Folates in rye:
Determination and enhancement by food processing

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ABSTRACT

The average daily intake of folate, one of the B vitamins, falls below recommendations among the Finnish population. Bread and cereals are the main sources of folate, rye being the most significant single source. Processing is a prerequisite for the consumption of whole grain rye; however, little is known about the effect of processing on folates. Moreover, data on the bioavailability of endogenous cereal folates are scarce. The aim of this study was to examine the variation in as well as the effect of fermentation, germination, and thermal processes on folate contents in rye. Bioavailability of endogenous rye folates was investigated in a four-week human intervention study. One of the objectives throughout the work was to optimise and evaluate analytical methods for determining folate contents in cereals.

Affinity chromatographic purification followed by high-performance liquid chromatography (HPLC) was a suitable method for analysing cereal products for folate vitamers, and microbiological assay with *Lactobacillus rhamnosus* reliably quantified the total folate. However, HPLC gave approximately 30% lower results than the microbiological assay.

The folate content of rye was high and could be further increased by targeted processing. The vitamer distribution of whole grain rye was characterised by a large proportion of formylated vitamers followed by 5-methyltetrahydrofolate. In sourdough fermentation of rye, the studied yeasts synthesized and lactic acid bacteria mainly depleted folate. Two endogenous bacteria isolated from rye flour were found to produce folate during fermentation. Inclusion of baker's yeast in sourdough fermentation raised the folate level so that the bread could contain more folate than the flour it was made of. Germination markedly increased the folate content of rye, with particularly high folate concentrations in hypocotylar roots. Thermal treatments caused significant folate losses but the preceding germination compensated well for the losses. In the bioavailability study, moderate amounts of endogenous folates in the form of different rye products and orange juice incorporated in the diet improved the folate status among healthy adults. Endogenous folates from rye and orange juice showed similar bioavailability to folic acid from fortified white bread.

In brief, it was shown that the folate content of rye can be enhanced manifold by optimising and combining food processing techniques. This offers some practical means to increase the daily intake of folate in a bioavailable form.

PREFACE

This study was carried out at the Department of Applied Chemistry and Microbiology, University of Helsinki during the years 2001–2008. I gratefully acknowledge the financial support by the EU-funded project "Folate: from food to functionality and optimal health" (FolateFuncHealth; QLRT-1999-00576), the Ministry of Agriculture and Forestry, the Finnish Funding Agency for Technology and Innovation (Tekes), The Research Foundation of Raisio Group Ltd., the Research Foundation of the University of Helsinki, and the Finnish Graduate School on Applied Bioscience (ABS).

I express my deepest gratitude to my supervisor, Professor Vieno Piironen, for all the support and enthusiasm. You have always managed to find time for discussions, and I sincerely thank you for thoroughly reading the manuscript and giving motivating criticism. My supervisor Dr. Liisa Vahteristo I wish to thank for leading me to the folate wonderland and being available whenever I needed encouragement. Thank you both for lots of invaluable advice but also for the freedom to study and letting me do my own mistakes.

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Finally, I owe my heartfelt thanks to my family, relatives, and friends. I am deeply grateful to my parents Kielo and Kari Kariluoto, my dear sisters Hanna and Minna and my wonderful brothers Antti and Matti as well as their families, if not always for understanding my work but for ever-continuing support, encouragement, and confidence. Warmest thanks belong to my husband Hung Nguyen and our daughters Ninh and Han for support, comfort, and all the love they have given. Without you this project may have been accomplished earlier but these years would not have been such a rich and joyous period in my life.

Helsinki, April 2008

Susanna Kariluoto

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their Roman numerals:

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

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

III Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., and Piironen, V. 2006. Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. International Journal of Food Microbiology 106: 137–143.

IV Kariluoto, S., Liukkonen, K-H, Myllymäki, O., Vahteristo, L., Kaukovirta-Norja, A., and Piironen, V. 2006. Effect of germination and thermal treatments on folates in rye. Journal of Agricultural and Food Chemistry 54: 9522–9528.

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

In addition, the same rye cultivars as studied in paper **I** were grown in the next season. Four rye cultivars were also grown both organically and conventionally. Samples from these experiments were analysed for total folate and the results were compared with those reported in paper **I**. The data have been included in this thesis.

Contribution of the author to papers I to V:

I - IV Susanna Kariluoto planned the study together with the other authors and was responsible for folate analyses. She had the main responsibility for interpreting the results and was the corresponding author of the paper.

V Susanna Kariluoto planned the study together with the other authors. She carried out part of the experimental work and participated in the preparation of the manuscript.

LIST OF ABBREVIATIONS

AACC International	previously known as the American Association of Cereal Chemists
AOAC	Association of Official Analytical Chemists
Ches	2-(N-cyclohexylamino)ethanesulfonic acid
CRM	certified reference material
CV	coefficient of variation
DAD	diode array detector
DFE	dietary folate equivalent
DM	dry matter
ELISA	enzyme linked immunosorbent assay
em	emission
EPBA	enzyme protein binding assay
ex	excitation
FAO	Food and Agriculture Organization of the United Nations
FBP	folate binding protein
FDA	U.S. Food and Drug Administration
FL	fluorescence
FSA	Food Standards Agency
FW	fresh weight
Hcy	homocysteine
Hepes	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
HK	hog kidney conjugase
HPLC	high-performance liquid chromatography
INFOODS	International Network of Food Data Systems
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	liquid chromatography – mass spectrometry
MA	microbiological assay
MTHFR	methylenetetrahydrofolate reductase
NTD	neural tube defect
RDA	recommended daily allowance
RIA	radioimmunoassay
RNI	reference nutrient intake
RPBA	radio protein binding assay
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAX	strong anion exchange
SPE	solid phase extraction
TYMS	thymidylate synthase
USDA	U.S. Department of Agriculture
UV	ultraviolet
WHO	World Health Organisation

H_4folate	tetrahydrofolic acid
5-CH_3-H_4folate	5-methyltetrahydrofolic acid
10-HCO-H_2folate	10-formyldihydrofolic acid
10-HCO-PGA	10-formylfolic acid
5-HCO-H_4folate	5-formyltetrahydrofolic acid
PGA	folic acid

CONTENTS

1. INTRODUCTION

In 1931 Dr. Lucy Wills successfully used yeast extract to treat macrocytic anaemia prevalent in pregnant Indian patients (Wills, 1931). Yeast contained the same antianaemia factor that was isolated from spinach in 1941, and the factor received the name folic acid after the Latin word folium, meaning leaf. Folate compounds belong to the vitamin B group, and folate is a generic term referring to derivatives of folic acid.

Megaloblastic anaemia is still a common consequence of folate deficiency, but recently more attention has been given to the effect of folate on preventing foetal neural tube defects (NTD). Folic acid supplementation is efficient in preventing the occurrence of NTDs (MRC Vitamin Study Research Group, 1991), but since NTDs develop during the first four weeks of pregnancy, prevention should be started before conception. Folate functionality is nowadays of great interest for several other reasons. Folate decreases plasma homocysteine, which has been proposed as a risk factor for cardiovascular disease (Boushey et al., 1995), ischemic heart disease, and stroke (Homocysteine Studies Collaboration, 2002). However, recent re-evaluation has revealed that the association of homocysteine with cardiovascular disease may be weaker than previously believed (B-Vitamin Treatment Trialists' Collaboration, 2006) but the role of homocysteine as a risk factor for stroke is more pronounced (Wang et al., 2007). Interestingly, plasma homocysteine seems to be a risk factor also for dementia and Alzheimer's disease (Seshadri et al., 2002). Dietary folate intake has been shown to be inversely associated with the risk of cardiovascular disease and stroke (Bazzano et al., 2002). Adequate folate intake also protects against colorectal cancer (Giovannucci et al., 1995). This said, the effect of folate on carcinogenesis may be of dual nature, depending on the dose, the folate form, and the timing (Sanderson et al., 2007).

Folate in food exists as various vitamers differing in oxidation status and single carbon substituents, and with a variable number of glutamyl residues. The labile nature of folate, differences in stabilities of vitamers, and low concentration levels make reliable analysis challenging. The properties of the food matrix also have to be addressed. Perhaps the most commonly used method in food folate analysis is microbiological assay, which provides a single figure, total folate, representing the sum of all folate derivatives. However, since vitamers differ in their chemical characteristics and possibly in their bioavailability, it is important to examine folate vitamer distribution. High-performance liquid chromatographic (HPLC) methods have been developed for this purpose, but the lack of specific purification methods has hindered effective use of HPLC. Affinity chromatography has been used with good results for purifying

and concentrating sample extracts (Seyoum and Selhub, 1993; Pfeiffer et al., 1997; Konings, 1999). Nevertheless, information about natural folate vitamers in food and especially in cereals is still scarce.

The average daily intake of folate among the Finnish population falls below recommendations. The main sources of dietary folate are bread and cereals, contributing over one third to the daily intake of folate (Männistö et al., 2003). Rye is the most significant single source of folate, providing approximately 11% of its daily intake (Laurinen, 2000). Most rye is consumed as rye bread baked from wholemeal flour. Consumers consider rye a healthy choice, and indeed, consumption of whole grain has been associated with a reduced risk of several chronic diseases. Processing is a prerequisite for the consumption of whole grain rye, and it may either negatively or positively affect folate contents. There is evidence that folate levels can be raised by means of selecting high-folate raw materials or applying certain bioprocesses (Liukkonen et al., 2003). However, studies conducted with rye are rare: germination studies have mainly been conducted with barley (Jägerstad et al., 2005), baking studies with wheat (Keagy et al., 1975), and fermentation studies with dairy products (Crittenden et al., 2002). Little is known about the effects of processing on rye. In countries such as Finland, where mandatory folic acid fortification is not practised and voluntary fortification is very limited, enhancement of natural folate contents could provide a means to improve the folate status of the population.

Bioavailability of food folate is not well understood and estimates of the bioavailability of endogenous food folate compared to folic acid vary markedly. Approximately 50% bioavailability has been reported for endogenous food folates (Hannon-Fletcher et al., 2004), but in some studies this figure has been up to 80% (Winkels et al., 2007). Moreover, considering the importance of cereals in the daily intake of folate, data on the bioavailability of cereal folates are surprisingly scarce. Fenech et al. (1999) reported good bioavailability of folate in wheat aleurone flour, and some studies have investigated the bioavailability of folic acid as a fortificant in cereal matrix (e.g. Witthöft et al., 2006).

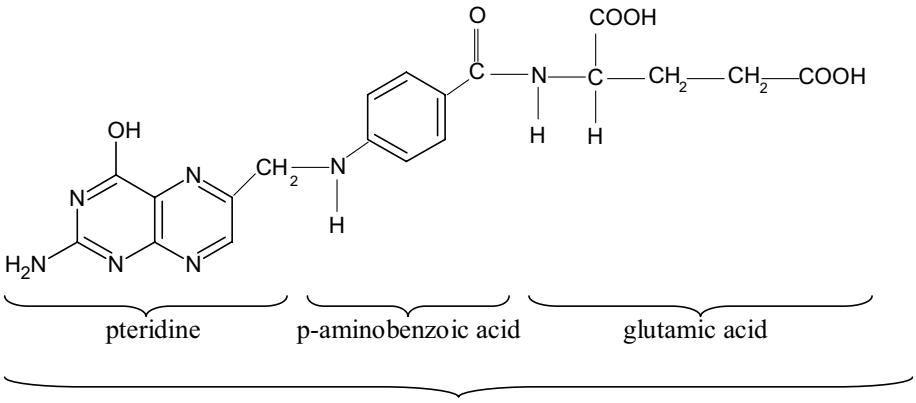
The first part of this thesis consists of a literature review focusing on the role of folate in human nutrition, the characteristics of folate in cereals, and the determination of food folate. The second part reviews the experimental section of the study. HPLC and microbiological methods were first optimised and validated for the cereal matrix and these methods were then applied to study folate contents in rye cultivars and processed rye. Finally, a human intervention study was conducted to investigate the bioavailability of rye folate.

2. LITERATURE REVIEW

2.1 Nomenclature and chemistry of folates

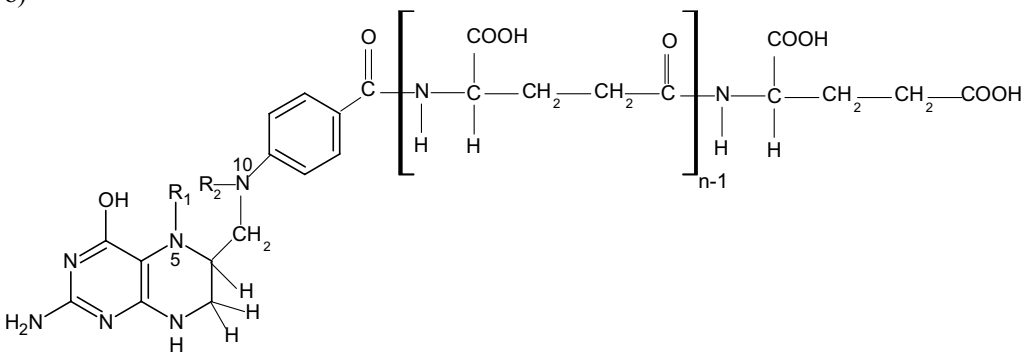
Folate is a generic term for compounds exhibiting similar chemical characteristics and biological activity to folic acid. The basic structure of folate is comprised of 2-amino-4-hydroxy-6-methylpterin (pteridine ring) linked through a methylene bridge to para-aminobenzoate which is conjugated with one or several L-glutamic acid residues with γ-peptide linkage (Figure 1).

a)



pteroylmonoglutamic acid (folic acid)

b)



polyglutamyl tetrahydrofolates

Vitamer	**R_1**	**R_2**
tetrahydrofolate	-H	-H
5-methyltetrahydrofolate	$-CH_3$	-H
5-formyltetrahydrofolate	-HCO	-H
10-formyltetrahydrofolate	-H	-HCO
5,10-methylenetetrahydrofolate	$-CH_2-$	
5,10-methenyltetrahydrofolate	–CH=	

Figure 1. Structures of a) folic acid, and b) polyglutamyl tetrahydrofolates.

Chemically-related compounds within the same vitamin family with comparable metabolic activities may be called vitamers (Combs, 1992). As regards folate, differences at three sites of the structure result in a large number of chemically-related species. Firstly, the pteridine ring can be fully oxidized (as in folic acid), partially reduced at the 7,8-position (H_2folate), or fully reduced. According to the recommendations of IUPAC, the reduced 5,6,7,8-tetrahydropteroylglutamic acid is called tetrahydrofolate and is abbreviated as H_4folate (Blakley, 1988). Secondly, tetrahydrofolate can be substituted at the N^5 or N^{10} position to form 5-methyl, 5-formyl, 5-formimino, and 10-formyl derivatives. In addition, N^5 and N^{10} can be bridged to form 5,10-methylene and 5,10-methenyl derivatives. Thirdly, the number of glutamyl residues varies greatly. Folates *in vivo* exist mainly as folylpolyglutamates that cannot pass membranes easily and may thus be the main coenzyme forms intracellularly. Many enzymes show greater affinity to folylpolyglutamates than to monoglutamates, and the length of the glutamyl side chain may regulate the flux of one-carbon units to different biochemical reactions (Schirch and Strong, 1989). There are two chiral centres in the fully reduced folates, the C^6 atom in the pteridine moiety and the α-C atom in the glutamyl moiety. The natural diastereoisomeres of H_4folate, 5-CH_3-H_4folate, and 5-HCO-H_4folate are [6S, αS] diastereoisomeres, whereas the naturally occurring forms of 10-HCO-H_4folate, 5,10-CH_2-H_4folate, and 5,10-CH^+-H_4folate are [6R, αS] diastereoisomeres.

All folates are sensitive to light. However, susceptibility to oxidative degradation varies: folic acid, the form used for fortification and in the pharmaceutical industry, is more resistant to oxidative stress than reduced folate forms (Hawkes and Villota, 1989). Substitution to the N^5 or N^{10} position increases the stability so that the order of stability in aqueous solutions is 5-HCO-H_4folate > 5-CH_3-H_4folate > 10-HCO-H_4folate > H_4folate (Eitenmiller and Landen, 1999). Oxidative cleavage leads to biologically inactive compounds (Gregory, 1996).

In addition to the folate form, the rate of degradation depends on pH, temperature, the buffer, and the presence of catalysts (e.g. trace elements) or antioxidants/reducing agents (Gregory, 1989). Depending on the pH, interconversions between folate forms are also possible. For instance, 10-HCO-H_4folate is prone to oxidation and is easily converted to 10-HCO-PGA and 10-HCO-H_2folate (Robinson, 1971; Maruyama et al., 1978), and 5-CH_3-H_4folate is oxidised to 5-CH_3-H_2folate (Maruyama et al., 1978). At mildly acidic pH 5,10-CH_2-H_4folate is readily dissociated to H_4folate, and in acidic conditions, 5-HCO-H_4folate and 10-HCO-H_4folate form 5,10-CH^+-H_4folate (Pfeiffer et al., 1997). Although several factors affecting folate stability have been recognised, knowledge of folate stability in complex systems such as foods is still limited.

2.2 Folate nutrition

2.2.1 Functions

Folate coenzymes function in various biosynthetic reactions that involve transfer of one-carbon units with different oxidation states. A one-carbon unit can be attached to either N^5 or N^{10} position or it can form a methylene or methenyl bridge between N^5 and N^{10}.

Folates are needed in the biosynthesis of DNA and RNA through the nucleotide synthesis cycle and in the metabolism of amino acids through the methylation cycle (Figure 2). The methylation balance (SAM/SAH) is an important control point of many other reactions, such as methylation of DNA, and phospholipid and neurotransmitter synthesis (Wagner, 1995). Synthesis of H_4folate from 5-CH_3-H_4folate is catalysed by a vitamin B_{12} -dependent enzyme, methionine synthase. If B_{12} is lacking (for instance, due to pernicious anaemia), a functional folate deficiency may occur, which leads to accumulation of 5-CH_3-H_4folate and homocysteine (Hcy). Synthetic folic acid is reduced to H_4folate without the functions of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase; its utilisation does not require vitamin B_{12}. Folic acid may thus mask the haematological clinical signs of vitamin B_{12} deficiency.

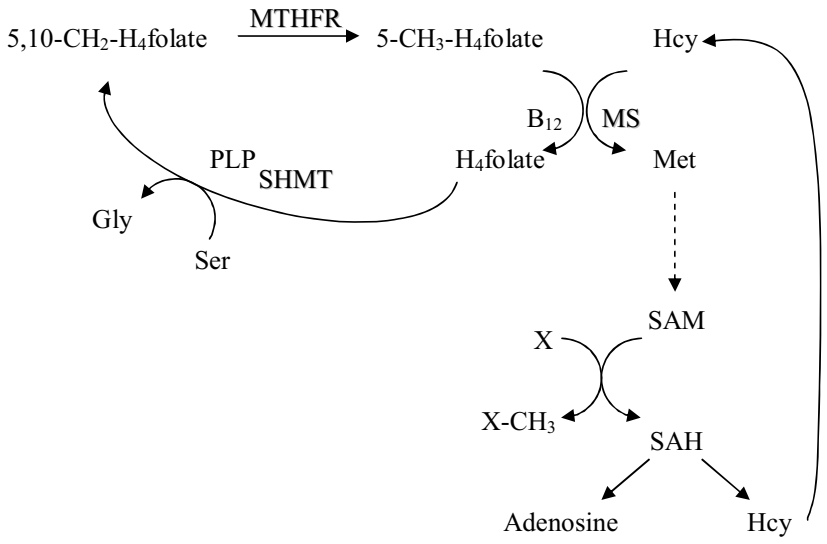


Figure 2. Methylation cycle.

Hcy	= homocysteine	SAM	= S-adenosyl methionine
Met	= methionine	SAH	= S-adenosyl homocysteine
Ser	= serine	X	= compound to be methylated
Gly	= glycine	X-CH_3	= methylated compound
PLP	= pyridoxal-5'-phosphate	MTHFR	= methylenetetrahydrofolate reductase
SHMT	= serine hydroxymethyltransferase	MS	= methionine synthase

2.2.2 Absorption and bioavailability

Folate bioavailability is mainly determined by the extent of absorption in the jejunum. Folate polyglutamates need to be hydrolyzed with γ-glutamyl hydrolase in the mucosa before absorption (Chandler et al., 1986). Monoglutamyl folates are absorbed mainly in jejunum by an active carrier-mediated, pH-dependent mechanism. At high concentrations a non-saturable diffusion-mediated transport dominates (Gregory, 2001). There are also high-affinity FBP folate receptors but their expression in small intestine is neglible. Recently, Qiu et al. (2006) identified a new proton-coupled folate transporter that, unlike the reduced folate carrier, is mutated in folate malabsorption patients.

After absorption folates are reduced to tetrahydro derivatives and methylated in the mucosal cells before entering the hepatic portal vein although it has been proposed that for folic acid the liver is the initial site of metabolism (Wright et al., 2007). Approximately 10 to 20% of the circulating 5-CH_3-H_4folate is retained by the liver (first pass effect). Folate undergoes a notable enterohepatic circulation and slow turnover with an estimated half-life of 100 days for the major folate pool (Gregory, 1995).

Several genetic variants in enzymes controlling folate absorption, transport and metabolism can have a significant effect on folate status and health (Gregory et al., 2005). For instance, Mitchell et al. (1997) estimated that at least 46% of the variation in red blood cell folate is due to genetic variance. Health-related and lifestyle factors also affect folate bioavailability. Some drugs such as anticonvulsants, cancer chemotherapeutic agents, and sulphasalazine (Bailey, 1988) as well as high chronic alcohol use and smoking (van den Berg et al., 2001) are known to affect folate metabolism and status. Folate absorption can also be impaired by inflammation or damage in the gastro-intestinal tract (Gregory, 1997).

Animal bioassays, mainly with rats, have been widely used to evaluate the bioavailability of folate (e.g. Clifford et al., 1991). However, their relevance has been questioned as knowledge on differences in folate deconjugation between animal species and humans has accumulated. In addition, basal diets differ from human diets both in their composition and matrix (Gregory, 2001). *In vitro* methods have been used to study potential inhibitors of the brush border hydrolase, and results have predicted a potential for reduced bioavailability (Gregory, 2001). An interesting approach to *in vitro* studies is the use of a simulated, dynamic gastro-intestinal tract in order to estimate the bioaccessibility of food folate (the fraction released from the food matrix and available for absorption) (Verwei et al., 2003).

In human volunteers, short-term studies with plasma folate or urinary excretion measurements are used to evaluate the bioavailability of dietary or supplemental folate. Reliable estimation of the response of plasma folate – the area under the curve (AUC) – requires several blood samples along a sufficient monitoring time. The sensitivity of the methods remains a problem: only foods/supplements with relatively high folate contents (> 300 µg/dose) can be assessed (Gregory, 2001). Folic acid is often used as the reference folate to determine the relative absorption of folate in test foods. However, Wright et al. (2005) recently showed that the kinetics of folic acid differ from those of other folates, and a significant fraction of folic acid may enter the portal circulation unaltered. In long-term protocols the folate status is reflected by changes in plasma folate, plasma homocysteine, and red cell folate concentrations – often in this order. The main advantage of long-term studies is that they are suitable for examining the bioavailability of the compound as a part of the diet. Folate bioavailability studies conducted with ileostomy volunteers (e.g. Konings et al., 2002; Witthöft et al., 2006) have further advantages, as they lack the interfering effect of microbial folate synthesis in the colon.

Stable isotope methods have also been used in folate bioavailability studies (Gregory et al., 1990a; Rogers et al., 1997; Finglas et al., 2002). Isotopic methods are specific and sensitive provided that the analytical method in measurement of the labeled tracer is accurate and that there is no loss of label during metabolism and analysis.

Estimates of the bioavailability of natural folates vary notably. Approximately 50% bioavailability of naturally-occurring folate forms relative to folic acid has been reported (Sauberlich et al., 1987; Hannon-Fletcher et al., 2004). However, higher estimates of 60 to 98%, have also been achieved (Brouwer et al., 1999a; Winkels et al., 2007). According to several human studies the bioavailability of folate polyglutamates is in the range of 50 to 80% relative to that of monoglutamates (Gregory et al., 2005), but equivalent bioavailabilities have also been determined (Wei et al., 1996). Much research has concentrated on the potential inhibition of brush border conjugase inhibitors. Organic acids have inhibited conjugase *in vitro* (Bhandari and Gregory, 1990; Wei and Gregory, 1998). Dietary fibre sources have in most cases had no adverse effect on folate bioavailability (Gregory, 1997). Interestingly, it has been proposed that non-starch polysaccharides may actually promote microbial folate synthesis and improve the folate status in human (Houghton et al., 1997).

The food matrix, cellular structure, and the chemical instability of tetrahydrofolates in the gastro-intestinal tract can also affect the bioavailability of folate (Seyoum and Selhub, 1998). In spinach the bioavailability of folate was better if the spinach matrix was disrupted (van het Hof et al., 1999). Binding to folate-binding proteins may protect folates and stabilise them (Jones and Nixon, 2002) but opposite results have also been achieved, especially for folic acid (Verwei et al., 2004; Witthöft et al., 2006).

2.2.3 Health impacts

Considering the crucial role that folate has in many biochemical reactions related to normal growth and cell differentiation, it is quite understandable that suboptimal intake of folate may lead to severe consequences. On the other hand, sufficient intake of folate has been shown to offer protection against certain diseases. Perhaps the most common but nowadays less discussed consequence of folate deficiency is megaloblastic anaemia, which is caused by the disruption of red blood cell formation and is characterised by abnormally large red blood cells.

Sufficient folate intake is especially important during pregnancy and has important implications for maternal, foetal and neonatal health. Folate especially decreases the risk of neural tube defects (NTDs). NTDs are classified into spina bifida (opening in the vertebral column protecting the spinal cord), anencephaly (absence of a major portion of the brain and skull), and encephalocele (a hernia of part of the brain and the meninges). NTDs occur during the first four weeks of pregnancy when neural plate closes and forms the neural tube. There is genetic variation in NTD prevalence. In Finland, the total prevalence of spina bifida and anecephaly in 1993–2004 was on average 7.4 cases/10 000 pregnancies per year, which is relatively low compared to other European populations (Stakes, 2006).

The mechanism underlying NTD is not well understood, but it is thought to be related to the reduced production of methionine, S-adenosylmethionine and nucleotides. Folic acid supplementation is efficient in preventing both the occurrence and recurrence of NTD (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992). Folate absorption tends to be lower than normal among women with an NTD-affected pregnancy (Neuhouser et al., 1998; Boddie et al., 2000). Interestingly, Taparia et al. (2007) presented a hypothesis that homocysteinylation of the folate receptor may be associated with the formation of autoantibodies against the folate receptor, inhibiting the transport of folate to the embryo. In addition, a common polymorphism of the 5,10-methylenetetrahydrofolate reductase gene (MTHFR) – thermolabile C677T – may increase the risk of NTD, but the evidence is not consistent (van der

Put et al., 1997; Molloy et al., 1998). The homozygous genotype C677TT is associated with lower folate and higher homocysteine levels than other variants of the MTHFR gene. Thus, some population subgroups may need an increased intake of folate.

Dietary folate is independently and inversely associated with the risk of cardiovascular disease and stroke (Bazzano et al., 2002). Folate administration decreases elevated plasma homocysteine, which in several studies and meta-analyses has been proposed as a risk factor for cardiovascular disease (Boushey et al., 1995; Wald et al., 2002), ischemic heart disease, and stroke (Homocysteine Studies Collaboration, 2002). However, there is ongoing debate over whether homocysteine is causally related to cardiovascular disease or whether it is merely a cause or a marker of an unfavourable status underlying vascular disease or folate deficiency. Recent evaluation of the randomised trials has casted doubts over the role of homocysteine as a risk factor for cardiovascular disease (B-Vitamin Treatment Trialists' Collaboration, 2006). In addition, Bazzano et al. (2006) concluded in their meta-analysis of randomised controlled trials that folic acid supplementation did not reduce risk for cardiovascular disease nor all-cause mortality among participants with a history of vascular disease. On the other hand, homocysteine is now more clearly viewed as a risk factor for stroke. Indeed, the U.S. mandatory folic acid fortification program accelerated the decline in stroke mortality (Yang et al., 2006), and a recent meta-analysis showed that folic acid supplementation reduced the risk of stroke by 18% (Wang et al., 2007).

The mechanism by which homocysteine could induce vascular disease, and a specific cut-off point for homocysteine concentration as regards cardiovascular disease have not been confirmed. Thus, estimates of the amount of folate needed for an optimum plasma homocysteine concentration vary considerably. Often the recommended levels of folate cannot even be reached by a normal, healthy diet. Wald et al. (2001a) recommended a daily dosage of 800 µg folic acid in order to prevent ischemic heart disease, whereas Ward et al. (1997) concluded that 200 µg supplemental folic acid per day was as effective in lowering plasma homocysteine as 400 µg per day. Brouwer et al. (1999a; 1999b) found that both supplemental folic acid (250 µg per day) and additional dietary folate (350 µg per day) were able to significantly reduce plasma homocysteine concentrations. Venn et al. (2002) reported that additional 100 µg of folic acid/day given as fortified breakfast cereal was as effective in reducing homocysteine concentration as higher levels, up to 300 µg per day.

The higher the starting homocysteine level is, the greater is the response to folate administration (Ward et al.,1997; Schorah et al., 1998). In addition, some genotypes, such as C677TT variant of MTHFR (Molloy et al., 1997; Silaste et al., 2001; Ashfield-Watt et al., 2002; Fohr et al., 2002) and TYMS 3/3 variant of thymidylate synthase (Trinh et al., 2002), modulate homocysteine and folate responses to supplemental and dietary folate. Individuals with C677TT genotype have a higher risk of coronary heart disease, particularly when their folate status is low (Klerk et al., 2002). Hyperhomocysteinaemia is also more common in elderly populations, which thus may need higher folate intakes in order to significantly decrease the risk of cardiovascular diseases (Rydlewicz et al., 2002). In Finland the prevalence of mild hyperhomocysteinaemia is 11% of the population (Alfthan et al., 2002). Among the Finnish population a low dietary intake of folate has been associated with a higher risk of acute coronary events (Voutilainen et al., 2001). High homocysteine concentrations have been shown to result in an elevated risk of stroke and cardiovascular disease mortality, whereas high serum folate levels decrease the risk of stroke and acute coronary events (Voutilainen et al., 2004; Virtanen et al., 2005).

High folate intake may decrease the risk of certain cancers. Interestingly, folate antagonists, methotrexate derivatives, are used in chemotherapy to prevent the replication and growth of cancer cells. Folate-related mechanisms in carcinogenesis include for instance the hypomethylation of DNA, miscorporation of uridylate for thymidylate in DNA, chromosome fragility and diminished DNA repair, and secondary choline deficiency (Mason, 1995). However, folate seems to have dual modulatory effects on carcinogenesis depending on the dose, the form of folate, and the timing of folate administration (Sanderson et al., 2007). For instance, Van Guelpen et al. (2005) found that low plasma folate concentration may protect against colorectal cancer, whereas a high intake of folate (attributable to supplemental folic acid) has been associated with an increased risk for breast cancer among postmenopausal women (Stolzenberg-Solomon et al., 2006).

Evidence for the protective effect of folate is strongest for colorectal cancer. Lashner et al. (1989) reported an inverse relationship between folate intake and the risk of colorectal adenomas or cancer in a case-control study. Large, prospective studies have also been able to demonstrate the protective effect of a sufficient folate intake against colorectal cancer (Giovannucci et al., 1995, 1998). In addition, there are some evidence that consumption of foods containing folate protect against pancreatic and oesophageal cancers (WCRF, 2007). However, the evidence for other cancer types is inconsistent. Folate may affect the risk of uterine cervical cancer (Butterworth et al., 1992) and breast cancer (Freudenheim et al., 1996). Cancers of the lungs and

brain have also been associated with poor folate status (Choi and Mason, 2000). Carcinogenesis seems to be modulated by gene-nutrient interactions. The thermolabile C677T variant of MTHFR may decrease the risk of colorectal cancer, depending on the folate status (Ma et al., 1997; Slattery et al., 1999), but increase the risk of endometrial cancer (Esteller et al., 1997).

Several other health benefits have recently aroused interest. In their large study with a long follow-up period, Seshadri et al. (2002) observed that plasma total Hcy was an independent risk factor for the development of dementia and Alzheimer's disease. However, this has not been confirmed universally (Ariogul et al., 2005). Ravaglia et al. (2005) stated that both elevated plasma Hcy and folate deficiency independently predict dementia and Alzheimer's disease, but argued whether the folate deficiency was rather a consequence of the decline in dietary habits among dementia patients. There are also indications that additional folate might improve age-related immune functions (Kemp et al., 2002; Field et al., 2006); however, human studies are still scarce. The effects may be different for dietary folate and folic acid supplements: unmetabolised folic acid in serum has been found to be inversely associated with natural killer cell cytotoxicity (Troen et al, 2006).

2.2.4 Recommendations and intake

Folate requirements change in the course of life due to physiological changes. In addition, as mentioned earlier, certain variants of key enzymes in folate and homocysteine metabolism can modulate the response of individuals to supplemental or food folate. The bioavailability of folate from different food sources also varies. Thus, converting a minimum requirement for folate into dietary recommendations is complicated.

In Finland the recommended daily intake of folate for adults is 300 µg. Women of reproductive age have a recommended a daily intake of 400 µg (NNC, 2005). In Nordic recommendations the recommended intake of folate for pregnant and lactating women is 500 µg (NNR, 2004); however, this recommendation has not been included in Finnish national nutritional recommendations since the problems caused by folate deficiency are rare and the higher level would have required a recommendation for the use of folic acid supplements. Table 1 presents some folate recommendations.

Table 1. Recommendations for intake of dietary folate (μg/day)

	Nordic countries (NNR, 2004)	United States (Food and Nutrition Board, 1998) Recommended daily allowance (RDA, as DFE[a])	United Kingdom (Department of Health, 1991; 2000) Reference nutrient intake (RNI)	FAO/WHO, 2004
Adults	300	400	200	400
Women capable of becoming pregnant	400	400 folic acid from supplements or fortified foods in addition to food folate	400 folic acid prior to conception and until 12th week of pregnancy; medicinal or food supplement	400
Pregnant women	500	600[b]	300	600
Lactating women	500	500	260	500

[a] Based on the estimated differences in the absorption of food folate and synthetic folic acid:
1 DFE (dietary folate equivalent)
= 1 μg food folate
= 0.6 μg folic acid from fortified food or supplement consumed with food
= 0.5 μg supplemental folic acid consumed without food

[b] 400 μg folic acid from supplements or fortified foods in addition to intake of food folate

The estimated daily intake of folate among the Finnish population is lower than the recommended level, being 273 ± 122 μg/day for men and 224 ± 98 μg/day for women. Intakes fall below recommendations even when adjusted for energy intake. Folate intake is especially low in the youngest adult groups (Männistö et al., 2003). The folate intake is close to the mean folate intake in Europe, which is 291 μg/day (197 to 326) for men and 247 μg/day (168 to 320) for women (de Bree et al., 1997). However, comparing intakes between countries is challenging due to variations in the quality of the food database and the methods used in recording food intake.

Cereal products are the main sources of dietary folate in Finland (Figure 3): they contribute 43% of the daily intake of folate for men and 36% for women (Männistö et al., 2003).

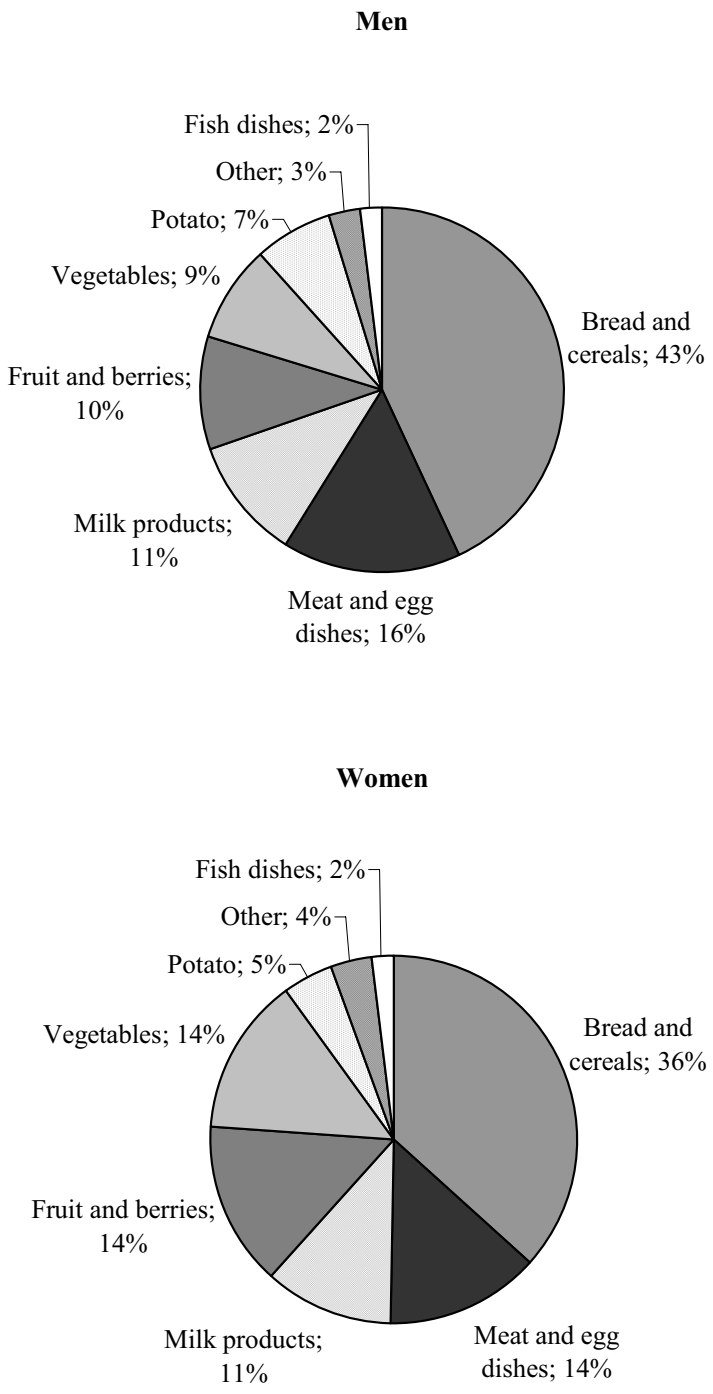


Figure 3. Contribution of different food groups to the folate intake among Finnish men and women (Männistö et al., 2003).

Rye is the single most significant source of folate in the Finnish diet, with a contribution of 11% to the daily folate intake (Laurinen, 2000). Most rye is consumed as rye bread, which is typically baked using wholemeal rye flour and wheat flour. Pregnant women and women planning pregnancy are encouraged to consume more of foods that are good folate sources: whole grain products, fresh vegetables, and fruits and berries (Männistö et al., 2003).

2.2.5 Fortification

Folic acid fortification has been launched or considered in several countries, including the UK, Canada, Australia, New Zealand, Ireland, the Czech Republic, and Chile. The U.S. Food and Drug Administration (FDA) started a folic acid fortification programme on 1 January 1998, in order to reduce the number of NTD cases. The U.S. fortification programme, aimed at an additional intake of 100 μg folate/day, has improved the folate status assessed from plasma folate and homocysteine values and reduced the prevalence of NTD. The fortification program virtually eliminated folate deficiency and efficiently lowered plasma homocysteine in the U.S. population: the prevalence of low serum folate concentrations decreased from 16% before to 0.5% after fortification, and 79% of the population had plasma homocysteine concentrations below 9 μmol/l (Pfeiffer et al., 2005). After the large initial increase in serum and red cell folate concentrations there was a slight decrease (Pfeiffer et al., 2007).

Honein et al. (2001) reported a 19% decline in the prevalence of NTD – less than the estimated 50%. The study of Williams et al. (2005) covered a longer period and characterised the differences among racial/ethnic groups. Williams et al. (2005) reported that the prevalence of spina bifida decreased 36% among Hispanic births and 34% among non-Hispanic white births but the decrease was only borderline statistically significant among black births. The decreases in the prevalence of anencephaly were 26% among Hispanic births and 29% among non-Hispanic white births. In order to obtain further decreases in the occurrence of NTD, increased fortification levels and encouragement of women to consume more folic acid have been suggested (Wald et al., 2001b; Brent and Oakley, 2005).

Fortification is in many respects an effective way to decrease NTD occurrence, since it results in a significant increase in folate intake from food sources without major dietary modifications. It also avoids the problem of poor compliance related to another commonly recommended strategy, supplementation with folic acid tablets. The proportion of unplanned pregnancies can be as high as 50%, which means that without fortification, in many cases the malformations may occur

before the pregnancy has been confirmed and sufficient folate intake secured (McNulty et al., 2000).

There are, however, concerns over the possible adverse effects of synthetic folic acid. Since the utilisation of folic acid does not require vitamin B_{12}, folic acid may prolong the diagnosis of B_{12} anaemia which continues to progress until irreversible neurological disorders occur. In addition, Stover and Garza (2002) raised the concern that excess folic acid may increase spontaneous abortions and increase the prevalence of the C677TT variant of the MTHFR gene. The thermolabile C677T variant of MTHFR is a risk factor for early pregnancy losses and may also affect the risk of some chronic diseases, particularly when associated with low dietary folate intake.

Biotransformation of folic acid is saturated in the region of 260 to 280 μg (Kelly et al., 1997) but regular intake of smaller doses may also result in the appearance of unmetabolised folic acid in serum (Wright et al., 2007). The long-term effects of unmetabolised folic acid are largely unknown (Lucock, 2004). However, some recent findings have raised debate over mandatory folic acid fortification. For instance, Troen et al. (2006) reported that concentrations of free folic acid in serum correlated with the reduction of natural killer cell cytotoxicity among postmenopausal women, which might promote carcinogenesis, and Cole et al. (2007) found that folic acid supplements may increase the risk for colorectal neoplasia.

There has been considerable variation in the folate contents of cereal products in the era of fortification (Johnston and Tamura, 2004); in addition, analysed folate contents have often been higher than labelled (Rader et al., 2000; Whittaker et al., 2001). The fortification programme would benefit from a systematic monitoring system that would include determinations of food folate contents, stability and bioavailability, the effect of fortification on the folate status, the effect of long-term exposure to folic acid as well as the vitamin B_{12} deficiency history (Neuhouser and Beresford, 2001; Rader, 2002; Johnston and Tamura, 2004).

2.3 Folate in cereals

2.3.1 Folate content of cereals and cereal products

Folate exists in foods as several vitamers, mostly in reduced forms. Unlike many vegetables that contain mainly 5-methyltetrahydrofolate (Gregory et al., 1984; Vahteristo et al., 1997a; Konings et al., 2001), cereal products often contain a large variety of vitamers including methyl and

formyl derivatives as well as unsubstituted tetrahydrofolate (Pfeiffer et al., 1997; Konings et al., 2001).

Folates in plant materials usually exist in polyglutamyl form, and cereals are no exception. Since *Lactobacillus rhamnosus*, the organism used in the microbiological assay of folate, mainly gives a response to mono-, di- and to a certain extent to triglutamates, the difference in samples analysed with and without conjugase reflects the amount of polyglutamyl folates in the sample. Konings et al. (2001) reported that 66 ± 27% of folates in breads exist in the polyglutamate form. This is fairly consistent with results of Arcot et al. (2002) showing a 44% proportion of undeconjugated folate in wheat bread and an average 60% proportion in twelve Australian wheats. Müller (1993) recorded 76.5% free folate (monoglutamate folate) in grains and 65.6% in bakery products, that is, 23.5% and 34.4% polyglutamates, respectively. However, as there are many confounding factors in the concept of "free" folate, the results should be considered only as rough and indicative estimates of the proportion of polyglutamyl folate.

Folate vitamers may differ in their polyglutamyl chain-length distributions and exist only with certain chain lengths (Zheng et al., 1992; Seyoum and Selhub, 1998). The role of polyglutamate synthesis is not well understood. The polyglutamate chain affects the affinity of the folate molecule for folate-dependent enzymes as well as the retention of the molecule in the cells (Schirch and Strong, 1989; Rebeille et al., 1994). Thus, the polyglutamyl chain length may have an important role in metabolic regulation. Little information is available on the precise polyglutamyl distribution in cereals. Ndaw et al. (2001) reported that in wheat flour the number of polyglutamyl residues varied from one to six.

Cereals, and especially whole grain products, contribute greatly to the intake of dietary folate and are generally rated as good sources of folate. There is considerable variation in folate contents between different food composition databases (Table 2). Folate contents differ markedly according to the grain species, cultivars, and growing conditions. In addition, sampling and analytical methods also vary. The use of grains requires various types of processing such as milling and fractionation, baking, and thermal treatments that have a significant effect on the folate contents of the end products.

Table 2. Total folate contents in selected cereals and cereal products according to Finnish, U.S., and UK food composition databases (µg/100 g edible part)[a]

	Fineli, 2006	USDA, 2005	FSA, 2002
Rye flour	72	60	78
Wheat flour, whole grain	50	44	57
Barley flour	19	8	–
Oats	46	56	60
Rice, brown	49	20	49
Rye bran	72	–	–
Wheat bran	195	79	260
Wheat germ	520	281	–
Oat bran	46	52	–
Rye bread	60.5	51[b]	24
Rye crispbread	73.1	22[b]	35
Wheat bread	37.2	41[b]	40
Pasta, cooked	6.9	7[b]	4
Rice, cooked	11.3	4	10

[a] Items chosen on the grounds of having similar ash contents
[b] Endogenous folate

Rye has a clearly higher folate content than the other main cereals. However, folate in rye has not been investigated as intensively as folate in wheat. Many studies have reported considerably higher folate contents in rye than those presented in Table 2: 92 µg/100 g (Cerna and Kas, 1983); 135 µg/100 g (Gujska and Kuncewicz, 2005); and 143 µg/100 g (Müller,1993).

Data on the variation between rye cultivars are almost non-existent, whereas the variation between wheat cultivars has been investigated in some studies. Keagy et al. (1980) found that wheat flours derived from soft wheat classes had significantly lower folate contents than those derived from hard classes, whereas Mullin and Jui (1986) reported that folate contents in brans derived from soft wheat classes were approximately 50% higher than in brans derived from hard classes. Arcot et al. (2002) reported folate contents of 80 to 114 µg/100 g for twelve Australian wheats from different receival sites, and in four Polish wheat cultivars collected from the same station folate contents varied from 33.6 µg/100 g to 40.3 µg/100 g (Gujska and Kuncewicz, 2005). In two Polish rye cultivars, folate contents were 123 and 135 µg/100 g (Gujska and Kuncewicz, 2005).

2.3.2 Effect of processing on cereal folates

Processing is a prerequisite for the increased consumption of whole grain rye. Along with the traditional baking of rye bread, the demand for new kinds of products such as snacks and

convenience foods is growing, which is offering challenges for food industry to maintain or improve the nutritional quality of cereal products. This may be achieved by minimal processing, but also by means of novel processing methods or combinations of processes.

Folates are not evenly distributed in the grain. For instance, Fenech et al. (1999) reported folate concentrations of 94 µg/100 g in wheat bran flour and 515 µg/100 g in aleurone flour. According to Arcot et al. (2002), wheat bran contained more than twice as much folate (160 µg/100 g dry matter) as the grains, and Mullin and Jui (1986) determined even higher folate contents (224 to 360 µg/100 g dry matter) in brans from different wheat classes. High folate concentrations are also found in the germ (Table 2). The large differences may be explained by differences in milling and especially in the proportion of the folate-rich aleurone layer in the fractions. In wheat milling the ash and protein contents of the flour generally correlate with the folate content (Keagy et al., 1980).

Rye grain is harder and different from that of wheat, and requires special milling and fractionation procedures. Although rye is mainly consumed as whole grain in Finland, in practice rye is first fractionated and the "whole grain" flour is then assembled from several fractions in order to obtain uniform quality. Rye bran has high folate contents, and folate contents between milling fractions may vary by up to ten-fold (Liukkonen et al., 2003). Hegedüs et al. (1985) showed that with an 81% extraction rate the folate concentration of rye flour had decreased by one third, and with a 75% extraction flour by almost two thirds compared to the folate concentration of the whole grain (65 µg/100 g dry matter).

The majority of rye in the Finnish diet is consumed as sourdough fermented bread and crisp breads. Baking studies have mainly been conducted with wheat, and data on folates in sourdough fermentation are scarce. It has been known for a long time that yeast contributes greatly to the folate content: as much as 53 to 65% of folate in dough can be of yeast origin and as a result bread can have a higher folate content than the flour it is made of (Butterfield and Calloway, 1972; Keagy et al., 1975). The folate content of yeast is high but also strain specific. Hjortmo et al. (2005) screened 44 yeast strains for folate and found a range of 4000 to 14500 µg/100 g dry matter. The baking method (straight-dough or sponge-dough) does not seem to affect the folate content of bread (Keagy et al., 1975). Folate losses during final bread baking typically vary between 20 and 34%, despite the high baking temperatures (Butterfield and Calloway, 1972; Osseyi et al., 2001; Arcot et al., 2002).

Sourdough is traditionally made by mixing rye flour with water and allowing it to ferment with or without added yeast. Commercial starters are readily available, and many bakeries use their own sourdoughs that are maintained by back-slopping. In addition to yeasts, lactic acid bacteria have been important microbes in sourdough baking. The production of lactic and acetic acids has a significant effect on flavour and shelf life. A low pH increases the solubility of pentosans, which improves the baking properties as rye flour is not able to form a gluten network (Lönner and Ahrne, 1995). Sourdough lactic acid bacteria have been studied for their effects on bread properties, but their role in folate production or consumption and interactions with other microbes need to be elucidated. So far, the production of folate by lactic acid bacteria has mainly been assessed in dairy products and *in vitro* experiments, and the results have been inconsistent. For instance, Rao et al. (1984) and Lin and Young (2000) showed that *Lactobacillus acidophilus* produced folate but Crittenden et al. (2002) found it to deplete folate. These differences might be explained by different growing conditions or strain characteristics.

Germination (or malting) is known to be accompanied by an accelerated synthesis of folates. Malting is mostly used in beer production, but malted cereals are also added to bakery products in order to obtain a certain flavour, colour, or texture. According to Jägerstad et al. (2005), malting of barley resulted in a 2- to 3-fold increase in the folate content, and folate levels in beer malts were 200 to 300 µg/100 g fresh weight. In industrially malted oats and wheat, folate concentrations were 70 µg and 140 µg/100 g, respectively, and in malted rye 140 to 330 µg/100 g.

Thermal treatments such as puffing or extrusion are used in order to create a crunchy texture or to avoid microbiological spoilage of germinated grains. Losses of heat-sensitive vitamins are inevitable, but strongly depend on the processing conditions, mostly on temperature, time, and the presence of oxygen. Håkansson et al. (1987) examined the effect of autoclaving and popping on folates in whole grain wheat and the effect of extrusion cooking on white flour. Autoclaving resulted in folate losses of 33 to 39%, depending on process conditions, whereas losses in popping were higher, 26 to 72%. Losses in extrusion cooking were moderate, 19 to 22%. In conclusion, processes involving a short exposure to high temperatures or low-temperature processes seem to retain folates better than those including a long exposure to high temperatures.

2.4 Determination of folates in food

Analysis of folates in foods is challenging due to several reasons that have to be addressed when choosing the method of analysis. Folate concentrations in biological materials are relatively low

and the folates occur in various forms exhibiting different stabilities. Folates are very susceptible to heat, light, and oxygen. Moreover, the effect of the food matrix is important: folate may be physically entrapped in the matrix, which can limit the extractability. Many biological materials contain endogenous conjugases and other enzymes that are able to cause interconversion between vitamers and changes in vitamer distribution.

The most commonly used techniques in food folate analysis are microbiological assays, ligand-binding assays, and high-performance liquid chromatographic (HPLC) methods. Nevertheless, the critical steps often take place well before the actual measurement. Ensuring folate stability during the storage of samples, extraction, enzyme treatments, and purification requires special attention. According to an inter-laboratory evaluation with 26 participating laboratories (Puwastien et al., 2005), standardising the methods for extraction and detection, and the use of certified reference materials are strongly recommended.

2.4.1 Sample extraction and enzyme treatments

Extraction of folates from the food matrix is typically performed by heat treatment (boiling in a waterbath or autoclaving) in buffers with added reducing agents. The pH of the buffer is in the range of neutral or alternatively mildly acidic or alkaline. A buffer-to-sample ratio of at least ten is recommended (10 ml of buffer to 1 g sample, on a dry matter basis). Ascorbic acid is the most commonly used reducing agent. The use of 2-mercaptoethanol together with ascorbate was first recommended by Wilson and Horne (1984) to block formaldehyde formation from ascorbate. Mercaptoethanol can form a hemithioacetal with formaldehyde and thus prevent interconversion of folates.

Heat treatment releases folates from the food matrix and folate-binding proteins (Gregory et al., 1990b). Samples can be homogenised either immediately before or right after the heat treatment. Hyun and Tamura (2005) stated that heat extraction is not necessary if trienzyme treatment is performed. Their procedure eliminates one step where folate destruction is possible; however, it might not be suitable for all types of folate analysis. Homogenisation disrupts the food matrix and releases folates as well as enzymes catalysing interconversion. Thus, if vitamer distribution is to be determined, it is recommended to homogenise the samples after heat treatment (denaturation of enzymes) or perform the homogenisation in hot buffer.

Most of the folates in foods are in polyglutamate forms. As many analytical methods can measure only mono- and diglutamates, polyglutamates therefore need to be hydrolysed using

conjugases (γ-glutamylhydrolases; EC 3.4.22.12). Conjugases with different pH optima and end products can be derived from several sources, the most common being human and rat plasma, chicken pancreas, and hog kidney. Chicken pancreas conjugase, which mainly produces diglutamates, is commercially available and is perhaps the most frequently used conjugase in microbiological assays. Rat serum is also readily available, easy to use, and suitable for HPLC analysis of monoglutamyl folates. Hog kidney conjugase, which produces folate monoglutamates, requires extraction and purification steps. The activity of the conjugase may vary considerably and some food components may inhibit the activity (Pedersen, 1988; Engelhardt and Gregory, 1990). It is highly recommended to test the activity of a particular batch of enzyme in the sample material and to use significantly more conjugase than theoretically needed (Pedersen, 1988).

The proportion of matrix-bound folates may be significant. For instance, Cerna and Kas (1983) found that approximately 40% of folates in wheat and rye are bound to starch, and Yon and Hyun (2003) reported on average 41% matrix-bound folate in cereals and grain products (63% for wheat flour and 39% for white bread). DeSouza and Eitenmiller (1990) and Martin et al. (1990) demonstrated that the use of α-amylase and protease in addition to conjugase resulted in higher measurable folate contents in microbiological and radioassays. The usefulness of the trienzyme treatment has further been verified by Pfeiffer et al. (1997) in HPLC analysis of cereal products, Rader et al. (1998) in microbiological assay of fortified cereals, DeVries et al. (2001) in microbiological assay of cereal products, and by Yon and Hyun (2003) in microbiological assay of several food items. However, some studies have not confirmed the necessity of trienzyme treatment (Shrestha et al., 2000; Ndaw, 2001; Iwatani and Arcot, 2003).

There are several differences in the trienzyme treatment conditions that can affect the effectiveness of the treatment: incubation time, optimum temperature and pH, and the order of enzyme addition. Furthermore, the optimal conditions for enzyme action may not be optimal regarding folate stability. For instance, overnight incubation with protease may destroy labile folate vitamers (Pfeiffer et al., 1997). It has been stated that trienzyme treatment should be optimised separately for each sample material (Aiso and Tamura, 1998; Engelhardt and Gregory, 1990), although Hyun and Tamura (2005) later presented a recommended procedure summarising the results of several enzyme treatment studies and providing practical advice in choosing the suitable method.

2.4.2 Microbiological assay

Microbiological assay has been regarded as a tedious, time-consuming method requiring special expertise (Tamura, 1998). However, a few modifications have greatly contributed to the practicability of microbiological methods. Firstly, cryoprotection in glycerol reduces the time needed for the assay, increases the reproducibility, and simplifies the maintenance of the organism (Grossowicz et al., 1981; Wilson and Horne, 1982). Secondly, the use of microtiter plates instead of test tubes increases the sample through-put, lowers the detection limit, and reduces time and effort as the plates can be read and results calculated automatically (Newman and Tsai, 1986; Horne and Patterson, 1988). Thirdly, the use of a chloramphenicol resistant strain has reduced the need for aseptic working conditions (Chanarin et al., 1972; O'Broin and Kelleher, 1992).

The microbiological method is the most widely used method in analysing the total folate content in foods, and is the method of choice in the existing official methods of folate analysis (Table 3). Microbiological assays are based on nutrient – in this case, folate – requirements of a certain microorganism. The growth of the organism is dependent on the folate content of the sample and can be measured turbidimetrically. The most common microorganism in folate analysis is *Lactobacillus rhamnosus* ATCC 7469.

Table 3. Official methods for folate analysis

	AOAC 944.12	AOAC 992.05	AACC 86-47	EN 14131
Application	folic acid (pteroylglutamic acid) in vitamin preparations	folic acid (pteroylglutamic acid) in infant formula	total folate in cereal products (using trienzyme extraction)	total folate in foodstuffs
Microorganism	*Enterococcus hirae* ATCC 8043	*L. rhamnosus* ATCC 7469	*L. rhamnosus* ATCC 7469	*L. rhamnosus* ATCC 7469
Reference	AOAC, 2006	AOAC, 2006	AACC, 2000	EN 14131, 2003

L. rhamnosus ATCC 7469 gives a response to mono,- di, and triglutamates (Tamura et al., 1972); thus, enzymatic deconjugation of long-chain folylpolyglutamates is necessary. The microorganism may exhibit significantly different responses to different folate vitamers (Newman and Tsai, 1986; Goli and Vanderslice, 1989). Phillips and Wright (1982) showed that the response of *L. rhamnosus* ATCC 7469 to 5-methyltetrahydrofolate was lower than its response to 5-formyltetrahydrofolate or folic acid, especially in low concentrations. However, when pH was adjusted to 6.2 instead of 6.8, there was no difference in the responses. Rader et al. (1998) also showed that the sensitivity was better at pH 6.2 than 6.7.

The microbiological method is often used as a reference method, or "gold standard", in the validation of analytical methods measuring individual vitamers. This provides that equal responses to different vitamers have been confirmed. However, there is a possibility that the growth of the microorganism is either enhanced or inhibited by non-folate compounds in the sample.

2.4.3 Ligand-binding methods

The competitive radio protein-binding assay (RPBA) is perhaps the most common ligand-binding assay in folate determinations. It is based on competition between radiolabelled folate in the analysis kit and unlabelled folate in the sample for the binding sites of folate binding protein (FBP). In the non-competitive assay the sample is incubated with FBP, and the remaining binding sites are then determined by titration with radiolabelled folate. The protein-binding assay can also be performed by using enzyme-labelled FBP (EPBA; enzyme protein-binding assay) in which the bound enzymatic activity is determined by measuring the conversion of substrate to product (Finglas et al., 1988). Protein binding assays have been widely used for analysing folate in serum and red blood cells. However, assay pH and matrix effects may strongly affect the results (van den Berg et al., 1994). Purification of sample extracts prior to analysis may be required to eliminate matrix effects (Strålsjö et al., 2003). On the other hand, the analysis of food folate is complicated, since the affinities of folate vitamers to FBP vary (Rauch et al., 1989; Finglas et al., 1993; Wigertz and Jägerstad, 1995; Strålsjö et al., 2002). Two folate forms not occurring in the nature, PGA and (6*R*)-5-CH_3-H_4folate, have been reported to have the highest affinities for bovine FBP (Nygren-Babol et al., 2005). Among natural folates, H_4folate had the highest affinity for bovine FBP, followed by 5-CH_3-H_4folate and 5-HCO-H_4folate. The dissociation rate constants also varied markedly (Nygren-Babol et al., 2005).

Ligand-binding methods also include immunoassays that are based on the interaction between an antibody and its target molecule (antigen). The enzyme-linked immunosorbent assay (ELISA) is highly specific and suitable for folate determination in a complex matrix (Finglas and Morgan, 1994), whereas the radioimmunoassay (RIA) is rather limited and mainly suitable for the analysis of folic acid (Keagy, 1985). A biosensor-based, continuous flow system has been validated for the determination of folic acid in fortified foods (Caselunghe and Lindeberg, 2000).

In general, biospecific assays are specific, rapid, and simple to perform. However, they are often less sensitive than the microbiological assay. The kits tend to give lower responses for other folate vitamers than folic acid, and several studies have reported poor correlations between

ligand-binding and microbiological assays (Klein and Kuo, 1981; Gregory et al., 1982; Rauch et al., 1989; Finglas et al., 1993).

2.4.4 Chromatographic assays

Chromatographic methods allow the determination of folate forms, either folate vitamers or folates with different numbers of glutamyl residues. In addition, HPLC has been used for "total folate" analysis. Several HPLC methods have been developed for the determination of folate vitamers in food (e.g.Wigertz and Jägerstad, 1995; Vahteristo et al., 1996a; Vahteristo et al., 1997a; Pfeiffer et al., 1997; Konings, 1999). The main limiting factor in the development of HPLC methods in food folate determination has been the lack of suitable and specific purification methods. The most common purification procedures include weak or strong anion exchange, cation exchange, and affinity chromatography. Affinity chromatography has proved to be an efficient method for concentrating and purifying sample extracts, especially in cereal folate analysis. Table 4 presents some examples of HPLC methods in analysing cereals and cereal products for folates.

Usually, HPLC methods are based on either reverse-phase or ion exchange separation. Polyglutamates can be separated and vitamers determined by an ion-pair HPLC method (Varela-Moreiras et al., 1991; Seyoum and Selhub, 1993). Chain length determination can also be performed by the cleavage of the C^9–N^{10} bond and analysis of the resulting para-aminobenzoylglutamates (Shane, 1980; Eto and Krumdieck, 1982).

The most common detection principles are UV, diode array, fluorescence, and electrochemical detection (see Table 4 and Bagley and Selhub, 2000). Fluorometric detection is more specific and sensitive than UV detection; however, the fluorescence activities of PGA and 5-HCO-H_4folate are low. Detection methods can be used in combinations. For instance, Vahteristo et al. (1997a) utilised UV and fluorescence dual detection in confirming peak identity and purity.

Table 4. HPLC methods for the determination of folates in cereal and cereal products

Sample	Enzyme treatments	Purification	Column	Conditions	Detection	Reference
Whole wheat flour	Hog kidney conjugase	DEAE-Sephadex A-25	μ-Bondapak-Phenyl (300 x 3.9 mm, 10 μm)	Acetonitrile – phosphate buffer, pH 2.3, gradient	FL ex/em 295/356 nm	Gregory et al., 1984
Rye and wheat grains, oatmeal, breads	Hog kidney conjugase	DEAE-Sephadex A-25	μ-Bondapak-Phenyl and μ-Bondapak-C_{18} (300 x 3.9 mm)	Acetonitrile – phosphate buffer, pH 2.3, isocratic or gradient	FL ex /em 292/356 nm and 365/450 nm (UV 280 nm)	Müller, 1993
White and wheat bread, rice, pasta, breakfast cereal	Rat plasma conjugase, α-amylase, and protease	Affinity chromatography	Phenomenex Ultremex C_{18} (250 x 4.6 mm, 5 μm)	Acetonitrile – phosphate buffer, pH 2.3, gradient	DAD 280 nm	Pfeiffer et al., 1997
Breakfast cereal	–	SPE, C_{18}	Hypersil ODS (125 x 3 mm, 3 μm)	Acetonitrile – acetic acid, isocratic	LC-MS, negative ion electrospray	Stokes and Webb, 1999
Breads, pastries and cakes, cereal products	α-amylase, protease, and rat plasma conjugase	Affinity chromatography	Vydac 201 TP 54 (250 x 4.6 mm, 5 μm)	Acetonitrile – phosphate buffer, pH 2.1, gradient	DAD 280 nm, FL ex/em 280/359 nm and 360/460 nm	Konings et al., 2001
Breadmaking (flour, sponge, bread)	α-amylase and rat plasma conjugase	SPE, SAX (Quaternary amine)	Microsorb-MV C_{18} (100 × 4.6 mm, 3 μm)	Methanol –phosphate buffer, pH 6.8, tetrabutylammonium dihydrogenphosphate (ion-pairing agent), isocratic	UV 280 nm, FL ex/em 290/350 nm or 450 nm	Osseyi et al., 2001

Breakfast rolls	α-amylase, protease, and hog kidney conjugase	SPE, SAX	Zorbax SB-C_8 (150 x 4.6 mm, 5 µm)	Acetonitrile – acetic acid, gradient	DAD 290 nm, FL ex/em 290/360 nm	Johansson et al., 2002
Wheat bread, fortified wheat flour, wholemeal bread, rice	Protease, α-amylase, and rat serum conjugase	SPE, SAX (Quaternary amine)	Phenomenex Aqua C_{18} (250 x 4.6 mm, 5 µm)	Acetonitrile – acetic acid, gradient	LC-MS-MS, stable isotope dilution assay, positive electrospray, selected reaction monitoring (SRM)	Freisleben et al., 2003; Rychlik, 2004
Whole grain wheat, triticale, and rye, ready-to-eat cereals, wheat flours, rye flour, oatmeal, barley groats, buckwheat groats; wheat and rye breads	Rat plasma conjugase, α-amylase, and protease	Affinity chromatography	Phenomenex Luna C_{18} (250 x 4.6 mm, 5 µm)	Acetonitrile – phosphate buffer, pH 2.3, gradient	UV 290 nm, FL ex/em 290/356 nm and 360/460 nm	Gujska and Kuncewicz, 2005; Gujska and Majewska, 2005

The need for laborious purification can be bypassed by using microbiological detection (Belz and Nau, 1998). In this method, folate vitamers are separated on an HPLC system equipped with a fraction collector. Fractions are collected into microtiter plates and a microbiological assay is then performed. The absorbances of fractions are plotted against fraction number, resulting in a chromatogram. On the other hand, Ndaw et al. (2001) developed a sensitive, specific, and quantitative HPLC method in which all the folate vitamers of the sample undergo a chemical precolumn conversion to 5-CH_3-H_4folate, which is then detected fluorometrically. Naturally, this method does not allow the determination of individual folate vitamers but offers a good alternative to the microbiological assay of total folate.

The weak point in many HPLC methods has been the lack of suitable internal standard that could correct for procedural losses. The development of mass spectrometric methods has provided promising approaches to folate determination; however, applications to food folate analysis are still scarce. Stokes and Webb (1999) developed an LC-MS method with negative ion electrospray for the separation and identification of four folate vitamers and tested the method on a multivitamin tablet, breakfast cereal, and beef and vegetable extract. Pawlosky et al. (2003) determined PGA and 5-CH_3-H_4folate in five reference materials, and Freisleben et al. (2003) and Rychlik (2004) have used stable isotope dilution assay for the quantification of five folate vitamers in meat, vegetables, orange juice, and cereals. Mass spectrometric detection, although requiring technical expertise, has the advantage of being accurate and highly specific, but low sensitivity to some folate vitamers and matrix-specific problems limit its use.

Although HPLC methods have been recognised as specific and free of inherent uncertainty related to biological measurements, their use requires good knowledge on the chemistry of folate vitamers. In addition, folate vitamer concentrations in foods may be low, thus impeding reliable determination. In summarising five intercomparison studies, Finglas et al. (1999) recommended the use of spectrometrically calibrated standards and fluorescence detection, as well as ensuring folate stability during extraction and clean-up procedures. They concluded that further work is needed for the HPLC analysis of vitamers other than 5-CH_3-H_4folate. Most of the food composition data for folate have been determined by microbiological assay, but HPLC has also been successfully used for this purpose (Vahteristo et al., 1996a & b; Vahteristo et al., 1997a & b; Konings et al., 2001) and will probably become more popular in the future.

3. OBJECTIVES OF THE STUDY

The main objective of the study was to examine how and to what extent it would be possible to enhance folate contents in rye and rye products. Specific objectives were to:

1) optimise and evaluate methods for determining total folate and folate vitamer contents in rye and rye products (**I, II**);

2) study genetic variation in folate contents in rye (**I**);

3) investigate the effect of food processing methods – fermentation, germination, and thermal treatments – on folates in rye (**II, III, IV**); and

4) examine the ability of endogenous folates to improve the folate status in healthy human volunteers (**V**).

4. MATERIALS AND METHODS

4.1 Study design and sampling

4.1.1 Variation in folate contents in rye (I)

Ten rye cultivars representing both population and hybrid cultivars were grown in two successive years, 1999 and 2000, in Jokioinen, Finland. The effect of the cultivation method was examined by determining total folates from four rye cultivars, Amilo, Anna, Bor 7068, and Picasso, grown both organically and conventionally at the same location in the same year. Total folate contents were measured microbiologically using chloramphenicol-resistant *Lactobacillus rhamnosus* (NCIB 10463) as the growth indicator organism, and the vitamer distribution of a commercial wholemeal rye flour was determined by HPLC.

4.1.2 Effect of processing on folates in rye (II–IV)

The samples in fermentation and baking experiments (**II**) are listed in Table 5. In the first fermentation experiment (**II**), three rye breads were baked using different fermentations: yeast fermentation, yeast and lactic acid bacteria fermentation, and lactic acid bacteria fermentation. In addition, three wheat breads were baked using different baking methods: the sponge-dough method, straight-dough method and straight-dough method with baking powder (without added yeast). Commercial flours were obtained from a Finnish milling company, and breads were baked in the Laboratory of Cereal Technology at the Department of Food Technology,

University of Helsinki, using the multistrain starter available. Total folate contents were analysed from flours and breads microbiologically by *Lactobacillus rhamnosus* ATCC 7469, and folate vitamer distribution in breads was determined by HPLC..

In the second experiment (**II**), rye sourdough fermentations with and without added baker's yeast were compared. Samples were taken at six points of the baking procedure (flour, sourdough start, sourdough end, dough, proofed dough, and bread) and analysed microbiologically for total folate. Two different starters, one a multistrain laboratory starter without added yeast and the other containing baker's yeast, *Lactobacillus brevis*, and *Lactobacillus plantarum*, and slightly different baking parameters were applied. In addition, fermentation with added baker's yeast, *L. brevis*, and *L. plantarum* was performed using two different rye cultivars, small-grain Akusti and large-grain Amilo. Folate vitamers were determined by HPLC from sourdough fermentation with added yeast.

Table 5. Sampling in the fermentation and baking experiments (**II**)

Experiment	Samples	Folate analysis method
Wheat baking	Wheat flour	Flours: MA
Sponge-dough method	Bread	Breads: MA, HPLC
Straight-dough method	Bread	
Baking powder leavening	Bread	
Rye baking	Rye flour	Flours: MA
Yeast fermentation	Bread	Breads: MA, HPLC
Yeast and lactic acid bacteria fermentation	Bread	
Lactic acid bacteria fermentation	Bread	
Rye sourdough fermentation with added baker's yeast; cultivar 'Akusti'	Flour	MA and HPLC
	Sourdough, start	
	Sourdough, end	
	Dough	
	Proofed dough	
	Bread	
Rye sourdough fermentation with added baker's yeast; cultivar 'Amilo'	Flour	MA
	Sourdough, start	
	Sourdough, end	
	Dough	
	Proofed dough	
	Bread	
Rye sourdough fermentation without added baker's yeast	Flour	MA and HPLC
	Sourdough, start	
	Sourdough, end	
	Dough	
	Proofed dough	
	Bread	

The microflora in rye sourdough fermentation was then studied at the test tube scale (**III**). Baker's yeast *Saccharomyces cerevisiae*, and three other yeasts, *Candida milleri*, *Saccharomyces cerevisiae* TS 146, and *Torulaspora delbrueckii* as well as four *Lactobacillus* spp., *L. acidophilus*, *L. brevis*, *L. plantarum*, and *L. sanfranciscensis* originally isolated from rye sourdough were examined for their folate production or consumption. The microorganisms were grown both in specific media (YPD or Elliker) and in sterile or non-sterile rye flour – water mixtures. Some other lactobacilli strains and three bacteria isolated from the non-sterilised rye flour were also screened for folate production. Total folates were analysed microbiologically by *L. rhamnosus* ATCC 7469.

Germination was also examined as a potential way to increase folate contents of rye. The increase in folate concentration during germination, the optimal germination conditions, and the effect of thermal treatments (extrusion, autoclaving and puffing, and IR and toasting) were studied (**IV**). Three germination variables, germination temperature (8, 15, and 22 ºC), time (3, 5, and 7 days) and drying temperature (50, 75, and 100 ºC), were included, and a central composite design was applied to arrange the experiments. Results were analysed using multiple regression methods. Thermal processing studies were performed with both native and germinated rye. Extrusion was performed using an APV MPF 19/25 twin screw extruder with die diameter of 3 mm. The temperatures of four heating/cooling barrel zones were 140ºC, 140ºC, 120ºC, and 20ºC, and the screw speed was 225 rpm. The retention time was about 30 s. The total load was 60% for native and 76% for germinated rye. In autoclaving and puffing grains were first mixed with water (1:1) and autoclaved at 120ºC for 0.5. The grains were then dried and toasted in a rack oven (Sveba Dahlin 900) at 175ºC for 30 min. In IR and toasting grains were first moistened to a moisture content of 20 to 30% at 80ºC. Grains were then heat-treated under radiated heat (375 W Osram lamps) for 5 min and heated in a rack oven at 200ºC for 10 min.

Folate analyses were performed using the microbiological method with *Lactobacillus rhamnosus* ATCC 7469; in addition, vitamer distributions of selected germinated and heat-treated samples were analysed by HPLC. From some germination batches the separated hypocotylar roots were collected and analysed for folates, both microbiologically and by HPLC.

4.1.3 Functionality of endogenous folates *in vivo* (V)

Healthy volunteers participated in a four-week intervention study (**V**). The intervention was conducted as a parallel study with two groups: a group receiving natural folates from rye and

orange juice (33 volunteers) and another receiving folic acid fortified white bread and apple juice (31 volunteers). Test foods, which were consumed as a part of the normal diet, provided the subjects with 184 µg folate/day in the rye group and 188 µg folate/day in the wheat group. Rye-based tests foods included different types of fresh and dried rye breads and a high-folate rye muesli manufactured from malted rye grain flakes. The change in the folate status of the volunteers was evaluated by the serum and red cell folate as well as plasma homocysteine at the beginning and end of the study period.

4.2 Folate analysis

An outline of folate analysis is presented in Figure 4. Analytical procedures were carried out under yellow or subdued light. Alternatively, samples and calibrants were covered with aluminium foil. Sample extracts were kept under nitrogen atmosphere whenever feasible.

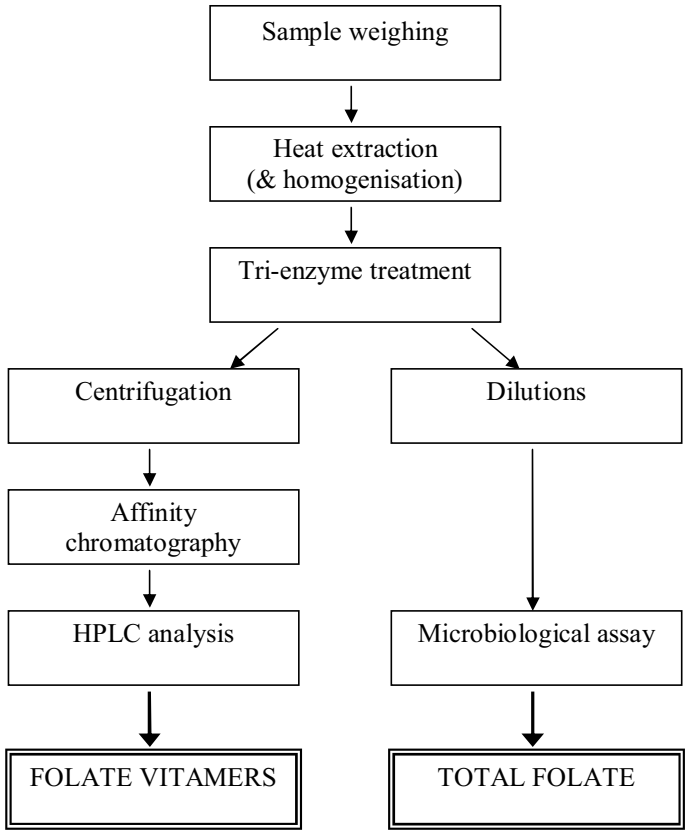


Figure 4. Schematic presentation of folate analysis.

4.2.1 Calibrants

Tetrahydrofolate was obtained from either Dr Schirck's Laboratories (Jona, Switzerland) (tetrahydrofolate trihydrochloride) or Eprova AG (Schaffhausen, Switzerland; [6S]-tetrahydrofolate, sodium salt). 5-Methyltetrahydrofolate ([6S]-5-CH_3-H_4folate, calcium salt) was obtained from Dr Schirck's Laboratories or Eprova AG. 5-Formyltetrahydrofolate ([6S]-5-HCO-H_4folate, sodium salt) was obtained from Eprova AG (Schaffhausen, Switzerland), and 10-formylfolic acid (10-HCO-PGA) and folic acid (PGA) from Dr Schirck's Laboratories (Jona, Switzerland). Calibrants were dissolved as described by van den Berg et al. (1994), and the purities were calculated using molar absorptivity coefficients at pH 7.0 (Table 6). Standard solutions, typically 10 to 120 µg folate/ml, were flushed with nitrogen and stored in 0.01M acetate buffer containing 1% (w/v) sodium ascorbate (pH 4.9) at –20 ºC.

10-Formyldihydrofolate (10-HCO-H_2folate) was synthesised from 5,10-methenyltetrahydrofolate hydrochloride ([6R,S]-5,10-CH^+-H_4folate, chlorine hydrochloride, Eprova AG) according to Pfeiffer et al. (1997) with a somewhat longer reaction time (2.5 hours). Standard solution was flushed with nitrogen and stored in 0.05M Tris/HCl (pH 8.4) at – 20 ºC.

Table 6. Molecular weights, molar absorption coefficients, and absorption maxima of folate vitamers (Blakley, 1969; Baggott et al., 1995)

Vitamer	Molecular weight (g/mol)	ε ($1 x mmol^{-1} x cm^{-1}$)	λ_{max} (nm)	pH
H_4folate	445.4	29.1	297	7.0
5-CH_3-H_4folate	459.4	31.7	290	7.0
10-HCO-H_2folate	471.4	34	234	7.4
10-HCO-PGA	469.4	20.9	269	7.0
5-HCO-H_4folate	473.4	37.2	285	7.0
PGA	441.4	27.6	282	7.0

4.2.2 Extraction and tri-enzyme treatment

The sample amount was 0.5 to 2 g, depending on the homogeneity of the sample and the estimated folate content. Samples (usually in duplicate) were weighed to plastic tubes, and 10 to 35 ml of extraction buffer (50 mM Ches, 50 mM Hepes, containing 2% sodium ascorbate and 10 mM 2-mercaptoethanol, pH 7.85) was added. The buffer-to-sample ratio was always at least 10. Samples were flushed with nitrogen, placed in a boiling water bath for 10 min, cooled on ice and homogenised, if necessary. Then, pH was adjusted to 4.9 with HCl and the extracts were again flushed with nitrogen. Extracts could be kept at –20 ºC for up to two weeks before analysis. A blank sample was analysed in each set of samples and the results were corrected accordingly.

Hog kidney conjugase (HK) was prepared from fresh kidneys according to Gregory et al. (1984) and its activity was tested according to Vahteristo et al. (1996a). Other enzymes, α-amylase (EC3.2.1.1) and protease (EC3.4.24.31), were obtained from Sigma (St. Louis, MO). Sample extracts were first incubated under a nitrogen atmosphere with α-amylase and HK for 3 h at 37 ºC in a waterbath. After that, the pH was adjusted to 7.0 with KOH and protease was added. Extracts were incubated under a nitrogen atmosphere for 1 h at 37 ºC, after which they were boiled for 5 min in a boiling waterbath to inactivate the enzymes, and cooled on ice. Extracts or aliquots of them for HPLC were centrifuged for 20 min at 13 000 rpm at 4 ºC. The remaining residue was suspended in 10 ml of extraction buffer and recentrifuged. Supernatants were then combined and filtered through 0.2 or 0.45 μm syringe filters.

4.2.3 Microbiological assay (MA)

At first (**I**), a chloramphenicol-resistant strain, *Lactobacillus rhamnosus* NCIB 10463, was used as the growth indicator organism. Cryoprotection and preparation of working inoculum were performed according to Molloy and Scott (1997). Eleven levels of the calibrant, corresponding to 0 to 50 pg of PGA, were pipetted into 96-well microtiter plates (Tissue culture treated; Costar Corporation, Cambridge, MA), four wells for each level. Sodium ascorbate (0.5%) was added to the calibrant-containing wells so that the final volume in each well before adding the inoculated medium was 100 μl. Two dilutions, typically varying from 1:400 to 1:50, were prepared from each sample to 0.5% (w/v) sodium ascorbate, and 100 μl of each dilution was pipetted into four wells. Inoculated medium was then added into each well (200 μl). After 42 h incubation at 37 ºC the optical densities of the wells were measured with a microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland) at 595 nm after 10 s mixing at 1150 rpm.

In studies **II** to **IV** , another *Lactobacillus* strain, *L. rhamnosus* ATCC 7469, was used as it was easier to grow and the incubation time was shorter. Although no systematic method comparison was performed, the two methods gave similar results for reference materials (data not published). *L. rhamnosus* was glycerol-cryoprotected according to the draft of European Standard for the determination of folate by microbiological assay (see EN 14131, 2003); however, instead of autoclaving the culture medium was sterile filtered (Acrodisc Syringe filters, 0.2 μm; Pall Gelman Laboratory, Ann Arbor, MI). Assay medium (Folic acid Casei Medium; Difco, Becton Dickinson & Co, Sparks, MD, USA) was used at 75% of the recommended strength, and 0.075 g of ascorbic acid was added to the medium after heating (Molloy and Scott, 1997). The pH was adjusted to 6.1 with acetic acid. To 1 ml of cryoprotected inoculum, 2.5 ml of sterile saline was

added (EN 14131, 2003), and 100 ml of culture medium was inoculated with 300 µl of this solution.

Sample extracts were diluted with 0.5% (w/v) sodium ascorbate, pH 6.1. Sample dilutions and the calibrant (eight levels, 0 to 80 pg 5-HCO-H_4folate/well) were pipetted as before. Inoculated medium (200 µl/well) was then added, and the plates were incubated for 18 to 20 h at 37ºC. Mixing the plates before the turbidometric measurement was not necessary.

4.2.4 Affinity chromatographic purification

Affinity columns were prepared by coupling folate binding protein (FBP) from bovine milk (Scripps Laboratories, CA, USA) to agarose (Affi-Gel 10; Bio-Rad Laboratories, Richmond, CA, USA) as described by Konings (1999). The gel volume per column was approximately 1.8 to 2 ml.

Columns were equilibrated with 10 ml of 0.1M potassium phosphate buffer (pH 7.0). Filtered sample extracts were applied to the columns slowly (0.35 to 0.6 ml/min). A vacuum manifolder was not usually needed. Columns were then washed with 5 ml of 0.025M potassium phosphate/1M NaCl (pH 7.0), followed by 5 ml of 0.025M potassium phosphate (pH 7.0). Folates were eluted with 4.95 ml of 0.02M trifluoroacetic acid/0.01M dithiothreitol to 5 ml volumetric flasks containing 30 µl 1M piperazine, 0.2% sodium ascorbate, and 5 µl 2-mercaptoethanol. After elution, the columns were washed with 1.5 ml of elution solution and with 10 ml of 0.1M potassium phosphate buffer (pH 7.0). Columns were stored in 0.1M potassium phosphate buffer containing 0.2% sodium azide (pH 7.0) at 4 ºC.

4.2.5 High-performance liquid chromatography (HPLC)

A Varian Vista 5500 liquid chromatograph and Waters 712 Satellite Wisp Autosampler with a cooler (Waters, Milford, MA, USA) were initially used (**I**). These were replaced by Waters 510 and 515 HPLC pumps and a Waters 717 plus Autosampler in later experiments (**II, IV**). In both systems the detection was carried out by a Waters 2487 Dual λ Absorbance detector set at 290 nm and by a Waters 470 fluorescence detector. The fluorescence detector was set at 290 nm excitation and 356 nm emission wavelengths for reduced folates, and 360 nm/460 nm for 10-HCO-PGA. The Waters Millennium 2020 Chromatography Manager data acquisition system was used to collect and calculate chromatographic data. Quantification of folate vitamers was based on an external standard method with peak areas plotted against concentrations. Calibrants (eight levels) were purified with affinity chromatography.

Folate monoglutamates were separated on a Shandon (ThermoQuest; Cheshire, UK) Hypersil ODS column (150 mm × 4.6 mm; 3 μm particle size). The column temperature was kept at 30 °C. Gradient elution was performed with acetonitrile and 30 mM potassium phosphate buffer, pH 2.2 at a flow rate of 0.9 mL/min. The gradient started at 5% (v/v) acetonitrile, which was maintained isocratically for the first 9 min and then raised to 7% within 13 min. Thereafter, the acetonitrile concentration was raised to 16% within 9 min and maintained for 2 min. Injection volumes were 25 to 200 μl. Peaks were identified by retention times, and their identities were confirmed by spiking purified sample extracts and by comparing the ratio of fluorescence and UV intensities of a certain peak to the respective ratio of the calibrant.

4.2.6 Method validation and quality assurance

The short-term stability of folates at different stages of analytical procedures was tested in order to justify the storage conditions (**II**). Mixtures of calibrants in affinity chromatography elution solution were kept at –20 ºC (freezer temperature) and 4 ºC (autosampler temperature), and the loss of folates was determined by HPLC. Folate stability was also tested for mixtures of calibrants in extraction buffer as such (pH 7.85) and after heat extraction with pH adjusted to 4.9.

Affinity chromatography testing included the FBP binding capacity tests with PGA, recoveries of calibrants, and binding of formylated vitamers to FBP-affinity columns (**I**). Affinity columns were loaded with different amounts of 5-HCO-H_4folate, 10-HCO-PGA, and 10-HCO-H_2folate, alone or in combination with other vitamers, so that the total folate load was approximately 10 to 50% of the capacity determined by overloading the columns with PGA.

Different gradients were tested in HPLC in order to optimise the peak separation and shape. Gradients were evaluated on the basis of resolution, relative retention, capacity factor, and tailing factor. Other HPLC validation data included numbers of theoretical plates, detection limits (signal-to-noise ratio ≥ 3; determined for calibrants), and linearity as well as within-day and between-day variation of the detector response and retention times.

Recovery tests were performed for rye fermentation samples (**II**) by spiking the samples before extraction with folate calibrants at a level of 60 to 120 μg/100 g and analysing the spiked and unspiked samples by HPLC. Recovery in the microbiological assay was studied by spiking Certified Reference Material (CRM) 121 (wholemeal flour; obtained from the Institute for Reference Materials and Measurements, Geel, Belgium) with a known amount of 5-HCO-

H_4folate; the latter was also analysed alone as a sample. Folate results for samples were not corrected for recovery values. Based on multiple analysis of CRM 121 a control chart was constructed and used in quality control of the microbiological assay (**II, III, IV**). Criteria for the minimum turbidity of the highest calibrant level and the maximum turbidity of the lowest calibrant level were also set in order to follow the repeatability of the growth of *L. rhamnosus* (**II**). Moreover, an integral part of the evaluation of method performance was the comparison between microbiological and HPLC results of both proficiency testing materials and processed rye samples (**II, IV**).

Method accuracy was evaluated not only by analysing certified reference materials but also by participating in proficiency tests organised by an EU-funded project "Folate: from food to functionality and optimal health" (FolateFuncHealth, QLRT-1999-00576), a Nordic comparison Norfolate, and FAO/INFOODS comparison. These intercomparisons included several foods with different folate concentrations and vitamer distribution patterns (Table 7).

Table 7. Overview of the interlaboratory studies participated in

	FolateFuncHealth	Norfolate	INFOODS
Year	2000	2000–2001	2001
Participants	8 European laboratories	6 Nordic laboratories	26 laboratories worldwide
Test materials	Four CRMs: 121 Wholemeal flour 421 Milk powder 485 Mixed vegetables 487 Pig's liver	Liver pate Freeze-dried broccoli Wholemeal flour (CRM) Milk powder (CRM)	Soybean flour Fish powder Breakfast cereal
Rounds	1	2	1
Folate levels	30 to 1340 µg/100 g DM	40 to 1230 µg/100 g	81 to 498 µg/100 g (MA)
Methods used by our laboratory	HPLC	MA	MA

In the intercomparison organised by the FolateFuncHealth project, each laboratory used its own method of analysis. The number of accepted results was 7 to 9 for each CRM, 3 to 4 of which were obtained by microbiological assay (MA) and 4 to 5 by HPLC. Two of the HPLC laboratories determined 5-methyltetrahydrofolate only, and one of the HPLC laboratories used microbiological detection (FolateFuncHealth, 2005).

In the Norfolate ring test, six laboratories analysed four samples, two of them CRMs, in two rounds. In each round, samples were to be analysed in duplicate on two different days. All the laboratories performed the analyses with the microbiological assay (Lindeberg et al., 2001).

In the proficiency test organised by FAO/INFOODS, three test materials were analysed, and the methods used included microbiological assay (22 laboratories), HPLC, LC-MS, and radioassay (Puwastien et al., 2005).

5. RESULTS

5.1 Validity of the analytical methods

5.1.1 Affinity chromatography and HPLC

The binding capacities of the affinity columns varied between 4.6 μg and 5.0 μg folic acid/ column, and the columns could be used for tens of times without considerable changes in the capacity. Recoveries of different vitamers in affinity chromatography were satisfactory, 83 to 102% of the binding capacity. However, the binding of 5-HCO-H_4folate was strongly affected by the total folate load. The recovery of 5-HCO-H_4folate was above 90% if the total folate load was kept under 25% of the column capacity, but decreased sharply if the total folate load was increased. 5-HCO-H_4folate alone, without the presence of other vitamers, was well bound to the FBP. The interfering effect of the total folate load on binding to FBP was not observed with other formylated vitamers.

The chosen conditions for HPLC offered satisfactory separation of six folate vitamers (**I**). Detection limits were 0.04 to 0.9 ng per injection, and the responses of the detectors were linear in the routine working concentration range. The detectors used for different vitamers, detection limits, and the main problems encountered are presented in Table 8. Between-day variations in the detector response and retention times expressed as CV% from six injections during three weeks were 1.7 to 10.4% and 2.4 to 7.4%, respectively. The HPLC method was to some extent able to separate isomers in the racemic mixture (6*R,S*) of 5-HCO-H_4folate. Thus, the natural 6*S* isomer of 5-HCO-H_4folate was chosen as the calibrant.

Table 8. Detection of folate vitamers

Vitamer	Detector	Detection limits; UV/FL (ng/injection)	Comments
H_4folate	UV/FL	0.6/0.08	FL more sensitive; UV peak often masked by an impurity (derived from affinity chromatography?)
5-CH_3-H_4folate	UV/FL	0.4/0.04	double peak in some processed samples
10-HCO-H_2folate	UV	0.8/-	UV only; sometimes masked by impurities
10-HCO-PGA	UV/FL	0.7/0.1	FL more sensitive; UV offered support in identification
5-HCO-H_4folate	UV/FL	0.6/0.6	FL more sensitive but peak often masked by impurities
PGA	UV	0.2/-	UV only

The stability of folates in the extraction buffer at –20 ºC was better after heat extraction and pH adjustment to 4.9 than in the extraction buffer as such (pH 7.85) (**II**). No significant loss of any folate vitamer occurred during the first two weeks. The short-term stability of folates in the affinity chromatography elution solution was also good: two days at 4 ºC or five days at –20 ºC did not result in folate losses.

5.1.2 Recovery studies and analysis of certified reference materials (CRMs)

Recovery of 5-HCO-H_4folate added to CRM 121 (wholemeal flour) or analysed like a sample was 105% in the microbiological assay (**II**). Recoveries of vitamers added to six rye fermentation samples and analysed by HPLC (**II**) were 72 to 104% (Table 9).

Table 9. Average recoveries of folate vitamers in sourdough baking samples (n = 6)

Vitamer	Recovery (%)
H_4folate	72 ± 5
5-CH_3-H_4folate	104 ± 18
10-HCO-H_2folate	84 ± 8
10-HCO-PGA	98 ± 16
5-HCO-H_4folate	83 ± 39
PGA	96 ± 7

In Study **II** a control chart was constructed based on microbiological analysis of CRM 121. The average folate content of CRM 121 was 51.1 ± 6.5 µg/100g DM (n = 10). Other CRMs were also analysed in proficiency testing programmes (Table 10).

Table 10. Analysed (our laboratory) and certified values for certified reference materials (µg/100 g DM)

	FolateFuncHealth 2000 HPLC (n = 4)	Norfolate 2000–2001 MA (n = 4)	Certified value
CRM 121 Wholemeal flour	31 ± 1	51 ± 2	50 ± 7
CRM 421 Milk powder[a]	87 ± 8	134 ± 16	142 ± 14
CRM 485 Mixed vegetables	241 ± 4	not included	315 ± 28
CRM 487 Pig's liver	1496 ± 76	not included	1340 ± 130

[a] Fortified with folic acid

Microbiological results agreed well with the certified values. Results obtained in our laboratory by HPLC were lower than those obtained by microbiological assay for the two matrices tested.

5.1.3 Proficiency tests

In addition to the analysis of certified reference materials, accuracy and performance were evaluated by participating in three proficiency tests.

In the intercomparison organised by the FolateFuncHealth project the average folate contents of four certified reference materials were lower than the certified values, and results obtained by HPLC were in most cases lower than results obtained by microbiological assay (Table 11). Within-laboratory variation was generally small (4 to 8%) whereas between-laboratory variation was large (22 to 39%; FolateFuncHealth, 2005). The values reported by our laboratory (obtained by HPLC) were 23 to 38% lower than the certified values except for CRM 487 (pig's liver).

Table 11. Analysed and certified values for four certified reference materials (µg/100 g DM) in the FolateFuncHealth intercomparison study

	HPLC; our laboratory (n = 4)	HPLC (n = 4–5)	MA (n = 3–4)	All (n = 7–9)	Certified value
CRM 121 Wholemeal flour	31 ± 1	30 ± 13	50 ± 10	39 ± 15	50 ± 7
CRM 421 Milk powder	87 ± 8	97 ± 19	134 ± 21	116 ± 27	142 ± 14
CRM 485 Mixed vegetables	241 ± 4	217 ± 49	296 ± 22	252 ± 56	315 ± 28
CRM 487 Pig's liver	1496 ± 76	1056 ± 540	1040 ± 170	1049 ± 400	1340 ± 130

In the Norfolate ring test the results obtained by our laboratory were highly repeatable and comparable with others. All the participating laboratories used microbiological assay for folate determination, and precision values were surprisingly good (Lindeberg et al., 2001).

In the proficiency test organised by FAO/INFOODS, among the 17 laboratories that used the microbiological assay with *L. rhamnosus*, the inter-laboratory coefficients of variation were 19% for soybean flour, 23% for fish powder and 18% for breakfast cereal after removal of extreme values. The results reported by our laboratory (Table 12) were well within the range of average values obtained by microbiological methods. Z-scores of our laboratory varied between -1.0 and 0.2 (Puwastien et al., 2005).

Table 12. Values for three test materials (µg/100 g) determined microbiologically with *L. rhamnosus* in the INFOODS intercomparison study (Puwastien et al., 2005)

	Mean of results obtained by our laboratory; tri-enzyme extraction (n = 3)	Mean of results with tri-enzyme extraction (n = 7)	Mean of all results with tri-, di-, or single enzyme extraction[a] (n = 13–15)
Soybean flour	267 ± 7	288 ± 69	277 ± 52
Fish powder	74 ± 6	100 ± 34	81 ± 19
Breakfast cereal	471 ± 8	515 ± 171	498 ± 92

[a] Extreme values removed

5.2 Folates in rye

5.2.1 Folates in rye grains (I)

Microbiologically determined folate contents of the ten rye cultivars grown at the same location varied from 68.9 µg/100 g to 85.3 µg/100 g (on dry matter basis) in 1999 and from 64.3 µg/100 g to 93.4 µg/100 g in 2000 (Figure 5). Average folate contents were 77.2 ± 5.9 µg/100 g in 1999 and 81.8 ± 9.1 µg/100 g in 2000 (70.0 ± 5.5 µg/100 g fresh weight and 73.8 ± 8.3 µg/100 g fresh weight, respecively). Coefficient of variation between the folate contents in rye cultivars was 8% in 1999 and 11% in 2000, and there was no statistically significant difference between the years.

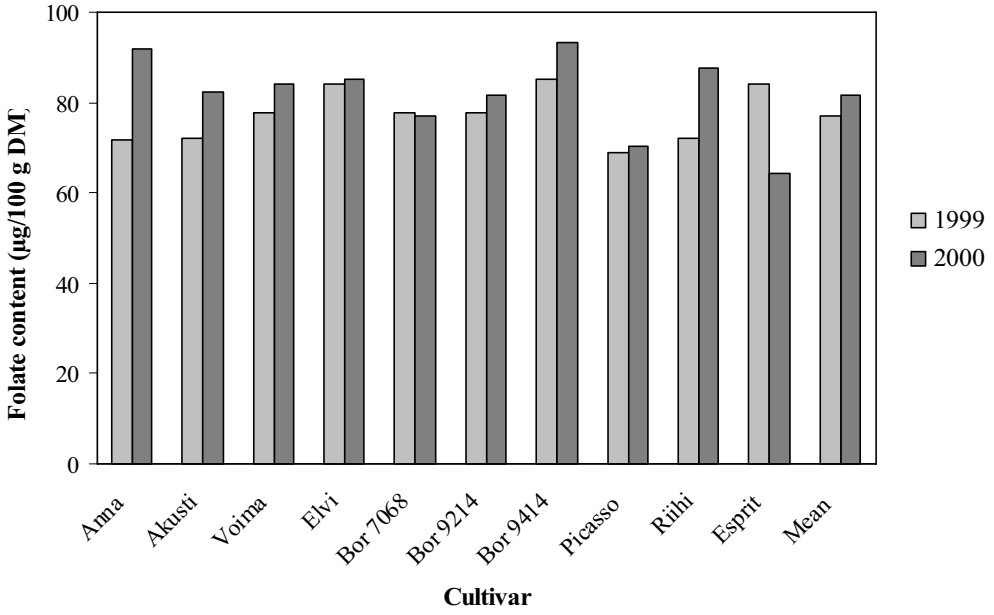


Figure 5. Total folate contents and their means in ten rye cultivars in two successive years (µg/100 g DM).

Total folate contents were somewhat higher in the organically than in conventionally grown cultivars but the difference (3 to 12%) was not statistically significant (Figure 6).

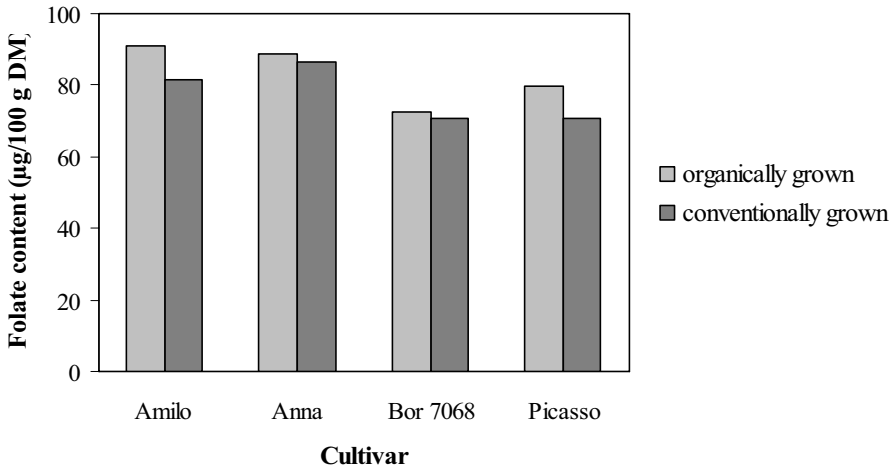


Figure 6. Total folate contents in organically and conventionally grown rye cultivars (µg/100 g DM).

The sum of vitamers in wholemeal rye flour analysed by HPLC was 53.1 ± 5.1 µg/100 g (n = 8). The predominant vitamers were 5-CH_3-H_4folate (26 ± 4% of the vitamer sum), 5-HCO-H_4folate (25 ± 5.4%), 10-HCO-H_2folate (26 ± 7.2%), and 10-HCO-PGA (15 ± 5%). Minor amounts of H_4folate and PGA were also detected. Thus, the formylated folates were the main vitamers in rye.

5.2.2 Fermentation and baking (II)

In the first experiment with rye and wheat breads baked using different fermentations and baking methods, the lowest folate contents were found in breads that were baked without added yeast. The total folate content of rye bread fermented with lactic acid bacteria was 29 µg/100 g DM, whereas the other two rye breads (yeast fermented, yeast and lactic acid bacteria fermented) contained 42 µg/100 g DM. Wheat bread leavened with baking powder had a folate content of 17 µg/100 g DM, and the other two wheat breads a folate level of 45 to 50 µg/100 g DM. Sponge-dough and straight-dough methods resulted in similar folate contents in wheat breads (50 and 45µg/100 g DM, respectively) .

The predominant vitamer in rye breads was 5-HCO-H_4folate, whereas in wheat breads 5-CH_3-H_4folate and 10-HCO-H_2folate were more abundant than 5-HCO-H_4folate. In rye and wheat breads baked without added yeast the concentrations of 5-CH_3-H_4folate and 5-HCO-H_4folate were low, and no H_4folate could be detected.

In the second experiment with rye sourdough fermentation the microbiologically determined folate contents were highest at the end of the fermentation if yeast was added; the increases during the fermentation phase were 54% and 128% in two separate fermentations. The two vitamers mainly responsible for the increase were 10-HCO-H_2folate and 5-CH_3-H_4folate. In contrast to fermentation with added yeast, the folate concentration during fermentation without added yeast remained unchanged (Figure 7).

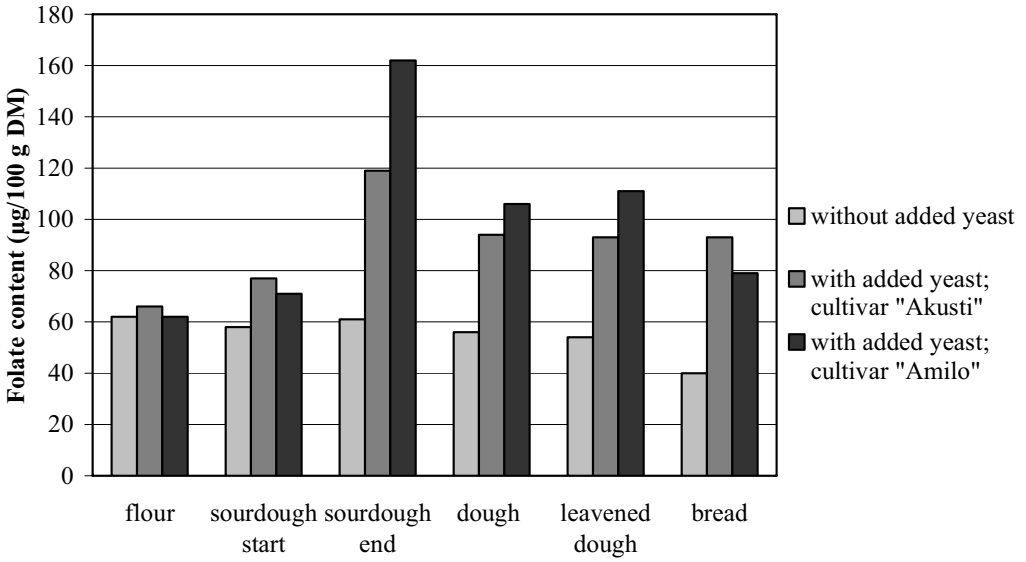


Figure 7. Total folate contents during sourdough baking of rye (µg/100 g DM).

The folate content decreased when the dough was mixed as the addition of other ingredients diluted the folate concentration. Proofing did not seem to affect the total folate content; however, the vitamer distribution pattern changed. For example, the amount of H_4folate increased three-fold and the amount of 10-HCO-H_2folate dropped by a half.

Baking losses were approximately 25%. H_4folate was almost completely destroyed, and the losses of 5-CH_3-H_4folate and 5-HCO-H_4folate were 67% and 76%, respectively. Levels of 10-HCO-H_2folate, PGA, and especially 10-HCO-PGA increased during the baking stage.

5.2.3 Yeasts and lactic acid bacteria in sourdough fermentation (III)

The four yeasts and four lactic acid bacteria did not excrete folates into the microbiological growth medium in significant amounts. In test tube fermentation experiments with sterilised rye

flour, *S. cerevisiae* ALKO 743 and *C. milleri* CBS 8195 produced similar amounts of folate: from an initial concentration of 6.5 µg/100 g to 22.3 ± 3.2 µg/100 g and 22.8 ± 4.8 µg/100 g after 19-hour incubation, respectively (Figure 8). Folate productions of *S. cerevisiae* TS 146 and *T. delbrueckii* TS 207 were significantly lower (15.4 ± 2.0 µg/100 g and 15.3 ± 4.2 µg/100 g). All four of the mainly studied lactic acid bacteria depleted folates: the folate concentration after 19 hours ranged from 2.9 to 4.2 µg/100 g.

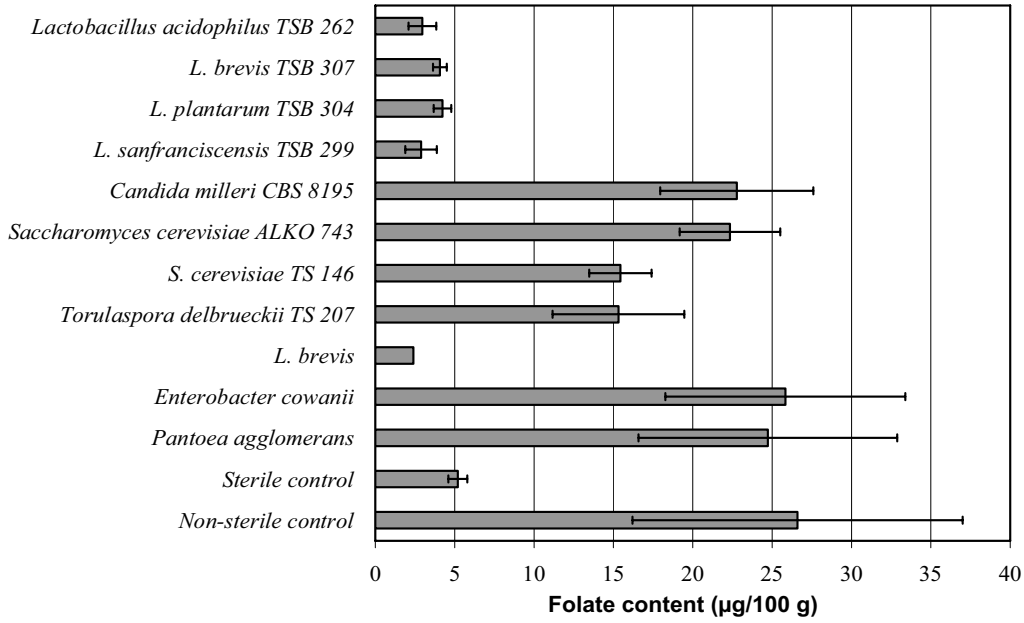


Figure 8. Total folate contents (µg/100 g FW) of monocultures of yeasts and bacteria grown in sterile rye flour – water at 30 °C for 19 h.

Only two of the additionally screened lactic acid bacteria, *L. bulgaricus* ABM 5096 and *S. thermophilus* ABM 5097, produced folate (8.1 ± 3.1 µg/100 g and 10.4 ± 2.3 µg/100 g).

Folate productions with a yeast alone and in pairwise combination with lactic acid bacteria were similar. Thus, combinations with the highest folate contents included *S. cerevisiae* ALKO 743 or *C. milleri* CBS 8195 and those with the lowest contents *S. cerevisiae* TS 146 or *T. delbrueckii* TS 207. The only exception was the lower folate concentrations of *C. milleri* CBS 8195 in cocultivation with *L. brevis* TSB 307 or *L. plantarum* TSB 304 than *C. milleri* CBS 8195 grown alone.

Fermentation of non-sterilised flour – water mixtures without any added microbes resulted in a three-fold increase in the folate content (note: there is a 5-fold difference in the folate contents of sterile and non-sterile controls in Fig. 8 but due to different starting levels, for non-sterile control the increase during 19 h fermentation was "only" 3-fold). Three types of bacteria were isolated and identified: *L. brevis*, *Enterobacter cowanii* and *Pantoea agglomerans*. *L. brevis* depleted folate in the fermentation of sterilised flour – water mixture, but the two other bacteria raised the folate level to that of the non-sterile control after 19 h of fermentation (Figure 8).

5.2.4 Germination and thermal processing (IV)

Germination increased the folate content by 1.7 to 3.8-fold, up to 250 µg/100 g DM (**IV**: Figure 1). The highest folate contents were achieved when rye grains were germinated at a moderate temperature (14 to 16°C) for 7 days. Increasing the drying temperature above 75 °C gradually reduced the folate contents. Germination was accompanied by increased contents of 5-CH_3-H_4folate and H_4folate, accounting for 49 to 55% and 23 to 25% of the sum of vitamers.

The folate concentrations in hypocotylar roots were 600 to 1180 µg/100 g DM. Roots from grains germinated at 22 °C contained less folates than those germinated at 15 °C. The main folate in roots was 5-CH_3-H_4folate (67 to 77% of the sum of the folate forms), whereas H_4folate contributed only to 4 to 10%.

Autoclaving and puffing decreased the folate contents by 45 to 54% compared to either germinated or non-germinated controls and IR followed by roasting by 35 to 50%. In extrusion the folate losses were 26 to 28%. Thermal treatments increased the proportion of PGA and decreased the amount of H_4folate. The sum of 10-HCO-H_2folate and 10-HCO-PGA constituted 20 to 22% of the sum of folate forms in germinated and heat-processed grains.

Folate levels in grains that were germinated and then heat-processed were higher than in native (non-germinated) grains (**IV**: Figure 2). Germinated, heat-processed grains contained relatively more 5-CH_3-H_4folate and less formylated folates than non-germinated grains. The relative proportion of 5-CH_3-H_4folate in germinated, thermally-processed samples was roughly the same as in the germinated, non-heat-processed control.

5.3 Bioavailability of endogenous folates *in vivo* (V)

Both in the group receiving natural folates from rye products and orange juice and in the group receiving folic acid from fortified bread, a statistically significant increase in serum folate was

observed after the four-week intervention period (Table 13). The increase was slightly greater in the folic acid fortified bread group than in the rye and orange juice group, but the difference was not statistically significant. The average increase in serum folate for those belonging to the lowest serum folate tertile at the beginning of the study was as high as 59 to 68%, whereas in the highest tertile the increase was only 12 to 14%. Red cell folate levels also increased significantly in both groups but the effect was not as dependent on the starting level as in the case of serum folate. Decreases in the plasma homocysteine concentrations were observed only in the highest tertiles of both groups. However, neither of the decreases was statistically significant.

Table 13. Folate intakes, plasma folate, red cell folate, and plasma total homocysteine concentrations before and after the intervention with either natural folates from rye & orange juice or with folic acid from fortified white bread

	Rye and orange juice group (n = 33)	Folic acid fortified bread group (n = 31)
Folate intake during the run-in period (μg/d)	277 ± 96	273 ± 94
Folate provided by test foods (μg/d)	184 ± 24	188 ± 8
Plasma folate (nmol/L)		
Beginning	9.9 ± 2.9	11.9 ± 5.3
End of diet	12.5 ± 3.6*	15.6 ± 5.8*
Difference	2.6 ± 3.2	3.7 ± 3.3
Increase (%)	26.1%	31.2%
Red cell folate (nmol/L)		
Beginning	561 ± 190	606 ± 224
End of diet	658 ± 227*	697 ± 250*
Difference	97 ± 92	91 ± 79
Increase (%)	17.3%	15.3%
Plasma Hcy (μmol/L)		
Beginning	8.5 ± 3.23	7.8 ± 2.37
End of diet	8.8 ± 2.54	7.8 ± 1.79
Difference	0.3	0
Increase (%)	3.5%	0%

* Statistically significant increase ($p<0.0001$)

6. DISCUSSION

6.1 Evaluation of the chosen methods for folate analysis in cereals

6.1.1 Laboratory proficiency

Our HPLC results in the intercomparison study organised by FolateFuncHealth were somewhat lower than the certified values and also lower than the average microbiological results, except for

CRM 487 (pig's liver), but nevertheless agreed well with the averages of all results and especially with the average HPLC values. Thus, the capability of our laboratory to perform food folate analysis (by HPLC) was in line with the general status of European laboratories.

The performance of our laboratory in microbiological assay was verified by the Nordic Norfolate ring test, and the INFOODS intercomparison study confirmed the quality of our analytical procedures on a broader scale. In the INFOODS proficiency test our results were well within the range of average values and the standard deviations were small. Z-scores indicating laboratory performance were good, $-2 < z < 2$ (Puwastien et al., 2005).

6.1.2 Comparison between the HPLC and microbiological methods

The detection limits in the optimised HPLC method were low and peak separation was generally good, allowing the quantification of folate vitamers in the samples. However, measurement uncertainty typically increases when working at low concentration levels, and even more so at levels near the detection limits (Horwitz et al., 1980). Although the repeatabilities of detector responses and retention times were good, even small changes in retention times occasionally disturbed peak identification and integration. As specific and selective as the affinity chromatography was, there were often peaks derived from sample impurities or reagents used in the analysis (e.g. ascorbate and mercaptoethanol). In these cases the dual detection system proved its usefulness: peak identification could be verified not only by spiking but also by comparing the ratios of UV and fluorescence responses of sample and standard peaks.

In HPLC analysis of fermentation samples the recoveries of individual folate vitamers were generally good, but somewhat low for H_4folate and even lower for 5-HCO-H_4folate in sponge and bread samples, reflecting problems with sample-derived impurities that masked the 5-HCO-H_4folate peak. Results were not corrected with recoveries because the recovery test were performed with folate monoglutamates, whereas a large proportion of folates in foods exist in the polyglutamate form. This means that results obtained by HPLC may in some cases be markedly below the true concentrations and should be considered rather as minimum values.

In the microbiological assay the repeatability of *L. rhamnosus* growth was generally good, and problems with contamination were extremely rare. The accuracy of the microbiological assay based on the analysis of CRM 121 (wholemeal flour) was good. The average folate content of CRM 121 calculated from ten separate determinations (51.1 ± 6.5 µg/100g DM; 12.7% CV) was

well within the certified value range (50 ± 7 µg/100g DM; 14% CV). The recoveries of 5-HCO-H_4folate, both added to CRM 121 and analysed as a sample, were also very good.

In the microbiological assay the total time required for the analysis was shorter and sample through-put higher than in HPLC analysis, despite the 18-hour incubation. In HPLC analysis a sample purification step was necessary, and sample runs as well as data processing required more time. Furthermore, if there was 10-HCO-PGA in the samples the purified extracts had to be re-run with a fluorescence detector set at different excitation and emission wavelengths. The equipment needed was simpler in the microbiological assay than in HPLC. However, both methods called for good laboratory skills, thoroughness, and expertise.

In general, there was a considerable, yet largely unidentified difference between microbiological and HPLC results. Results obtained by HPLC were approximately one third lower than those obtained by the microbiological method (Table 14). For instance, in CRM 121 (wholemeal flour) the sum of folate vitamers was 61% of the microbiologically determined total folate content and in CRM 421 (milk powder) 65%. Microbiological results were well in line with certified values. In this context it should be noted that the certified values were originally determined by microbiological assay.

The same pattern was found in the FolateFuncHealth intercomparison: it seemed that HPLC methods may underestimate folate contents by approximately 20 to 30% (FolateFuncHealth, 2005). Results for CRMs obtained by HPLC were lower than the microbiological results, except for CRM 487. Pig's liver contains a high proportion of H_4folate (Vahteristo et al., 1996b) that may have been destroyed during the long incubation period in the microbiological assay. Again, the microbiological results showed a relatively good agreement with certified values. Konings et al. (2001) also reported that folate contents analysed by HPLC were approximately 25% lower than contents in food composition tables established by using the microbiological method. However, according Pfeiffer et al. (1997), folate contents in cereals determined by HPLC correlated well with the microbiologically determined folate contents.

Table 14. Folate contents (µg/100 g DM) determined by HPLC and microbiological method, and their ratios for selected samples

Sample	HPLC result (µg/100 g DM)[a]	MA result (µg/100 g DM)	HPLC/MA (%)
Samples without thermal treatments			
CRM 121	31	51	61%
CRM 421	87	134	65%
Rye flour	71	66	108 %
Sourdough, start	75	77	97 %
Sourdough, end	86	119	72 %
Dough	73	94	78 %
Proofed dough	70	93	75 %
Germinated rye; 3 days at 15ºC	71	122	58 %
Germinated rye; 5 days at 15ºC	90	130	69 %
Germinated rye; 7 days at 15ºC	94	122	77 %
Average			76% ± 16%
Thermally treated samples			
Wheat bread; sponge dough	41	50	82 %
Wheat bread; straight dough	42	45	93 %
Wheat bread; baking powder	21	17	124 %
Rye bread; yeast fermented	24	42	57 %
Rye bread; yeast and LAB fermented[b]	30	42	71 %
Rye bread; LAB fermented	15	29	52 %
Rye bread	41	93	44 %
Extruded rye; native	38	48	79 %
Extruded rye; germinated	82	105	78 %
Autoclaved & puffed rye; native	18	30	60 %
Autoclaved & puffed rye; germinated	45	81	56 %
IR & roasted rye; native	14	42	33 %
IR & roasted rye; germinated	24	73	33 %
Average			66% ± 25%
Average of all			71% ± 22%

[a] Sum of vitamers; the figures would be somewhat higher if calculated as 5-HCO-H_4folate
[b] LAB = lactic acid bacteria

In germinated samples (**IV**) the sum of the measured vitamers varied from 58 to 77% of the total folate content obtained by microbiological assay (Table 14), but was lower in thermally-processed samples (33 to 79%). Correlation coefficient between the results obtained by the two methods was 0.79 for all the samples presented in Table 14 but only 0.59 for thermally treated samples. Bland-Altman analysis confirmed that the two methods were producing different results. The mean difference was 22.7 µg/100g (95% confidence interval 15.2 to 30.2 µg/100g; limits of agreement -11.2 to 56.5 µg/100g). There was no systematic relationship between the difference and the folate content.

Folate concentrations approaching the quantitation limits cause uncertainty to the results but in the thermally processed grain samples the difference between HPLC and microbiological results was not dependent on low folate concentrations as such. The best agreement between the two methods was found in samples with the smallest folate losses, regardless of the folate concentrations. High temperatures may induce the formation of compounds, either folate or non-folate, that are not quantitated by HPLC but give a response in the microbiological assay. In samples taken at different stages of fermentation and baking (**II**) the sum of vitamers varied from 72 to 124% of the total folate content but was only 44 to 71% in rye breads, which also supports the hypothesis of the role of elevated temperatures.

During food processing, in the extraction, and under the acidic conditions in HPLC some folate vitamers may convert to others. Thus, unidentified folate vitamers may exist that are not quantitated at all. Chromatographic methods with specific physical and chemical detection principles do not share the weakness of inherent uncertainty related to biological measurements such as in the microbiological assay. Nevertheless, they only measure the vitamers that they are calibrated for. On the other hand, it is possible that *L. rhamnosus* used in the microbiological assay gives unequal responses to different vitamers or gives response to non-folate compounds. However, results obtained by the combined LC-MA assay in the FolateFuncHealth intercomparison showed no non-folate peaks in the chromatograms of four CRMs (Kariluoto et al., 2002).

HPLC methods may not be ideal for determining total folate contents and are not well suited to routine folate determination, which was also a major finding of a set of intercomparison studies (Finglas et al., 1999). If acquiring data on individual folate vitamers is not considered necessary, microbiological assay is in most cases the method of choice. However, HPLC has certainly proved its importance for various research purposes where data on folate vitamer concentrations and distribution are needed. The wide variation among laboratories in intercomparison studies obviously calls for further standardisation of methods, starting from the sample storage and pre-treatments. Any laboratory performing food folate analysis should, if feasible, make use of CRMs and participate in collaboration studies.

6.2 Folates in rye

6.2.1 Folates in rye grains (I)

The folate content of rye was generally high compared to other main cereals. The average of folate contents of ten rye cultivars, 70.0 ± 5.5 µg/100 g fresh weight in 1999 and 73.8 ± 8.3

µg/100 g in 2000, agreed well with the value 72 µg/100 g for rye flour in the Finnish national food composition database (Fineli, 2006). Folate contents were generally higher in 2000 than in 1999 and the range of folate contents was wider in 2000. However, neither of these differences was statistically significant. For wheats, large variations have been reported (Arcot et al., 2002: 80 to 114 µg/100 g; Gujska and Kuncewicz, 2005: 34 to 40 µg/100 g).

Cultivar Bor 9414 had the highest folate content both in 1999 and 2000, and cultivar Picasso had the lowest folate content in 1999 and the second lowest in 2000. Thus, it seems that the effect of genotype on folate content may in some cases be greater than the effect of growing conditions. Conventionally and organically grown cultivars had similar folate contents.

The main vitamer in rye flour was 5-CH_3-H_4folate (approximately one fourth of the vitamer sum), but the vitamer distribution pattern was distinctively characterised by a high proportion of various formylated vitamers that altogether accounted for two thirds of the vitamer sum. This pattern is very different from that of most vegetables, where the predominant vitamer is 5-CH_3-H_4folate and other vitamers typically account for 0 to 20% of the vitamer sum (Vahteristo et al., 1997a; Konings et al., 2001). The high proportions of formyl and methyl folates in cereal products have also been shown by Pfeiffer et al. (1997) and Konings et al. (2001).

6.2.2 Effect of fermentation and baking on folates (II–III)

In Study **II**, the inclusion of added yeast clearly resulted in higher folate content of both rye and wheat breads than would have been expected based on the folate content of flour. Thus, the folates in bread derive not only from the flour but also from the added yeast. This has earlier been shown in wheat breads (Butterfield and Calloway, 1972; Keagy et al., 1975) but now also in rye baking, where the absence of gluten and acidity of the dough make it different from wheat baking. The folate contents of rye breads baked with added yeast were 45% higher than the folate content of bread baked without added yeast. In wheat breads the difference between breads baked with and without added yeast was even greater, 165 to 194%. The folate content of baker's yeast is high. For instance, Patring and Jastrebova (2007) reported a folate content of 3520 µg/100 g dry yeast. The increase in folate content during sourdough fermentation was linked not only to adding folate-rich baker's yeast *per se*, but also to folate synthesis during the fermentation phase.

During the fermentation, the concentrations of 5-CH_3-H_4folate and 10-HCO-H_2folate, in particular, markedly increased. The 5-HCO-H_4folate concentration also increased, but the

difference was not statistically significant. The two most important vitamers of yeast are 5-CH_3-H_4folate and H_4folate (Seyoum and Selhub, 1998; Patring and Jastrebova, 2007). The increase in the 5-CH_3-H_4folate concentration during fermentation and the strikingly low contents of 5-CH_3-H_4folate in rye and wheat breads baked without added yeast agree with the finding that the concentration of 5-CH_3-H_4folate increases with an increasing growth rate of yeast but the concentration of H_4folate remains rather constant (Hjortmo et al., 2007).

After fermentation the dough was mixed and the addition of other ingredients diluted the folate concentration. Proofing modified the vitamer distribution pattern but did not affect the total folate concentration. Baking losses in general (approximately 25%) were comparable with those in previous studies (Butterfield and Calloway, 1972; Keagy et al., 1975; Osseyi et al., 2001; Arcot et al., 2002). Although losses of H_4folate, 5-CH_3-H_4folate, and 5-HCO-H_4folate were great, concentrations of 10-HCO-H_2folate, 10-HCO-PGA, and PGA increased. In the analytical system 10-HCO-H_4folate could not be quantitated as such, but since 10-HCO-H_4folate is easily converted 10-HCO-PGA and 10-HCO-H_2folate (Robinson, 1971; Maruyama et al., 1978), these two vitamers reflect the original amount of 10-HCO-H_4folate. The oxidation of 10-HCO-H_4folate may have taken place during processing or analysis, or both. The results were largely in agreement with the study of Osseyi et al. (2001) reporting vitamer changes in wheat baking. However, Osseyi et al. (2001) observed an increase in the 5-CH_3-H_4folate concentration after baking, whereas we found a significant loss (67%). In thermally-processed rye grains the concentration of 5-CH_3-H_4folate also decreased (**IV**). The reason for this discrepancy remains unclear.

Study **III** further elucidated the role of yeasts and lactic acid bacteria in sourdough baking. The yeasts and lactic acid bacteria studied differed in their abilities to produce or consume folates. All the yeasts produced folate (from 6.5 µg/100 g in sterile rye flour – water mixture up to 22.8 µg/100 g after fermentation), *S. cerevisiae* ALKO 743 and *C. milleri* CBS 8195 being the best producers. Lactic acid bacteria mainly depleted folate. *L. bulgaricus* ABM 5096 and *S. thermophilus* ABM 5097 produced folate in the tested conditions, but still much less than yeasts. Hjortmo et al. (2005) found that the folate production of cultivated yeast strains was highly strain dependent, the total folate content varying 3.6-fold. It also seemed that *S. cerevisiae* and closely-related species were better folate producers than the other species studied, which is in accordance with the results of Study **III**. Large variations in yeast folate contents at different growth stages have been reported. Fast growing cells seem to have higher folate demands compared to slowly

growing cultures (Hjortmo et al., 2007). Thus, it would have been useful to measure the folate content more often during the 19-hour incubation and possibly apply longer incubation times.

Data on folate production or consumption by lactic acid bacteria are rather scarce. The potential of lactic acid bacteria as folate producers has mainly been studied in dairy products, but next to nothing is known about their role in folate production during the fermentation of cereals. Furthermore, results are often controversial since differences in strains and culture conditions induce variability in the results (Lin and Young, 2000; Sybesma et al., 2003). For instance, in Study **IV**, *L. bulgaricus* ABM 5096 and *S. thermophilus* ABM 5097 produced folates, whereas in milk *L. bulgaricus* has been found to consume folates (Rao et al., 1984). However, folate production by *L. bulgaricus* has also been reported (Lin and Young, 2000). Likewise, in Study **IV**, *L. acidophilus* consumed folates as earlier observed by Alm (1982) and Crittenden et al. (2002), but several studies have also reported folate production (Rao et al., 1984; Lin and Young, 2000). *S. thermophilus* has been shown to produce folates (Rao et al., 1984; Sybesma et al., 2003).

Fermentation of sterile rye flour with pairwise combinations of yeasts and lactic acid bacteria resulted in similar or slightly lower folate levels than in fermentations with yeasts alone. Thus, in this test tube fermentation, yeasts were responsible for the increase in the folate concentration. Although folate-consuming lactic acid bacteria were present in the sourdough, they could not affect the folate concentration much. One reason is that yeasts did not excrete folates. This observation is confirmed by Hjortmo et al. (2005), who reported very low folate concentrations in spent media, probably originating from lysed cells. Another reason is that the consumption of folate by lactic acid bacteria was moderate. In the sterile rye flour – water mixture lactic acid bacteria depleted 0.8 to 1.7 μg folate, which is of same magnitude (0.9 to 2 μg) as previously determined with *L. rhamnosus* LC-705 and *L. rhamnosus* GG (Turbic et al., 2002).

Folate levels after fermentation of sterile flour with *Enterobacter cowanii* ABM 5061 or *Pantoea agglomerans* ABM 5062 were similar to levels in the fermented non-sterile control. These two endogenous bacteria isolated from rye flour were as good folate producers as yeasts and seemed to excrete folates into the medium. Nevertheless, non-sterile flour – water mixture inoculated with lactic acid bacteria showed no increase in folate concentration in spite of the simultaneous presence of folate-producing bacteria. Lactic acid bacteria may have consumed folates produced by the endogenous bacteria or retarded their growth, for instance by lowering the pH and

competing for nutrients. The latter option seems more likely, as the number of inoculated lactic acid bacteria was after all higher than the number of endogenous bacteria.

Endogenous (lactic acid) bacteria may in certain circumstances have a significant effect on folate levels. Katina et al. (2007) assessed folate contents during the fermentation of rye bran and recorded the highest folate concentrations when the growth of endogenous lactic acid bacteria was pronounced. The growth of yeast could not merely explain the observed increase in folate concentration.

Fermentation affects also other vitamins. For instance, Batifoulier et al. (2005) reported that long yeast fermentations in wheat breadmaking led to an increase in thiamine and riboflavine contents, and the loss of pyridoxine occurring in baking was compensated with fermentation by yeast or using sourdough fermentation. Tocopherol and tocotrienol concentrations, however, have been found to decrease during rye sourdough fermentation (Liukkonen et al., 2003). Bread and other cereals are the main sources of thiamine in Finland, with their 33% contribution to the daily thiamine intake. Their contribution to riboflavine and pyridoxine intake is lower (18 to 19%) but still significant (Männistö et al., 2003). Increased consumption of fermented cereals could thus improve the intake of several B vitamins.

6.2.3 Effect of germination and thermal processing on folates (IV)

Germination temperature affected the rate of germination, which in turn largely explained the increase in folate content during germination. The higher the temperature, the less time was needed to reach 100% germination. However, a prolonged germination time could also decrease the folate content as a consequence of folate destruction by oxidation. In the subsequent drying, moderate temperatures retained folates rather well. Folate contents of germinated rye grains were higher than folate contents reported for malted wheat and oats and of same magnitude as barley beer malts (Jägerstad et al., 2005). The maximum increase in folate content in germinated rye (3.8-fold) was in good accordance with the 3.4-fold increase reported for germinated wheat (Koehler et al., 2007).

The folate concentration in hypocotylar roots was high, and removal of roots clearly resulted in lower folate contents in germinated rye grains. Folate contents of roots were 6- to10-fold higher than in germinated grains and 10- to 19-fold higher than in native (non-germinated) grains. Although the proportion of roots was only 5 to 10% of total germinated grain weight, they contributed 30 to 50% of the folate content and thus markedly influenced the total folate content

of germinated grain. Roots often loosen easily during processing and are then removed. From another point of view, this indicates that a folate-rich fraction could be separated, collected and perhaps used in other food products. The bitterness brings about some problems but, as shown by Yang et al. (2001), steeping and germination conditions might be optimised to achieve acceptable sensory properties.

Germination altered the folate vitamer distribution in rye grains by increasing the proportions of 5-CH_3-H_4folate and H_4folate and decreasing the proportion of formylated folates. The folate content and vitamer distribution pattern in roots were similar to those of other proliferating tissues, such as pea cotyledons (Roos and Cossins, 1971), radish cotyledons, wheat leaves, pea leaves, and spinach leaves (Spronk and Cossins, 1972).

As noticed earlier, formylated folates are often the main vitamers in cereal products whereas in vegetables 5-CH_3-H_4folate typically dominates (approximately 90%). The content of 5-CH_3-H_4folate in germinated grains and hypocotylar roots was higher than in cereal grains but lower than in vegetables. In rye flour, 5-CH_3-H_4folate accounted for 26% of the vitamer sum, in germinated grain for 49 to 55%, and in roots for 67 to 77%. Koehler et al. (2007) also noted that the folate increase during germination was mainly attributable to the increasing amount of 5-CH_3-H_4folate. The changes in 5-CH_3-H_4folate and H_4folate proportions during germination reflect the change of grain into a metabolically active photosynthetic organism. Interestingly, the combined proportions of 5-CH_3-H_4folate and H_4folate were similar in both roots and in the respective germinated grains. Plants are capable of *de novo* synthesis of H_4folate, which is then converted to other folate compounds needed for normal growth. Various methylation reactions and the biosynthesis of methionine use 5-CH_3-H_4folate as the coenzyme (Roos and Cossins, 1971; Rebeille et al., 2006).

All the thermal treatments resulted in significant folate losses in both germinated and non-germinated rye grains. The greatest losses were caused by autoclaving and puffing, where high temperatures were applied together with the presence of oxygen. IR treatment followed by roasting also resulted in notable losses. In extrusion, however, the retention of folate was much better, in spite of the high temperature, since the processing time was very short. This evidence together suggests that processes with a short exposure to low or high temperatures retain folates considerably better than processes involving longer exposure to high temperatures or a long processing time. Studies on thermal processing of whole-grain wheat and white flour support this conclusion (Håkansson et al., 1987).

Compared to rye flour (**I**), non-germinated, heat-processed grains underwent similar changes in folate vitamer distribution as in rye bread baking (**II**): the proportions of H_4folate, 5-CH_3-H_4folate, and 5-HCO-H_4folate decreased, whereas the proportions of 10-HCO-H_2folate, 10-HCO-PGA, and PGA increased.

The amount of 5-CH_3-H_4folate markedly increased during germination, which could also be seen in germinated, heat-processed grains: germinated, thermally-processed grains contained relatively more 5-CH_3-H_4folate and less formylated folates than non-germinated, thermally-processed grains. Although folates were very sensitive to heat, the rise in folate content during germination ensured a high folate concentration in thermally-processed germinated rye grains. Folate contents in germinated and subsequently thermally-processed grains always exceeded the folate content of the native (non-germinated) grain.

6.3 Bioavailability of endogenous folates (V)

The results clearly demonstrated that a diet with natural folates of rye and orange juice favourably affected the folate status in healthy volunteers. The amounts of folate provided by the test foods were moderate, even rather small (184 µg folate/day in rye group and 188 µg folate/day in wheat group), and the estimated increase in the daily intake of folate (endogenous or added folic acid) was 120 to 130 µg. Wald et al. (2001b) reported a change of 2.1 nmol/l in serum folate levels for each 100 µg of folic acid (administrated in tablet form). In Study **V** the increases in serum folate concentrations were 2.6 nmol/l for the rye group and 3.7 nmol/l for the fortified wheat group. The increases in serum folate were of the same magnitude or somewhat higher than the 1.8 and 2.6 nmol/l reported for 200 µg of spinach and yeast folate, respectively, after a 30-day intervention period (Hannon-Fletcher, 2004), but lower than 4.3 nmol/l (after 30 days intervention) and 5.5 nmol/l (after 60 days intervention) reported for 166 µg of folic acid from fortified breakfast rolls (Johansson et al., 2002). Our results are also in line with those reported by Ashfield-Watt et al. (2003): additional intake of 50 µg folate from fruit and vegetables resulted in a 2.76 nmol/l increase and additional intake of 100 µg folic acid from fortified foods in a 2.97 nmol/l increase in serum folate during 4-month intervention period.

The effect of the dietary intervention on red cell folate was not as constant and dependent on the starting level as in the case of serum folate. This is understandable since the duration of the treatment was only 3 to 4 weeks. However, there was a clear, significant increase in red cell folate in both groups after the intervention whereas Johansson et al. (2002) found a significant inrease in red cell folate only after 90 days intervention.

Based on the changes in serum and red cell folate, endogenous folates showed good bioavailability that was similar to folic acid from fortified white bread. This was somewhat unexpected, as several studies have reported reduced bioavailability of natural folates compared to folic acid (Cuskelly et al., 1996; Hannon-Fletcher et al., 2004). However, one of the few clinical trials conducted with cereal products showed the bioavailability of folate in wheat aleurone flour to be as good as the bioavailability of folic acid administered in a tablet with wheat bran cereal (Fenech et al., 1999). Interestingly, non-starch polysaccharides in the diet may also promote microbial folate synthesis in the gut and thus improve the folate status (Houghton et al., 1997).

Study **V** failed to show a homocysteine lowering effect of additional folate intake. A daily folic acid intake of the same magnitude (Ward et al., 1997; Venn et al., 2002; Johansson et al., 2002) as well as an additional 350 μg of natural folate from vegetables and citrus fruit (Brouwer et al., 1999a) have earlier been shown to reduce plasma homocysteine. However, the homocysteine lowering effect of folate/folic acid was not seen in the study of Ashfield-Watt et al. (2003). The response is dependent on the starting homocysteine level (Schorah et al., 1998; Ward et al.,1997). In our study the starting plasma homocysteine concentrations were already low and were not lowered further by moderate amounts of additional folate.

6.4 Overview of the potential for enhancement of folate content

Processing, which is a prerequisite for the consumption of whole grain rye, is often accompanied by folate losses. In addition to improving folate retention by optimising food processing methods (in other words, minimising vitamin losses), folate contents may be maintained or even enhanced by means of certain processes that increase the folate concentration. Traditional processes may be transferred to new applications, novel processing methods may be developed, or different types of process may be combined.

Folate contents in rye cultivars did not markedly differ. However, folate is unevenly distributed in the rye kernel, and milling fractions of rye may have 10-fold differences in their folate concentrations (Liukkonen et al., 2003). This enables the tailoring of rye flour or bran ingredients for various purposes. Table 15 summarises the positive impacts of food processing methods on the folate contents of rye that were investigated in this study.

Table 15. Overview of processing methods for enhancing the folate contents of rye products

	Folate increase	Average folate content	Remarks
Bread baking	1.3 to 1.4-fold	40 to 80 µg/100 g DM	Compared to flour
Sourdough fermentation phase in baking; with added yeast	1.8 to 2.6-fold	120 to 160 µg/100 g DM	Compared to flour
In vitro fermentation with added yeasts (and lactic acid bacteria)	1.9 to 3.4-fold	12 to 23 µg/100 g FW	
Spontaneous *in vitro* fermentation	3.5-fold	26 µg/100 g FW	Large variation
Germination	1.7 to 3.8-fold	160 to 250 µg/100 g DM	
Germination and successive thermal treatments	1.1 to 1.6-fold	70 to 105 µg/100 g DM	Compared to native grain
Germination; hypocotylar roots	6 to 10-fold	600 to 1180 µg/100 g DM	Compared to germinated grain
	10 to 19-fold		Compared to native grain

Metabolic engineering of lactic acid bacteria offers promising possibilities to enhance folate levels by the over-expression of enzymes related to tetrahydrofolate biosynthesis and by decreasing the proportion of potentially less bioavailable polyglutamate folate (Sybesma et al., 2003; Wegkamp et al., 2004). However, consumer attitudes towards genetically-modified microorganisms are often doubtful, which may hamper the development of food applications. Instead, as shown by the results in Table 15, folate contents can be significantly increased by conventional food processing techniques. Additional enhancement is even possible by combining processes. For instance, Katina et al. (2007) successfully applied fermentation of native and peeled rye bran in order to improve the technological properties and to elevate the levels of bioactive compounds, including folate. The fermentation of germinated rye may also result in increased folate concentrations, considering that germinated grain offers good growing conditions for yeasts. Clearly, specific attention has to be paid to avoiding any microbiological risks.

The average consumption of rye in Finland is 45 g/d per capita according to the Balance Sheet for Food Commodities (2007). Slightly different figures were obtained in the national FINDIET 2002 Study, 61 g/d for men and 39 g/d for women (Männistö et al., 2003). Most of the rye is consumed as rye bread, the average consumption being 100 g/d among men and 66 g/d among women (Männistö et al., 2003). Enhancement of the folate content of bread by processing is illustrated by Table 16, which compares the contribution of three types of rye bread to the recommended daily intake of folate. The impact of increasing rye bread consumption by one third is also calculated. Compared to the consumption of rye bread baked without fermentation (without added baker's yeast), the consumption of bread with fermentation including baker's yeast or bread with germinated rye results in a 1.7- to 1.8-fold increase in folate intake. A simultaneous increase in bread consumption by one third would more than double the folate intake.

Table 16. Contribution of rye bread consumption to the intake of folate

	Folate provided by 100 g of bread (% of the recommendation; men)[a]	Folate provided by 133 g of bread (% of the recommendation; men)	Folate provided by 66 g of bread (% of the recommendation; women)	Folate provided by 88 g of bread (% of the recommendation; women)
Rye bread without sourdough fermentation with added yeast[b]	30 µg (10%)	40 µg (13%)	20 µg (5%)	26 µg (7%)
Rye bread with sourdough fermentation with added yeast[c]	55 µg (18%)	73 µg (24%)	36 µg (9%)	48 µg (12%)
Rye bread with one third of the rye germinated[d]	51 µg (17%)	68 µg (23%)	34 µg (8%)	45 µg (11%)

[a] Recommended daily intake of folate: 300 µg/d for men and 400 µg/d for women
[b] Estimated folate content 30 µg/100 g FW
[c] Estimated folate content 55 µg/100 g FW
[d] Estimated folate content 51 µg/100 g FW: 100 g of rye bread corresponds to approximately 63 g of rye flour. Folate content of rye flour is 70 µg/100 g, and germination is assumed to increase the folate content 2.7-fold. If one third of the flour is germinated and baking loss is 25%, the folate content of bread is approximately 51 µg/100 g.

Another possibility to increase the folate intake from rye could be the development of new types of product, such as breakfast cereals or snacks with germinated and heat-processed rye, to targeted population groups. For instance, the rye muesli developed for Study **V** had a folate

content as high as 188 µg/100 g. Rye already has a positive, healthy image, and has been an important source of whole grain in the Finnish diet for a long time.

7. CONCLUSIONS AND RECOMMENDATIONS

Affinity chromatography proved to be an efficient purification method allowing the use of HPLC in analysing rye and rye products for folates, even though folate peaks, 5-HCO-H_4folate especially, were sometimes masked by compounds from the sample matrix or reagents. In general, HPLC methods are not yet at the stage for routine folate analysis, but could surely be more widely used provided that attention is paid to method validation. The development of mass spectrometric methods will certainly reveal new information on food folates in the coming years. Nevertheless, microbiological assay still has its place when there is no need for data on folate vitamers.

Microbiological and HPLC results differed, and it seemed that HPLC methods may underestimate total folate by approximately one third. Part of this underestimation in our results could be due to not performing any recovery corrections. This and other reasons for the discrepancy are evidently complex and require further examination. Based on the results of this study, the use of reference materials, either certified or in-house, as well as participation in interlaboratory studies can be regarded as essential for every laboratory determining food folate.

New data were obtaibed on the folate content and its variation in rye cultivars. The folate content of rye was rather high, over 70 µg/100 g on dry matter basis. Results indicated that the genotype may in some cases have a significant effect on the folate content. However, further studies are needed to evaluate the degree of this variation. Conventionally and organically grown cultivars had similar folate contents. The folate vitamer distribution pattern of rye was characterised by a large proportion of formylated vitamers in addition to 5-CH_3-H_4folate typically found in plant materials.

The folate content of rye products could be significantly enhanced by food processing. Yeasts were able to synthesize folate in test tube fermentation, but most of the studied lactic acid bacteria depleted folate. Nevertheless, the consumption of folate by lactic acid bacteria in co-cultivations with yeasts seemed to be very moderate and affected folate concentration only to a limited extent. Two endogenous bacteria isolated from rye flour produced similar amounts of folate as yeasts. The potential of endogenous microorganisms that could be exploited in food

applications is worthy of study. In addition, the effects of food matrix on folate production by microorganisms requires further examination. The use of baker's yeast (*Saccharomyces cerevisiae*) in sourdough fermentation markedly increased the folate content of bread so that the bread had a higher folate content than the flour it was made of. Thus, as regards folate intake, it is recommendable to include yeast in baking.

Germination was even more efficient than fermentation in raising folate levels in rye. The highest folate levels were achieved with long germination at moderate temperatures (14 to 16 °C) and drying temperatures below 75 °C. Germination also altered the folate vitamer distribution: the most abundant vitamers in germinated grain were 5-CH_3-H_4folate and H_4folate, as compared to formylated vitamers in native grain. The folate concentration in hypocotylar roots was particularly high, and the possible use of this folate-rich fraction would be an interesting topic for further experimental research. All the thermal treatments studied caused significant folate losses (26 to 54%). Despite losses, folate contents in germinated grains after thermal processing exceeded the folate content of the native (non-germinated) grain. Folate retention was fairly good in processes with a short-term exposure to low or high temperatures.

The results of the bioavailability study showed that a diet including natural folates of rye and orange juice improved the folate status among healthy adults. In this study a favourable effect was seen with rather small amounts of additional folate, either natural folates (rye group; 184 ± 24 µg folate from test foods per day) or synthetic folic acid (wheat group; 188 ± 8 µg folate from test foods per day). Based on the results of this study, natural folates from rye and orange juice are as bioavailable as folic acid from fortified white bread.

In summary, the folate contents of rye and rye products can be markedly increased by certain food processing methods. Folate levels can be raised by fermentation or germination so that enhanced folate concentrations may compensate for folate losses at later stages of food processing. Further research on optimising and combining processing techniques is encouraged.

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