swedish nutritional advice on folate status of healthy women was assessed in a 12-week randomised controlled intervention trial. By combining a human plasma area under the curve (AUC)/ileostomy model with a stable isotope technique, the bioavailability of wholemeal bread fortified with reduced folate (5-CH_3-H_4folate) or folic acid was determined. *In vitro* effects of the food matrix on bioaccessibility and uptake of reduced folates were studied using a dynamic gastrointestinal model (TIM) and Caco-2 cells.

The intervention breakfast diet (contributing ~1/3 of recommended daily folate intake) significantly improved folate status of the women, indicating a high folate bioavailability. The bread intervention diet (contributing ~1/5 of recommended daily folate intake) maintained folate and decreased homocysteine concentrations.

Based on folate content in stomal effluent, the bioavailability of both folate fortificants tested was similarly high (~90%) but plasma kinetics differed significantly depending on ingested folate form. Data from *in vitro* TIM and Caco-2 cell experiments showed an inhibitory effect (~25%) of the bread matrix on bioaccessibility and uptake of reduced folates, which was higher than *in vivo* findings.

Overall, data from these *in vivo* and *in vitro* studies suggest that the bioavailability of reduced folate is high and comparable to that of synthetic folic acid. Food matrix effects, *e.g.* of bread or a typical breakfast meal, on folate bioavailability seem negligible at physiological folate intake doses.

Keywords: folates, folic acid, intervention trial, plasma AUC/ileostomy model, TNO gastrointestinal model, Caco-2 cell, food folate intake stable isotope-labell

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To all of you interested in folates

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This thesis is based on the work contained in the following papers, which are referred to in the text by their Roman numerals:

I Öhrvik V, Olsson J, Sundberg B and Witthöft CM (2009). Effect of two pieces of nutritional advice on folate status in Swedish women: A randomised controlled dietary intervention trial. *American Journal of Clinical Nutrition* 89(4), 1053-1058.

II Öhrvik V, Büttner B, Rychlik M, Lundin E and Witthöft CM. Folate absorption from breads and a meal assessed with a novel human stable isotope AUC/ileostomy model. *Manuscript*.

III Öhrvik V and Witthöft CM (2008) Orange juice is a good folate source in respect to folate content and stability during storage and simulated digestion. *European Journal of Nutrition* (47), 92-98.

IV Öhrvik V, Öhrvik H, Tallkvist J and Witthöft CM. Folates in bread – retention during bread-making and *in vitro* bioaccessibility. *Submitted*.

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The contribution of Veronica Öhrvik to the papers included in this thesis was as follows:

I Planned and conducted the human trial together with main supervisor. Performed food and statistical analyses and had main responsibility for writing and revising the paper.

II Planned and conducted the human trial together with main supervisor. Carried out food analyses and participated in preparation of clinical samples for analyses. Evaluated analytical data for plasma and stomal effluent, and performed kinetical and statistical analysis. Had main responsibility for writing the manuscript.

III Planned experiments with main supervisor and performed stability experiments, laboratory analyses, evaluated results and had main responsibility for writing and revising the paper.

IV Planned experiments with main supervisor. Performed baking experiments, assisted in Caco-2 cell experiments, carried out laboratory analyses and evaluated results. Had main responsibility for writing the paper.

Abbreviations

AUC	Area under the curve
CV	Coefficient of variance
EC	Electrochemical
FBP	Folate-binding proteins
FLD	Fluorescence detector
FFQ	Food frequency questionnaire
FR	Folate receptor
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
MS	Mass spectrometry
NTD	Neural tube defect
PCFT	Protein-coupled folate transporter
Q_1 and Q_3	1st quartile and 3d quartile
RC	Reliability coefficient
RFC	Reduced folate carrier
SAX	Strong anion exchange
SIDA	Stable isotope dilution assay
SPE	Solid phase extraction
tHcy and homocysteine	Total plasma homocysteine
(6S)-H_4folate	Tetrahydrofolate
(6S)-5-CH_3-H_4folate	5-methyl-tetrahydrofolate
(6S)-5-HCO-H_4folate	5-formyl-tetrahydrofolate
(6R)-5,10-CH+-H_4folate	5,10-methylene-tetrahydrofolate
(6R)-10-HCO-H_4folate	10-formyl-tetrahydrofolate
(6R)-10-HCO-H_2folate	10-formyl-dihydrofolate
10-HCO-folic acid	10-formylfolic acid

1 Background

Nearly 80 years ago, Lucy Wills treated anaemic pregnant Indian women with a yeast extract (Wills *et al.*, 1937). The active compound in the yeast was later identified as folate, at that time called antianaemia factor (Pfiffner *et al.*, 1945). As folates are required for cellular replication and thereby production of red blood cells, folate deficiency causes anaemia.

Folate requirement is now known to be higher in life-stages with intense cell division, *e.g.* during pregnancy. The preventive effect of a high folic acid intake against neural tube defects (NTD) is considered one of the most important nutritional discoveries between 1976-2006 (Katan *et al.*, 2009). This is due to the severity of NTD rather than the incidence rate, which is ~0.1% of births in Europe (Busby *et al.*, 2005). Children born with NTD often die before the age of 5 (~50%) or have mobility and intellectual disabilities (Sutton *et al.*, 2008).

An inadequate intake of folates is the only risk factor for NTD that can be directly affected (Mitchell *et al.*, 2004). Unfortunately, folates and vitamin D are the only vitamins for which a varied diet cannot be assumed to completely cover the nutrient requirements of the entire population in the Nordic countries (NNR, 2005). Reported folate intake in women of childbearing age is lower than recommended in Scandinavia (NNR, 2005) but also in several other countries world-wide, *e.g.* the Netherlands (van de Rest *et al.*, 2007) and Thailand (Sirikulchayanonta *et al.*, 2004).

Fruit and vegetables are good folate sources and are commonly consumed by the Swedish population (Becker & Pearson, 2003). However, the most popular fruit and vegetables, *i.e.* tomato, apple and pear, hardly contain any folates. In addition, consumption of very good folate sources, such as pulses, is almost negligible (Becker & Pearson, 2003). Intake of folates is further reduced by folates being greatly unstable molecules (Figure 1), resulting in

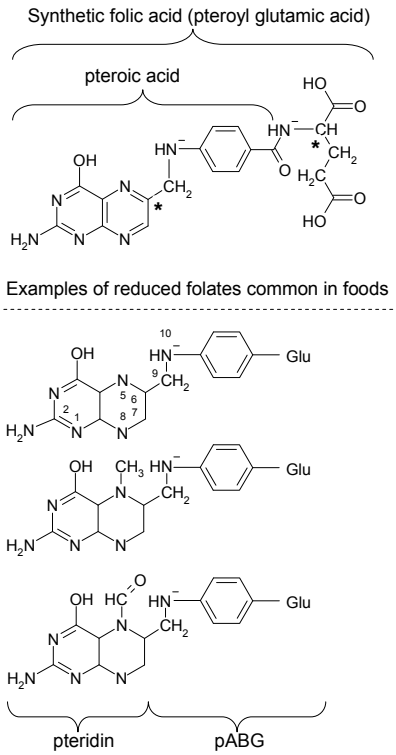
average 30% losses of vitamin activity during food processing (Bergström, 1994).

The most stable folate form, *i.e.* folic acid (Figure 1), is cheap to synthesise and is thus frequently used for food fortification and in supplements. National introduction of folic acid-fortified staple foods, *e.g.* in the USA and Canada, has effectively reduced the prevalence of NTD (Mosley *et al.*, 2009; Liu *et al.*, 2004). On the other hand fortification is questioned, as a high folic acid intake is suggested to accelerate development of *e.g.* colorectal cancer (Kim, 2007).

Recommendations are met more easily through consumption of folic acid-fortified foods, partly due to the higher bioavailability of folic acid compared with food folates. A food folate bioavailability value of 50% of that of folic acid supplements is commonly assumed when establishing recommendations and upper limits of intake e.g. at Centre for Disease Control in USA (CDC, 2009) and in the Nordic Nutrition Recommendations (NNR, 2005). The 50% level must be considered a rough estimate, as data on food folate bioavailability vary substantially from 30% (Hannon-Fletcher *et al.*, 2004) to 98% (Brouwer *et al.*, 1999). The large discrepancy in estimated bioavailability could be caused by different test foods, *e.g.* the food matrix may entrap folates and food components may affect folate stability and inhibit absorption. Different absorption and distribution kinetics for food (reduced) folates and folic acid have also been shown (Wright *et al.*, 2005). Moreover, differences in study design, study population and analytical methods may affect estimated bioavailability.

1.1 Metabolism of folates

Unlike plants and bacteria, mammals cannot synthesise folates and hence intake through food or supplements is required (Figure 2). Assessment of data on human *in vivo* folate production, distribution and excretion (Figure 2) is difficult and hence the limited data summarised in Figure 2 mostly originate from ingestion of radio- or stable isotope-labelled folic acid and are not always representative.



The IUPAC nomenclature (Blakley, 1988) of folates is 'a group of heterocyclic compounds based on a 4-[(pteridin-6-ylmethyl)amino]benzoic acid skeleton conjugated with one or more L-glutamate units'. Over 150 folate forms exist in theory, but in most animals and plants less than 50 are found. In nature, folates exist in reduced form but during sample preparation and storage folates may be partly or totally oxidised.

Causes of **structural variations** are:
1. Oxidation state of the pterin ring.
2. Number of glutamate (Glu) residues, often 3-11.
3. One-carbon substituents at N-5 and/or N-10.
4. Diastereoisomery, two chiral centres (*) exist and determine folate activity. For H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate, (6S) has vitamin activity, while for 10-HCO-H_4folate, (6R) has. Oxidisation state and substitution lead to increased stability of the folate molecule.

Vitamin activity may be lost by oxidative cleaving at the 9C-10N bond resulting in a pterin and para-aminobenzoylglutamate (pABG) molecule.

Many folate forms are prone to **interconversion**, *e.g.* caused by oxygen or changes in pH. For folates often found in foods, common interconversions are:

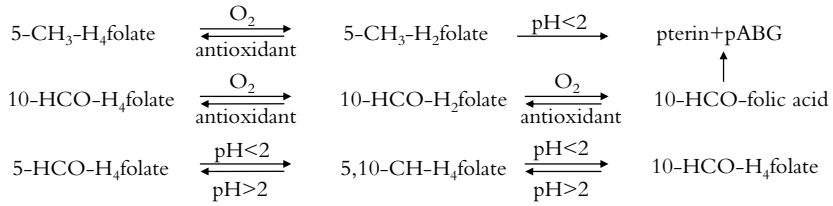


Figure 1. Structure, nomenclature and interconversion of synthetic folic acid and common food folate forms summarised from Gregory (1996) and Blakley (1988).

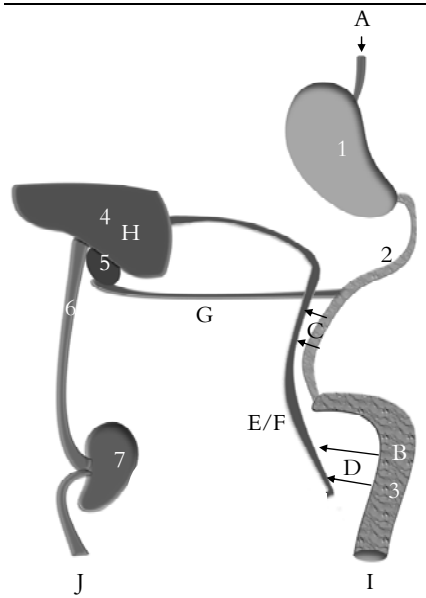
	Sources	Body pools	Excretion
	**A. Dietary intake**[1,2] USA[2]: ~715-3135 nmol/day (Choumenkovitch *et al.*, 2002). European countries: ~420-1050 nmol/day (Dhonukshe-Rutten *et al.*, 2009). **B. Microbial synthesis** ~25-100 nmol/day in infants (Kim *et al.*, 2004). **C. Absorption of food folate** ~50-98% (Winkels *et al.*, 2007; Witthöft *et al.*, 2006; Lin *et al.*, 2004; Konings *et al.*, 2002; Sauberlich *et al.*, 1987). **D. Absorption in colon** Physiological doses (684 nmol) are absorbed in colon after caecal infusion. (Aufreiter *et al.*, 2009)	**E. Plasma**[1] USA[2]: ~28 nmol/L (McDowell *et al.*, 2008). European countries: ~7-20 nmol/L (Dhonukshe-Rutten *et al.*, 2009). **F. Erythrocytes**[1] USA:~ 600 nmol/L (McDowell *et al.*, 2008). Denmark: ~820 nmol/L (Bor *et al.*, 2008). **G. Bile** ~90 nmol/L (Gregory & Quinlivan, 2002). Reabsorption of bile folate is estimated to be 96% (Lin *et al.*, 2004). **H. Liver** ~18±6 nmol/g (Hoppner & Lampi, 1980) (liver weight ~1350 g). Assumed to comprise ~50% of the folate pool (Gregory *et al.*, 1998).	**I. Faeces**[1] ~415 nmol/day adults (Lin *et al.*, 2004), ~100 nmol/day infants (Kim *et al.*, 2004). **J. Urine** ~3-75 nmol intact folate /day (Gregory *et al.*, 1998). ~330 nmol pABG/day (Gregory *et al.*, 1998). <5 % of an ingested folate dose is excreted as intact folate (Lin *et al.*, 2004; Witthöft *et al.*, 2003; Rogers *et al.*, 1997)

Figure 2. Schematic overview of the human folate pool, estimated sources of intake and excretion [1]Conversion of g to mol by multiplying by 0.441^{-1}. [2]Post-fortification (after 1998). [2]Recommended daily intake is 300-400 μg /day (700-900 nmol). 1 = stomach; 2 = small intestine; 3 = colon; 4= liver; 5 = gall bladder; 6 = circulation; 7 = kidney

Folate absorption

Folates are absorbed as monoglutamyl folates, mainly in the jejunum after cleavage of the polyglutamic chain by intestinal γ-glutamyl hydrolase (Halsted, 1990). Folate absorption is optimal at pH 6.3 (Russell *et al.*, 1979). As folates are charged and thereby hydrophilic in the neutral pH of the intestine (Figure 3), passive diffusion across the cell-membrane is limited (Zhao *et al.*, 2009). Instead, nearly all absorption (Figure 3) occurs actively by:

1. Transport across the luminal membrane by highly specific transporters, mainly the protein-coupled folate transporter (PCFT) (Qiu *et al.*, 2006).
2. Retention within the enterocyte, where absorbed folates are converted into 5-CH_3-H_4folate by dihydrofolate reductase (DHFR) and methyltetrahydrofolate reductase (MTHFR) (Chanarin & Perry, 1969).
3. Transport across the basolateral membrane into plasma by less specific transporters, *e.g.* multidrug resistance associated proteins (MRP) (Assaraf, 2006).

The affinities of folate receptors and carriers (transporters) vary with pH and thus different transporters are important in different cells (Figure 3). In addition, affinities vary between folate forms, being higher to reduced folates than folic acid for both PCFT and RFC (Zhao *et al.*, 2009). In contrast, affinity to folic acid is higher for FR α & ß (Zhao *et al.*, 2009; Assaraf, 2006; Nygren-Babol *et al.*, 2005).

The glutamic chain added by folylpolyglutamate synthetase (Figure 3, H) causes cellular retention of folates by decreasing affinity to the efflux transporters (Figure 3, I), as well as increasing binding affinity to enzymes in folate metabolism (Quinlivan *et al.*, 2006).

Folate absorption may also occur in the colon (Figure 2) (Aufreiter *et al.*, 2009). Although colon absorption rate has been found to be ~50 times lower than estimated in the small intestine, it may contribute significantly to total folate absorption since the transit time in the colon is longer (~15 times) and due to the presence of microbial folate production (Aufreiter *et al.*, 2009). Nearly 70 years ago, microbial synthesis of folates in mammals was suggested (Mitchell *et al.*, 1941), which was later estimated to about 25-100 nmol/d in infants (Kim *et al.*, 2004). The relevance for folate status of folates synthesised in the colon is unknown but bacterially synthesised folates has been shown to be bioavailable in humans (Camilo *et al.*, 1996) and pigs (Asrar & O'Connor, 2005).

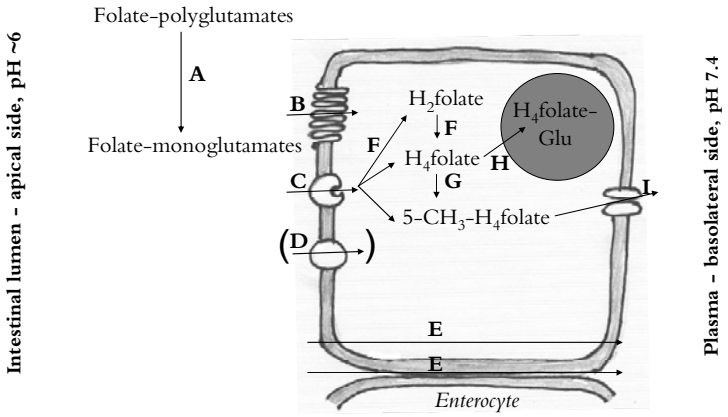


Figure 3. Intestinal absorption of food folates (modified from review by Zhao *et al.,* 2009)

A. γ-glutamyl hydrolase, cleaves the polyglutamic chain.

B. Reduced folate carrier (RFC), expressed in *e.g.* the colon, small intestine, liver, kidney and blood brain barrier and has pH optimum 7.4.

C. Protein-coupled folate transporter (PCFT), expressed in *e.g.* the small intestine, liver, kidney, placenta, brain and proximal colon and has pH optimum 5.5.

D. Folate receptors (FR) α & ß, FRα expressed in *e.g.* the kidney, uterus and placenta and FRß in *e.g.* the placenta, spleen and thymus. FR have pH optimum 6.0-6.5.

E. Passive diffusion is limited.

F. Dihydrofolate reductase (DHFR).

G. Methylenetetrahydrofolate reductase (MTHFR).

H. Folylpolyglutamate synthetase (FPGS).

I. Multidrug resistance associated proteins (MRP).

Distribution and physiological function of folates

After absorption, folates are transported to the liver (Figure 2), which is estimated to comprise 50% of the total folate body pool (Gregory *et al.*, 1998; Herbert, 1987). The liver folate pool is partly secreted (intact or as inactive metabolites) into bile (Herbert, 1987). Reabsorption of folates from bile in the gastrointestinal tract (Figure 2) is suggested to blunt between-meal fluctuations in folate supply to cells (Lin *et al.*, 2004). However, estimations of folate content in bile vary greatly from 227 nmol/d (Herbert, 1987) to 5300 nmol/d (Lin *et al.*, 2004). As a consequence, the estimated size of the total human body pool of folate (Figure 2) varies from 17 to 225 mmol (Lin *et al.*, 2004; Gregory *et al.*, 2001; Herbert, 1987).

Between 10-20% of absorbed folates are estimated to be retained in the liver in the so-called first-pass effect (Gregory, 1995). Remaining absorbed

folates are transported to other tissues with the systemic circulation. In plasma, folates are partly protein-bound, mainly to albumin (50% of bound folates) but also to soluble folate binding proteins (Shane, 1995).

In the cells, folate polyglutamates are retained and used in the nucleus as substrates in both nucleotide synthesis and the methylation cycle (Figure 4). H_4folate-polyglutamates are required for pyrimidine and purine synthesis, and thus DNA and RNA production, by providing one-carbon atoms.

H_4folate-polyglutamates are also involved in amino acid synthesis by accepting a one-carbon group (-CH_3) from the amino acid serine, which is then converted into glycine (Wagner, 1995).

In the methylation cycle, 5-CH_3-H_4folate donates a one-carbon group (-CH_3) to vitamin B12, which transfers it to homocysteine (Figure 4). Accumulation of homocysteine in the cell is thereby avoided and synthesis of amino acids maintained (Wagner, 1995).

As folates are involved in cell replication, the first signs of deficiency appear in tissues with rapid cell turnover and in life stages with increased cell division, such as during pregnancy (Tamura & Picciano, 2006). Folate deficiency results in so-called megaloblastic anaemia caused by impaired synthesis of red blood cells (erythrocytes) due to lack of DNA (Herbert, 1987).

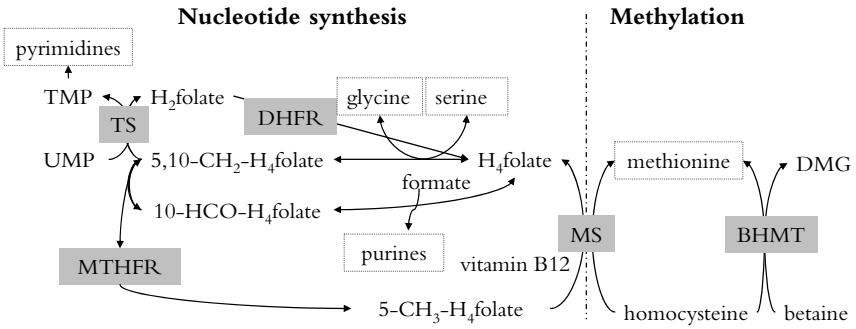


Figure 4. Folate involvement in nucleotide synthesis and the methylation cycle (Reed *et al.*, 2006). End-products are in boxes. Enzymes are shaded

BHMT = betaine-homocysteine methyltransferase; DHFR = dihydrofolate reductase; DMG = dimethylglycine; MS = methionine synthase; MTHFR = methylenetetrahydrofolate reductase; TMP = thymidine monophosphate; TS = thymidylate synthase; UMP = uridine monophosphate.

Several factors are known to affect cellular folate metabolism:

- A polymorphism in the enzyme methylenetetrahydrofolate reductase (677C→T, Figure 3, G), which reduces enzyme activity to below 20% (Rozen, 2004) and thus increases the need for food folate or folic acid intake. Prevalence of this polymorphism varies worldwide from zero up to 32% (Wilcken *et al.*, 2003). In Sweden, below 10% of the population are carriers of the 677C→T-allele (Johansson *et al.*, 2007; Ruiz *et al.*, 2007; Brattström *et al.*, 1998).
- A polymorphism in RFC (80A→G, Figure 3, B) is a risk factor for NTD (Morin *et al.*, 2003), either by interfering with intestinal absorption or folate transport, or both (Gregory *et al.*, 2005).
- The very rare disease 'hereditary folate malabsorption' is caused by insufficient absorption and transport of folate due to lack of PCFT (Figure 3, C) (Qiu *et al.*, 2006).
- Deficiency of vitamin B12 reduces the activity of methionine synthase (Figure 4, MS). Homocysteine and 5-CH_3-H_4folate are then accumulated in the cell, resulting in the so-called methyl trap and folate deficiency (Reed *et al.*, 2006).

Excretion of folates

About 0.3-0.8% of the folate body pool has been estimated to be excreted intact or as catabolites daily (Gregory *et al.*, 2001; Gregory *et al.*, 1998). Excretion of intact folate with urine is as low as ~5% of ingested folates (Lin *et al.*, 2004; Witthöft *et al.*, 2003; Pfeiffer *et al.*, 1997a; Rogers *et al.*, 1997), due to folates being efficiently reabsorbed from primary urine by renal FRα (Zhao *et al.*, 2009). At higher intakes of folates the excretion of intact folates increases. For example, in volunteers consuming 850 μg (1925 nmol) folic acid daily for 12 weeks, as much as 19% of total urinary folate excretion originated from the dose (isotope-labelled folic acid) (Gregory *et al.*, 2001). The majority of folates excreted with urine (>300 nmol/day) occur as catabolites (Figure 2) and this fraction is not affected by *e.g.* increased daily folate intake (454-907 nmol/d) for 10 weeks (Gregory *et al.,* 1998).

In human faeces, folate content is about 400 nmol/day (Lin *et al.*, 2004; Gregory & Quinlivan, 2002). Folates found in faeces originate from bacterial production, lysed enterocytes and gastrointestinal secretions such as bile (Gregory & Quinlivan, 2002). The 400 nmol folate excreted with faeces and stomal effluents daily contains about 20% non-absorbed food folates (Witthöft *et al.*, 2006; Lin *et al.*, 2004; Konings *et al.*, 2002), which indicates incomplete bioavailability of folates.

1.2 Bioavailability of folates

When formulating nutrition recommendations, a food folate bioavailability of 50% of that of folic acid is commonly used (CDC, 2009; NNR, 2005). One of the most cited references for this estimate is a 92-day trial assessing the minimum food folate intake required for normalisation of folate status after depletion (Sauberlich *et al.*, 1987). Although the size of doses of food folate (n=3) and folic acid (n=4) varied greatly in that trial, it was estimated that availability of food folates was 50% of that of synthetic folic acid consumed with food (Sauberlich *et al.*, 1987). Since that trial, several intervention studies assessing folate bioavailability have been carried out but the results are inconclusive (Table 1a and 1b). Variations in study design and definition of bioavailability complicate comparisons between long-term (Table 1a and 1b) and short-term (Table 2) experiments.

Definitions used in this thesis are:

- Folate absorption, *in vivo* - process by which folates proceed from site of administration to site of measurement (usually plasma) (Tozer & Rowland, 2006).
- Folate bioavailability, *in vivo* - fraction of ingested folate that is absorbed and can be used for metabolic processes (Melse-Boonstra *et al.*, 2004a).
- Folate bioaccessibility, *in vitro* – fraction of a given amount folate that is released from [food] matrix and available for absorption (Minekus *et al.*, 1995).

Long-term bioavailability of folate

Physiological effects of folates can only be studied using long-term intervention trials. Most intervention trials with folate have a duration of 4 to 16 weeks and measure effects of the intervention diet as changes in folate status parameters (Table 1a and 1b). The most commonly used parameters are:

- Fasting serum folate.
- Erythrocyte folate, which reflects the average folate status during the lifespan of the erythrocyte (~120 days).
- Plasma total homocysteine, which is inversely associated to folate status (see Figure 4).

More than one status parameter is commonly assessed, which is appropriate since the sensitivity of parameters varies. For example, in a 4-week intervention trial the estimated bioavailability of food folate compared with folic acid was 78% based on serum folate concentrations, 98% based on erythrocyte folate concentrations and only 60% based on homocysteine concentrations (Brouwer *et al.*, 1999).

Several intervention trials report a low bioavailability of food folates compared with folic acid (Table 1a) (Hannon-Fletcher *et al.*, 2004; Riddell *et al.*, 2000; Cuskelly *et al.*, 1996). However, the data are inconclusive as many other intervention studies show good bioavailability of food folates (Table 1b), either compared with a control group receiving no additional folate (Broekmans *et al.*, 2000) or folic acid-fortified foods or supplements (Winkels *et al.*, 2007; Fenech *et al.*, 2005; Vahteristo *et al.*, 2002). For example, bioavailability of equimolar doses of food folates (from rye products and orange juice) and folic acid (from fortified wheat bread) is equivalent based on similar improvements of folate status (n=64 women) (Vahteristo *et al.*, 2002).

Folate intervention trials can be hampered by uncontrolled consumption of other foods that are fortified or naturally rich in folates and lack of compliance. Hence it can be assumed that interventions with pharmaceutical preparations are less biased than those with food folate. For example, consumption of an intervention diet for several weeks may cause problems with compliance, *e.g.* during travel or if the diet needs to be purchased by the volunteers themselves, as in some intervention trials (Ashfield-Watt *et al.*, 2003; Riddell *et al.*, 2000).

Table 1a. *Trials with food folate interventions having no significant effects on folate status, listed in order of increasing intervention dose*

Intervention diet	Dose (total[1]) folate μg/day	W	N[2]	Age	Erythrocyte folate (nmol/L) Baseline	End	p/s-folate (nmol/L) Baseline	End	tHcy (μmol/L) Baseline	End	Reference
Control	2	4	35, F	41±3[3]			15±9[3]	13	11±4[3]	10	Bogers *et*
5-a-day (fruit/veg)	43		36, F	42±4[3]			17±9[3]	16	10±3[3]	10	*al.*,2007
Control	0 (236)	16	43	-			26±8[3]	25	9±3[3]	9	Ashfield-
5-a-day (fruit/veg)	63 (306)		41	-			26±7[3]	28♦	10±4[3]	9	Watt *et al.*, 2003
Control	0 (186)[3]		18, M	23-39[4]			16±9[3]	15	12±4[3]	12	Hannon-
Spinach	200 (384)	4	18, M	23-39[4]			14±5[3]	15	12±3[3]	12	Fletcher *et*
Yeast	200 (411)		19, M	23-39[4]			15±7[3]	18	12±3[3]	11	*al.*, 2004
Control	23 (210)	12	9, F	17-40[4]	709±157[3,5]	729[5]					Cuskelly *et*
High folate diet	201 (410)		10, F	17-40[4]	797±200[3,5]	857[5]					*al.*, 1996
Control	0 (*227*)	12	15	36-71[4]	539±166[3]	537	15 (12-18)[6]	14[6]	12 (11-14)[6]	12[6]	Riddell *et*
High folate diet	~350 (*707*)		15	36-71[4]	571±162[3]	643	15 (13-17)[6]	22[6]♦	11 (9-12)[6]	10[6]	*al.*, 2000

F = female; M = men; W = week; Veg = vegetables. [1]Estimated by dietary recall *(total folate intake not reported but estimated as dose + folate intake at screening)*. [2]Female and men unless otherwise stated. [3]Mean±SD. [4]Age reported as inclusion criteria. [5]Erythrocyte folate concentrations given in μg/L, calculated to nmol/L (0.460^{-1} nmol/μg). [6]Geometric means (95% CI). ♦Significant effect compared with control ($P<0.05$)

Table 1b. *Trials with food folate interventions having significant effects on folate status, listed in order of increasing intervention dose*

Intervention diet	Dose (total[1]) folate μg/day	W	N[2]	Age	Erythrocyte folate (nmol/L) Baseline	End	p/s-folate (nmol/L) Baseline[2]	End	tHcy (μmol/L) Baseline[2]	End	Reference
Low folate diet	0 (131)	4	23	50±4[3]			13±6[3]	12	14±6[3]	13	Broekmans
500 g veg and fruits	97 (228)	4	24	49±6[3]			16±10[3]	16♦	12±4[3]	11♦	*et al.*, 2000
Folic acid-fortified bread	188 (*461*)	4	31	36±13[3]	606±224[3]	697★	12±5[3]	16★			Vahteristo *et*
Rye and orange juice	184 (*461*)		33	36±13[3]	561±190[3]	658★	10±3[3]	13★			*al.*, 2002
Folic acid	150 (221)[4]	4	15	19-48[5]			12±4[3]	15	10±2[3]	9	Winkels *et*
Veg, fruits and liver pâté	331 (*427*)[4]		29	19-41[5]			12±4[3]	18♦	10±3[3]	9	*al.*, 2007
Control	0 (242)	4	14	60±15[3]			18 (15-22)[6]	18[6]	14 (12-16)[6]	14[6]	Venn *et al.*,
High folate diet	355 (618)		20	58±18[3]			18 (16-19)[6]	25[6]♦	12 (11-13)[6]	11[6]♦	2002
Control	0 (210)[4]	4	22	23±8[3]	347±79[3]	345	13±3[3]	13	10±3[3]	11	Brouwer *et*
Veg and fruits	350 (560)[4]		23	23±8[3]	338±81[3]	400♦	14±3[3]	20♦	11±5[3]	10♦	*al.*, 1999
Pericarp flour bread	223 (436)	16	25	48-56[5]	497 (414-581)[6]	578[6]	13 (11-15)[6]	14[6]	10 (8-11)[6]	8[6]	Fenech *et*
Aleurone flour bread	615 (836)		25	46-54[5]	509 (434-584)[6]	768[6]♦	13 (10-16)[6]	27[6]♦	9 (8-10)[6]	7[6]♦	*al.*, 2005

Nd = no data; W = week; Veg = vegetables. [1]Estimated by dietary recall *(total folate intake not reported but estimated as dose + folate intake at screening).* [2]Female and men. [3]Mean±SD. [4]Folate dose is analysed and not estimated using dietary recall. [5]Range. [6]Geometric means (95% CI). ★Significant effect within the group compared with baseline (P<0.05) ♦Significant effect compared with control (P<0.05)

Short-term bioavailability of folate

Short-term bioavailability of food folate has been found to be incomplete compared with that of folic acid (Tamura & Stokstad, 1973). Since then, bioavailability has been extensively studied using a number of approaches for assessment, for example:

- *Plasma area under the curve (AUC)*

Post-dose plasma AUC is assumed to correspond to the folate fraction absorbed from a single dose. Data are commonly presented as relative absorption, which is the ratio between plasma AUC after a test dose and an oral or intravenous dose of folic acid (Table 2). This approach is only appropriate if the absorption and metabolism of folate forms in the test and reference dose are the same, which has been questioned for 5-CH_3-H_4folate and 5-HCO-H_4folate versus folic acid (Wright *et al.*, 2005). Therefore, so-called apparent absorption accounting for the folate distribution volume in the body has been determined in some trials (Table 2) (Witthöft *et al.*, 2006; Wright *et al.*, 2005).

- *Urinary folate excretion ratio*

Folate bioavailability is assumed to correspond to the excretion in urine of [^{13}C]-labelled folate from an oral dose relative to that of [^{2}H]-folic acid from an intravenous dose. By using this so-called dual-label stable-isotope protocol, folate absorption of food folate and added fortificants has been estimated to be 37-153% (Finglas *et al.*, 2002; Pfeiffer *et al.*, 1997a; Rogers *et al.*, 1997; Wei *et al.*, 1996; Gregory *et al.*, 1991).

- *Oral-faecal balance technique*

Folate bioavailability is assumed to be inversely correlated to folate content in stomal effluent or faeces, *i.e.* non-absorbed folate, after a given dose, commonly related to that after a folic acid supplement. Not all folates found in stomal effluents and faeces originate from non-absorbed ingested folate (Gregory & Quinlivan, 2002). Use of stable isotope-labelling allows to distinguish folate from the dose from endogenous folates from body stores (Lin *et al.*, 2004; Buchholz *et al.*, 1999). To account for folate in stomal effluents not deriving from the dose baseline excretion on a folate-free study day was assessed (Witthöft *et al.*, 2006; Witthöft *et al.*, 2003). Another approach is to report stomal effluent content after a certain dose relative to that after a dose of folic acid (Konings *et al.*, 2002). Folate bioavailability from single doses of food folate has been estimated to be 50-90% by use of

ileostomy models (Witthöft *et al.*, 2006; Witthöft *et al.*, 2003; Konings *et al.*, 2002; Wigertz, 1997).

A common criticism of short-term protocols is the use of nonphysiological doses, or large portions of test foods that are not likely to be consumed in a meal by the general population, *e.g.* 30 grams of yeast (Tamura & Stokstad, 1973) or 500 g of spinach (Prinz-Langenohl *et al.*, 1999). Most models seem to be insufficiently sensitive to assess folate bioavailability from a single food under physiological conditions. To improve sensitivity, folate body stores can be presaturated with supplemental folic acid (Table 2) to minimise and standardise hepatic retention of absorbed folate from the dose (Tamura & Stokstad, 1973). Use of stable isotope-labelled folates (Table 2) also increases sensitivity, as it is possible to distinguish labelled folate from the dose and unlabelled folate from endogenous body stores (Wright *et al.*, 2005; Wright *et al.*, 2003; Rogers *et al.*, 1997). However, unless intrinsic labelling is used (Wright *et al.*, 2005), no data on absorption of native folates from a specific food matrix are obtained.

Spinach is often used as a test food (Table 2) due to its high native folate content of nearly 200 μg/100g (NFA, 2009a). Similar post-dose plasma AUCs have been found after ingestion of nearly equimolar doses of spinach folate and supplemental folic acid (Table 2) (Konings *et al.*, 2002; Prinz-Langenohl *et al.*, 1999), which is surprising with respect to the general assumption of lower bioavailability of food folates. Furthermore, estimated apparent absorption is reported to be significantly higher after ingestion of intrinsically stable isotope-labelled spinach compared with supplemental folic acid (Table 2) (Wright *et al.,* 2005). Data on folate absorption from foods other than spinach are sparse, but plasma AUCs after ingestion of strawberries, broccoli and bread made from aleurone flour are reported to be similar to those for folate supplements (Table 2) (Witthöft *et al.*, 2003; Fenech *et al.*, 1999).

Table 2. *Trials using plasma concentrations to assess relative food folate or fortificant bioavailability*

	Dose nmol	App abs[1] or AUC	Sampling duration (nr/4h)[2]	Female + men	Age[3]	Reference
Apparent absorption (%) from supplements, fortified foods and food folate						
$^{13}C_6$-folic acid	634	24%	8h (8)	14[4]	33±2	Wright *et al.* 2005
$^{13}C_6$-5-HCO-H$_4$folate	500	38%	8h (8)	14[4]	33±2	
spinach $^{15}N_{1-7}$folate	588	44%	8h (8)	14[4]	33±2	
5-CH$_3$-H$_4$folate in fermented milk[5]	450[6]	86%	10h (7)	1+8	51-79	Witthöft *et al.* 2006
Folic acid in bread[5]	491[6]	74%	10h (7)	1+8	51-79	
Yeast[5]	155[6]	80%	10h (7)	1+8	51-79	
Size of AUC (h·nmol/L) from supplements and fortified foods						
$^{13}C_6$-folic acid	634	19	8h (8)	10[4]	31±1	Wright *et al.* 2003
Folic acid[5]	1134	146	10h (6)	0+13	26±6	Pentieva *et al.* 2004
Folic acid	1134	40	10h (6)	6+6	36-69	Konings *et al.* 2002
Folic acid	1193	37, 49[7]	7h (3)	8+8	20-50	Fenech *et al.* 1999
Folic acid	907	62	10h (4)	10+10	27±3	Prinz-Langenohl *et al.* 1999
$^{13}C_6$-folic acid & *$^{2}H_2$-folic acid,* i.v.	1010 226	-	9h (6)	2+2	20-35	Rogers *et al.* 1997
$^{13}C_6$-5-HCO-H$_4$folate	500	42	8h (8)	10[4]	31±1	Wright *et al.* 2003
5-CH$_3$-H$_4$folate[5]	1088	142	10h (6)	0+13	26±6	Pentieva *et al.* 2004
5-CH$_3$-H$_4$folate[5]	830[6]	44, 88[6]	10h (7)	0+2	55, 77	Witthöft *et al.* 2003
5-CH$_3$-H$_4$folate i.m.[5]	830[6]	111, 144[6]	10h (7)	0+2	55, 77	
Size of AUC (h·nmol/L) from food folates						
Broccoli[5]	440	27, 41[6]	10h (7)	0+2	55, 77	Witthöft *et al.* 2003
Strawberries[5]	450	32, 41[6]	10h (7)	0+2	55, 77	
Spinach, monoglu-	820	31	10h (6)	6+6	36-69	Konings *et al.* 2002
Spinach, polyglu-	990	27	10h (6)	6+6	36-69	
Spinach	544& 1088	41, 71	10h (4)	10+10	27±3	Prinz-Langenohl *et al.* 1999
Aleurone flour	1167	46, 38[7]	7h (3)	8+8	20-50	Fenech *et al.* 1999
Wheat bran	213	8, 6[7]	7h (3)	8+8	20-50	

[1]App abs = apparent absorption, estimated by kinetic modelling (Wright *et al.*, 2005).
[2]Number of sampling occasions 0-4 h post-dose in brackets. [3]Mean+SD or range. [4]No data on gender of volunteers. [5]Presaturation with ~1 mg (Witthöft *et al.,* 2006) or 5 mg (Pentieva *et al.,* 2004) folic acid/day from day -9 to day -2 prior to each test day. [6]Conversion factor from μg to nmol: 0.441^{-1} nmol/μg (folic acid doses) and 0.460^{-1} nmol/μg (plasma folate and 5-CH$_3$-H$_4$folate doses). [7]Female and men reported separately

By measurement of folate content in faeces or stomal effluents, the non-absorbed fraction after ingestion of folate-rich foods, fortified foods or supplements has been estimated at 8-23% (Witthöft *et al.*, 2006; Konings *et al.*, 2002; Lin *et al.*, 2004; Buchholz *et al.*, 1999), indicating a high folate bioavailability. A gap of up to 25% between absorbed (55-86%) and non-absorbed folates has been reported (Witthöft *et al.*, 2006). This could be explained by analytical errors, incomplete sample collection or, as suggested by Wright *et al.* (2005), hepatic folate retention during first passage. Wright *et al.* (2005) estimated the first-pass effect to be as much as 73% of absorbed folic acid, and 52% of absorbed intrinsically labelled spinach folate.

Factors affecting bioavailability of folates

Bioavailability of folate may be affected by so-called extrinsic factors, *e.g.* the food matrix and folate form, as well as host-related intrinsic factors such as gastrointestinal pH.

The effect of the cereal matrix on folate bioavailability has been extensively studied as it is an appropriate matrix for folic acid fortification. Findings from short-term trials indicate an inhibitory effect of dietary fibre on folate bioavailability. For example, the relative increase in serum folate concentrations after single doses of 1000 μg (2265 nmol) folic acid was only ~30% from a fortified whole wheat bread, which was significantly lower than the 50-60% assessed after ingestion of an eqimolar folic acid dose from fortified polished rice and maize porridge (Colman *et al.*, 1975).

The negative effect of dietary fibre content was confirmed using a dual-label stable-isotope protocol in which relative urinary excretion ratio (page 24) of physiological doses of folic acid (225 μg, 510 nmol) was lower from bran flakes rich in dietary fibre (~0.4) than from white bread (~0.7) (Finglas *et al.*, 2002). However, in long-term trials, cereal foods rich in folate and dietary fibre have been reported to be as efficient as folic acid in improving the folate status of volunteers (Table 1b) (Fenech *et al.*, 2005; Vahteristo *et al.*, 2002). Furthermore, ingestion of dietary fibre has been correlated to serum folate concentrations in female adolescents (Houghton *et al.*, 1997) and to folate intake in post-menopausal women (Ericson *et al.*, 2007).

Folate bioavailability from a single dose of 600 mL orange juice (840 μg/1900 nmol) has also been shown to be low (~30%) compared with an oral dose of folic acid (Tamura & Stokstad, 1973). This was assumed to be caused by a lowering of pH due to the large amount of ingested orange juice (Tamura & Stokstad, 1973). Wei & Gregory (1998) suggested that it was the organic anions in the orange juice that inhibited γ-glutamyl hydrolase activity and thereby potentially folate absorption. The possibility

that γ-glutamyl hydrolase might be a limiting factor in folate absorption *in vivo* was also indicated in a 12-week intervention study where bioavailability of folate polyglutamates was ~65% of that of folate monoglutamates (Melse-Boonstra *et al.*, 2004b). Furthermore, phenolic compounds in wine, beer and tea have been shown to reduce folic acid uptake into Caco-2 cells (Lemos *et al.*, 2007).

Other food components suggested to affect folate bioavailability are folate-binding proteins (FBP). The FBP present in *e.g.* plasma and milk have been suggested to stabilise folates in the gastrointestinal tract (Jones & Nixon, 2002). However, by using an *in vitro* model assessing release and stability of folates during passage through a simulated gastrointestinal tract (TIM), addition of FBP has been shown to reduce folate bioaccessibility from *e.g.* yoghurt (Arkbåge *et al.*, 2003). This finding was confirmed *in vivo*, since apparent absorption of 5-CH_3-H_4folate fortificant was 16% lower and relative stomal excretion 9% higher when FBP was added to fermented milk (Witthöft *et al.*, 2006).

As previously mentioned, the bioavailability of folic acid supplements is considered superior. This might be explained by lack of matrix effects and that folic acid has no polyglutamic chain and hence γ-glutamyl hydrolase cannot be a limiting factor.

Host-related intrinsic factors such as gastric pH and folate status may also affect absorption. Drugs or diseases causing increased gastric pH, *e.g.* antacids or atrophic gastritis, reduce folate absorption *in vivo* (Russell *et al.*, 1988) and use of Caco-2 cells has shown that folic acid uptake is optimal at pH ~5.5 (Mason *et al.*, 1990). High exposure to folic acid can also reduce uptake by down-regulation of genes coding for folate absorption and transport (PCFT, RFC and FR, Figure 3, B-D) in Caco-2 and renal cells (Ashokkumar *et al.*, 2007).

To summarise, several factors affecting human folate bioavailability have been found using *in vivo* and *in vitro* models. The potential underlying mechanisms were identified using *in vitro* and animal models.

1.3 Folate intake and status

National intake studies in northern Europe indicate insufficient folate intake among women of childbearing age (Dhonukshe-Rutten *et al.*, 2009). In Sweden, folate intake in this particular group is estimated to be about 60% of the recommended level (400 μg daily) (NNR, 2005; Becker & Pearson, 2003). However, population-based estimates of folate intake are only as good as the national food data used (Gregory *et al.*, 2005) and these data vary as a result of different methods for folate quantification, adjustment for preparation losses and due to natural variations in folate content in food products (Williamson & Buttriss, 2007; Hakala *et al.*, 2003).

Data on folate intake in the Swedish population (Table 3) are rather comprehensive due to the national intake surveys (Becker & Pearson, 2003; Becker, 1994) and the large cohorts (Ericson *et al.*, 2007; Van Guelphen, 2006; Larsson *et al.*, 2004). Folate intake can be assessed using different methods, *e.g.* weighed food records, where volunteers weigh all foods and beverages consumed over a specific time period. A less burdensome method for volunteers is dietary recall interviews, which rely on the volunteers' memory. Such interviews require skilled interviewers and are consequently costly for large studies (Pufulete *et al.*, 2002). Therefore Food Frequency Questionnaires (FFQ) validated against other dietary assessment methods or biochemical parameters are commonly used for assessment of folate intake in epidemiology. FFQ are distributed to volunteers who mark intake frequency of common foods during a certain time period. Intake of nutrients is estimated by multiplying frequency by typical portion sizes and nutrient content in food (derived from food composition tables). As shown in Table 3, folate intake estimated using FFQ are similar to those using other methods, which is partly due to use of the same food database (NFA, 2009a).

Recommended folate intake for women of childbearing age is 400 μg daily (NNR, 2005), an intake only reported for vegans (Table 3) (Larsson & Johansson, 2002). For adults the recommended daily intake is 300 μg (NNR, 2005) since this level has been shown sufficient to maintain good folate status (Prinz-Langenohl *et al.*, 1999; Cuskelly *et al.*, 1996; Gregory, 1995; Herbert, 1987; Sauberlich *et al.*, 1987).

Folate intake by pregnant women (Åden *et al.*, 2007) is only about 50% of the recommended 500 μg/day (NNR, 2005). The higher recommended intake for pregnant and nursing women is based on a metabolic study (84-d) where about 600 μg dietary folate equivalents (*i.e.* food folates) maintained

erythrocyte folate status in pregnant women (Caudill *et al.*, 1997). Although the Swedish National Food Administration recommends that all women who might become pregnant consume folic acid supplements, none of the women in the trial by Åden *et al.* (2007) consumed folic acid supplements prior to pregnancy and only 12% consumed such supplements during pregnancy.

Table 3. *Estimated folate intake in Swedish women*

Method	Name of study	N	Region	µg/day	Reference
National intake surveys					
7 d record	Riksmaten	626	Sweden	211 (142-294)[1]	Becker & Pearson, 2003
7 d record	HULK	804	Sweden	183 (126-267)[1]	Becker, 1994
Cohort studies					
Diet-history method[2]	Malmö Diet and Cancer cohort	11307	Malmö	287±318[3]	Ericson *et al.*, 2007
65-84 FFQ	NSHDS	826	Umeå	226 (179-274)[4]	Van Guelphen, 2006
28 d record	The Swedish Mammography Cohort	129	Uppsala/ Västman-land	211[4]	Larsson *et al.*, 2004
96 FFQ		61084		178 (155-204)[4]	Larsson *et al.*, 2004
Intake studies					
84 FFQ	-	49	Umeå	225±91[3,5]	Åden *et al.*, 2007
24 h recall	-	50	Umeå	260±80[3,6]	Åden *et al.*, 2007
Diet-history interview	-	30	Umeå	473±187[3,7]	Larsson & Johansson, 2002
		30	Umeå	226±73[3,8]	
Intervention study					
FFQ	-	15	Uppsala	209	Johansson *et al.*, 2002
		14	Uppsala	221	Johansson *et al.*, 2002

HULK = HUshållens Livsmedelsutgifter och Kostvanor; NSHDS = Northern Sweden Health and Disease Study. [1]1st and 9th deciles. [2]Combination of 7-day menu-book, 168 item FFQ and diet-history interview. [3]Mean±SD. [4]Median (Q_1 and Q_3) [5]Prior to pregnancy. [6]Mid-gestation. [7]Vegans. [8]Omnivores

Data on folate status in Europe are less comprehensive, but for women of childbearing age high average erythrocyte folate concentrations (>800 nmol/L) are reported from Ireland (Flynn *et al.*, 2008) and Denmark (Bor *et al.*, 2008), whereas concentrations in Germany (Thamm *et al.*, 1999) are similar to those in the USA (~600 nmol/L) (McDowell *et al.*, 2008).

In the literature review carried out before a decision was made on whether to introduce folic acid fortification in Sweden (SBU, 2007), it was noted that data on the Swedish population were limited and the authors only refered to data from one case-control study (George *et al.*, 2002), one cohort (Wahlin *et al.*, 2002) and one intervention trial (Johansson *et al.*, 2002). Of these, only the case-control study could be considered representative, as intervention trials are not representative and as Wahlin *et al.* (2002) presented data which were not corrected for non-fasting sample collection. Table 4 summarises reported data on plasma folate and plasma total homocysteine in Sweden.

For the representative studies, plasma folate concentrations were about 9 nmol/L (deficiency <7 nmol/L) but in one cohort (NSHDS) nearly 1/3 of the participants were folate-deficient (Van Guelphen, 2006). For homocysteine, elevated concentrations - possibly due to chronic renal disease or malabsorption of vitamin B12 (Carmel *et al.*, 2003) - were reported in study populations >70 years (Lewerin *et al.*, 2005; Björkegren & Svardsudd, 2003) and occurred in as many as 50% of volunteers with normal folate status in one intervention trial (Lewerin *et al.*, 2005). Therefore elevated homocysteine concentrations in the elderly must not be used as a marker of increased risk of cardiovascular disease (Carmel *et al.*, 2003).

Table 4. *Concentrations of plasma (or serum) folate and plasma total homocysteine as reported in Swedish trials. Mean±SD if not otherwise stated*

Study type	N	% Men	Age	Region	Folate nmol/L[1]	tHcy µmol/L[1]	Reference
Cohort	194	0	16±4[2]	County of Uppsala and Västmanland	-	9±4[2]	Ruiz *et al.*, 2007
European Youth Heart Study	185	100	16±4[2]		-	10±4[2]	
	138	0	10±4[2]		-	7±1[2]	
	163	100	10±4[2]		-	6±1[2]	
Cohort	329	0	<60	Umeå	9 (7-12)[3]	9 (8-11)[3,4]	Van Guelphen, 2006
NSHDS	826	0	25-74[5]	Umeå	9 (6-12)[3]	9 (6-12)[3,4]	
	1791	100	25-74[5]	Umeå	8 (6-11)[3]	10 (9-12)[3,4]	
Cohort *Skara Population Study*	571	0	63±13[2]	Skara	-	10±4[2,6]	Björck *et al.*, 2006
	537	100	63±13[2]	Skara	-	11±4[2,6]	
Cohort *Betula Project*	961	47	35-80[7]	Umeå	18±11[2,8]	18±11[2,8]	Wahlin *et al.* , 2001
Cohort	161	43	77±6[2]	Älvkarleby	9±4[2]	18±7[2]	Björkegren & Svardsudd, 2003
Case-control	583	0	30	Uppsala	9[2,9]	-	George *et al.*, 2006
Case-control	921	0	-	Uppsala	9±5[2,9]	9±5[2]	George *et al.*, 2002
Intervention	126	38	76±5[2]	Göteborg	16±6[2]	18±6[2]	Lewerin *et al.* 2005
	69	44	76±4[2]	Göteborg	16±5[2]	16±5[2]	
Intervention	15	0	23-50[7]	Uppsala	16±5[2]	9±2[2]	Johansson *et al.*, 2002
	14	0	23-50[7]	Uppsala	19±5[2]	8±2[2]	

NSHDS, Northern Sweden Health and Disease Study. [1]Concentrations of folate and tHcy were determined with radiobinding assay unless otherwise stated. [2]Mean±SD. [3]Median (Q_1 and Q_3). [4]Partly HPLC (n=190). [5]80% between 49-61 years. [6]Determined using HPLC. [7]Range. [8]Not fasting. [9]Within 6 weeks after delivery

Folic acid fortification

It has proven difficult to reach a food folate intake associated with a low risk of neural tube defects. Therefore the authorities in many countries aim to increase the population's intake of folate or folic acid by *e.g.* information campaigns and fortification programmes, either voluntary or mandatory. Each method has its benefits and drawbacks, and an alternative might be good in one country but less good in another depending on initial folate status and prevalence of NTD.

In the USA, Canada, Costa Rica and Chile, average folic acid intake has been increased by mandatory folic acid fortification of staple foods. Incidence of NTDs has decreased significantly (Mosley *et al.*, 2009; Castilla *et al.*, 2003), especially in areas with a high prevalence prior to fortification. For example, in Newfoundland, Canada, a 78% reduction (from 44/10 000) has been observed (Liu *et al.*, 2004). Concerns have been raised that high intake of synthetic folic acid will result in unmetabolised folic acid in plasma and adverse health effects (Smith *et al.*, 2008). For example, unmetabolised folic acid in fasting plasma samples was found in 75% of non-supplement users (n=705, Framingham Offspring Cohort) (Kalmbach *et al.*, 2008). However, the implications of this are still under discussion (Smith *et al.*, 2008).

Introduction of folic acid fortification in Sweden would bring about an estimated 25% reduction in abortions due to neural tube defects (current rate ~80/year) and births with neural tube defects (current rate ~20-25/year) (SBU, 2007). However, due to concerns regarding the ambiguous role of folic acid in development of colorectal cancer, fortification has not been introduced (NFA, 2009b).

1.4 Folate quantification in foods and clinical samples

Four tons of spinach was used to isolate folate for the first time and hence the authors suggested the name folic acid from the Latin word *folium*, meaning leaf (Mitchell *et al.*, 1941). Since then, a number of different methods for determination of folate content in clinical samples and foods have been developed (Figure 5), as reviewed by Quinlivan *et al.* (2006). The most suitable method depends on the type of sample and level of information needed, as well as time and financial restraints (Figure 5). Depending on method, folate data may vary. For example, folate data (food and clinical samples) obtained by HPLC methods are reported to be up to 30% lower than those obtained by microbiological assays (Konings *et al.*,

2001; Finglas *et al.*, 1996; Gregory *et al.*, 1982) and ~30% higher than data on clinical samples obtained by radioassays (Pfeiffer *et al.*, 2004).

The great structural variations in folates (Gregory, 1996) complicate determination of folate content, since response (microbiological and radioassays) and sensitivity (HPLC methods) can vary between forms (Figure 5). Another challenge during folate measurements is to avoid oxidative cleavage and preserve the reduction of the pteridin ring, which is commonly done by use of antioxidants, avoidance of UV-light and overlaying samples with gas.

Folate concentrations were determined in this thesis using HPLC methods, in which the cellular structures of the sample are disrupted by homogenisation and heat extraction or by enzymatic degradation (*e.g.* the so-called trienzyme method), resulting in folates being released from the matrix by leakage (Gregory, 1996). Prior to quantification, purification is required, *e.g.* by solid phase extraction or affinity chromatography (Selhub, 1989). Affinity chromatography is more sensitive and more selective due to the covalent binding of FBP to folates, which is more specific and stronger than *e.g.* the ion-binding in strong anion exchange columns (SPE method). However, the selectivity of the HPLC method depends mainly on the detector used and is superior for EC and MSMS followed by MS and FLD (Quinlivan *et al.*, 2006; Bagley & Selhub, 2000).

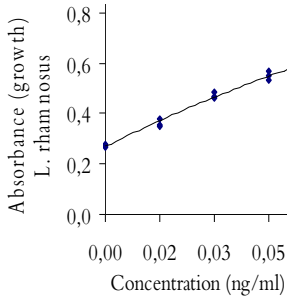
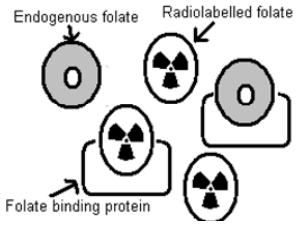
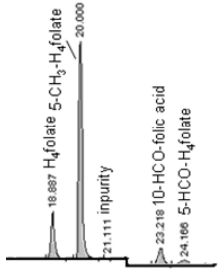
	Microbiological assay	Radioassay	HPLC (FL, UV, MS, MSMS, EC)
Principle	Growth of microorganisms (*e.g.* L. rhamnosus) is dependent on exogenous folate. Measured absorbance (growth) correlate to folate content in the sample. 	Radioactivity is measured after competive binding between sample folates and a known amount of radiolabelled folate to folate binding protein (RBPA) or specific antibodies (RIA). 	Peak areas of individual folates are measured using various detectors (FL, UV, MS, EC) after separation by differences in binding strength to the stationary phase. 
Main use	Food	Clinical samples	Food and clinical samples
Main advantage	The only AOAC method. Sensitive.	High sample throughput.	Quantification of individual folates.
Drawbacks	Growth response may vary with assay pH and polyglutamic chain length. Antifolates and antibacterial drugs will affect results.	Variable affinity between different folate forms. Not suitable for foods as binding is not selective for e.g. stereoisomers with vitamin activity.	Expensive and high demands on extract purity. Limited number of folate standards commercially available.

Figure 5. Overview of folate quantification methods summarised from Quinlivan *et al.* (2006). HPLC = high performance liquid chromatography; FL = fluorescence; UV = ultraviolet; MS = mass spectrometry; EC = electrochemical.

2 Objectives

The overall objective of this thesis was to assess folate bioavailability. *In vivo* models to assess long-term effects of food folate on status and short-term absorption of fortificants were complemented with less expensive but well established *in vitro* models (Table 5). The same test foods, analytical measurements and statistical evaluations were used whenever possible to enable comparison between study results (Table 5). Specific objectives were:

- To determine effects on folate status in women of an intervention involving two pieces of nutritional advice, a food (wholemeal bread) and a meal (breakfast).
- To determine folate bioavailability after ingestion of single doses of wholemeal bread fortified with either 5-CH_3-H_4folate or folic acid using a novel stable-isotope AUC/ileostomy model.
- To determine the bioavailability of folic acid ingested with wholemeal bread or as part of a complete breakfast meal using a novel stable-isotope AUC/ileostomy model.
- To determine *in vitro* bioaccessibility of endogenous folates from breads, a complete breakfast meal and orange juice using the dynamic TNO gastrointestinal model TIM.
- To assess effects of bread matrix on *in vitro* uptake and transport of the dominant food folate 5-CH_3-H_4folate using a novel stable isotope/Caco-2 cell model.

Table 5. *Overview of trials included in thesis*

	In vivo		***In vitro***	
Model	Intervention	Plasma AUC/ileostomy	Gastrointestinal TIM	Caco-2 cells
Paper	I	II	III&IV	IV
Objectives	Assess effects of two pieces of dietary advice on folate status in women.	Assess absorbed and non-absorbed fraction after ingestion of $^{13}C_5$-5-CH_3-H_4folate or $^{13}C_5$-folic acid (from fortified bread or meal).	Assess bioaccessibility of 5-CH_3-H_4folate and 10-HCO-folic acid from orange juice, wholemeal and ryebran breads and a breakfast meal.	Assess effects of wholemeal bread matrix on relative uptake of $^{13}C_5$-5-CH_3-H_4folate.
Folate doses	0, 70, 125 μg/d	200 μg/portion	30-40 μg/gastric intake[1]	0.2 μg/well (0.5 ml)[1]
Used test foods				
-breakfast[2]	√	√	√ (IV)	-
-wholemeal bread	√	√	√ (IV)	√
-orange juice	-	-	√ (III)	-
Analysis of food samples	LC-MS[3]&MA[4]	LC-MS[3]	HPLC-FLD[5]	LC-MS[3]
Analysis of clinical or TIM & Caco-2 cells samples	Radioassay[6]	SIDA/LC-MSMS[7]	HPLC-FLD[5]	LC-MS[3]
N	17 women/group (3)	8 ileostomists	2 independent trials/food	6 wells/test digests (2)
Statistical analysis	Wilcoxon-Mann-Whitney rank-sum test	Q-statistics	Q-statistics	Wilcoxon-Mann-Whitney rank-sum test

[1]Given amount of folate. [2]Contained wholemeal bread with liver-pâté, dairy product with cereals, orange juice and a kiwi. [3]In house folate determination using Agilent 1100 Series (Patring & Jastrebova, 2007). [4]At accredited laboratory (National Food Administration, Uppsala, Sweden). [5]In house folate determination using Agilent 1100 Series (Jastrebova *et al.*, 2003). [6]At accredited laboratory (Quintiles AB, Uppsala, Sweden). [7]At Bioanalytik, Weihenstephan, Germany (Freisleben *et al.*, 2003).

3 Material and methods

3.1 Folate quantification

Clinical samples were externally analysed using LC-MSMS or radioassays (Table 5), for details see papers I and II. *In vitro* and food (Table 6) samples were analysed using in-house HPLC methods. Test foods were analysed to determine doses and to account for batch and brand variations as well as potential storage losses (Table 6).

Test foods and doses

Test foods used in the *in vivo* and *in vitro* studies are listed in Table 6. The breakfast (Table 6, Figure 6) was composed with minor modifications according to Swedish Nutrition Recommendations Objectified (SNÖ) (Enghardt-Barbieri & Lindvall, 2005). (6S)-$^{13}C_5$-5-CH_3-H_4folate (calcium salt) and $^{13}C_5$-folic acid, produced according to GLP/pharmaceutical standards, were purchased from Merck Eprova AG (Schaffhausen, Switzerland). The content of unlabelled folate in the standards was <1% as detected by LC-MS (Patring & Jastrebova, 2007). Wholemeal breads (Table 6) were baked with the labelled standards (II, IV) at the Lantmännen test bakery, Järna, Sweden, as described in manuscript IV.

Table 6. *Test foods used for* in vivo *and* in vitro *trials*

Test foods	Paper[1]	Folate content checked for effect of:		
		Brand	Batch	Storage (conditions)
Breads (Lantmännen), stored at -20°C				
- wholemeal (8% dietary fibre)	I	√	√	√ (-20°C, 4 months)
- wholemeal, fortified with labelled folate (test bakery)	II,IV		√	
- wholemeal rye (8% dietary fibre)	I	√	√	√ (-20°C, 4 months)
- B3, made of B3-flour (test bakery)	IV	√		
- rye bran (test bakery)	IV	√		
Juices (JO-Bolaget), stored at 5-8°C				
- orange juice diluted from concentrate	III	√		√ (8°C, 7 weeks)
- orange juice ready to drink	I,II,III	√	√	√ (5°C, 4 months)
- apple juice ready to drink	I			
Breakfast cereals, stored at 5-8°C (rye flakes) or ambient temperature (breakfast cereals)				
- rye flakes (test bakery, Lantmännen)	I			√ (5°C, 4 months)
- breakfast cereals (17% fibre, Coop)	II,IV			
Other foods, stored at 5-8°C				
- kiwi	I,IV		√	
- liver pâté (38% liver, ICA)	I		√	
- liver pâté (41% liver, Scan)	II,IV			
- fermented milk (3% fat, Norrrmejerier)	II			
- fermented milk (0.5% fat, Arla)	IV			

[1]See Table 5. Food from Lantmännen (Järna, Sweden) and JO-Bolaget (Stockholm, Sweden) were obtained directly from the producers. The foods from Coop (Stockholm, Sweden); ICA (Solna, Sweden); Scan (Johanneshov, Sweden); Norrrmejerier (Umeå, Sweden) and Arla (Stockholm, Sweden) were purchased at local supermarkets.

Analytical methods

In the intervention trial (I), the concentrations of serum folate, erythrocyte folate and plasma total homocysteine were externally analysed at a routine laboratory (Table 5) (Quintiles AB, Uppsala, Sweden) accredited by Swedac (Swedish Board for Accreditation and Conformity). Serum and erythrocyte folate concentrations were measured using Immunoassay Plus (BioRad, Sundbyberg, Sweden) and plasma total homocysteine using a CardiacMark 1/2/3 (BioRad).

In the ileostomy/plasma AUC trial (II), plasma and stomal effluents were externally analysed using a SIDA method with LC-MSMS detection modified from Freisleben *et al.* (2003). Analyses were carried out in duplicate with duplicate injection at Bioanalytik Weihenstephan, Technische Universität München, Germany.

In the Caco-2 cell trial (IV), folates in the samples were stabilised with antioxidants (0.2% (w/v) ascorbate and 0.01% (v/v) 2,3-dimercapto-1-propanol) directly after the experiment ended. Sample extracts were purified by centrifugation (10 000 rpm, 10 min) and strong anion exchange chromatography prior to LC-MS quantification as described in manuscript IV.

TIM samples (III, IV), test foods and doses (I-IV) were analysed using methods based on the work of Patring & Jastrebova (2007), Jastrebova *et al.* (2003), Witthöft *et al.* (2003) and Johansson *et al.* (2002). In brief, all foods and *in vitro* TIM samples were homogenised and heat-extracted in phosphate buffer (pH 6.1) containing ascorbate and 2,3-dimercapto-1-propanol. For foods rich in starch, such as bread, thermostable α-amylase was added during heat extraction. After centrifugation the supernatant was incubated with rat serum (and α-amylase again for starchy foods) and thereafter purified on either SAX or affinity columns.

Folate content was quantified using external calibration and an HPLC-FLD/UV method (Jastrebova *et al.*, 2003) or LC-MS method (Patring & Jastrebova, 2007). By using different wavelengths, quantification of 10-HCO-folic acid in FLD was enabled (Pfeiffer *et al.*, 1997b). The LC-MS method was adjusted to enable quantification of 10-HCO-H_2folate, [6S]-$^{13}C_5$-5-CH_3-H_4folate, [6S]-$^{13}C_5$-5-HCO-H_4folate and $^{13}C_5$-folic acid.

3.2 Intervention study with two pieces of nutritional advice (I)

A randomised controlled parallel intervention study (Figure 6, I) was carried out. Folate status in healthy women was determined after intervention with two pieces of nutritional advice: a food recommendation (175 g fibre-rich bread, 70 μg folate (159 nmol)) and a complete meal (breakfast, 125 μg folate (283 nmol)) (Enghardt-Barbieri & Lindvall, 2005). The control group received apple juice (0 μg additional folate/day) (Figure 6).

A total of 51 women who fulfilled the inclusion criteria (Figure 6), *i.e.* normal range of biochemical parameters (I), non-pregnant and non-nursing, were randomly assigned to the different groups.

Folate status was assessed by concentrations of erythrocyte folate, serum folate and plasma total homocysteine at baseline, week 8 and week 12 of the trial (Figure 6). Fasting blood sampling was carried out at Good Food Practice, Uppsala, Sweden, and the study protocol was approved by the Regional Ethical Review Board in Uppsala, Sweden.

Estimation of folate intake using FFQ

To estimate changes in food folate intake during the trial, a 71-item Food Frequency Questionnaire (FFQ) was completed by volunteers at screening (3 month recall) and during the intervention trial (2 month recall) (Figure 6). The software Dietist XP version 3.0 (Kost och näringsdata, Bromma, Sweden), using data from the Swedish food composition database (NFA, 2006) and food industries, was used for calculations of folate intake.

3.3 Plasma AUC/ileostomy study using stable isotope-labelled folate (II)

A crossover trial with three independent study days separated by a wash-out period of 2-5 weeks was carried out. On study days, the ileostomy volunteers (n=8) were randomly assigned wholemeal bread fortified with ~450 nmol (200 μg) of either (6S)-$^{13}C_5$-5-CH_3-H_4folate (bread only) or $^{13}C_5$-folic acid (bread only or as part of a complete breakfast containing feremented milk, breakfast cereals, orange juice and liver pâté). Post-dose plasma was collected for determination of plasma kinetics and stomal effluent for determination of the non-absorbed fraction of labelled folate.

Upsala Nya Tidning Sunday 25th of June 2006

Volunteers needed to an intervention trial. Inclusion critera:
- healthy women aged 25-60 years,
- non-nursing, non-pregnant, not planning pregnancy and
- not consuming folic acid supplements or fortified foods.

Interview	General health Dietary habits Study purpose
Screening	Folate status Health check FFQ (n=67)
Baseline	Folate status (n=51)

INTERVENTION

Control (n=17)	Bread (n=17)	Breakfast (n=17)
Apple juice 250 ml)	Wholemeal bread (175 g, 5 slices)	Orange juice (250 ml), wholemeal bread (70 g), liver pateé (~15 g), breakfast cereals (30 g), kiwi (~60 g) and dairy product (own choice).
Breakfast of own choice		
0 µg folate	70 µg folate (159 nmol)	125 µg folate (283 nmol)

Week 8	Folate status FFQ (n=51)

INTERVENTION

Control (n=17)	Bread (n=17)	Breakfast (n=17)

Week 12	Folate status Health check (n=51)

Figure 6. Outline of intervention trial.

During cannulation, a fasting baseline plasma sample was collected. Meals and sampling of plasma and stomal effluents were strictly standardised (Witthöft *et al.*, 2003). Post-dose blood samples were collected at 20, 40, 60, 90, 120, 180, 240, 360, 480, 600 and 720 minutes. Stomal effluent bags were changed every 2 hours during 12 h post-dose and by free choice between 12-24 h post-dose. This trial was carried out at the Centre for Clinical Research (KFC) in Umeå, Sweden and the study protocol was approved by the Regional Ethical Review Board in Uppsala, Sweden.

3.4 Folate bioaccessibility assessed in the *in vitro* gastrointestinal model TIM (III, IV)

In vitro studies were carried out with the dynamic, computer-controlled gastrointestinal model TIM (Minekus *et al.*, 1995) to assess folate bioaccessibility (fraction of given folate available for absorption) from orange juice, two sorts of bread and a complete breakfast (Table 5).

In brief, the so-called gastric intake, prepared by mixing the test food with a buffer solution, was placed in the gastric compartment of TIM on two separate days as described by Verwei *et al.* (2003). The gastric intake was mixed with electrolytes and enzymes during transport through the compartments of stomach, duodenum, jejunum and ileum (Minekus *et al.*, 1995). Bioaccessible fractions (from jejunum and ileum compartments) and non-bioaccessible fractions (from ileal deliveries) were collected for folate quantification during the 5 h experiment.

3.5 Uptake of $^{13}C_5$-5-CH_3-H_4folate into Caco-2 cells (IV)

Caco-2 cells were used to assess the effect of bread matrix on uptake and transport of stable isotope-labelled (6S)-$^{13}C_5$-5-CH_3-H_4folate. Caco-2 cells, deriving from a human colon carcinoma cell line, differentiate spontaneously into cells resembling absorptive enterocytes (Hubatsch *et al.*, 2007). Polarised Caco-2 cell monolayers are suitable for folate uptake studies since known folate transporters (Figure 3, PCFT, RFC, MRP) are expressed at the cells (Ashokkumar *et al.*, 2007).

Digests of wholemeal bread fortified with $^{13}C_5$-5-CH_3-H_4folate (Table 5) and a $^{13}C_5$-5-CH_3-H_4folate standard solution were prepared by enzymatic treatment in concentrations according to Salovaara *et al.* (Salovaara *et al.*, 2003), under simulated gastrointestinal conditions. The uptake and transport experiment was performed under sterile conditions using fully differentiated cells, at a pH of 6.3 at the luminal side and 7.4 at the basolateral side.

In brief, the digests containing 0.4-0.5 μmol $^{13}C_5$-5-CH_3-H_4folate were added to the apical chambers and incubated for 60 min at 37°C in the dark under gentle shaking. Concentrations of labelled and unlabelled folate were measured in extracts from the lysed cells, the apical side and the basolateral side. Content of labelled folate polyglutamates in lysed cells was estimated by determination of folate content prior to and after deconjugation.

3.6 Calculations and statistics

The reliability coefficient (RC, intra class correlation) (Fleiss, 1986) shows the ratio of between subject variability to total variability. RC was estimated for each folate status parameter for volunteers in the control group in the intervention trial (I) as:

$$RC = (BMS-WMS)/(BMS+(n-1)*WMS) \quad (1)$$

where BMS is the between subject mean square error, WMS is within subject mean square error and n is number of replicates within subject.

The $AUC_{0\rightarrow12}$ (median and range) of plasma data (II) was calculated using the trapezoidal method (Tozer & Rowland, 2006). Because $^{13}C_5$-5-CH_3-H_4folate had not reached baseline concentrations (0 nmol) at the last blood sampling point (C_{720}), $AUC_{12\rightarrow\infty}$ was estimated by extrapolation from the last seven data points by log-linear regression analysis (Tozer & Rowland, 2006). Folate recovery (mass balance) of the plasma AUC/ileostomy model for the labelled folate forms was estimated as:

$$R = (C_{max}*V+F_{non\text{-}absorbed})/F_{dose} \quad (2)$$

where C_{max} is the highest measured concentration of labelled folate in plasma after given dose, V the distribution volume of folic acid (0.389 L/kg bodyweight as estimated (Loew *et al.*, 1987)), $F_{non\text{-}absorbed}$ the labelled folate content in the stomal effluent (0-24 h) and F_{dose} the labelled folate content in dose.

Folate bioaccessibility as assessed by TIM gastrointestinal model (III,IV) was calculated using data from two independent TIM runs, each analysed in duplicate as:

$$\text{Bioaccessibility} = F_{\text{dialysate}}/F_{\text{food portion}} \quad (3)$$

where $F_{dialysate}$ and $F_{food\ portion}$ are folate content in dialysates (jejunal and ileal) and the TIM food portion, respectively.

Folate recovery (mass balance) of the TIM model was calculated as:

$$R = (F_{\text{dialysate}}+F_{\text{ileal delivery}}+F_{\text{residues}})/F_{\text{food portion}} \quad (4)$$

where $F_{ileal\ \ delivery}$ and $F_{residues}$ are folate content in the ileal delivery ('non-absorbed' fraction) and the residues (folate content in the model after the experiment) (Verwei *et al.*, 2003), respectively.

Folate uptake into Caco-2 cells (IV) was calculated using data on [6S]-$^{13}C_5$-5-CH_3-H_4folate from six wells as:

$$\text{Uptake} = (F_{\text{lysed Caco-2 cells}}+F_{\text{basolateral}})/F_{\text{digest}} \quad (5)$$

where $F_{lysed\ \ Caco\text{-}2\ \ cells}$, $F_{basolateral}$ and F_{digest} are $^{13}C_5$-5-CH_3-H_4folate content in lysed Caco-2 cells, basolateral samples and digests, respectively.

Folate transport into Caco-2 cells (IV) was calculated using data on $^{13}C_5$-5-CH_3-H_4folate from six wells as:

$$\text{Transport} = F_{\text{basolateral}}/F_{\text{digest}} \quad (6)$$

Folate recovery (mass balance) of the Caco-2 cell model was calculated as:

$$R = (F_{\text{apical}}+F_{\text{lysed Caco-2 cells}}+F_{\text{basolateral}})/F_{\text{digest}} \quad (7)$$

where F_{apical} is $^{13}C_5$-5-CH_3-H_4folate content in apical samples.

Analytical surrogate recovery (Thompson *et al.*, 2002) was calculated as:

$$R = (F_{\text{sample+analyte}}-F_{\text{sample}})/F_{\text{analyte}} \quad (8)$$

where $F_{sample+analyte}$ is the folate content in the sample with surrogate analyte (spike) added, F_{sample} the content in the non-spiked sample and $F_{analyte}$ the added amount of surrogate analyte.

Data on concentrations and content in clinical samples (plasma, erythrocyte and stomal effluents) were expressed as median and 1st (Q_1) and 3d (Q_3) quartile (I) or range (II). Folate contents in TIM and food samples were expressed as individual values (n=2, III) or mean±root mean square error or standard deviation (n>2, IV). Folate contents in Caco-2 cell samples (IV) were expressed as median and range. Since data were usually not normally distributed, non-parametric methods were used as standard.

The Wilcoxon-Mann-Whitney rank-sum test was used to compare changes from baseline in active groups with the control group, *i.e.* intervention groups (I) and Caco-2 cell digests (IV) (Table 5). General linear model was used to assess BMS and WMS (equation 1). Q-statistics (Putt & Chinchilli, 2004; Öhrvik, 1998) for non-parametric analysis of high order cross-over trials was used to compare different study days in the plasma AUC/ileostomy study (II, Table 5). Kruskal Wallis test was used to compare doses in the plasma AUC/ileostomy trial. Minitab® ver 15.1.0.0 (Minitab Ltd., Coventry, UK) was used for these analyses. The Jonckheere-Terpstra test (StatXact-4 ver 4.0.1, Cytel Software Corporation, Cambridge, USA) was used to test for dose-response effects in the intervention trial (I). Associations (status- and biochemical parameters at screening of the intervention trial) were assessed by Spearman's correlation coefficients (SAS ver 9.2, Cary, USA). A two-sided $p<0.05$ was regarded as statistically significant.

4 Results and discussion

The objective of this thesis was to assess folate bioavailability using different *in vivo* and *in vitro* models but similar test foods and analytical methods. The main findings were:

- Regular consumption of a breakfast following SNÖ recommendations (I) (Enghardt-Barbieri & Lindvall, 2005) improved folate status significantly. After the trial, more than 50% of volunteers had a status associated with the lowest risk of an NTD-affected pregnancy (compared with ~25% prior to the trial).
- Regular consumption of 5 slices of wholemeal bread daily (I) maintained erythrocyte and serum folate concentrations and reduced plasma total homocysteine concentrations significantly.
- Post-dose kinetics in peripheral plasma (II) differed significantly between folate forms. As labelled folate content in stomal effluents did not differ significantly between study days the difference could not be explained by differing bioavailability.
- Bioavailability of 5-CH_3-H_4folate fortificant was as high as the bioavailability of folic acid fortificant from wholegrain bread (~90%), based on content of labelled folate in stomal effluent (II).
- Bioavailability of folic acid fortificant from bread was similar after ingestion of folic acid with bread alone, and as part of a breakfast meal, based on content of labelled folate in stomal effluent (II). Post-dose kinetics in peripheral plasma was also not affected by the breakfast meal.
- Foods rich in dietary fibre had some inhibitory effect (~25%) on folate bioaccessibility (III, IV) and uptake (IV) *in vitro* but not *in vivo* as less than 10% of the labelled folate dose was found in stomal effluents (II).
- Colon cells (Caco-2 cells) not only facilitated transport across the cell membranes but also retained folate within the cells by conjugation of a polyglutamate chain (IV).

4.1 Intervention study with two pieces of nutritional advice (I)

After 12 weeks of regular consumption of either a complete breakfast (additional 125 μg folate (283 nmol)) or 5 slices of wholemeal bread daily (additional 70 μg folate (159 nmol)) (Figure 6), the effects of the intervention diets were observed as:

- >20% increase in median erythrocyte folate concentrations for the breakfast group, which was significant compared with changes in the control group (-18%) (Table 7).
- >20% reduction in plasma total homocysteine concentrations for both active groups (Table 7).

In the control group (apple juice, 0 μg folate), folate status parameters were stable, although a trend ($p=0.07$) of decreasing erythrocyte folate concentrations was observed (Table 7), possibly reflecting a seasonal effect as observed by others (Hao *et al.*, 2003; McKinley *et al.*, 2001).

Findings from trials using intervention diets with food folate are inconclusive, as some report no significant effects (Table 1a) while others report significant effects (Table 1b). Figures 7a-c show responses to intervention doses of trials in Table 1a and 1b and that in Paper I. Responses in erythrocyte and serum folate concentrations in (I) were in agreement with those in previous trials (Figure 7 a-b). However, the observed increase in serum folate of 2 nmol/L after consumption of the breakfast was not significant ($p=0.06$, Table 7). Therefore no conclusions can be drawn on the effect of the intervention on serum folate.

Despite low food folate doses of ~100 μg/day in (I), plasma total homocysteine concentrations decreased significantly, with levels similar to responses observed in trials with intervention doses >400 μg food folate (Figure 7c).

The pronounced decrease in homocysteine might partly be explained by our volunteers being women, who respond more efficiently to supplemental folic acid (Homocysteine Lowering Trialists, 2005). Except for Bogers *et al.* (Bogers *et al.*, 2007), all other trials had volunteers of both genders or male only (Hannon-Fletcher *et al.,* 2004). For homocysteine lowering a strongly significant dose-response effect (Table 7) was found, so folate was essential in the reduction of plasma total homocysteine concentrations. However, other food components involved in methylation of homocysteine (Figure 4), *e.g.* vitamin B12 (as provided by the liver pâté) and betaine (as provided by the wholemeal bread) might have contributed to the pronounced decrease.

Table 7. *Concentrations of erythrocyte folate, serum folate and plasma total homocysteine at baseline, week 8 and week 12 of the intervention trial*

	Group	Control 0 µg/d (n=17)		Bread 70 µg/d (n=17)			Breakfast 125 µg/d (n=17)			Dose-response
		median	Q_1, Q_3	median	Q_1, Q_3	vs. control[1]	median	Q_1, Q_3	vs. control[1]	effect week 12[2]
Ery-folate	Baseline	900	647, 1079	855	635, 992		805	742, 909		
(nmol/L)	Week 8	838	621, 1065	805	683, 998	p=0.58	856	740, 1112	p=0.095	
	Week 12	735	641, 892	703	629, 839	p=0.78	990	763, 1058	p=0.019	p=0.017
S-folate	Baseline	12	11, 15	12	9, 16		12	10, 16		
(nmol/L)	Week 8	13	11, 15	11	10, 15	p=1.00	14	12, 21	p=0.074	
	Week 12	12	9, 15	12	10, 16	p=0.23	13	12, 20	p=0.058	p=0.054
tHcy	Baseline	9.3	8.6, 11.1	9.1	7.9, 11.0		8.7	7.3, 10.3		
(µmol/L)	Week 8	9.9	9.0, 12.6	9.2	8.5, 11.6	p=0.35	9.0	7.3, 9.5	p=0.070	
	Week 12	9.6	7.6, 11.6	7.2	6.9, 8.5	p=0.076	6.7	5.6, 7.4	P<0.001	p<0.001

Ery-folate = erythrocyte folate; S-folate = serum folate; tHcy = plasma total homocysteine. [1]Differences in changes from baseline between the control and each active group were tested for using Wilcoxon-Mann-Whitney rank-sum test. [2]Dose response effects at week 12 were tested for using Jonckheere-Terpstra test.

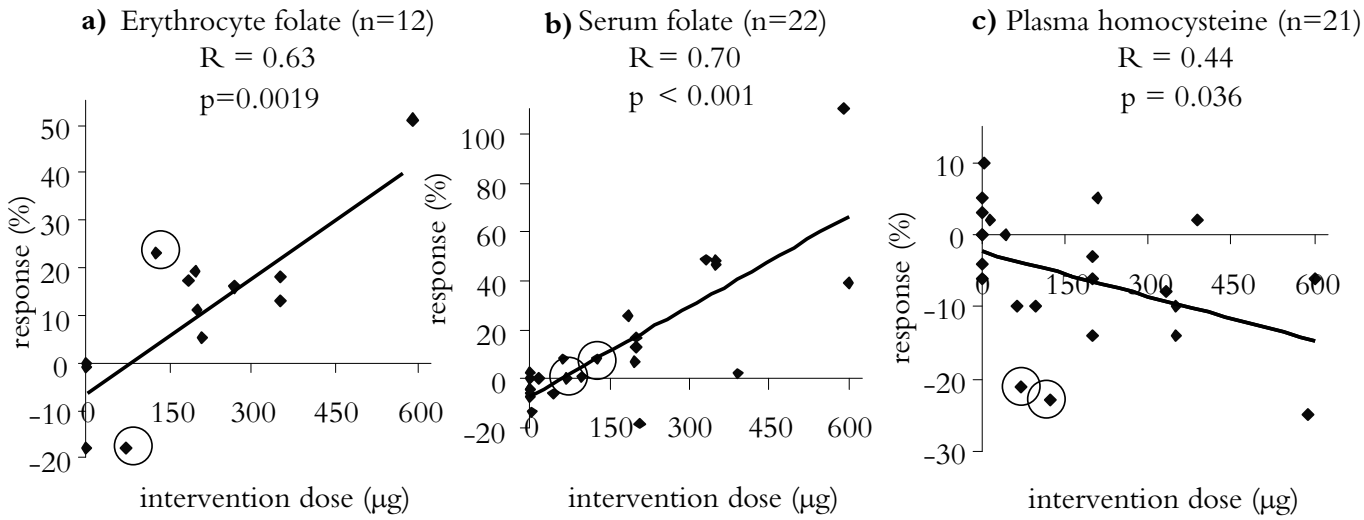


Figure 7 a-c. Response (%) in concentrations of a) erythrocyte folate, b) serum folate and c) plasma total homocysteine to intervention doses (µg/day) and Spearman's correlation coefficients. Data are based on trials (n=6-10) listed in Table 1a and 1b and the Paper I. Responses (n=4) from the intervention doses in this trial (I) were marked with a ring

The health-related benefit of homocysteine lowering is under discussion. In a meta-analysis (12 clinical trials, n=16958) folic acid supplements were found to be ineffective in the prevention of cardiovascular disease among persons with a history of vascular diseases (Bazzano *et al.*, 2006). Nonetheless, data from meta-analyses suggest a preventive effect of homocysteine lowering (>20%) on stroke (Saposnik *et al.*, 2009; Wang *et al.*, 2007; Bazzano *et al.*, 2006).

Findings on folate bioavailability from different trials vary depending on the status parameter used for assessment (see section 1.2). In this trial (I) all status parameter seemed appropriate, based on reliability coefficients - estimated from the control group (Table 8) - within the range reported by others (0.65-0.97) (McKinley *et al.*, 2001; Clarke *et al.*, 1998; Garg *et al.*, 1997).

Table 8. *Evaluation of the folate status parameters in the intervention trial*

	Ery-folate	S-folate	tHcy
Reliability coefficients (equation 1)	0.698	0.648	0.694
Time (week) until significant response from a dose of 125 μg/day (n=17)	>8 week	n.a.	8 week
Correlations to the other parameters at screening (n=67)			
-to serum folate	0.770***	-	-
-to plasma total homocysteine	-0.266*	-0.281*	-
-to serum vitamin B12	0.130	0.111	-0.373**
-to haemoglobin	0.326*	0.238	-0.151

n.a. = non applicable. Spearman's correlation coefficient, * $p<0.05$, ** $p<0.01$, *** $p>0.001$.

To summarise, intervention with the breakfast meal improved folate status, whereas 5 slices of wholemeal bread maintained folate status. In an Irish study, the risk of an NTD-affected pregnancy was found to be eightfold higher for women having erythrocyte folate concentrations below ~340 nmol/L compared with those having concentrations above ~900 nmol/L ($p<0.001$) (Daly *et al.*, 1995). After regular consumption of the breakfast for 12 weeks, the number of volunteers with erythrocyte folate concentrations >900 nmol/L more than doubled from 24% to 59% (I).

Folate intake estimations using FFQ (I)

At screening of volunteers for the intervention study (Figure 6, I), median folate intake was estimated to be 279 μg/day (n=67), which is close to the recommended daily intake for adults (300 μg/day). However, folate intake varied significantly with age (Figure 8, p=0.04) and was only 244 μg/day for women of childbearing age (26-44 years). Only one volunteer (aged 26-44 years) reached the recommended daily intake for this particular group (400 μg/day). This is in agreement with data from the latest Swedish food intake survey (Becker & Pearson, 2003), but contradictory to findings from the Swedish Mammography Cohort (Larsson *et al.*, 2004), where a higher folate intake was associated with lower age (Table 3).

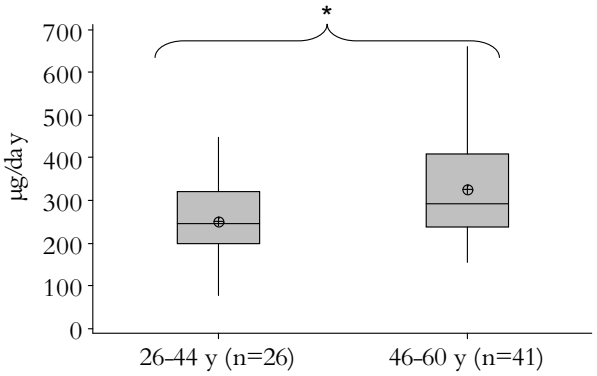


Figure 8. Boxplots of estimated folate intake (μg/day) in women, grouped by age. Mean levels are marked by circles, the lines represent the Q_1, Q_2 and Q_3.

Folate from wholemeal bread, broccoli, dishes with beans, orange juice and liver pâté contributed nearly 50% of the median folate intake in volunteers at screening (n=67 women). Compared with national intake data (Becker & Pearson, 2003), the contribution from cereal products and beans was higher in the volunteers (I) while animal products contributed less (Figure 9).

The intake estimation with the FFQ used in the intervention trial (I) was limited by several factors. Firstly, the study population (volunteers) was limited in size (n=67) and not representative of the general population. Secondly, the FFQ was not validated against another dietary assessment method or status parameters. Estimated folate intake did not correlate to measured concentrations of serum folate, erythrocyte folate or plasma total homocysteine (I) as reported for several validated FFQ (Brantsaeter *et al.*, 2008; Konstantinova *et al.*, 2007; Owens *et al.*, 2007; van de Rest *et al.*,

2007; Hickling *et al.*, 2005; Pufulete *et al.*, 2002). However, for the purpose of assessing changes in dietary folate intake during the intervention trial the FFQ (I) was considered sufficient.

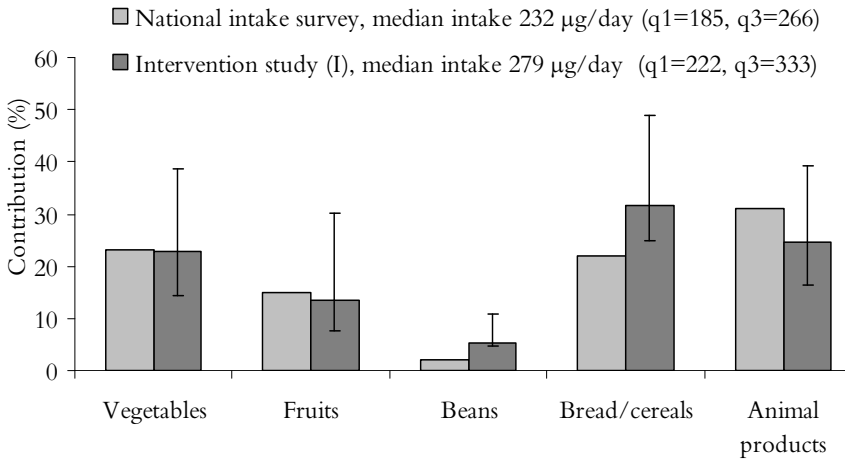


Figure 9. Contribution (%) from food groups to folate intake. Comparison of data from Riksmaten 1997-1998, dietary recall, n= 215 women and men (Becker & Pearson, 2003) and from the screening of the intervention trial 2006 (I), 71-item FFQ, n=67 women, (median ($Q_1$and Q_3))

Folate intake and status of volunteers at screening for the intervention trial

Estimated folate intake and folate status presented in this thesis are based on data from women screened to participate in the intervention trial (I). Hence neither data on intake nor status are representative for Sweden, but could possibly reflect a health-interested population of women. Some features of the women (n=67) were:

- None of the women was folate-deficient, defined as serum folate concentrations below 7 nmol/L and erythrocyte folate concentrations below 363 nmol/L (Herbert & Das, 1994).
- Although volunteers were instructed to avoid folic acid-fortified foods and supplements for two months prior to the screening, erythrocyte folate concentrations were about 30% higher than for US women in the post-fortification era (McDowell *et al.*, 2008).
- 30% of the women had a folate status associated with the lowest risk of NTD (>900 nmol/L) (Table 9) (Daly *et al.*, 1995).
- For women of childbearing age there was a large gap of 40% between estimated (240 µg/day) (Figure 8) and recommended (400 µg/day) (NNR, 2005) folate intake.

In Table 9, concentrations of erythrocyte folate and serum folate of women in the Uppsala area at screening (Öhrvik *et al.*, 2009; Johansson *et al.*, 2002) are ranged according to estimated risk of neural tube defects as determined by Daly *et al.* (1995).

About 70% of the women in both studies had already at screening erythrocyte folate concentrations associated with a low risk (0.15%) of an NTD-affected birth (and pregnancy). After the intervention trial median erythrocyte folate concentrations (Table 7) were >900 nmol/L for women in the breakfast group. Thus demonstrating that only minor additional folate intake (~125 μg/day), as achieved by following SNÖ recommendations (Enghardt-Barbieri & Lindvall, 2005), is required to reach erythrocyte folate concentrations associated with the lowest NTD risk (Table 9) (Daly *et al.*, 1995).

Table 9. *Folate status of women in Uppsala, grouped by estimated NTD risk at screening*

nmol/L	Risk of NTD/1000 births[1]	2006 (n=67)[2]	2001 (n=33)[3]
Erythrocyte folate concentrations		%	%
0-339	6.6	0	0
340-452	3.2	6	0
453-679	2.3	27	30
680-905	1.6	37	30
>906	0.8	30	40
Serum folate concentrations			
0-4.4	3.7	0	0
4.5-6.7	2.6	1	0
6.8-11.2	1.9	24	15
11.3-15.8	1.5	45	21
>15.9	0.9	30	64

[1]Estimated in Ireland (where termination of pregnancies is prohibited) according to Daly *et al.* (1995). [2]Öhrvik *et al.* (2009). [3]With kind permission of Johansson *et al.* (2002).

4.2 Plasma AUC/ileostomy study using stable isotope-labelled folate (II)

In this randomised cross-over study, the plasma kinetics of labelled folate were studied 12 hours after ingestion of either [6S]-$^{13}C_5$-5-CH_3-H_4folate or $^{13}C_5$-folic acid. Independent of the folate form ingested, a plasma curve with $^{13}C_5$-5-CH_3-H_4folate was obtained (Figure 10). No other labelled folate forms were detected. Bioconversion of a dose of 450 nmol $^{13}C_5$-folic acid into $^{13}C_5$-5-CH_3-H_4folate (Figure 3) thereby appeared complete.

Median plasma $AUC_{0\rightarrow\infty}$ (equation 2) was 89 (48-111) h·nmol/L after ingestion of $^{13}C_5$-5-CH_3-H_4folate-fortified wholemeal bread. This was nearly twice the estimated $AUC_{0\rightarrow\infty}$ after ingestion of $^{13}C_5$-folic acid-fortified wholemeal bread, which was 50 (38-81) h·nmol/L (Table 10, Figure 10). T_{max} and C_{max} also varied significantly depending on folate form in the dose (Table 10, Figure 10). Consumption of the $^{13}C_5$-folic acid-fortified wholemeal bread with a breakfast meal instead of bread alone resulted in similar median plasma $AUC_{0\rightarrow\infty}$, which was 47 (26-119) h·nmol/L, T_{max} and C_{max} (Table 10, Figure 10).

Table 10. *Kinetic parameters of $^{13}C_5$-5-CH_3-H_4folate in plasma after single test doses in random order. Median (range) of 8 ileostomists*

	Bread fortified with $^{13}C_5$-5-CH_3-H_4folate	Bread fortified with $^{13}C_5$-folic acid	Bread fortified with $^{13}C_5$-folic acid as part of complete breakfast	p
($^{13}C_5$)-dose (nmol)	464 (450-467)	451 (448-472)	462 (448-471)	0.61[1]
($^{13}C_5$)-$AUC_{0\rightarrow\infty}$ (h·nmol/L)[2]	0.20 (0.10-0.24)	0.11 (0.08-0.18)	0.10 (0.05-0.25)	0.0053[3]
($^{13}C_5$)-C_{max} (nmol/L)	10.6 (6.3-16.5)	3.1 (1.7-4.5)	3.3 (2.1-5.7)	<0.001[3]
($^{13}C_5$)-T_{max} (min)	40 (40-90)	150 (90-240)	240 (120-240)	0.003[3]

[1] Differences in test doses were controlled using Kruskal Wallis test. [2] Dose-normalised $^{13}C_5$-$AUC_{0\rightarrow\infty}$ (AUC/dose). [3]Differences in response to test doses were tested using Q-statistics for non-parametric analysis of high order cross-over trials (Putt & Chinchilli, 2004; Öhrvik, 1998). $^{13}C_5$-$AUC_{12\rightarrow\infty}$ was estimated by extrapolation using log-linear regression analysis of the plasma concentrations in the elimination phase (Tozer & Rowland, 2006) (section 3.6).

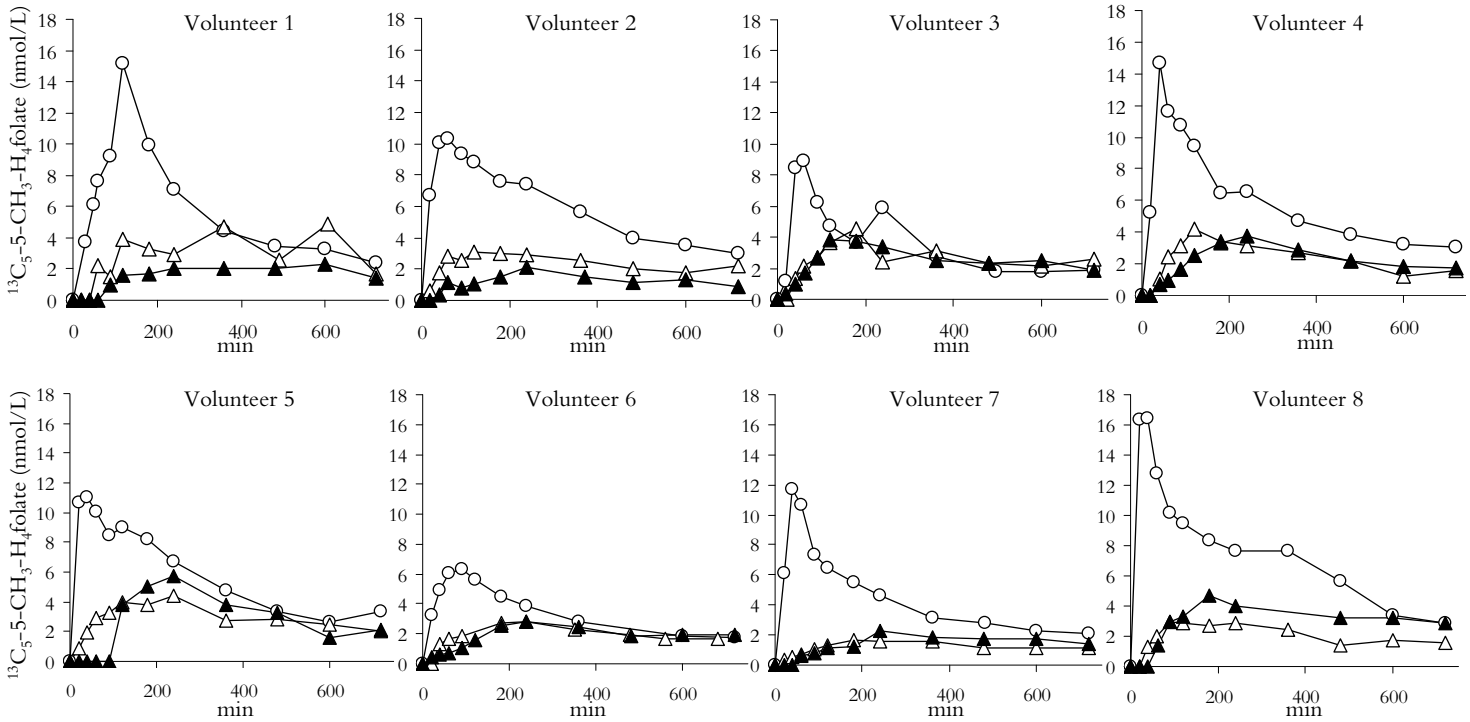


Figure 10. Individual plasma curves of $^{13}C_5$-5-CH_3-H_4 folate after single test doses of $^{13}C_5$-5-CH_3-H_4 folate-fortified bread (circle), $^{13}C_5$-folic acid- fortified bread (unfilled triangle) and a breakfast meal containing $^{13}C_5$-folic acid-fortified bread (filled triangle).

Discrepancies in post-dose kinetics between doses of $^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-folic acid (Table 10, Figure 10) might partly be explained by a less precise estimate of plasma AUCs after ingestion of $^{13}C_5$-folic acid doses. At T_{max} after ingestion of folic acid doses, sampling interval was less frequent (every 60 min, Figure 10) than after ingestion of $^{13}C_5$-5-CH_3-H_4folate (every 20 min, Figure 10). Hence, C_{max} and $AUC_{0\rightarrow\infty}$ might have been underestimated after ingestion of $^{13}C_5$-folic acid. In addition, the delayed T_{max} also resulted in a less distinct curve shape (Figure 10) resulting in an $AUC_{0\rightarrow12}$ of only about 55% of the total $AUC_{0\rightarrow\infty}$ after extrapolation.

In the stomal effluents collected during 24 h post-dose, the ingested labelled forms ($^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-folic acid) and unlabelled H_4folate, 5-CH_3-H_4folate and 10-HCO-folic acid were found (Figure 10). The content of labelled folate in stomal effluent varied from 2-9% of the given dose (Table 11) and did not differ significantly (p=0.15) with respect to folate form in dose or how the dose was ingested (with bread only or as part of a breakfast).

Table 11. *Content of folate (nmol) in stomal effluents collected during 24 h after oral doses of ~450 nmol folate. Median (range) of 8 ileostomists*

	Bread fortified with [6S]-$^{13}C_5$-5-CH_3-H_4folate	Bread fortified with $^{13}C_5$-folic acid	Bread fortified with $^{13}C_5$-folic acid as part of a breakfast
($^{13}C_5$)-5-CH_3-H_4folate	13.1 (9.8-31.2)	0.5 (0.1-1.0)	0.7 (0-5.0)
($^{13}C_5$)-folic acid	not detected	24.5 (8.7-32.1)	25.3 (7.7-37.3)
Relative ($^{13}C_5$)-labelled folate content as % of dose[1]	2.8 (2.1-6.7)	5.4 (1.9-7.2)	5.6 (1.7-9.0)
Unlabelled folate content[1,2] (nmol)	99 (52-178)	106 (43-148)	103 (46-166)

[1]No significant differences in non-absorbed labelled folate were found between the test meals using Q-statistics for non-parametric analysis of high order cross-over trials (Putt & Chinchilli, 2004; Öhrvik, 1998). [2]Unlabelled folate content in stomal effluents collected during 12 h post-dose only (stomal collection from 12-24 h post-dose was not standardised). Unlabelled folate intake during 0-12 h was 140 nmol for study days with bread and 380 nmol for the study day with the breakfast meal, including folates from thest foods and other meals.

A minimal fraction of the stomal effluent after ingestion of $^{13}C_5$-folic acid was $^{13}C_5$-5-CH_3-H_4folate (about 0.6 nmol/24 h, Table 11). By LC-MS analysis (II) it was confirmed that the $^{13}C_5$-folic acid-fortified bread contained no $^{13}C_5$-5-CH_3-H_4folate. Therefore it can be hypothesised that $^{13}C_5$-5-CH_3-H_4folate detected in stomal effluents originated from bioconverted $^{13}C_5$-folic acid. Bioconverted $^{13}C_5$-folic acid might have ended up in the stomal effluent after release from either lysed enterocytes or newly produced bile in the gastrointestinal tract. According to previous estimates, at least 10% of daily folate intake is secreted into bile (Gregory & Quinlivan, 2002) and nearly completely (96%) reabsorbed in the gastrointestinal tract (Lin *et al.*, 2004). In the plasma AUC/ileostomy model low fat diets were used on study days (12 h post-dose), which minimised bile flow and thereby folate excretion into the gastrointestinal tract.

Volunteers' total folate intake of labelled and unlabelled folate by test foods and other meals on the study days (t=0-12h) was ~590 (bread days) and ~830 nmol (breakfast days) of which 60-80% (~450 nmol) was labelled. Median labelled folate content in stomal effluents was only 17% (5-36) (Table 11). Hence, it can be assumed that the majority of folates in stomal effluents do not originate from non-absorbed recent dietary intake but possibly from lysed enterocytes and gastrointestinal secretions such as bile (Gregory & Quinlivan, 2002).

Stomal effluents collected during 12 h post-dose contained 103 (43-178) nmol unlabelled folate (Figure 11) and the level did not differ significantly between study days (Table 11) (p=0.53).

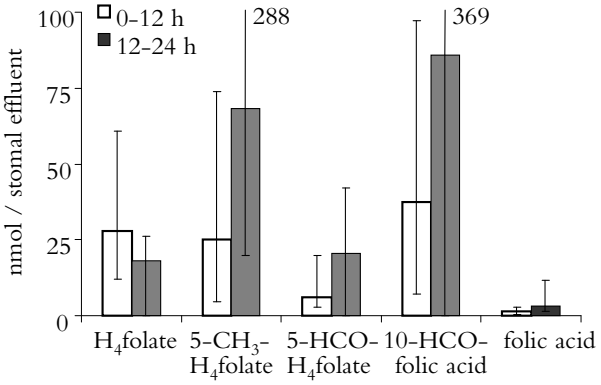


Figure 11. Content (nmol) of unlabelled folates in stomal effluents during 0-12 h and 12-24 h post-dose. Median content (range) is based on data from 8 volunteers on three independent test days (n=24), each analysed as duplicates. Volunteers' diet during 12-24 h post-dose was not standardised

Differences in absorption kinetics between reduced folates and folic acid have been reported by others (Wright *et al.*, 2005; Wright *et al.*, 2003; Konings *et al.*, 2002). Wright *et al.* (2005) postulated that bioconversion of folic acid occurs in the liver and not in the enterocytes as generally accepted. Hence a greater hepatic retention of folic acid, caused by liver enzymes having greater affinity to folic acid than reduced folates, would explain the difference in appearance in peripheral plasma (Wright *et al.*, 2005). Wright *et al.* (2005) estimated hepatic retention of absorbed folates in volunteers without presaturation of body stores. For absorbed $^{13}C_5$-folic acid hepatic retention was estimated as high as 73%, which was significantly higher than after ingestion of $^{13}C_5$-5-HCO-H_4folate (58%) and intrinsically labelled spinach folate (52%) (Wright *et al.*, 2005).

Variable affinity of folate transporters – thereby variable mobility – might contribute to different kinetics in peripheral plasma *in vivo*. For example, the affinity of PCFT (Figure 3), which is assumed to facilitate the main absorption of folates in the small intestine, is threefold lower to folic acid than to reduced folates (at pH 6.5) (Qiu *et al.*, 2006). Within the cells, folic acid might be more retained, since the cellular efflux facilitated by multidrug associated proteins (MRP, Figure 3) is two- to 10-fold higher for reduced folates than folic acid (Assaraf, 2006). Furthermore, transport via systemic circulation might differ, *e.g.* affinity of PCFT to folic acid is negligible in plasma (pH 7.4), whereas reduced folates may be efficiently transported (Qiu *et al.*, 2006).

The discussion above is hypothetical but could partly explain the findings of Wright *et al.* (2005; 2003) and findings of the current trial (II). However, this needs to be further studied using isolated supplements.

4.3 Folate bioaccessibility assessed in the *in vitro* gastrointestinal model TIM (III, IV)

The *in vitro* TIM experiments revealed high folate bioaccessibility (equation 3), 75% in bread (IV), 95% in the breakfast (IV) and 100% in the orange juice (III) (Figure 12). Hence, folates appeared to be both released from the food matrix and stable during passage through the simulated gastrointestinal tract. Bioaccessibility of breakfast folate was similar to that of orange juice folate (Figure 12) and orange juice contributed with 60% of the folate content in the breakfast.

Bioaccessibility data were in agreement with previous TIM data for beverages (80-100%) and solid foods (75-85%) (Verwei, 2004; Verwei *et al.*, 2004; Arkbåge *et al.*, 2003; Verwei *et al.*, 2003).

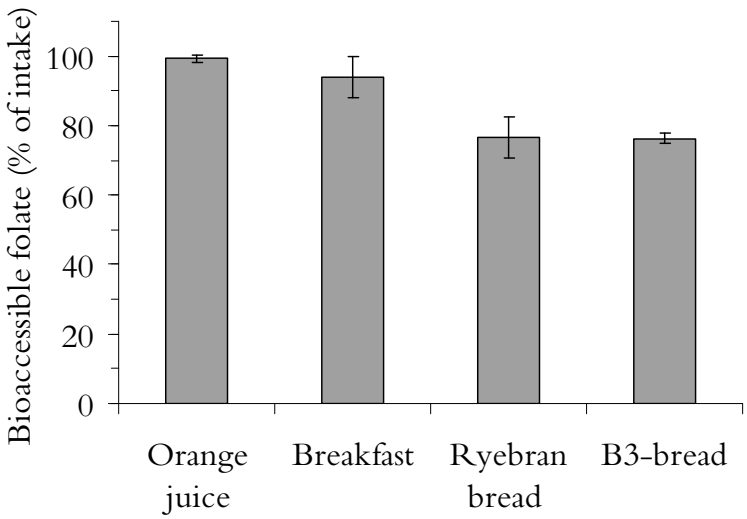


Figure 12. Bioaccessible folate as percentage of folate content in test foods (for details see Table 6). Mean ± root mean square error of two TIM trials (each analysed in duplicate, n=4)

As discussed in section 1.2, several matrix-related factors may inhibit folate absorption. For example Tamura *et al.* (1973) found incomplete *in vivo* availability of folates in orange juice, probably caused by reduced deconjugation of folate-polyglutamates (Wei & Gregory, 1998). Data from the TIM trial with orange juice indicate that folate bioaccessibility was complete (Figure 12).

The lower folate bioaccessibility from wholemeal breads (~75%) compared with orange juice (100%) (Figure 12) is probably due to dietary

fibres, which are suggested to inhibit folate absorption *e.g.* by entrapment (Melse-Boonstra *et al.*, 2004a). Data on the effect of dietary fibre on folat bioaccessibility as assessed in the TIM model are sparse. However, Verwei (2004) report a folate bioaccessibility of 87% in hard bread (knäckebröd).

The TIM experiments indicated good folate *in vitro* bioavailability from the breakfast (Figure 12). This was also confirmed by findings in the intervention trial, where the breakfast significantly improved folate status of the women (I).

To summarise, folate bioaccessibility was high for all tested foods (Table 5). Whereas *in vitro* bioaccessibility for folates from the wholemeal bread rich in dietary fibre tended to be at the lower range at 75%, a 3-month intervention with this type of bread could maintain volunteers' good folate status.

4.4 Uptake of $^{13}C_5$-5-CH_3-H_4folate into Caco-2 cells (IV)

Uptake into Caco-2 cells (equation 5, section 3.6) and transport across monolayers (equation 6) was determined for $^{13}C_5$-5-CH_3-H_4folate. Uptake of labelled folate fortificant from the bread matrix digest (15±2%) was significantly lower than from the folate standard digest (22±3%) ($p<0.05$). However, transport across the cells (6±1% & 6±2%) did not differ significantly ($p=0.32$).

Folates are polyglutamylated to increase retention within the cells (Figure 3, H). The 15% increase in $^{13}C_5$-5-CH_3-H_4folate content in lysed cells after incubation with rat serum (deconjugation) indicates that cellular storage of labelled folate occurred already within the one-hour experiment.

Others have mainly studied uptake mechanisms of the oxidised folate form folic acid into Caco-2 cells (Subramanian *et al.*, 2008; Ashokkumar *et al.*, 2007; Mason *et al.*, 1990) or effects of dietary compounds on folic acid uptake (Lemos *et al.,* 2004; Verwei *et al.,* 2005). However, Verwei *et al.* (2005) also report uptake and transport of the reduced folate form 5-CH_3-H_4folate into Caco-2 cells. Measured uptake of radiolabelled 5-CH_3-H_4folate (3%) in that trial (Verwei *et al.*, 2005) was sevenfold lower than in the current trial (22%, IV). Verwei *et al.* (2005) carried out the experiment at a higher pH of 7.4, where affinity of PCFT to folates is much lower than at pH values around 6 (Mason *et al.*, 1990; Russell *et al.*, 1979). Furthermore, Verwei *et al.* (2005) used a racemic (6R/6S) 5-CH_3-H_4folate mixture and it is possible that uptake of the diastereoisomer without biological activity (6R, Figure 1) is lower.

Recently, Aufreiter *et al.*, (2009) reported *in vivo* absorption of folates in the human colon. After caecal infusion of [6S]-$^{13}C_5$-5-HCO-H_4folate they found $^{13}C_5$-5-CH_3-H_4folate and minor amounts of $^{13}C_5$-5-HCO-H_4folate in peripheral plasma, indicating a conversion of $^{13}C_5$-5-HCO-H_4folate in the colon- and/or livercells. According to Wright *et al.* (2005; 2003) conversion occurs in the liver instead of the gastrointestinal tract as is generally accepted. Using the Caco-2 cell/stable isotope model (IV) folate conversion in colon cells can be studied.

To summarise, data from the Caco-2 cell trial showed that dietary fibre had an inhibitory effect on uptake of 5-CH_3-H_4folate in colon cells and that some 5-CH_3-H_4folate was retained as folate polyglutamate within the cells.

4.5 Folate quantification

Quantification of folate content in foods and clinical samples is challenging, mainly due to the low initial concentrations in the samples and interfering matrix compounds. For HPLC methods the sensitivity of the instrument can also be limiting.

Folate content in test foods and clinical samples

Examples of folate content and dominant forms in test foods are shown in Table 12. Discrepancies to reference values (national food composition database or determined at an accredited laboratory) can partly be explained by methodological differences. Folate data in food composition tables are usually expressed as total folate content from microbiological assay. Results from HPLC are usually expressed as sum of analysed individual folates - often only 2-6 forms (Jastrebova *et al.*, 2003; Pfeiffer *et al.*, 1997b) – and are therefore commonly lower than results from microbiological assays (Konings *et al.*, 2001; Gregory *et al.*, 1982). This is probably why the folate content of the certified reference material CRM 121 determined with the in-house method was 74% of the reference value determined using microbiological assay (Table 12).

Although the microbiological assay is considered the golden standard, it has some limitations (Figure 5). Folate content in a breakfast extract (Table 12) as assessed by microbiological assay and an in-house LC-MS method (Patring & Jastrebova, 2007) indicates that H_4folate was lost during the sample pretreatment for the microbiological assay. This was probably due to the total incubation time, which was sevenfold longer (~22h) for the microbiological assay used at the accrediated laboratory. The longer incubation most likely also explain the substantial increase of oxidised folates

(10-HCO-folic acid), which was observed in this sample (Table 12). Hence, microbiological assays might be less appropriate for analysis of foods rich in H_4folate, such as liver pâté, as demonstrated by the twice as high folate content in liver pâté analysed by LC-MS (Table 12).

Table 12. Folate content as mean and SD (μg/100g) in test foods used for *in vivo* and *in vitro* trials measured by HPLC[1]

	Trial	n[2]	H_4folate	5-CH_3-H_4folate	5-HCO-H_4folate	10-HCO-folic acid	Sum as folic acid	Ref.
Wholemeal bread	I,II,IV	6	traces	7±1	18±6	13±3	45±4[3]	42±3[4]
Wholemeal rye bread	I	2	1±1	6±0	19±4	3±0	27±0[5]	40±4[4]
B3-bread	IV	3	traces	16±1	16±2	14±4	43±4[6]	16-44[7]
Rye bran bread	IV	3	traces	4±1	19±4	18±1	39±4	16-44[7]
Orange juice, from concentrate	III	5	traces	25±2	nd	nd	24±2	15-30[7]
Orange juice, ready to drink	I,III	4	traces	21±4	nd	nd	20±4	15-30[7]
Rye bran cereal	I	2	traces	5±0	7±0	6±0	54±2[8]	56±2[4]
Kiwi	I,II,IV	4	1±1	22±2	nd	traces	22±2	42[7]
Liver pâté	I,II,IV	4	77±4	20±4	nd	nd	96±1	36[7]
Breakfast	I,II,IV	4	6±0	19±0	8±1	2±0	33±1	29±1[4]
Breakfast[9]	I,II,IV	2	nd	14±1	7±1	14±2	35±1	
CRM whole-meal flour 121		3	4±1	8±1	17±0	9±1	38±1	50±7[10]

Ref = reference value; Traces = peak areas below limit of quantification (0.3-4 ng/mL); nd = not detected. [1]Patring & Jastrebova, 2007; Jastrebova *et al.*, 2003; Witthöft *et al.*, 2003; Johansson *et al.*, 2002. [2]Analytical n (as example from one batch of each food and meal). [3]10-HCO-H_2folate (9±2 μg/100g) included into sum of folate. [4]Data presented as mean±SD were analysed using accredited microbiological assay (National Food Administration). [5]10-HCO-H_2folate was not included into sum of folate due to coeluting peaks. [6]10-HCO-H_2folate (8±2 μg/100g) included into sum of folate. [7]Range (or individual values) of food composition database values for similar products (NFA, 2009a). [8]10-HCO-H_2folate (39±1 μg/100g) included into sum of folate. [9]LC-MS quantification of the sample extract prepared for microbiological assay at accredited laboratory of National Food Administration (Uppsala, Sweden). [10]Certified reference value by microbiological assay (n=15, replicates 73).

It is important when carrying out intervention trials to have control on effects from storage and batch variation on folate content in test foods (Table 6). Due to the duration of the intervention trial (I), different batches of kiwi and liver pâté had to be used. Furthermore, wholemeal breads, rye bran cereal, orange juice and apple juice had to be stored ~4 months at 5°C or -

20ºC. Therefore, variations in folate content due to storage and differences in brands and batches were checked (Table 6).

No crucial variations in folate content of test foods due to use of different batches or storage was found. For wholemeal bread folate content was similar between batches (I,II) (Figure 13) and no losses were found during the four months of storage at -20°C (I) (data not shown).

Folate content in orange juice was stable during storage in the intervention trial (4 months, 5°C). No difference in folate content between orange juice brands (Table 12) and batches (Figure 13) were found. Folate content in kiwi and liver pâté (purchased at three occasions in October, December and February) varied by almost 20% between batches (Figure 13). However, as the contribution of these foods to total folate intake from the breakfast was below 25%, the batch variations (~20%) did not affect the intervention doses substantially. This is demonstrated by a lower batch than analytical variation for the breakfast meal (intra-assay CV, Figure 13).

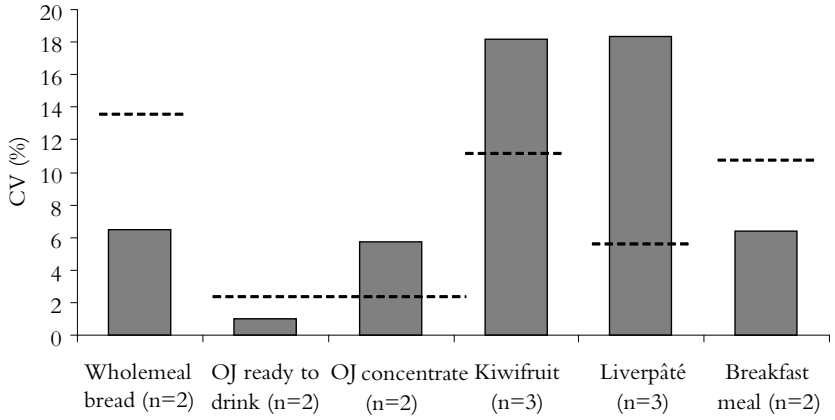


Figure 13. Batch variation (staples, n=2-3) and analytical variation (----, based on intra-assay CV, n=3-4) in breakfast ingredients and a composite sample. OJ = orange juice

Quality parameters of analytical methods

Data on folate content in foods were considered acceptable if the precision under repeatability conditions (intra-assay CV) was below 10% for the sum of folates (Table 13). For individual forms with a low content in foods, a CV of up to 20% was accepted (Table 13). Due to the low folate concentrations in some of the plasma and stomal effluent samples an intra-assay CV of up to 25% (n=4) was accepted.

Table 13. Quality parameters of the methods used

	HPLC-FLD/MS	SIDA-LC-MSMS
Lowest quantifiable amount	0.4-11.2 μg/100g[1]	1 nmol/L (plasma)[2], 64 pmol/g (stom effl)[2]
Intra-assay CV % (n=4)	<10 (1-20)[3]	<5
Inter-assay CV % (n=4)	<20 (2-25)[3]	<20
Recovery % (equation 8)	97±10 (77-127)[3]	Not applicable

Stom effl = stomal effluent. [1]Lowest quantifiable amount expressed in μg/100g (accounting for dilution during sample preparation). Calibration levels (at least 6) that differed <20% from the theoretical value of the calibration curve (with R>0.999) were used for the calibration. [2]Limit of detection. [3]Range for individual folate forms.

4.6 Suitability of folate bioavailability models

Main findings from the *in vivo* and *in vitro* models are summarised for comparison (Table 14). Choice of method to assess bioavailability depends on the aim.

If the aim is to examine physiological effects long-term intervention trials assessing more than one status parameter are preferable. The dietary intervention (I) affected two of three status parameters, erythrocyte folate and homocysteine concentrations, but not serum folate concentrations. As a similar RC was estimated for all parameters (Table 8), the lack of response in serum folate concentrations is probably due to fastened serum folate being less sensitive to minor changes in food folate intake.

Many intervention trials have assessed the effect of food folate on folate status and results are inconclusive (Table 1a and 1b). However, several of these trials were carried out using unphysiologically high doses and total folate intake by interventions were often far above recommendations (Fenech *et al.*, 2005; Venn *et al.*, 2002; Riddell *et al.*, 2000). More trials with intervention diets likely to be consumed by the general population and in physiological range would possibly result in more consistent data on folate bioavailability.

For the plasma AUC/ileostomy model (II), the TIM model (III, IV) and the Caco-2 cell model (IV), recoveries from given amounts of folate were calculated. A recovery different from 100% (Table 14) may indicate incomplete sampling, losses during storage and sample preparation and analytical errors. It could be questioned to calculate recovery for the plasma AUC/ileostomy model (equation 2, section 3.6), as the estimation of absorbed folate using C_{max}*V in peripheral plasma is a rough measure (Tozer & Rowland, 2006). Furthermore, the low recovery for folic acid (<30%, Table 14) can be explained by physiological reasons other than above named and supports the hypothesis of Wright *et al.* (2005; 2003) regarding liver retention.

The plasma AUC/ileostomy model (II) was used to assess food matrix effects and differences in absorption kinetics between oxidised and reduced folates (Table 10). The model could also be used to prove enterohepatic circulation. Post-dose secretion from oral doses of labelled folate (*e.g.* $^{13}C_5$-5-HCO-H_4folate and $^{13}C_5$-folic acid) into bile is reflected by $^{13}C_5$-5-CH_3-H_4folate content in stomal effluent. It could be hypothesised that $^{13}C_5$-5-CH_3-H_4folate content in bile differs when minimising or stimulating bile secretion and variation of biliary folate excretion could be assessed from stomal folate content.

For the TIM model (III,IV) recovery was considered satisfactory and was confirming findings by others (Verwei, 2004), indicating a well functioning model. Furthermore the TIM model is a rather cheap and easy tool to study the effects of food matrix on folate bioaccessibility. Other purposes for which the model can be used are systematic assessments of effects of food processing on folate bioaccessibility.

The novel Caco-2 cell/stable isotope model (IV) was used to assess matrix effects from the wholemeal bread on cellular folate uptake and transport. The model could further be used to study whether methylation and reduction of folates could occur in colon cells by application of *e.g.* $^{13}C_5$-5-HCO-H_4folate and $^{13}C_5$-folic acid. Furthermore, this model could be used to detect whether rate of transport across, and storage within, the colon cells varies between reduced folate forms and folic acid.

Table 14. *Summary of results and evaluation of the bioavailability models used in this thesis*

	In vivo		***In vitro***	
Model	Long-term intervention	Plasma AUC/ileostomy	Gastrointestinal TIM	Caco-2 cells
Paper	I	II	III&IV	IV
Main result	Low doses of food folate (125 μg/day) significantly improved folate status to a nutritionally relevant level according to Daly *et al.* (1995).	Plasma kinetics differed significantly between $^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-folic acid. Bioavailabilities of both folates were similar from different matrices and around 90%.	Folate bioaccessibility from wholemeal bread was significantly lower than from orange juice. Bioaccessibility of folates from a breakfast and orange juice was high (>90%).	Wholemeal bread matrix significantly reduced relative uptake of $^{13}C_5$-5-CH_3-H_4folate into Caco-2 cells.
Minimum effective dose	70 μg/d (tHcy) 125 μg/d (ery-Folate)	200 μg (labelled folate)	>15 μg of each folate form of interest /100 g food	>0.2 μg/well
Model recovery[1]	Not applicable	82±25% (5-CH_3-H_4folate, n=8) 28±9% (folic acid, n=16)	93±13% (n=4)	93±10% (n=11)
Advantages of the model	Physiological effects on folate status can be studied. Results may be applicable to specific populations.	The combination of methods strengthens results. Use of stable isotopes improves sensitivity and specificity.	Full compliance and lack of biological variation results in reproducible results from few experiments. No ethical limitations.	Information on mechanisms can be assessed.
Limitations of the model	Unless a dose-response effect is established, effects as result from other food components cannot be excluded. Problems with compliance.	Data from ileostomists can only with care be applied to the general population.	Findings need to be confirmed *in vivo*.	This cell line originates from one individual. Tumourgenic cell line.

[1]Equations 2, 4 and 7 (section 3.6).

5 Concluding remarks and future perspectives

Epidemiological studies (Table 3) confirm that intake of folate in Sweden is among the lowest in Europe (Dhonukshe-Rutten *et al.*, 2009; Becker & Pearson, 2003). On the other hand, the prevalence of folate deficiency and neural tube defects is no higher in Sweden than in other Western countries (SBU, 2007; Busby *et al.*, 2005), including those with mandatory folic acid fortification (McDowell *et al.*, 2008). Obviously factors other than daily folate intake, which comprises only up to 4% of the folate body pool (Lin *et al.*, 2004; Gregory *et al.*, 2001; Herbert, 1987) affect folate status.

Folate homeostasis is known to be well regulated, *e.g.* deficiency increases the expression of folate transporters in the small intestine and the proportion of protein-bound folates in plasma, resulting in increased absorption and reduced excretion (Zhao *et al.*, 2009). Moreover, the colon microflora may affect folate status positively (Camilo *et al.*, 1996). As shown by several intervention trials, folate intake (Table 1a and 1b) and folate bioavailability (Hannon-Fletcher *et al.*, 2004; Cuskelly *et al.*, 1996) both affect folate status. However, *in vivo* data on factors affecting folate status are limited. *E.g.* findings on the bioavailability of folates are partly contradictory and data on the human colon microflora scarce (Aufreiter *et al.*, 2009; Kim *et al.*, 2004).

The bioavailability of supplemental folic acid is considered superior to that of reduced folates (Sauberlich *et al.*, 1987). In addition, folic acid is the most stable and cheapest form to synthesise and is therefore used for fortification purposes. However, a high intake of folic acid has been associated not only with health benefits but also adverse health effects, *e.g.* colorectal cancer as reviewed by Smith *et al.* (2008). Although these health effects are not well documented, they emphasise the need to understand the metabolism and bioavailability of both food folate and synthetic folic acid.

In short-term trials without presaturation of body stores, a difference in post-dose plasma kinetics has been observed between reduced folates deriving from foods, and supplemental folic acid (Wright *et al.*, 2005). This was confirmed by findings from the plasma AUC/ileostomy trial (II) after ingestion of reduced 5-CH_3-H_4folate and folic acid.

It remains unclear from findings in this thesis whether greater retention of folic acid in the liver can explain the difference in plasma kinetics between the folate forms, as suggested by Wright *et al.,* (2005; 2003).

Studies on transport of food folates have not been prioritised in molecular research, where the focus is merely on antifolate drugs. The main folate transporter in the small intestine, PCFT, was isolated only three years ago (Qiu *et al.*, 2006). It may be that the hitherto rather unexplored field of molecular research may reveal in future explanations regarding the difference in plasma kinetics of reduced folate and folic acid. As previously discussed, some folate transporters have substantially higher affinity to reduced folates (PCFT, RCF), whereas others have higher affinity to folic acid (FR).

The question from a public health perspective is whether differences in transporters can result in different tissues distribution of reduced folates and folic acid. This could partly explain the ambiguous role of folic acid in colorectal cancer (Kim, 2007). As malignant tumours are known to overexpress FR (Sega & Low, 2008) and FR has 10-fold higher affinity to folic acid than reduced folates (Kelemen, 2006), tumour growth is possibly accelerated by a high folic acid ingestion.

Median plasma folate concentration in American women (post-fortification) is ~25 nmol/L (McDowell *et al.*, 2008) and twice as high as in Swedish women (Table 4). In contrast, the limited Swedish data on erythrocyte folate concentrations (Table 9) show the level to be higher or at least similar to that of American women (~600 nmol/L) (McDowell *et al.*, 2008). Could this be due to folic acid being a relative greater part of total folate intake in the USA (>200 μg/day) (Kalmbach *et al.*, 2008; Quinlivan & Gregory, 2003) but a minor contributor to total folate intake in Sweden?

Intervention studies with supplemental 5-CH_3-H_4folate have shown that reduced folates can improve erythrocyte folate concentrations more or equally efficient as folic acid (Lamers *et al.*, 2006). Knowledge on post-dose folate distribution of different folates within the human body is still limited and further mechanistic studies - using doses of biological active food folate diastereoisomers and not racemates - are therefore needed.

The superior bioavailability of folic acid was not confirmed in the plasma AUC/ileostomy trial (II). Based on labeled folate content in stomal effluent the bioavailability appeared to be over 90% for both, $^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-folic acid. Release of the added fortificants ($^{13}C_5$-5-CH_3-H_4folate and $^{13}C_5$-folic acid) does not necessarily reflect release of endogenous food folates in the wholemeal bread accurately. Only by use of intrinsic labelling could this be studied.

The pronounced lowering (>20%) of plasma total homocysteine concentration after consumption of five slices wholemeal bread (containing an additional folate dose of only ~70 μg/day) exemplifies a challenge of nutritional studies. As no significant effect was observed in the other folate status parameters, *i.e.* serum and erythrocyte folate concentrations, additive effects from intake of folate and other bread components, *e.g.* betain, could have caused this pronounced reduction in homocysteine concentrations (Alfthan *et al.*, 2004). It could also be hypothesised that dietary fibre from the wholemeal bread affected the colon microflora (Keagy, 1990), which in turn could affect human folate status.

In the post-dose stomal effluents, labelled folates comprised only one-fifth of the total folate content. Unlabelled folates in stomal effluents are suggested to originate from bile and other secretions, sloughed enterocytes and possibly to a small extent the colon microflora (Gregory & Quinlivan, 2002), but the contribution of each of these sources is unknown.

To conclude, folate research has hitherto identified the important physiological function of folates and the clinical implications of an insufficient intake. Furthermore, data on estimated intake of folates indicate that it may be inadequate in subgroups of the population but this was not always associated with an inadequate folate status.

Folate bioavailability from different test foods and a complete meal was in this thesis estimated to be about 75-90% and positive effects from both interventions on folate status of healthy women could be demonstrated. This is in agreement with several recent intervention trials by others (Table 1b) and indicates that the general assumption of an inferior bioavailability of only 50 % of food folate (CDC, 2009; NNR, 2005) compared to supplemental folic acid might need to be reconsidered. It is therefore desirable that research focuses to identify factors affecting folate status, *e.g.* folate bioavailability, and the importance of these.

References

Åden, E., Johansson, I. & Håglin, L. (2007). Energy and nutrients in self-reported diet before and at week 18-22 of pregnancy. *Scandinavian Journal of Food & Nutrition* 51(2), 67-73.

Alfthan, G., Tapani, K., Nissinen, K., Saarela, J. & Aro, A. (2004). The effect of low doses of betaine on plasma homocysteine in healthy volunteers. *British Journal of Nutrition* 92(4), 665-669.

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(11), 3678-3683.

Ashfield-Watt, P.A.L., Whiting, J.M., Clark, Z.E., Moat, S.J., Newcombe, R.G., Burr, M.L. & McDowell, I.F.W. (2003). A comparison of the effect of advice to eat either '5-a-day' fruit and vegetables or folic acid-fortified foods on plasma folate and homocysteine. *European Journal of Clinical Nutrition* 57(2), 316-323.

Ashokkumar, B., Mohammed, Z.M., Vaziri, N.D. & Said, H.M. (2007). Effect of folate oversupplementation on folate uptake by human intestinal and renal epithelial cells. *Americal Journal of Clinical Nutrition* 86(1), 159-166.

Asrar, F.M. & O'Connor, D.L. (2005). Bacterially synthesized folate and supplemental folic acid are absorbed across the large intestine of piglets. *The Journal of Nutritional Biochemistry* 16(10), 587.

Assaraf, Y.G. (2006). The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. *Drug Resistance Updates* 9(4-5), 227-246.

Aufreiter, S., Gregory, J.F., III, Pfeiffer, C.M., Fazili, Z., Kim, Y.-I., Marcon, N., Kamalaporn, P., Pencharz, P.B. & O'Connor, D.L. (2009). Folate is absorbed across the colon of adults: evidence from cecal infusion of 13C-labeled [6S]-5-formyltetrahydrofolic acid. *American Journal Of Clinical Nutrition*, ajcn.2008.27345.

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

Bazzano, L.A., Reynolds, K., Holder, K.N. & He, J. (2006). Effect of folic acid supplementation on risk of cardiovascular diseases - A meta-analysis of randomized controlled trials. *Journal Of The American Medical Association* 296(22), 2720-2726.

Becker, W. (1994). *Kostvanor och näringsintag i Sverige 1989. Resultat från undersökningen Hushållens livsmedelsutgifter och kostvanor (HULK)*. Uppsala: National Food Administration.

Becker, W. & Pearson, M. (2003). *Riksmaten 1997-98, Kostvanor och näringsintag i Sverige - Metod och resultatanalys*. Uppsala: National Food Administration.

Bergström, L. (1994). *Nutrient losses and gains in the preparation of foods*. Uppsala: National Food Administration.

Björkegren, K. & Svardsudd, K. (2003). Reported symptoms and clinical findings in relation to serum cobalamin, folate, methylmalonic acid and total homocysteine among elderly Swedes: a population-based study. *Journal of Internal Medicine* 254(4), 343-352.

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(1), 605-607.

Bogers, R.P., Dagnelie, P.C., Bast, A., van Leeuwen, M., van Klaveren, J.D. & van den Brandt, P.A. (2007). Effect of increased vegetable and fruit consumption on plasma folate and homocysteine concentrations. *Nutrition* 23(2), 97-102.

Bor, M.V., Wulff, A.M., Nexo, E. & Krarup, H. (2008). Infrequency of low red blood cell (RBC) folate levels despite no folate fortification program: a study based on results from routine requests for RBC folate. *Clinical Chemistry and Laboratory Medicine* 46(3), 401-404.

Brantsaeter, A.L., Haugen, M., Alexander, J. & Meltzer, H.M. (2008). Validity of a new food frequency questionnaire for pregnant women in the Norwegian Mother and Child Cohort Study (MoBa). *Maternal and Child Nutrition* 4(1), 28-43.

Brattström, L., Zhang, Y., Hurtig, M., Refsum, H., Östensson, S., Fransson, L., Jonés, K., Landgren, F., Brudin, L. & Ueland, P.M. (1998). A common methylenetetrahydrofolate reductase gene mutation and longevity. *Atherosclerosis* 141(2), 315-319.

Broekmans, W.M.R., Klopping-Ketelaars, I.A.A., Schuurman, C.R.W.C., Verhagen, H., van den Berg, H., Kok, F.J. & van Poppel, G. (2000). Fruits and vegetables increase plasma carotenoids and vitamins and decrease homocysteine in humans. *Journal of Nutrition* 130(6), 1578-1583.

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

Buchholz, B.A., Arjomand, A., Dueker, S.R., Schneider, P.D., Clifford, A.J. & Vogel, J.S. (1999). Intrinsic erythrocyte labeling and attomole pharmacokinetic tracing of14C-labeled folic acid with accelerator mass spectrometry. *Analytical Biochemistry* 269(2), 348-352.

Busby, A., Armstrong, B., Dolk, H., Armstrong, N., Haeusler, M., Berghold, A., Gillerot, Y., Baguette, A., Gjerga, R., Barisic, I., Christiansen, M., Goujard, J., Steinbicker, V., Rosch, C., McDonnell, R., Scarano, G., Calzolari, E., Neville, A., Cocchi, G., Bianca, S., Gatt, M., De Walle, H., Braz, P., Latos-Bielenska, A., Gener, B.,

Portillor, I., Addor, M.C., Abramsky, L., Ritvanen, A., Robert-Gnansia, E., Daltveit, A.K., Aneren, G., Olars, B. & Edwards, G. (2005). Preventing neural tube defects in Europe: A missed opportunity. *Reproductive Toxicology* 20(3), 393-402.

Camilo, E., Zimmerman, J., Mason, J.B., Golner, B., Russell, R., Selhub, J. & Rosenberg, I.H. (1996). Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. *Gastroenterology* 110(4), 991-998.

Carmel, R., Green, R., Rosenblatt, D.S. & Watkins, D. (2003). Update on cobalamin, folate, and homocysteine. *Hematology* 2003(1), 62-81.

Castilla, E., Orioli, I., Lopez-Camelo, J., Dutra, M. & Nazer-Herrera, J. (2003). Preliminary data on changes in neural tube defect prevalence rates after folic acid fortification in South America. *American Journal of Medical Genetics Part A* 123A(2), 123-128.

Caudill, M.A., Cruz, A.C., Gregory, J.F., 3rd, Hutson, A.D. & Bailey, L.B. (1997). Folate status response to controlled folate intake in pregnant women. *Journal of Nutrition* 127(12), 2363-70.

CDC (2009). *Water-Soluble Vitamins & Related Biochemical Compounds* [online]. Centers for Disease Control and Prevention Available from: http://www.cdc.gov/nutritionreport/part_1.html [Accessed July 13th].

Chanarin, I. & Perry, J. (1969). Evidence for reduction and methylation of folate in intestine during normal absorption. *The Lancet* 2(7624), 776-778.

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(9), 2792-2798.

Clarke, R., Woodhouse, P., Ulvik, A., Frost, C., Sherliker, P., Refsum, H., Ueland, P.M. & Khaw, K.-T. (1998). Variability and determinants of total homocysteine concentrations in plasma in an elderly population. *Clinical Chemistry* 44(1), 102-107.

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(5), 459-464.

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.

Daly, L.E., Kirke, P.N., Molloy, A., Weir, D.G. & Scott, J.M. (1995). Folate levels and neural-tube defects - implications for prevention. *Journal Of The American Medical Association* 274(21), 1698-1702.

Dhonukshe-Rutten, R.A.M., de Vries, J.H.M., de Bree, A., van der Put, N., van Staveren, W.A. & de Groot, L. (2009). Dietary intake and status of folate and vitamin B12 and their association with homocysteine and cardiovascular disease in European populations. *European Journal of Clinical Nutrition* 63(1), 18-30.

Enghardt-Barbieri, H. & Lindvall, C. (2005). *Swedish Nutrition Recommendations Objectified (SNO) – Basis for general advice on food consumption for healthy adults*. Uppsala: Sweden National Food Administration.

Ericson, U., Sonestedt, E., Gullberg, B., Olsson, H. & Wirfält, E. (2007). High folate intake is associated with lower breast cancer incidence in postmenopausal women in the

Malmo Diet and Cancer cohort. *American Journal Of Clinical Nutrition* 86(2), 434-443.

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.

Fenech, M., Noakes, M., Clifton, P. & Topping, D. (2005). Aleurone flour increases red-cell folate and lowers plasma homocyst(e)ine substantially in man. *British Journal of Nutrition* 93(03), 353-360.

Finglas, P.M., van den Berg, H. & de Froidmont-Görtz, I. (1996). Improvements in the determination of vitamins in foods: method intercomparison studies and preparation of certified reference material (CRMs). *Food Chemistry* 57(1), 91-94.

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(5), 936-939.

Fleiss, J. (1986). *The design and analysis of clinical experiments*. New York: John Wiley and sons inc.

Flynn, M.A.T., Anderson, W.A., Burke, S.J. & Reilly, A. (2008). Folic acid food fortification: the Irish experience. *Proceedings of the nutrition society* 67(4), 381-389.

Garg, U.C., Zheng, Z.-J., Folsom, A.R., Moyer, Y.S., Tsai, M.Y., McGovern, P. & Eckfeldt, J.H. (1997). Short-term and long-term variability of plasma homocysteine measurement. *Clinical Chemistry* 43(1), 141-145.

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. *Journal Of The American Medical Association* 288(15), 1867-1873.

Gregory, J.F. (1995). The Bioavailability of Folate. In: Bailey, L.B. (Ed.) *Folate in Health and Disease*. New York: Marcel Deeker. pp. 195-235.

Gregory, J.F. (1996). Folate. In: Fennema, O.R. (Ed.) *Food Chemistry*. New York: Marcel & Dekker. pp. 590-616.

Gregory, J.F., Bhandari, S.D., Bailey, L.B., Toth, J.P., Baumgartner, T.G. & Cerda, J.J. (1991). Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human-subjects. *American Journal Of Clinical Nutrition* 53(3), 736-740.

Gregory, J.F., Caudill, M.A., Opalko, F.J. & Bailey, L.B. (2001). Kinetics of folate turnover in pregnant women (second trimester) and nonpregnant controls during folic acid supplementation: Stable-isotopic labeling of plasma folate, urinary folate and folate catabolites shows subtle effects of pregnancy on turnover of folate pools. *Journal of Nutrition* 131(7), 1928-1937.

Gregory, J.F., Day, B.P.F. & Ristow, K.A. (1982). Comparison of high performance liquid chromatographic, radiometric and *lactobacillus casei* methods for the determination of folacin in selected foods. *Journal of Food Science* 47(5), 1568-1571.

Gregory, J.F. & Quinlivan, E.P. (2002). In vivo kinetics of folate metabolism. *Annual Review of Nutrition* 22, 199-220.

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

Gregory, J.F., Williamson, J., Liao, J.F., Bailey, L.B. & Toth, J.P. (1998). Kinetic model of folate metabolism in nonpregnant women consuming [H-2(2)]folic acid: Isotopic labeling of urinary folate and the catabolite para-acetamidobenzoylglutamate indicates slow, intake-dependent, turnover of folate pools. *Journal of Nutrition* 128(11), 1896-1906.

Hakala, P., Knuts, L.R., Vuorinen, A., Hammar, N. & Becker, W. (2003). Comparison of nutrient intake data calculated on the basis of two different databases. Results and experiences from a Swedish-Finnish study. *European Journal of Clinical Nutrition* 57(9), 1035-1044.

Halsted, C.H. (1990). Intestinal Absorption of Dietary Folates. In: Picciano, M.F*., et al.* (Eds.) *Folic acid methabolism in health and disease*. New York: Wiley-Liss. pp. 23-46.

Hannon-Fletcher, M.P., Armstrong, N.C., Scott, J.M., Pentieva, K., Bradbury, I., Ward, M., Strain, J.J., Dunn, A.A., Molloy, A.M., Kerr, M.A. & McNulty, H. (2004). Determining bioavailability of food folates in a controlled intervention study. *American Journal Of Clinical Nutrition* 80(4), 911-918.

Hao, L., Ma, J., Stampfer, M.J., Ren, A., Tian, Y.H., Tang, Y., Willett, W.C. & Li, Z. (2003). Geographical, seasonal and gender differences in folate status among chinese adults. *Journal of Nutrition* 133(11), 3630-3635.

Herbert, V. (1987). Recommended dietary intakes (RDI) of folate in humans. *American Journal Of Clinical Nutrition* 45(4), 661-670.

Herbert, V. & Das, K.C. (1994). Folic acid and Vitamin B_{12}. In: Shils, M*., et al.* (Eds.) *Modern Nutrition in Health and Disease*. Philadelphia: Lea & Febiger.1). pp. 402-425.

Hickling, S., Knuiman, M., Jamrozik, K. & Hung, J. (2005). A rapid dietary assessment tool to determine intake of folate was developed and validated. *Journal of Clinical Epidemiology* 58(8), 802-808.

Homocysteine Lowering Trialists, C. (2005). Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. *American Journal Of Clinical Nutrition* 82(4), 806-812.

Hoppner, K. & Lampi, B. (1980). Folate levels in human liver from autopsies in Canada. *American Journal Of Clinical Nutrition* 33(4), 862-864.

Houghton, L.A., Green, T.J., Donovan, U.M., Gibson, R.S., Stephen, A.M. & O'Connor, D.L. (1997). Association between dietary fiber intake and the folate status of a group of female adolescents. *American Journal Of Clinical Nutrition* 66(6), 1414-1421.

Hubatsch, I., Ragnarsson, E.G.E. & Artursson, P. (2007). Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols* 2(9), 2111-2119.

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.

Johansson, M., Van Guelpen, B., Hultdin, J., Wiklund, F., Adami, H.-O., Bälter, K., Grönberg, H. & Stattin, P. (2007). The MTHFR 677C→T polymorphism and risk of prostate cancer: results from the CAPS study. *Cancer Causes and Control* 18(10), 1169-1174.

Johansson, M., Witthöft, C.M., Bruce, Å. & 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(6), 279-286.

Jones, M.L. & Nixon, P.F. (2002). Tetrahydrofolates are great stabilized by binding to bovine milk folate-binding protein. *Journal of Nutrition* 132(9), 2690-2694.

Kalmbach, R.D., Choumenkovitch, S.F., Troen, A.M., D'Agostino, R., Jacques, P.F. & Selhub, J. (2008). Circulating folic acid in plasma: relation to folic acid fortification. *American Journal Of Clinical Nutrition* 88(3), 763-768.

Katan, M.B., Boekschoten, M.V., Connor, W.E., Mensink, R.P., Seidell, J., Vessby, B. & Willett, W. (2009). Which are the greatest recent discoveries and the greatest future challenges in nutrition? *European Journal of Clinical Nutrition* 63(1), 2-10.

Keagy, P.M. (1990). Animal assays for folate bioavailability: a critical evaluation. In: Picciano, M.F*., et al*. (Eds.) *Folic acid methabolism in health and disease*. New York: Wiley-Liss.13). pp. 139-150.

Kelemen, L.E. (2006). The role of folate receptor alpha in cancer development, progression and treatment: Cause, consequence or innocent bystander? *International Journal of Cancer* 119(2), 243-250.

Kim, T.H., Yang, J., Darling, P.B. & O'Connor, D.L. (2004). A large pool of available folate exists in the large intestine of human infants and piglets. *Journal of Nutrition* 134(6), 1389-1394.

Kim, Y.I. (2007). Folate and colorectal cancer: An evidence-based critical review. *Molecular Nutrition & Food Research* 51(3), 267-292.

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

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(3), 235-242.

Konstantinova, S.V., Vollset, S.E., Berstad, P., Ueland, P.M., Drevon, C.A., Refsum, H. & Tell, G.S. (2007). Dietary predictors of plasma total homocysteine in the Hordaland Homocysteine Study. *British Journal of Nutrition* 98(1), 201-210.

Lamers, Y., Prinz-Langenohl, R., Bramswig, S. & Pietrzik, K. (2006). Red blood cell folate concentrations increase more after supplementation with [6S]-5-methyltetrahydrofolate than with folic acid in women of childbearing age. *American Journal Of Clinical Nutrition* 84(1), 156-161.

Larsson, C.L. & Johansson, G.K. (2002). Dietary intake and nutritional status of young vegans and omnivores in Sweden. *American Journal Of Clinical Nutrition* 76(1), 100-106.

Larsson, S.C., Giovannucci, E. & Wolk, A. (2004). Dietary folate intake and incidence of ovarian cancer: The Swedish Mammography Cohort. *Journal of the National Cancer Institute* 96(5), 396-402.

Lemos, C., Peters, G., Jansen, G., Martel, F. & Calhau, C. (2007). Modulation of folate uptake in cultured human colon adenocarcinoma Caco-2 cells by dietary compounds. *European Journal of Nutrition* 46(6), 329-336.

Lewerin, C., Matousek, M., Steen, G., Johansson, B., Steen, B. & Nilsson-Ehle, H. (2005). Significant correlations of plasma homocysteine and serum methylmalonic acid with movement and cognitive performance in elderly subjects but no improvement from short-term vitamin therapy: a placebo-controlled randomized study. *American Journal Of Clinical Nutrition* 81(5), 1155-1162.

Lin, Y., Dueker, S.R., Follett, J.R., Fadel, J.G., Arjomand, A., Schneider, P.D., Miller, J.W., Green, R., Buchholz, B.A., Vogel, J.S., Phair, R.D. & Clifford, A.J. (2004). Quantitation of in vivo human folate metabolism. *American Journal Of Clinical Nutrition* 80(3), 680-691.

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(1), 20.

Loew, D., Eberhardt, A., Heseker, H. & Kübler, W. (1987). Zur Plasmakinetik und Elimination von Folsäure. *Journal of Molecular Medicine* 65(11), 520-524.

Mason, J.B., Shoda, R., Haskell, M., Selhub, J. & Rosenberg, I.H. (1990). Carrier affinity as a mechanism for the pH-dependence of folate transport in the small-intestine. *Biochimica Et Biophysica Acta* 1024(2), 331-335.

McDowell, M., Lacher, D., Pfeiffer, C., Mulinare, J., Picciano, M., Rader, J., Yetley, E., Kennedy-Stephenson, J. & Johnson, C. (2008). Blood folate levels: the latest NHANES results. NCHS Data Brief.

McKinley, M.C., Strain, J.J., McPartlin, J., Scott, J.M. & McNulty, H. (2001). Plasma homocysteine is not subject to seasonal variation. *Clinical Chemistry* 47(8), 1430-1436.

Melse-Boonstra, A., Verhoef, P. & West, C. (2004a). Quantifying folate bioavailability: a critical appraisal of methods. *Current Opinion in Clinical Nutrition and Metabolic Care* 7(5), 539-545.

Melse-Boonstra, A., West, C.E., Katan, M.B., Kok, F.J. & Verhoef, P. (2004b). Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. *American Journal Of Clinical Nutrition* 79(3), 424-429.

Minekus, M., Marteau, P., Havenaar, R. & Huis in`t Veld, J. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *ATLA* 23, 197-209.

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

Mitchell, L.E., Adzick, N.S., Melchionne, J., Pasquariello, P.S., Sutton, L.N. & Whitehead, A.S. (2004). Spina bifida. *The Lancet* 364(9448), 1885-1895.

Morin, I., Devlin, A.M., Leclerc, D., Sabbaghian, N., Halsted, C.H., Finnell, R. & Rozen, R. (2003). Evaluation of genetic variants in the reduced folate carrier and in glutamate carboxypeptidase II for spina bifida risk. *Molecular Genetics and Metabolism* 79(3), 197-200.

Mosley, B.S., Cleves, M.A., Siega-Riz, A.M., Shaw, G.M., Canfield, M.A., Waller, D.K., Werler, M.M., Hobbs, C.A. & for the National Birth Defects Prevention, S. (2009). Neural tube defects and maternal folate intake among pregnancies

conceived after folic acid fortification in the United States. *American Journal of Epidemiology* 169(1), 9-17.

NFA (2006). *Food Composition Table* [online]. Uppsala National Food Administration Available from: http://192.121.81.11/livsmedelsok/sok.aspx?lang=2 [Accessed 10 April].

NFA (2009a). *Food Composition Table* [online]. Uppsala National Food Administration Available from: http://www.slv.se/en-gb/Group1/Food-and-Nutrition/The-Food-Database/ [Accessed 19 May].

NFA (2009b). *Varför inför inte Sverige obligatorisk folsyraberikning? (Why Sweden does not introduce mandatory folic acid fortification?)* [online]. Uppsala National Food Administration Available from: http://www.slv.se/sv/Fragor--svar/Fragor-och-svar/Mat-vid-graviditet/Fragor-och-svar-om-folsyra/ [Accessed 22 July].

NNR (2005). *Nordic Nutrition Recommendations NNR 2004 integrating nutrition and physical activity*. Stockholm: Nordic Council of Ministers.

Nygren-Babol, L., Sternesjö, A., Jägerstad, M. & Björck, L. (2005). Affinity and rate constants for interactions of bovine folate-binding protein and folate derivatives determined by optical biosensor technology. Effect of stereoselectivity. *Journal of Agricultural and Food Chemistry* 53(13), 5473-5478.

Owens, J.E., Holstege, D.M. & Clifford, A.J. (2007). Comparison of two dietary folate intake instruments and their validation by RBC folate. *Journal of Agricultural and Food Chemistry* 55(9), 3737-3740.

Patring, J.D.M. & Jastrebova, J.A. (2007). Application of liquid chromatography-electrospray ionisation mass spectrometry for determination of dietary folates: Effects of buffer nature and mobile phase composition on sensitivity and selectivity. *Journal of Chromatography A* 1143(1-2), 72-82.

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(2), 423-432.

Pfeiffer, C.M., Rogers, L.M., Bailey, L.B. & Gregory, J.F. (1997a). 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. *Americal Journal of Clinical Nutrition* 66, 1388-1397.

Pfeiffer, C.M., Rogers, L.M. & Gregory, J.F. (1997b). 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(2), 407-413.

Pfiffner, J.J., Calkins, D.G., Odell, B.L., Bloom, E.S., Brown, R.A., Campbell, C.J. & Bird, O.D. (1945). Isolation of an Antianemia Factor (Vitamin-Bc Conjugate) in Crystalline Form from Yeast. *Science* 102(2644), 228-230.

Prinz-Langenohl, R., Brönstrup, A., Thorand, B., Hages, M. & Pietrzik, K. (1999). Availability of food folate in humans. *Journal of Nutrition* 129(4), 913-916.

Pufulete, M., Emery, P.W., Nelson, M. & Sanders, T.A.B. (2002). Validation of a short food frequency questionnaire to assess folate intake. *British Journal of Nutrition* 87(4), 383-390.

Putt, M. & Chinchilli, V. (2004). Nonparametric approaches to the analysis of crossover studies. *Statistical Science* 19(4), 712-719.

Qiu, A., Jansen, M., Sakaris, A., Min, S.H., Chattopadhyay, S., Tsai, E., Sandoval, C., Zhao, R., Akabas, M.H. & Goldman, I.D. (2006). Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell* 127(5), 917-928.

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(1), 221-225.

Quinlivan, E.P., Hanson, A.D. & Gregory, J.F. (2006). The analysis of folate and its metabolic precursors in biological samples. *Analytical Biochemistry* 348(2), 163-184.

Reed, M.C., Nijhout, H.F., Neuhouser, M.L., Gregory, J.E., Shane, B., James, S.J., Boynton, A. & Ulrich, C.M. (2006). A mathematical model gives insights into nutritional and genetic aspects of folate-mediated one-carbon metabolism. *Journal of Nutrition* 136(10), 2653-2661.

Riddell, L.J., Chisholm, A., Williams, S. & Mann, J.I. (2000). Dietary strategies for lowering homocysteine concentrations. *Americal Journal of Clinical Nutrition* 71(6), 1448-1454.

Rogers, L.M., Pfeiffer, C.M., Bailey, L.B. & Gregory, J.F. (1997). A dual-label stable-isotopic protocol is suitable for determination of folate bioavailability in humans: Evaluation of urinary excretion and plasma folate kinetics of intravenous and oral doses of [C-13(5)] and [H-2(2)]folic acid. *Journal of Nutrition* 127(12), 2321-2327.

Rozen, R. (2004). Folate and genetics. *Journal of Food Science* 69(1), S65-S67.

Ruiz, J.R., Hurtig-Wennlof, A., Ortega, F.B., Patterson, E., Nilsson, T.K., Castillo, M.J. & Sjostrom, M. (2007). Homocysteine levels in children and adolescents are associated with the methylenetetrahydrofolate reductase 677C > T genotype, but not with physical activity, fitness or fatness: The European Youth Heart Study. *British Journal of Nutrition* 97(2), 255-262.

Russell, R.M., Dhar, G.J., Dutta, S.K. & Rosenberg, I.H. (1979). Influence of intraluminal pH on folate absorption - studies in control subjects and in patients with pancreatic insufficiency. *Journal of Laboratory and Clinical Medicine* 93(3), 428-436.

Russell, R.M., Golner, B.B., Krasinski, S.D., Sadowski, J.A., Suter, P.M. & Braun, C.L. (1988). Effect of antacid and H-2-receptor antagonists on the intestinal-absorption of folic acid. *Journal of Laboratory and Clinical Medicine* 112(4), 458-463.

Salovaara, S., Larsson Alminger, M., Eklund-Jonsson, C., Andlid, T. & Sandberg, A.-S. (2003). Prolonged Transit Time through the Stomach and Small Intestine Improves Iron Dialyzability and Uptake in Vitro. *Journal of Agricultural and Food Chemistry* 51(17), 5131-5136.

Saposnik, G., Ray, J.G., Sheridan, P., McQueen, M., Lonn, E. & the, H.I. (2009). Homocysteine-lowering therapy and stroke risk, severity, and disability: additional findings from the HOPE 2 trial. *Stroke* 40(4), 1365-1372.

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

SBU (2007). *Nyttan att berika mjöl med folsyra i syfte att minska risken för neural rörsdefekter. En systematisk litteraturöversikt. (Benefits of fortifying flour with folic acid to reduce risk of*

neural tube defects). Stockholm, Sweden: SBU The Swedish Council on Technology Assessment in Health.

Sega, E.I. & Low, P.S. (2008). Tumor detection using folate receptor-targeted imaging agents. *Cancer and Metastasis Reviews* 27(4), 655-664.

Selhub, J. (1989). Determination of tissue folate composition by affinity chromatography followed by high-pressure ion pair liquid chromatography. *Analytical Biochemistry* 182(1), 84-93.

Shane, B. (1995). Folate Chemistry and Metabolism. In: Bailey, L.B. (Ed.) *Folate in healthband disease*. New York: Marcel Dekker. (Clinical nutrition in health and disease 1). pp. 1-21.

Sirikulchayanonta, C., Madjupa, K., Chongsuwat, R. & Pandii, W. (2004). Do Thai women of child bearing age need pre-conceptional supplementation of dietary folate? *Asia Pacific Journal of Clinical Nutrition* 13(1), 69-73.

Smith, A.D., Kim, Y.-I. & Refsum, H. (2008). Is folic acid good for everyone? *Americal Journal of Clinical Nutrition* 87(3), 517-533.

Subramanian, V.S., Reidling, J.C. & Said, H.M. (2008). Differentiation-dependent regulation of the intestinal folate uptake process: studies with Caco-2 cells and native mouse intestine. *American Journal of Physiology - Cell Physiology* 295(3), 828-835.

Sutton, M., Daly, L.E. & Kirke, P.N. (2008). Survival and Disability in a Cohort of Neural Tube Defect Births in Dublin, Ireland. *Birth Defects Research Part a-Clinical and Molecular Teratology* 82(10), 701-709.

Tamura, T. & Picciano, M.F. (2006). Folate and human reproduction. *Americal Journal of Clinical Nutrition* 83(5), 993-1016.

Tamura, T. & Stokstad, E.L. (1973). Availability of food folate in man. *British Journal of Haematology* 25(4), 513-532.

Thamm, M., Mensink, G.B.M. & Thierfelder, W. (1999). Folsäureversorgung von Frauen im gebärfähigen Alter. *Gesundheitswesen* 61, S207-S212.

Thompson, M., Ellison, S.L.R. & Wood, R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis - (IUPAC technical report). *Pure and Applied Chemistry* 74(5), 835-855.

Tozer, T. & Rowland, M. (2006). *Introduction to pharmacokinetics and pharmacodynamics*. Baltimore: Lippincott Williams & Wilkins.

Wagner, C. (1995). Biochemical Role of Folate in Cellular Metabolism. In: Bailey, L.B. (Ed.) *Folate in Health and Disease*. New York: Marcel Dekker. (Clinical Nutrition in health and disease 1). pp. 23-42.

Wahlin, Å., Bäckman, L., Hultdin, J., Adolfsson, R. & Nilsson, L.-G. (2002). Reference values for serum levels of vitamin B12 and folic acid in a population-based sample of adults between 35 and 80 years of age. *Public Health Nutrition* 5(3), 505-511.

Vahteristo, L., Kariluoto, S., Barlund, S., Karkkainen, 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(6), 271-278.

van de Rest, O., Durga, J., Verhoef, P., Melse-Boonstra, A. & Brants, H.A.M. (2007). Validation of a food frequency questionnaire to assess folate intake of Dutch elderly people. *British Journal of Nutrition* 98(5), 1014-1020.

Van Guelphen, B. (2006). *Folate in cancer and cardiovascular disease - prospective studies from the population-based Northern Sweden Health and Disease Study*. Diss. Umeå University. Umeå.

Wang, X.B., Qin, X.H., Demirtas, H., Li, J.P., Mao, G.Y., Huo, Y., Sun, N.L., Liu, L.H. & Xu, X.P. (2007). Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *The Lancet* 369(9576), 1876-1882.

Wei, M.-M. & Gregory, J.F., III (1998). Organic acids in selected food inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: Potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *Journal of Agricultural and Food Chemistry* 46(1), 211-219.

Wei, M.M., Bailey, L.B., Toth, J.P. & Gregory, J.F. (1996). Bioavailability for humans of deuterium-labeled monoglutamyl and polyglutamyl folates is affected by selected foods. *Journal of Nutrition* 126(12), 3100-3108.

Venn, B.J., Mann, J.I., Williams, S.M., Riddell, L.J., Chisholm, A., Harper, M.J. & Aitken, W. (2002). Dietary counseling to increase natural folate intake: a randomized, placebo-controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. *Americal Journal of Clinical Nutrition* 76(4), 758-765.

Verwei, M. (2004). *Bioaccessibility of folate from several liquid and solid food products*. Diss. Wageningen University. Wageningen.

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(7), 2377-2383.

Verwei, M., Arkbåge, K., Mocking, H., Havenaar, R. & Groten, J. (2004). The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model. *Journal of Nutrition* 134(1), 31-37.

Verwei, M., van den Berg, H., Havenaar, R. & Groten, J.P. (2005). Effect of folate-binding protein on intestinal transport of folic acid and 5-methyltetrahydrofolate across Caco-2 cells. *European Journal of Nutrition* 44(4), 242-249.

Wigertz, K. (1997). *Milk folates - Characterisation and availability*. Diss. Lund University. Lund.

Wilcken, B., Bamforth, F., Li, Z., Zhu, H., Ritvanen, A., Redlund, M., Stoll, C., Alembik, Y., Dott, B., Czeizel, A.E., Gelman-Kohan, Z., Scarano, G., Bianca, S., Ettore, G., Tenconi, R., Bellato, S., Scala, I., Mutchinick, O.M., Lopez, M.A., de Walle, H., Hofstra, R., Joutchenko, L., Kavteladze, L., Bermejo, E., Martinez-Frias, M.L., Gallagher, M., Erickson, J.D., Vollset, S.E., Mastroiacovo, P., Andria, G. & Botto, L.D. (2003). Geographical and ethnic variation of the 677C > T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. *Journal of Medical Genetics* 40(8), 619-625.

Williamson, C.S. & Buttriss, J.L. (2007). EuroFIR Congress 2006: Food information databank systems - everything you ever wanted to know. *Trends in Food Science & Technology* 18(8), 398-406.

Wills, L., Clutterbuck, P.W. & Evans, B.D.F. (1937). A new factor in the production and cure of macrocytic anaemias and its relation to other haemopoietic principles curative in pernicious anaemia. *Biochemical Journal* 31, 2136-2147.

Winkels, R.M., Brouwer, I.A., Siebelink, E., Katan, M.B. & Verhoef, P. (2007). Bioavailability of food folates is 80% of that of folic acid. *American Journal Of Clinical Nutrition* 85(2), 465-473.

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., Arkbåge, K., Johansson, M., Lundin, E., Berglund, G., Zhang, J.X., Lennernäs, H. & Dainty, J.R. (2006). Folate absorption from folate-fortified and processed foods using a human ileostomy model. *British Journal of Nutrition* 95(1), 181-187.

Wright, A.J.A., Finglas, P.M., Dainty, J.R., Hart, D.J., Wolfe, C.A., Southon, S. & Gregory, J.F. (2003). Single oral doses of C-13 forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies. *British Journal of Nutrition* 90(2), 363-371.

Wright, A.J.A., Finglas, P.M., Dainty, J.R., Wolfe, C.A., Hart, D.J., Wright, D.M. & Gregory, J.F. (2005). Differential kinetic behavior and distribution for pteroylglutamic acid and reduced folates: a revised hypothesis of the primary site of PteGlu metabolism in humans. *Journal of Nutrition* 135(3), 619-623.

Zhao, R., Matherly, L.H. & Goldman, I.D. (2009). Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Reviews in Molecular Medicine* 11(e4), doi:10.1017/S1462399409000969.

Åden, E., Johansson, I. & Håglin, L. (2007). Energy and nutrients in self-reported diet before and at week 18-22 of pregnancy. *Scandinavian Journal of Food & Nutrition* 51(2), 67-73.

Öhrvik, J. (1998). Nonparametric methods in crossover trials. *Biometrical Journal* 40, 771-789.

Öhrvik, V.E., Olsson, J.C., Sundberg, B.E. & Witthöft, C.M. (2009). Effect of 2 pieces of nutritional advice on folate status in Swedish women: a randomized controlled trial. *Americal Journal of Clinical Nutrition* 89(4), 1053-1058.

Acknowledgements

This work was funded by the Swedish research council FORMAS. Cerealia R&D and JO-bolaget are further acknowledged for donation of foods and financial contribution. Conference and study visits were supported EuroFIR grant, Hjelms foundation, LiFT scholarship and Knut and Alice Wallenberg Foundation.

I would like to express my appreciation to my group of supervisors, in particular my main supervisor, Cornelia Witthöft, for sharing all your knowledge and for you never ending enthusiasm and support. My co-supervisor, Margaretha Jägerstad, for enthusiasing discussions. My co-supervisor in Germany, Michael Rychlik, for sharing your expertise in folate analysis.

Several others have contributed irreplaceable to this thesis without having their names on the papers. I therefore wish to greatly acknowledge:

All fantastic and collaborative volunteers in Uppsala and Umeå,

The research nurses: Lena Uddstål (Clinical Research Centra in Umeå) and Eva Broman & Nilla Forss (Good Food Practise in Uppsala) for an excellent job with the volunteers,

Jelena Jastrebova, Johan Patring, Madelene Johansson, Karin Arkbåge, Lena Strålsjö, Cornelia and Margaretha for all work on analytical methods, which I had use of,

Gerd Berglund for all superb arrangements in the ileostomy trial,

Maria Hällgren and Eva-Lena Andersson for enormous help with FFQ,

John Öhrvik for inspiring discussions on scientific methods,

Rikard Landberg, for encouraging feedback on the thesis (and for all bile)

and a huge thanks to Afaf Kamal-Eldin, for spending more than one night reading drafts and always finding solutions.