Development and Validation of Chromatographic Methods to Study Folate Derivatives Produced by Yeasts

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Abstract

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Folate, or folic acid, is an important water soluble B vitamin that exists in many different forms. Its presence is necessary for synthesis of DNA and RNA and methylation of homocysteine to methionine. Deficiency of folate is closely connected to increased risk of neural tube defects, a serious birth defect, and development of anaemia.

Humans cannot synthesise folate by themselves and therefore depend on an adequate supply through food intake. However, the recommended daily intake of folate is not reached by many people, especially women in fertile ages.

This thesis aimed to investigate possibilities for enhancing folate production from yeast for use as biofortificants in food to increase the natural folate content.

To quantify folate, analytical methods were developed and validated. This involved optimisation of sample pre-treatment steps, HPLC methods and LC-MS methods. The main findings were that folate analysis of yeast could be facilitated by excluding an SPE step prior to HPLC analysis, that sample handling and choice of antioxidant greatly influenced folate stability, that choice of reversed-phase column considerably affected folate separation/retention and that LC-MS provided a powerful tool for yeast folate analysis.

These developed analytical methods were used to investigate differences between yeast strains to produce folates. Furthermore, effect of cultivation conditions on folate content in yeast was studied. The main conclusions from these experiments were that careful selection of yeast strain may considerably increase folate content in food. It was also shown that folate concentration in yeast was significantly increased by optimising the cultivation procedure. The folate derivatives that were found to exist in yeast were tetrahydrofolate, 5-methyltetrahydrofolate, 10-formylfolic acid and 5-formyltetrahydrofolate.

The findings in this thesis show that there are great possibilities for increasing folate content in yeast-fermented foods, e.g. bread and dairy products, if a proper yeast strain is used under optimal growth conditions in appropriate culturing media.

Keywords: folates, analysis, HPLC, LC-MS, method development, sample pre-treatment, yeast, food, cultivation conditions, biofortification

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In 1903 the Russian botanist, Mikhail S. Tswett, first applied adsorption chromatography to the separation of plant pigments, using a hydrocarbon solvent and inulin powder (a carbohydrate) as stationary phase. The separation of coloured bands led to the name chromatography, from the Greek words chromatos, meaning “colour” and “graphia” meaning "writing" i.e. literally “colour writing”.

Daniel C. Harris

Sjömannen ber inte om medvind, han lär sig segla.

Gustaf Lindborg (1875-1923)

Upptäckter är resultatet av planmässigt famlande.

Carl Friedrich Gauss (1777-1855)

List of abbreviations

5-CH_3-H_4folate	5-methyl-tetrahydrofolate
5-HCO-H_4folate	5-formyl-tetrahydrofolate
10-HCO-H_4folate	10-formyl-tetrahydrofolate
10-HCO-H_2folate	10-formyl-dihydrofolate
10-HCO-folic acid	10-formyl-folic acid
5,10-CH^+=H_4folate	5,10-methylene-tetrahydrofolate
5,10-CH_2-H_4folate	5,10-methenyl-tetrahydrofolate
AA	ascorbic acid
ACN	acetonitrile
APCI	atmospheric pressure chemical ionisation
BAL	2,3-dimercapto-1-propanol
Ches	2-(N-cyclohexylamino)ethanesulfonic acid
CP	chicken pancreas
DAD	diode array detector
ESI	electrospray ionisation
FBP	folate binding proteins
FD	fluorescence detector
H_2folate	dihydrofolate
HAc	acetic acid
H_4folate	tetrahydrofolate
Hepes	N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid
HFo	formic acid
HK	hog kidney
HPLC	high performance liquid chromatography
LC	liquid chromatography
LOD	limit of determination
LOQ	limit of quantification
MA	microbiological assay
MCE	2-mercaptoethanol
MS	mass spectrometry
MTX	methotrexate
MW	molecular weight
NTD	neural tube defect
OD	optical density
pABG	*para*-aminobenzoylglutamic acid
PhB	phosphate buffer
$PteGlu_3$	pteroyltri-γ-L-glutamic acid
RDI	recommended daily intake
RP	reversed phase
RS	rat serum
RPBA	radio protein-binding assay
R^2	coefficient of correlation
SAX	strong anion exchange
SD	standard deviation
SPE	solid phase extraction
UV	ultraviolet

Contents

Appendix

Papers I-VII
This thesis is based on the following papers, which are referred to by their Roman numerals. Reprints were published with kind permission of respective publisher concerned:

I. Patring, J.D.M., Jastrebova, J.A., Hjortmo, S.B., Andlid, T.A. & Jagerstad, I.M. 2005. Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection. *Journal of Agricultural and Food Chemistry 53*, 2406-2411.

II. Patring, J.D.M., Johansson, M., Yazynina, E. & Jastrebova, J. 2005. Evaluation of impact of different antioxidants on stability of dietary folates during food sample preparation and storage of extracts prior to analysis. *Analytica Chimica Acta 553*, 36-42.

III. Patring, J.D.M., Lanina, S.A. & Jastrebova, J.A. 2006. Applicability of alkyl-bonded ultra-pure silica stationary phases for gradient reversed-phase HPLC of folates with conventional and volatile buffers under highly aqueous conditions. *Journal of Separation Science 29*, 889-904.

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

V. Hjortmo, S., Patring, J., Jastrebova, J. & Andlid, T. 2005. Inherent biodiversity of folate content and composition in yeasts. *Trends in Food Science & Technology 16*, 311-316.

VI. Patring, J.D.M., Hjortmo, S.B., Jastrebova, J.A., Svensson, U.K., Andlid, T.A. & Jagerstad, I.M. 2006. Characterization and quantification of folates produced by yeast strains isolated from kefir granules. *European Food Research and Technology 223*, 633-637.

VII. Hjortmo, S., Patring, J., & Andlid, T., Effect of cultivation conditions on folate content in S. cerevisiae. *Basic manuscript to be complemented with further data*.

My contributions to the papers were:

- **I.** Planned the experiment together with Dr Jelena Jastrebova. Performed all the laboratory work. Wrote manuscript together with co-authors.
- **II.** Planned the experiment together with Dr Jelena Jastrebova. Performed all the laboratory work. Had the main responsibility for writing the manuscript.
- **III.** Planned the work together with Dr Jelena Jastrebova. Performed most of the laboratory work. Participated in writing of manuscript together with co-authors.
- **IV.** Planned the work and wrote manuscript together with Dr Jelena Jastrebova. Performed all the laboratory work.
- **V.** Performed extraction, deconjugation and analysis of yeast folates. Participated in writing the manuscript.
- **VI.** Planned the experiment together with co-authors. Performed the extraction, deconjugation and analysis of yeast folates and evaluation of the analytical results. Wrote the article together with co-authors.
- **VII.** Performed deconjugation and analyses of yeast folates.

1 Introduction

1.1 Background

Folate is a group of B vitamins related to folic acid that has received considerable interest due to its health promoting effects. Its first observed therapeutic effects in 1931 was curing of macrocytic anaemia, a common disease in India during late pregnancy, by the use of yeast extract (Lucock, 2000). However, at the time, this effect was not associated to the unknown substance folate. In the article resulting from this discovery, the author made a connection between the curative effect and "yeast", and called the article "Treatment of pernicious anaemia of pregnancy and tropical anaemia with special reference to yeast extract as curative agent" (Willis, 1931).

From 1931 and later, other substances were isolated from yeast and liver and were also shown to cure induced anaemia in monkeys and to be essential growth factors for *Lactobacillus casei*. Because "folate" was an unknown factor, the "therapeutic factor" was called by different names e.g. "Vitamin M", "Vitamin B_c" and "*L. casei* factor" (Ball, 1998). In 1941 a research group purified an acidic substance from 4 tons of spinach leaves, which was shown to be a growth factor for rats and *Lactobacillus casei* (Mitchell *et al.*, 1941). They called this factor "folic acid" from the Latin word "folium" meaning leaf, a trivial term that persists today. In 1940 and 1943 this "factor" was also isolated from liver and yeast (Snell & Peterson, 1940; Stokstad, 1943), but was only first identified and synthesised in 1945 (Angier *et al.*, 1945).

According to Selhub & Rosenberg (1996), the first half of the 20th century can be classified as the era of identification of all the known vitamins and research in identifying deficiency diseases. The second half can be classified as when new research on absorption, metabolism and assay characteristics of folate led to new insights of its importance to prevent cancer, heart disease, stroke and birth defects.

Among all these health promoting effects of folate, the correlation of good folate status to decreased risk of neural tube defects is the most well-known. Besides heart defect, NTDs are the most common form of malformation worldwide (Bjorklund & Gordon, 2006). Worldwide 300,000 - 400,000 infants are born every year with NTDs (Whitney *et al.*, 1994). The first connection that diet could be connected to neural tube defects came from examining the Dutch famine in the Winter of 1944, caused by Nazi occupiers (Bjorklund & Gordon, 2006). The incident of NTDs doubled among those conceived during this time. Some studies have pointed out that folate is the key factor in preventing NTDs as reviewed by Lucock, 2000.

A key factor in the science of folate is access to reliable analytical tools. However, analysis of folate has always been a complicated and troublesome activity due to several causes, e.g. folate exists in ppb-levels and in different

forms; many of them are sensitive to degradation/interconversions. These factors make both sample preparation and analysis challenging.

Different analytical methods have been used during the years, most frequently "microbiological assay"; however despite being a highly sensitive method microbiological assay cannot distinguish between different folate derivatives. During the last 20 years chromatographic methods (mostly with UV and fluorescence detectors) have been more commonly used, making differentiation between different folate forms possible but at the loss of sensitivity. However, since 1999 tremendous progress has been made using mass spectrometry as detection technique with improved sensitivity and specificity. Nevertheless, many issues, problems and uncertainties remain regarding the application of these methods for folate analysis.

Because many people do not reach the recommended daily intake of folate, efforts have been made to increase folate content in food; for example, some countries fortify food with folic acid, the synthetic form of folate.

Yeast is an important component during several food manufacturing processes, with a remarkable ability to synthesise folate. One part of this work aimed therefore to investigate ways to enhance folate production using different yeast strains and bio-process conditions. With this knowledge, novel products with enhanced folate content using yeast as ingredients can be developed. To achieve this, analytical methods for folate analysis in yeast needed to be developed, which involved studies of the chromatographic behaviour of folates, stability, sample pre-treatment and detection optimisation.

1.2 Chemical structure and nomenclature of folate

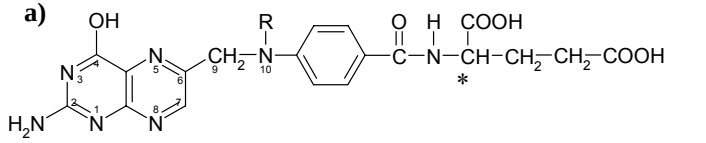
The IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) has defined recommendations on how nomenclature of folates should be used (Blakley, 1988). This nomenclature is complicated due to the many different forms possible. Theoretically more than 150 different forms of folates exist (Baugh & Krumdieck, 1971) although less than 50 are probably present in animal and plant tissue (Gregory, 1989). All folates are built up of a basic unit of 4-[(pteridine-6-ylmethyl)amino]benzoic acid skeleton conjugated with L-glutamic acid (**Figure 1 a-d**), often abbreviated to "pteroate" (abbreviated Pte). Pteroate can be futher divided into smaller units consisting of a pteridine ring and *para*-aminbenzoate (Eitenmiller & Landen, 1999).

The structural differences of the vitamin group of folate can roughly be divided into four main characters that give folate its unique properties. Firstly, folates differ in levels of reduction of the pteridine ring. Folates containing fully reduced pteridine rings (**Figure 1 d**) are called "tetrahydrofolates", those partially reduced (**Figure 1 b-c**) are called "dihydrofolates", and those fully oxidised are called "folic acid" (**Figure 1 a**). Secondly, folate differs in substituents located in the N-5 and N-10 position of the pteridine ring and can consist of hydrogen (H), methyl (CH_3), formyl (HCO), formimino (NHCH), methenyl (CH^+) and methylene (CH_2).

Folate derivatives are named depending on the nature of the substituent and reduction level of pteridine ring. For example a tetrahydrofolate, dihydrofolate and fully oxidised folate with a –HCO-substitution at N10-position is thus called 10-formyltetrahydrofolate (10-HCO-H_4folate), 10-formyldihydrofolate (10-HCO-H_2folate) and 10-formylfolic acid (10-HCO-folic acid) (**Figure 1 a, b, d**). Thirdly, the L-glutamate can be conjugated in repetitive units via γ-peptide linkage of the carboxyl group. These glutamates are called e.g. diglutamate and triglutamate, depending on the number of residues. For example, 5-methyltetrahydrofolate with 3 glutamate moieties is called 5-methyltetrahydropteroyltriglutamate (i.e. 5-CH_3-H_4PteGlu$_3$). Sometimes the term folate polyglutamates is used, meaning all folates with more than one glutamate residue regardless of any particular substituent. Fourthly, all fully reduced folates derived from tetrahydrofolate have two chiral centres. They are the α-C in the glutamic acid moiety and the C atom in position 6 of the pteridine ring. Thus, four diastereoisomers exist: [6S, αS], [6S, αR], [6R, αS] and [6R, αR]. The naturally biologically active form of the diastereoisomer is [6S, αS] for tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate) and 5-formyl tetrahydrofolate (5-HCO-H_4folate). The active form is [6R, αS] for 10-HCO-tetrahydrofolate (10-HCO-H_4folate), 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) and 5,10-methenyltetrahydrofolate (5,10-CH^+=H_4folate) (Groehn & Moser, 1999).

How folate is named often causes confusion when it is used. It is worth stressing that the name "folate" means all fully reduced or partially reduced forms of folates regardless of length of the glutamic residue. However, the term folates may sometimes be used in a generic sense, meaning any derivatives of folate or mixture of them, having various levels of reduction of the pteridine ring, one-carbon substitutions and a number of glutamate residues (Blakley, 1988). It is therefore also important to consider the context what the supposed meaning of "folate" is. Folic acid is virtually unknown in nature because very little tetrahydrofolate or dihydrofolate loses its hydrogen to form folic acid while remaining uncleaved (Scott *et al.*, 2000). However, folic acid can be manufactured synthetically and is used for fortification purposes in food or supplements such as tablets.

a)

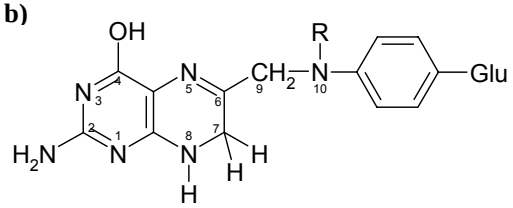


Pteridine ring — *para*-amino-benzoate — Glutamic acid (Glu)

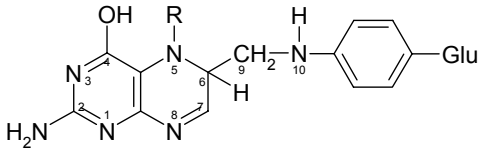
Pteroate (Pte)

para-aminobenzoylglutamic acid (PABG)

b)



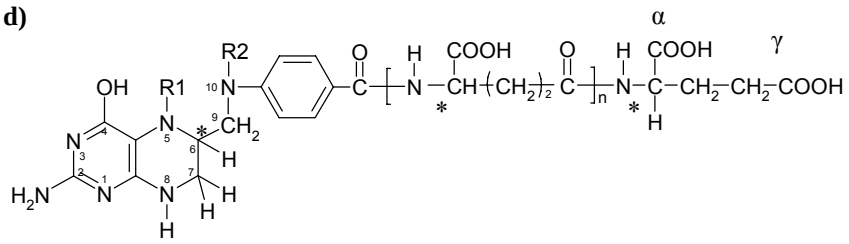
c)



a) Fully oxidized pteridine ring	
Substituent (R)	Folate derivative
-H	Folic acid
-HCO	10-formyl-folic acid (10-HCO-folic acid)

b & c) Partially oxidized pteridine ring	
b) Substituent (R)	Folate derivative
-H	Dihydrofolate (H_2folate)
-HCO	10-formyldihydrofolate (10-HCO-H_2folate)
c) Substituent (R)	
-CH_3	5-methyldihydrofolate (5-CH_3-H_2folate)
-HCO	5-formyldihydrofolate (5-HCO-H_2folate)

d)



* = tetrahedral stereocentres

d) Fully reduced pteridine ring		
Substituent (R1)	Substituent (R2)	Folate derivative
-H	-H	Tetrahydrofolate (H_4folate)
$-CH_3$	-H	5-methyltetrahydrofolate (5-CH_3-H_4folate)
-CH=NH	-H	5-formiminotetrahydrofolate (10-NHCH-H_4folate)
-HCO	-H	5-formyltetrahydrofolate (5-HCO-H_4folate)
-H	-HCO	10-formyltetrahydrofolate (10-HCO-H_4folate)
	Bridge R1-R2	
	$-CH_2-$	5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate)
	$-CH^+=$	5,10-methenyltetrahydrofolate (5,10-CH^+=H_4folate)

Figure 1 a-d. Structure and name of some folate derivatives with fully oxidised, partially oxidised and fully reduced pteridine ring. The number of extra glutamate units (n) can vary from 0 (folate monoglutamates) to 7 (folate polyglutamates).

1.3 The role of folate and associated health effects

Folates serve as donors and acceptors of one-carbon units in important metabolic reactions. Two processes of major importance are the synthesis of the base pairs of the nucleotides, DNA and RNA, and methylation of homocysteine to methionine **(Figure 2)**. As such, folate is inevitably wherever cell replication occurs. Especially important is its presence during rapid cell division, such as erythrocytes produced by the bone marrow. A deficiency in folate is therefore closely associated with development of anaemia. Moreover folate deficiency is associated with increased risk of the serious birth defect "neural tube defects" (NTDs) (Bjorklund & Gordon, 2006; Czeizel & Dudas, 1992; Laurence *et al.*, 1981; Medical *et al.*, 1991). Occurrence rates for NTD vary depending on geographic area, socioeconomic status and ethnic background (Pitkin, 2007). For example, in Europe (excluding UK and Ireland) there are a prevalence of around 10 cases of NTD / 10,000 pregnancies (Busby *et al.*, 2005) whereas in a region of China a prevalence was found to be 138.7 cases / 10,000 pregnancies, which is among the highest prevalences worldwide (Li *et al.*, 2006).

Plasma homocysteine is also closely connected to folate status, with accumulation of homocysteine if the folate status is poor due to reduction in the methylation cycle. High homocysteine level is an independent risk factor for development of cardiovascular disease and stroke (Lucock, 2000; Refsum *et al.*, 1998; Scott & Weir, 1996; Selhub *et al.*, 1996; Wald *et al.*, 1998). It must however be kept in mind that elevated plasma homocysteine levels can also be a result of other B-vitamin deficiencies, e.g. vitamin B_{12} or vitamin B_6 (Refsum *et al.*, 1998; Selhub & Rosenberg, 1996) or due to homocystinuria, an inborn error of metabolism. Homocystinuria results in methionine synthase deficiency due to an inability to synthesise the enzyme methionine synthase which catalyses the transfer of a methyl group from 5-CH_3-H_4folate to homocysteine, generating tetrahydrofolate and methionine as illustrated in **Figure 2** (Refsum *et al.*, 1998; Swanson *et al.*, 2001).

The way to measure folate status in humans is to quantify plasma or serum folate levels, where 5-CH_3-H_4folate is the dominating form. Plasma contains pteroyl-γ-glutamyl hydrolase (conjugase) and therefore all folates present in plasma are monoglutamates (Lucock, 2000). Serum folate levels less than 3 ng/ml (6.8 nmol/l) indicate a folate deficiency (poor), 3-5 ng/ml (6.7-11.1 nmol/l) a marginal deficiency and levels above 5 ng/ml adequate status (Sauberlich, 1990). However, measuring folate levels in erythrocytes (red blood cells) gives a more accurate picture not influenced by recent intake of folate, reflecting folate intake of the past 2-3 months (de Bree *et al.*, 1997). Levels of erythrocyte folate of <160 ng/ml (356 nmol/l) indicate a folate deficiency, and levels above 200 ng/ml (445 nmol/l) indicate acceptable folate status (Sauberlich, 1990). The practical implications of these groupings are e.g. that there is a more than eightfold increased risk of foetal NTDs as erythrocyte folate status falls from adequate to poor (Daly *et al.*, 1995). During erythrocyte formation, folates are incorporated, but these folates have no known metabolic function and are thought to function as a storage reservoir and long-term buffer for maintaining folate homeostasis. The

erythrocyte folates occur mainly as polyglutamates of 5-CH_3-H_4folate and 5-HCO-H_4folate (Lucock, 2000).

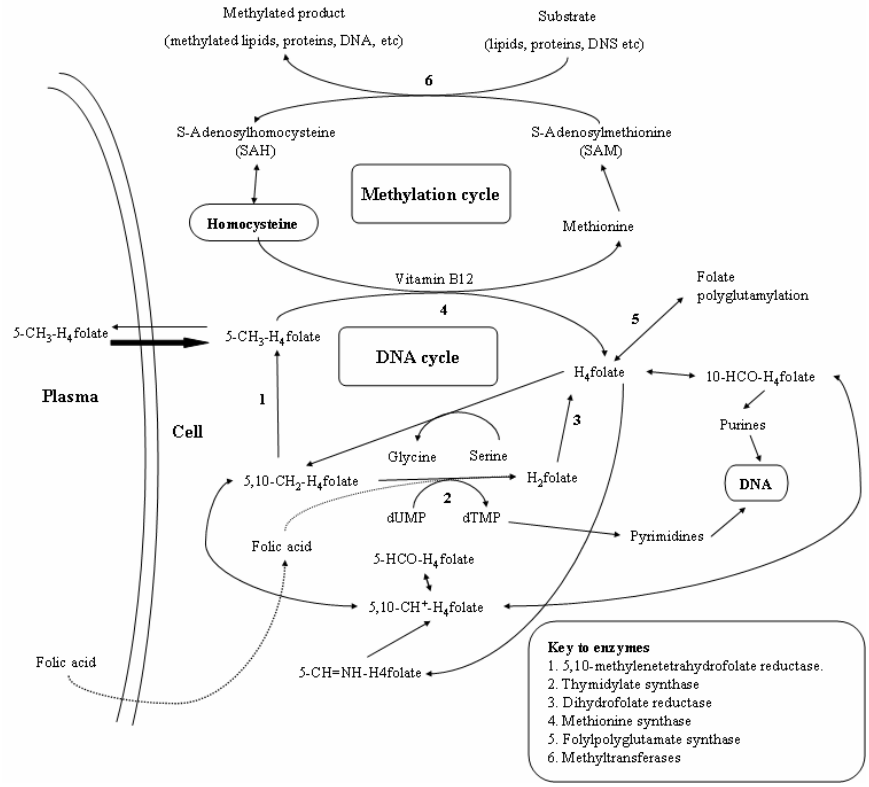


Figure 2. Folate functions in the cell. Modified from Scott, Rebeille & Fletcher (2000) and Lucock (2000).

1.4 Nutritional aspects of folate

Humans do not have the ability to synthesise folate and must therefore rely on adequate intake from other sources, primary from food. In Sweden the recommended daily intake (RDI) of folate is 300 µg for adults but 400 µg for fertile women and 500 µg for pregnant and lactating women (Becker *et al.*, 2004). As seen from **Figure 3**, most people do not reach the RDI of folate, except from vegetarians and vegans, according to a Swedish dietary survey, "HULK", from 1989 (Livsmedelsverket, 1994); similar findings were presented in a later dietary survey, "Riksmaten", done in 1997-1998 (Livsmedelsverket, 2002). However, inadequate folate intake is not just a Swedish phenomenon, it is worldwide. Folate intake differs between countries due to differences in dietary habits; for example, a European North-South gradient in dietary folate intake has been observed with the highest folate intake in Mediterranean countries and the lowest intake in Sweden, the UK, and Ireland (de Bree *et al.*, 1997). However, comparison of folate intake between different countries is complicated due to absence of reliable data for folates in different food products, which in turn is due to uncertainty of analytical

methods and lack of methodological control (de Bree *et al.*, 1997; Kariluoto *et al.*, 2002; Konings *et al.*, 2001; Konings *et al.*, 2002). Folate occurs in a wide range of food, and the most folate-rich foods are yeast, liver, leafy vegetables, beans and peas and fruits, e.g. orange and avocado (Witthöft *et al.*, 1999). According to a Swedish survey, different food sources contribute to the folate intake in the following way, expressed as relative percentages: vegetables (15%), fruit, berries and fruit juices (15%), cereals (22%), dairy products (16%), meat, fish and egg (16%) and potatoes (8%) (Livsmedelsverket, 2002).

Folate is present in food mainly as reduced polyglutamates. For example it has been estimated that approximately 75% of the folate content in American diets is present as polyglutamates (Sauberlich *et al.*, 1987). They need to be deconjugated (hydrolysed) before absorption by a lysosomal γ-glutamyl hydrolase enzyme (conjugase) that is present in intestines at the human brush border membrane. Intestinal folate absorption occurs over this membrane of the jejunum, and during passage into the blood plasma all folates become reduced and methylated (Brody, 1991). After passage into the cell, 5-CH_3-H_4folate is converted to H_4folate and thereafter to other forms depending on metabolic processes **(Figure 2)**. The knowledge about bioavailability of folate is limited (de Bree *et al.*, 1997). Several factors, i.e. the folate derivative, type of food and food composition, food processing, digestive physiology and drug intake may influence folate bioavailability (Gregory, 1997). The mean bioavailability of polyglutamates relative to monoglutamates is about 75% according to Gregory (1997). However, the data on folate bioavailability are contradictory and it was recently proposed that the degree of absorption is not related to the extent of folate conjugation (McKillop *et al.*, 2006). In a mixed diet, endogenous food folate has a bioavailability of less than 50% as compared to synthetic folic acid (Sauberlich *et al.*, 1987) but new data suggest it to be about 80% (Winkels *et al.*, 2007).

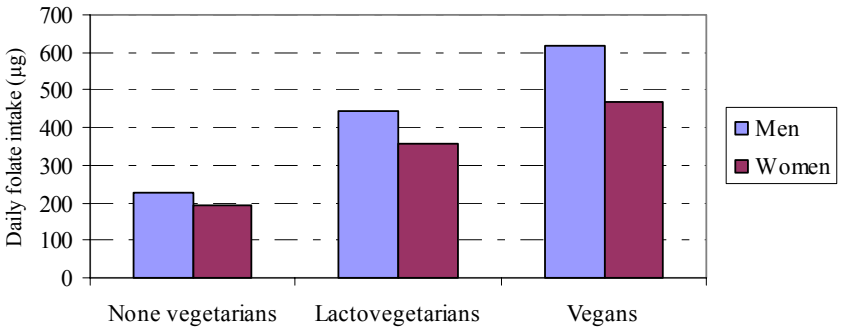


Figure 3. The daily average intake of folates by Swedish people (Livsmedelsverket, 1994).

1.5 Analytical methods

Folate analysis is difficult due to the many different folate derivatives, variable length of polyglutamate chain and instability. The choice of method depends on the final use of data. If the purpose is metabolic and bioavailability studies, chromatographic methods are necessary due to the need for detailed understanding of the effect of various folate derivatives (Scott *et al.*, 2000). On the other hand, if only total folate content is wanted, e.g. for labelling food products, microbiological assay has traditionally been the method of choice. Depending on the method chosen, different prerequisites during sample pre-treatment are needed.

1.5.1 Microbiological assay (MA)

Microbiological assay is based on use of a micro-organism that ultimately needs folate for growth. Theoretically its growth (read spectrophotometrically by using a UV detector) is logarithmically proportional to folate content in the sample, and traditionally folic acid is used as calibrant (Martin, 1995). Despite several drawbacks, the use of MA is regarded as the premium method of choice in folate analysis for determining total folate content. This is probably due to the high sensitivity of the method, which is its primary advantage (Arcot & Shrestha, 2005; Hawkes & Villota, 1989). The drawbacks are that it is tedious, liable to microbial and extraneous folate contamination, requires analytical skills (Quinlivan *et al.*, 2006), and that the narrowness of the logarithmic calibration curve (0.2-1.0 ng folic acid/ml) results in a need to estimate folate content in sample beforehand and a recommendation to make at least three different dilutions of each sample (Scott *et al.*, 2000).

There are several reports on implications, e.g. differences in growth response for different bacteria. The most frequently used bacteria in MA, *Lactobacillus rhamnosus* ATCC no. 7469 (formerly known as *Lactobacillus casei*), responds to all natural folate derivatives found in food, in contrast to many other bacteria investigated (Eitenmiller & Landen, 1999). However, if the polyglutamate chain of folate derivative is longer than 3 glutamate moieties, the response decreases drastically with increased length (Tamura *et al.*, 1972). This makes it necessary to convert all folate polyglutamates to mono- or diglutamate. However, it was observed that for reduced folate derivatives (5-CH_3-H_4folate and 5-HCO-H_4folate) and folic acid, the uptake of diglutamate in *L. rhamnosus* is not as active as for monoglutamate. This resulted in a lower growth response as compared to monoglutamate (Goli & Vanderslice, 1992; Phillips & Wright, 1983). Furthermore, the ability to respond equally, on a molar basis, to all different folate monoglutamates has been debated. Some researchers have found equal responses for different folate monoglutamates, provided at an appropriate pH (McKillop *et al.*, 2003; O'Broin *et al.*, 1975; Shane *et al.*, 1980) whereas others have observed the opposite (Goli & Vanderslice, 1989; Philips & Wright, 1982; Reingold & Picciano, 1982); this has lead some authors to stress that “the need for another method of analysis is even more apparent” (Reingold & Picciano, 1982).

The differences in growth response for folic acid and 5-CH_3-H_4folate were shown to depend on pH, buffer capacity and the folate concentration in sample, and could be smoothed by changing the initial pH of growth medium from 6.8 to 6.2 and by assuring high buffer capacity (Philips & Wright, 1982). Furthermore, non-folate substances in food matrices have been observed to promote growth of *Lactobacillus rhamnosus* (Hutner *et al.*, 1958; Rickes *et al.*, 1949; Snell, 1948). The growth rate of *L. rhamnosus* is greatly stimulated in the presence of aspargine, serine and glutamic acid, which are closely related metabolically to streptogenin (protein hydrolysates) (Rickes *et al.*, 1949; Sprince & Woolley, 1945), which is a growth-stimulating factor for certain microorganisms and laboratory animals. A large number of peptides have streptogenin activity, and may therefore serve as a simple accessible source of amino acids (http://www.mercksource.com/pp/us/cns/cns_home.jsp 25-Jan 2007). The formulation of folic acid depleted basal medium that is used for incubation of *L. rhamnosus* in MA is based particularly on folic acid analysis in blood serum (http://www.bd.com/ds/technicalCenter/inserts/Folic_Acid_Casei_Medium.pdf 23-Jan 2007 (Baker *et al.*, 1959; Flynn *et al.*, 1951; Waters *et al.*, 1961). Because the matrix of serum is totally different from that of food, the growth of *L. rhamnosus* in extracts from food may give incorrect results. On the other hand, the increased growth rate observed for *L. rhamnosus* when ascorbic acid is used (Herbert, 1961) was proved not to be a growth factor of *L. rhamnosus*. Instead, it is a result of increased stability of folic acid during incubation of the assay (Waters *et al.*, 1961).

All these uncertainties have resulted in that the officially recognised AOAC method for analysis of folate, a microbiological assay (AOAC International 992.05 and 944.12) (International, 2000) is only approved for the analysis of folic acid in vitamin preparations and infant formula. There is still no method that has been officially approved for the measurement of naturally occurring folates in foods.

1.5.2 Radioprotein-binding assay (RPBA)

An alternative method for folate analysis is radioactive-labelled protein binding assays (RPBA), based on a competition between folate in sample with radiolabelled folate for binding sites on a folate-binding protein. The concentration of folate in sample corresponds to the relationship between protein-bound and free species of the labelled folate. The RPBA method was developed shortly after the identification of folate binding proteins (Waxman *et al.*, 1971). The application to food analysis is, in spite of the convenience, limited because of problems with variations of ligand binding affinity for the multiple folate forms present in many foodstuffs (Shane *et al.*, 1980; Strålsjö *et al.*, 2002). Especially low affinity to folate binding proteins (as compared to folic acid) has been observed for 5-HCO-H_4folate (Gregory *et al.*, 1982; Strålsjö *et al.*, 2002), whereas H_4folate has a much higher affinity (Strålsjö *et al.*, 2002) and the affinity for 5-CH_3-H_4folate is in the same range as for folic acid presupposing that a pH of 9.3 is used in the assay (Givas & Gutcho, 1975; Strålsjö *et al.*, 2002). This stresses the necessity for having only one dominating form of folate in the sample, which rules out the use of RPBA as a method of choice for analysing natural folate in many food items

(Ball, 1998). Furthermore, several more limitations have been observed for RPBA such as low precision between assays and laboratories, and poor agreement with the microbiological assay (De Souza & Eitenmiller, 1990; Finglas *et al.*, 1993; Shane *et al.*, 1980; van den Berg *et al.*, 1994). On the other hand, because 5-CH_3-H_4folate is almost the only form present in blood (except from folic acid under certain conditions) RPBA is a good-working method for the assay of folate in blood and serum, and has today mainly replaced the use of MA, recognised as an important clinical tool to evaluate the folate status of a patient and to diagnose megaloblastic anaemia (Eitenmiller & Landen, 1999). Compared to MA, RPBA is less tedious, less expensive, less time consuming and less influenced by possible presence of antibiotics or substances that can promote growth of *Lactobacillus casei* in MA (Ball, 1998). However, as stated by Eitenmiller & Landen (1999) the failure to obtain similar protein binding with all folate derivatives with RPBA in food analysis leads to the need of good LC methods.

1.5.3 Chromatographic methods (HPLC)

One of the main objectives in chromatographic methods is to separate and quantify the different forms of folates, which is important due to the different degree of bioavailability depending on the one-carbon substituents (Gregory *et al.*, 1992; Hawkes & Villota, 1989). The detection is usually based on UV and fluorescence detectors, but in recent years use of mass spectrometric methods has become more common. Separation techniques used have included size exclusion, ion-exchange and also reversed phase chromatography**,** whose first application in food folate analysis was reported in 1981 (Day & Gregory, 1981). However, as early as in 1975 RP-HPLC was regarded as an excellent alternative to ion-exchange chromatography for analysis of folate derivatives (Bush *et al.*, 1979). It was discovered that retention of folates increases as the pH decreases because the carboxylic groups get more and more undissociated (Bush *et al.*, 1979) and hence it was argued that selectivity could be drastically modulated by manipulating pH of the eluent. As shown by the authors, the elution order was proportional to the number of glutamate residues when the carboxylic groups of folate polyglutamates were undissociated at low pH (due to increased hydrophobocity). However, it became inversed as pH gradually was increased and the groups became dissociated. Since 1992 most of the folate methods published have used reversed-phase chromatography with C18-columns (Eitenmiller & Landen, 1999; Hawkes & Villota, 1989; Vahteristo & Finglas, 2000). The retention in reversed-phase chromatography largely depends on the degree of hydrophobocity of folate, which is influenced by the degree of ionisation and the different one-carbon substituents. Folates are multifunctional compounds with at least two carboxylic groups (i.e. for the monoglutamatic forms) with pK_a around 3.1-3.5 for the strongest acid group (α-carboxyl group). To render these carboxyl groups uncharged, a mobile phase buffer with pH 2-3 has commonly been used to achieve proper separation and retention. However, this acidic pH in mobile phase buffers is highly unsuitable for identification and quantification of some folate derivatives, such as 10-HCO-H_4folate and 5,10-CH_2-H_4folate, due to rapidly conversion to 5,10-CH^+=H_4folate and H_4folate (Blakley, 1960; Wilson & Horne, 1984) as stated by several authors (Pfeiffer *et al.*, 1997; Vahteristo *et al.*, 1997b).

The surface chemistry of reversed-phase stationary phases is important for separation and peak shapes of folates, and hence the choice of column may considerably influence the chromatographic result. Despite importance of this topic, extensive column comparative studies for folate analysis have been limited. However, one study found that alkyl-bonded stationary phases exhibited better chromatographic performance than phenyl-bonded phases and polar-bonded (cyano) stationary phase (Johansson *et al.*, 2005). It was also found that buffer of low ionic strength, i.e. acetate buffer, negatively influenced peak shape in comparison to phosphate buffer with higher ionic strength.

Use of acetonitrile gradient results in increased resolution and narrower peak widths in comparison to isocratic mode as reviewed by Gregory (1984) and shown by Bush et al. (1979). For most cases in food analysis, however, it is not possible to separate a complex mixture of folate derivatives with different length of the polyglutamate chain. A deconjugation step is therefore also necessary with use of HPLC, in the same way as for MA and RPBA. By using this step it is possible to limit the number of folate derivatives to be analysed to only a few folate monoglutamates. In this case the concentration of each folate derivative corresponds to the sum of all mono- and polyglutamates, which makes their separation easier. This also results in less demand of detector sensitivity because the folate content to be determined is not as low as when deconjugation step is omitted. Furthermore, the work to prepare all the different folate standards would be overwhelming both in terms of time and cost. Therefore, prior to separation by chromatography, folate polyglutamates are usually deconjugated to monoglutamates to simplify analysis and improve detection limit and achieve adequate separation. The addition of deconjugation step allows to avoid use of numerous polyglutamate standards (all of them are not commercially available). However, an alternative to deconjugation is to convert all different folate pteroylpolyglutamate derivatives to 5-CH_3-H_4folate and separate these polyglutamates without a proceeding deconjugation (Ndaw *et al.*, 2001). In this way information about the folate polyglutamate content in food can be obtained, but simultaneously information about different folate derivatives is lost.

The main disadvantage of HPLC is the need of sample purification before analysis, which is both tedious and complicated. However, the use of automated SPE robots may facilitate this part in the future. Another limitation is the low sensitivity for some folate forms compared to MA by use of UV or fluorescence detector. The introduction of mass spectrometric detectors in 1999 has nonetheless improved sensitivity and specificity of folate analysis.

1.6 Sample pre-treatment and pitfalls

Sample treatment before HPLC or LC-MS analysis can roughly be divided into three different stages: extraction, deconjugation and sample purification, each of which has its own challenges. Sample pre-treatment is an important step because "regardless of the analytical procedure, the accuracy of analysis is highly dependent on the merits of the preparative methods employed" (Gregory *et al.*, 1990). Moreover the choice and handling of folate standards is critical for the final result. In this section these different aspects will therefore be discussed.

1.6.1 Stability, interconversion and choice of folate standards

The degree of folate stability differs between different forms and is pH dependent. Substitution at the N^5 position improves stability of folate, rendering it less susceptible for C^9-N^{10} bond cleavage. This may be because these substituents prevent resonance forms of the molecule, which are associated with oxidative degradation of folates. They may also constitute steric hindrance for oxidative reagents to approach the molecule (Gregory, 1989). The susceptibility for degradation of folate was found to be in the following order: folic acid < 5-HCO-H_4folate < 5-CH_3-H_4folate < 10-HCO-H_4folate ≤ H_4folate (O'Broin *et al.*, 1975). However, as discussed by the authors, the indication that 10-HCO-H_4folate is more stable than H_4folate, could depend on conversion of 10-HCO-H_4folate to the more stable forms of 5-HCO-H_4folate and 10-HCO-folic acid, a hypothesis not possible to substantiate by their use of microbiological assay. The length of glutamate chain does not seem to influence folate stability according to both Gregory (1996) and Matella et al. (2005).

5-CH_3-H_4folate can at low or neutral pH be oxidised to 5-CH_3-H_2folate, which in turn degrades to an unidentified pterin and pABG in acidic medium. However, in the presence of antioxidants (ascorbate or thiols or both) 5-CH_3-H_2folate is rapidly reduced back to 5-CH_3-H_4folate, as reviewed by Gregory (1996). Stability of 5-CH_3-H_4folate increases as pH decreases from 6 to 4, which is probably due to increased protonation at N^5 position (pK_a = 5.2) (Gregory, 1996). However, conflicting results of pH dependences have been reported (O'Broin *et al.*, 1975; Paine-Wilson & Chen, 1979).

H_4folate is more stable between pH 1-2 and 7-12 than between pH 4-6 (Gregory, 1996; O'Broin *et al.*, 1975; Paine-Wilson & Chen, 1979) but even in the more favourable pH range H_4folate is extremely unstable (Gregory, 1996). H_2folate is highly labile even in the presence of ascorbic acid under nitrogen atmosphere (O'Broin *et al.*, 1975). When H_4folate or H_2folate is degraded, they generally lose their side chain, resulting in pABG and pterin-6-carboxylic acid as the main degradation products (Gregory, 1996; Hawkes & Villota, 1989) but even formation of folic acid has been reported (Smith *et al.*, 2006; Wilson & Horne, 1984).

5,10-CH_2-H_4folate is more stable in the presence of oxygen than H_4folate (Blakley, 1969; Hawkes & Villota, 1989; Osborn *et al.*, 1960) but at pH < 9.5

5,10-CH_2-H_4folate dissociates to H_4folate and formaldehyde, even in the presence of 2-mercaptoethanol (Osborn *et al.*, 1960; Quinlivan *et al.*, 2006; Smith *et al.*, 2006; Wilson & Horne, 1984). The reversed process, formation of 5,10-CH_2-H_4folate from H_4folate in the presence of formaldehyde, is 40 times more favourable when the N^5 position of H_4folate is unprotonated (pK_a = 4.82) (i.e. at pH ≥ 7) (Gregory, 1989) as also indicated by Osborn et al. (1960).

Compared to other folate forms, 5-HCO-H_4folate is relatively stable to oxidation and thermal degradation because the pteridine ring is stabilised by the formyl-substituent group. 10-HCO-H_4folate is easily oxidised to either the stable form 10-formylfolic acid or to a pterin and pABG (Gregory, 1996). However, there is no loss of vitamin function if it degrades to 10-formylfolic acid since this form was proved to exhibit biological activity (Gregory *et al.*, 1984a). Heat causes inevitable conversion of 10-HCO-H_4folate to 5-HCO-H_4folate as reviewed by Gregory (1989).

5,10-CH^+=H_4folate is formed at acidic pH < 4 by conversion of 10-HCO-H_4folate and 5-HCO-H_4folate after loss of a water molecule. Conversely, at pH > 4.5 5,10-CH^+=H_4folate is mainly hydrolysed to 5-HCO-H_4folate (Baggott, 2000).

5-CH=NH-H_4folate, like 5-HCO-H_4folate, is relatively unaffected by oxidation in presence of oxygen. However, it is easily hydrolysed resulting in loss of ammonia and it has a half-life of less than 1 min at 100 °C at pH 5-9, as reviewed by Blakley, 1969. In acid solution it is also easily converted to 5,10-CH^+=H_4folate (Quinlivan *et al.*, 2006).

Because of the interconversions and stability problems, there are limitations about which folate forms can be found in food extracts. As mentioned above, 10-HCO-H_4folate, H_2folate and 5-CH=NH-H_4folate are highly unstable. Furthermore, the pH used during pre-treatment of food samples is often in the range 4.5 - 7.85. Therefore 5,10-CH^+=H_4folate mostly converts to 5-HCO-H_4folate and 5,10-CH_2-H_4folate converts to H_4folate. Thus, the folate forms usually expected to be found in sample extracts are 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate. Moreover folic acid and 10-formylfolic acid are of interest because folic acid is commonly used in fortification purposes and 10-HCO-folic acid is a possible degradation product of 10-HCO-H_4folate. In this work we therefore used folate standards of 5-CH_3-H_4folate, H_4folate, 5-HCO-H_4folate, 10-formylfolic acid and folic acid, which is in accordance with other groups (Finglas *et al.*, 1999; Freisleben *et al.*, 2003a; Freisleben *et al.*, 2003b; Ginting & Arcot, 2004; Osseyi *et al.*, 2001; Vahteristo *et al.*, 1997a; Vahteristo *et al.*, 1997b; Zhang *et al.*, 2005). A recent report showed that only 5-HCO-H_4folate, 5-CH_3-H_4folate, folic acid and H_4folate are stable enough as standards in buffers, excluding the use of 10-HCO-H_4folate, 5,10-CH_2-H_4folate, 5,10-CH^+=H4folate and H_2folate (Smith *et al.*, 2006). However, to accurately quantify even these more labile forms during analysis of erythrocytes, the authors thought it was preferable to report comprehensive results for interconverting folates rather than for individual derivatives. Hence the different folate derivatives was divided into three different groups: unsubstituted (folic acid and H_2folate), non-methylH_4folate (5-HCO-H_4folate, H_4folate, 10-

HCO-H_4folate, 5,10-CH_2-H_4folate, 5,10-CH^+=H4folate) and 5-CH_3-H_4folate (respectively based on the rationale that interconversions were restricted to within the folate groups.

The choice of folate standards for calibration is sometimes contradictory and not based on stability data for different folate forms. There are reports on findings of 10-HCO-H_4folate, H_2folate, 5,10-CH^+=H_4folate in yeast and spinach (Belz & Nau, 1998; McKillop *et al.*, 2003). In mouse plasma and erythrocytes 10-HCO-H_4folate, H_2folate, 5,10-CH^+=H_4folate and 5,10-CH_2-H_4folate was found (Belz & Nau, 1998). These forms were found despite the use of buffers which could not provide good stability. There are also several reports on the presence of 10-HCO-H_2folate in food after sample extraction, especially extracts derived from bread (Gujska & Majewska, 2005; Kariluoto *et al.*, 2001; Kariluoto *et al.*, 2004; Pfeiffer *et al.*, 1997; Ruggeri *et al.*, 1999) from chickpeas (Ruggeri *et al.*, 1999) and vegetables (Konings *et al.*, 2001). These findings were considered to be an artefact from sample preparation because of high susceptibility of 10-HCO-H_4folate and 10-HCO-H_2folate to degradation (Freisleben *et al.*, 2003b). Indeed, neither 10-HCO-H_4folate nor 10-HCO-H_2folate was found in a wide range of food, e.g. bread and vegetables (Freisleben *et al.*, 2003b; Konings, 1999). Nevertheless, there are indications that 10-HCO-H_2folate is the biological active form, not 10-HCO-folic acid, and therefore 10-HCO-H_2folate might be present naturally in nature (Baggott & Johanning, 1999; Baggott & Tamura, 2001). However, prolonged air oxidation in solution of 10-HCO-H_2folate resulted in oxidation to 10-HCO-folic acid; furthermore, the susceptibility to oxidation and/or degradation of 10-HCO-H_2folate seem to be pronounced since it was only stable for 3 days when stored at -70 ºC (Baggott *et al.*, 1995).

In an attempt to overcome these problems, two buffers with different pH were used to extract liver folate before analysis on HPLC coupled to microbiological assay (Horne, 2001). Buffer 1 (pH 10) was used to determine 5,10-CH_2-H_4folate and buffer 2 (pH 7.85) to determine 5-CH_3-H_4folate, H_4folate, 5-HCO-H_4folate and 10-HCO-H_4folate.

1.6.2 Extraction procedure

Because folates are sensitive to oxidation under exposure to heat, light and air as reviewed by Ruddick et al. (1980) and shown by other researchers (O'Broin *et al.*, 1975; Paine-Wilson & Chen, 1979) there is a need to use antioxidants to retard degradation. Usually ascorbic acid has been used as antioxidant because it was proved to retard degradation of folates, especially H_4folate (Chen & Cooper, 1979; Jastrebova *et al.*, 2003; O'Broin *et al.*, 1975; Waters *et al.*, 1961). However, it was reported that heated ascorbate solution resulted in interconversions of folates. The formation of 5,10-CH_2-H_4folate from H_4folate was probably because of formation of formaldehyde due to degradation of ascorbic acid during heating, which then reacted with H_4folate (Wilson & Horne, 1983). The combined use of 2% ascorbic acid/ascorbate and 0.1% 2-mercaptoethanol was later shown to eliminate interconversions for some folate derivatives, especially for H_4folate, because formaldehyde reacts with 2-mercaptoethanol to form hemithioacetal which then

removes formaldehyde from the system (Wilson & Horne, 1984). Nevertheless, with this system 5,10-CH_2-H_4folate was still converted to H_4folate, and H_2folate was still sensitive to degradation and was converted partially to folic acid. However, even though interconversions could be prevented for some folate derivatives by combined use of antioxidants, this was not enough to completely protect against degradation during heating (recovery varied 69-72%). Worth mentioning in this context is that use of 2,3-dimercaptopropanol as early as in 1960 was shown to protect H_4folate much better from degradation than 2-mercaptoethanol (Blakley, 1960) but that finding sank into oblivion with time. Irrespective, the superiority of the combined use of ascorbic acid and 2-mercaptoethanol during sample treatment were later confirmed (Pfeiffer *et al.*, 1997; Vahteristo *et al.*, 1996) with increased stability especially for H_4folate, but also for 5-CH_3-H_4folate 5-HCO-H_4folate. 2-mercaptoethanol has indeed been the most commonly used antioxidant, but even dithiothreitol has sometimes been used (Lucock *et al.*, 1993; Nelson *et al.*, 2006).

Another way to retard degradation is to decrease dissolved oxygen concentration in sample solution as shown for 5-CH_3-H_4folate (Ruddick *et al.*, 1980). Use of nitrogen gas flushed to folate standard solution prior to boiling was shown to retard degradation of folates (Chen & Cooper, 1979; Vahteristo *et al.*, 1996). As an alternative to nitrogen, argon gas can also be used (Freisleben *et al.*, 2003a).

The most common procedure to extract folate is thermal treatment of sample in either acetate buffer pH 4.5-5, phosphate buffer pH 6-7 or Ches/Hepes buffer pH 7-8 (Eitenmiller & Landen, 1999) to denature folate-binding proteins and enzymes that may promote folate degradation or interconversions (Gregory, 1989). The choice of buffer system depends on the pH optimum of the conjugase source used during the deconjugation step (Gregory *et al.*, 1990). The evaluation of extraction efficiency of Ches/Hepes buffer pH 7.85 and phosphate buffer pH 6.0 by use of similar antioxidant revealed no significant differences (Vahteristo *et al.*, 1997a).

The ratio of buffer volume to the amount of sample was shown to be important for good recovery during the extraction step, and may vary depending on the food matrix composition (de Quiros *et al.*, 2004; Zhang *et al.*, 2005). Moreover a second homogenisation and centrifugation step of the pellets improved the yield of folate (8-51%), but the grade of improvement depended on food matrix and buffer used. Use of buffer with different pH (phosphate buffer with 1% ascorbate + 0.1% 2-mercaptoethanol) was shown to influence the recovery of folate, with different pH optimum for different folate derivatives (Zhang *et al.*, 2005). Therefore, it seems difficult to use a pH optimal for all folate derivatives, but a suitable compromise seemed to be a buffer with pH around 6-7. However, the use of 2% ascorbate instead of 1% in phosphate buffer can be more optimal because it was shown to significantly retard degradation of H_4folate (Jastrebova *et al.*, 2003).

It was reported that during heat treatment in 0.05 M acetate buffer with 1% ascorbic acid, pH 4.9, 10-HCO-H_4folate partially converted to 5-HCO-H_4folate. This interconversion was completed after 60 min of heat treatment and a 60-min

heating time was therefore selected to simplify quantification of formyl-H_4folates (Gregory *et al.*, 1984b). However, it was observed that the total folate content was decreased after 60 min of boiling instead of 10 min of boiling and therefore 60 min of boiling was rejected (Vahteristo *et al.*, 1996), which was also confirmed in another study (Zhang *et al.*, 2005).

An additional procedure to release matrix-bonded folates is the so-called "tri-enzyme treatment". In addition to heat treatment, the combination of protease and α-amylase was shown to increase measurable folates from complex food matrices by use of a microbiological method (Martin *et al.*, 1990), which might be due to binding of folates to protein and carbohydrates in foods. Therefore, foods high in protein and carbohydrate levels showed a tendency to liberate more folate after these enzyme treatments since they can degrade proteins and starch (De Souza & Eitenmiller, 1990; Pfeiffer *et al.*, 1997; Ruggeri *et al.*, 1999).

Use of tri-enzyme treatment prior to folate determination is suggested essential to obtain true folate content in food (Aiso & Tamura, 1998). This viewpoint is supported by several experimental investigations that have compared the tri-enzyme method with mono-enzyme treatmeant in different food products (Johnston *et al.*, 2002; Lim *et al.*, 1998; Martin *et al.*, 1990; Pfeiffer *et al.*, 1997; Tamura *et al.*, 1997).

Despite many reports concluding that tri-enzyme treatment is superior, others conclude the opposite. In recent years it has also been realised that food folate values do not always increase after tri-enzyme extraction as reviewed by Huyn & Tamura (2005). For example, when an autoclavation step was introduced for 10 min at 121°C during folate extraction of spinach, fortified white bread, whole grain wheat cereal and oat, corn and wheat cereal, the tri-enzyme treatment was significantly more efficient only for fortified white bread (Shrestha *et al.*, 2000). Moreover no differences could be shown in folate content for yeast, spinach, beef liver, beef fillet and peas by comparing tri-enzyme treatment with only heat and mono-enzyme treatment (Ndaw *et al.*, 2001).

Contradictory results exist as well. Higher folate content was found in spinach by use of tri-enzyme method (Aiso & Tamura, 1998; Martin *et al.*, 1990) but mono-enzyme treatment was in another study found to be advantageous for spinach and broccoli (Iwatani *et al.*, 2003).

Use of protease with chromatography as analytical method may indeed also be unfavourable since that treatment was found to result in very high background noise (Zhang *et al.*, 2005). There is also a risk of increased folate degradation due to prolonged incubation time. Use of 16 h incubation with protease instead of 1 h (after previous incubation of 4 h with α-amylase and rat plasma) resulted in 20% decrease of total folate content, and the use of such a long treatment time was therefore not recommended (Pfeiffer *et al.*, 1997). A similar result was obtained during deconjugation with chicken pancreas for 20 h instead of 1 h (Pedersen, 1988).

The tri-enzyme treatment is thus a contradictory area, which needs more research. The necessity of this procedure for all food matrixes is still questionable. In bioanalysis, for instance, SPE, liquid-liquid extraction and protein precipitation are sucessfully used to break matrix-bindings of the analyte by careful selection of pH, solvent and buffers (Ascoli *et al.*, 2006; Brockman *et al.*, 2007; Misl'anová & Hutta, 2003; Musteata & Pawliszyn, 2007; Musteata *et al.*, 2006; Rossi & Wright, 1997; Wiltshire, 2000). Indeed, "the goal of sample preparation is to eliminate interfering compounds from the matrix using a minimum number of steps, resulting in reproducible methodology" (Musteata & Pawliszyn, 2007). Therefore the development of simplier techniques to release matrix-bonded folates may be of interest.

1.6.3 Deconjugation

The deconjugation process in which the glutamatic resin is cleaved is enzyme driven. The most common conjugase sources used are rat plasma/serum or human plasma, chicken pancreas and hog kidney. The final product of the deconjugation step are folate monoglutamates except for chicken pancreas (folate diglutamates) as shown by Goli & Vanderslice (1992). This makes chicken pancreas primarily useless if chromatographic methods are to be used, due to lack of diglutamatic folate standards, but is on the other hand often applied when MA is used.

Plasma conjugase is believed to be an exopeptidase that trunctates the polyglutamyl chain successively to yield single glutamates, whereas chicken pancreas conjugase, an endopeptidase, truncates the chain as a whole at the di-glutamate-triglutamate junction (Goli & Vanderslice, 1992). However, chicken pancreas seems to contain also a small amount of exopeptidase that can convert folate polyglutamates to monoglutamate (Goli & Vanderslice, 1992; Leichter *et al.*, 1977). Use of rat plasma/serum instead of human plasma is favourable due to its higher conjugase activity (Vahteristo *et al.*, 1996; Zhang *et al.*, 2005). A comparison of rat plasma/serum, hog kidney and human plasma showed that rat plasma/serum has the highest conjugase activity (Doherty & Beecher, 2003; Ndaw *et al.*, 2001). Rat serum/plasma is also commonly used today because it is easily available and has low endogenous folate (Tamura, 1998). In comparison of hog kidney and chicken pancreas at different concentrations, hog kidney was shown to give the highest folate values at maximal conjugase activity, which, according to the authors, could be due to the different modes of transport used by *L. casei* for the different deconjugation products (Phillips & Wright, 1983). However, later research found no differences of folate levels for these two enzyme treatments (Kirsch & Chen, 1984; Pedersen, 1988).

Selection of buffer seems to be important since inhibition of hog kidney conjugase was noticed in acetate buffer, but not in phosphate buffer (Vahteristo *et al.*, 1996). The pH optimum for optimal rat serum/plasma conjugase activity has in several studies been found to be around 6-7 in phosphate buffer (Doherty & Beecher, 2003; Horne *et al.*, 1981; Zhang *et al.*, 2005) and 7-7.5 in Ches/Hepes buffer (Horne *et al.*, 1981) but for human plasma conjugase it is at pH 4.5 (Goli &

Vanderslice, 1992). However, it was recommended to use a pH around 6-7 for human plasma irrespective due to observed folic acid degradation at pH 4.5; still, it was stressed that human plasma should be in excess to obtain optimal results at these conditions (Goli & Vanderslice, 1992). Further research revealed chicken pancreas, in phosphate buffer pH 6.1, to significantly increase folate level in comparison to human plasma, in phosphate buffer pH 4.5, when using MA (Shrestha *et al.*, 2000).

The pH optimum for hog kidney conjugase was found to be in the interval of 4.3-4.6 (Bird *et al.*, 1946; Engelhardt & Gregory, 1990; Goli & Vanderslice, 1992; Pedersen, 1988) and 4.8-5.0 (Vahteristo *et al.*, 1996) but the stability of hog kidney conjugase was found optimal around pH 5-7 (Engelhardt & Gregory, 1990). The conjugase activity of chicken pancreas was shown to depend on both type of buffer and pH. In tris-HCl buffer the pH optimum is 8-8.5 (Kirsch & Chen, 1984; Leichter *et al.*, 1977). In phosphate buffers it was found to be 5.6 (Kirsch & Chen, 1984) which is in contrast to another report claiming a pH optimum of 7 (Bird *et al.*, 1946). In an evalutation to find the optimal buffer for deconjugation with chicken pancreas, spinach extract was used in several buffers at their respective optimal pH. No difference in deconjugation rate could be found for tris-HCl, potassium phospahte-NaOH and sodium phosphate after 1 h of incubation (Kirsch & Chen, 1984).

More recently, large variations in conjugase activity (decrease 42% of conjugase activity) were found between different batches of rat plasma (Rychlik *et al.*, 2007). By combined use of chicken pancreas and rat plasma complete deconjugation was nearly achieved (96%). Increased rat plasma conjugase activity was also achieved by addition of 2-mercaptoethanol in buffer, whereas there was a 93% loss of conjugase activity in rat plasma if 2-mercaptoethanol was not added (Horne *et al.*, 1981). Many other different antioxidants, i.e. ascorbic acid and 2,3-dimercaptopropanol, and specific enzymes (ribonuclease and thymononucleodepolymerase) were also shown to increase conjugase activity (Mims *et al.*, 1947). On the other hand, the presence of citrate in sample was shown to strongly inhibit the conjugase activity of chicken pancreas or human plasma or both (Goli & Vanderslice, 1992; Gregory *et al.*, 1990; Kirsch & Chen, 1984). Other salts (acetate, tartarate, chloride and oxalate) also have inhibitory effects for human plasma, chicken pancreas and hog kidney, but to much less extent than citrate (Goli & Vanderslice, 1992; Vahteristo *et al.*, 1996). Yeast extracts exhibited strong inhibitory effect to hog kidney conjugase (Bird *et al.*, 1946), which was shown to depend on the nucleic acids in yeast (Mims *et al.*, 1947).

However, different conjugase sources exhibit different sensitivity to the same inhibitor (Kirsch & Chen, 1984; Ndaw *et al.*, 2001), and chicken pancreas conjugase was for example shown not to be inhibited by nucleic acid of yeast (Mims *et al.*, 1947). It is difficult to give a general advice of conjugase amount needed for complete deconjugation in food sample extracts since food contains different components with inhibitory effects (Engelhardt & Gregory, 1990; Goli & Vanderslice, 1992; Gregory *et al.*, 1990). For example, deconjugation of 5 µM

$PteGlu_3$ was completed after 60 min with hog kidney in acetate buffer, whereas 180 min was required when spiking the same amount of $PteGlu_3$ to a liver extract using the same buffer system. This was suggested to depend partly on presence of anionic polysaccharides. Increasing the concentration of conjugase was shown to overcome the inhibitory effects (Engelhardt & Gregory, 1990). It is therefore adviseable to add conjugase and spike with $PteGlu_3$ in sample extracts to evaluate conjugase activity as shown by Rychlik et al. (2007). One recently developed way to avoid the conjugase inhibitory effect is to purify food extracts by SPE before the deconjugation step (Rychlik *et al.*, 2007), a procedure usually done after the deconjugation step.

1.6.4 Sample Purification

As mentioned earlier (Gregory, 1984; Rebello, 1987), methods for folate analysis with chromatography gained interest in the late 1970s but had limited applications for analysis of biological samples due to lack of sensitivity and specificity, partly due to inadequate sample clean up. Efforts in the early 1980s with sample clean up resulted in considerable dilution of sample, which made it necessary to incorporate an overnight lyophilisation step prior to HPLC for quantification with a UV detector (Duch *et al.*, 1983). Lyophilisation step was however regarded not applicable to other biological materials than liver and kidney due to the limited sensitivity of UV detection (Gregory *et al.*, 1984b). The time to prepare the bio-beads columns used for purification (Duch *et al.*, 1983; Gregory *et al.*, 1982; Gregory *et al.*, 1984b) was also time consuming and considered the most rate limited step of the chromatographic method (Gregory *et al.*, 1984b).

However, with the introduction of commercial solid phase extraction columns (SPE columns) in the beginning of 1980s, the preparative step disappeared. The SPE columns commonly used in folate analysis are strong anion exchange columns (SAX) (Finglas *et al.*, 1999; Nilsson *et al.*, 2004), which consist of positively charged aliphatic quaternary amine group bonded to silica surface. During application of sample to column at appropriate pH (over pH 5.5, i.e. 2 pH units above the pKa of α-COOH-group), negatively charged folate are electrostatically attracted to the positively charged quaternary amine group and sample impurities of non-anionic character pass through. The first use of SAX was in 1984 but was only applied to analysis of folic acid (Schieffer *et al.*, 1984). In another study, the use of weak anion exchange columns (NH_2-sorbent) was reported (Rebello, 1987), but later it was recommended to use SAX sorbent instead of NH_2 sorbent because SAX cartridges make the method more robust since this sorbent is much less affected by small changes in matrix than that of NH_2 (Vahteristo *et al.*, 1996). Use of weak anion exchange during folate analysis has been limited. Another alternative for sample purification is the use of reversed-phase SPE columns (Nelson *et al.*, 2001; Nilsson *et al.*, 2004; Pfeiffer *et al.*, 2004; Stokes & Webb, 1999). By use of these sorbents, folates are retained based on their hydrophobicity and need therefore to be in uncharged form. Depending on the sorbent used (e.g. anion exchange and non-polar sorbents), the pH of the conditioning solution, sample extract and elution buffer must differ so that folates to be retained and eluted. Use of reversed-phase (C18) adsorbents for

sample purification was recommended because there was no need to use high salt concentrations of the eluent, which are not compatible with stable ESI MS (Nelson *et al.*, 2005). However, this problem can be overcome by directing the effluent to waste at the beginning of run programme thereby obtaining a stable spray (Freisleben *et al.*, 2003b).

Folates are also sensitive to degradation during the SPE procedure. With use of solutions with low pH during the SPE procedure with reversed-phase columns, it was shown that H_4folate degrades significantly in columns (21%). This could be circumvented by addition of 1% ascorbic acid to the solutions applied during washing and conditioning steps, resulting in only 5% degradation (Nilsson *et al.*, 2004).

The selectivity of counter-ion during both retention and elution procedure is important during the SAX procedure. Selectivity can be defined as the degree to which the counter-ion can compete with other counter-ions on an ion-exchange sorbent. Isolate elution is facilated by buffers containing high selectivity counter-ions that can drive the isolate off of the charged sorbent. Chloride ion has for example stronger selectivity than phosphate ion. Choosing a counter-ion with high selectivity decreases the volume needed to be applied during the elution step. By use of phosphate ions during the elution step it was not possible to concentrate the sample (Rebello, 1987), but with chloride ions it was possible (Vahteristo *et al.*, 1996). Moreover the concentration of the counter-ion influences the performance. Evaluation of different elution buffers and sorbents showed that use of 10% NaCl as counter ion instead of 5% NaCl in 0.1 M acetate buffer resulted in more effective and complete elution of folates (Vahteristo *et al.*, 1996) as also illustrated by Nelson et al. (2005).

Recovery tests have proved use of SAX columns to be quantitatively reliable when using 10% sodium chloride (Jastrebova *et al.*, 2003; Vahteristo *et al.*, 1996). However, despite working well in sample purification of folates, use of SAX is not always enough selective for some folate forms, especially for 5-HCO-H_4folate and 10-HCO-folic acid (Nilsson *et al.*, 2004). This is due to interfering impurities in sample matrix that co-elute both in SAX and HPLC system, resulting in noisy chromatograms with a possible masking of the peak(s) of interest (Ginting & Arcot, 2004; Ruggeri *et al.*, 1999; Vahteristo *et al.*, 1997b). The consecutive use of a SAX cartridge followed by application of the resulting eluent to an unpolar cartridge (phenyl endcapped) resulted in much cleaner extracts (i.e. chromatograms) and minimised interferences, especially around the retention time for 5-HCO-H_4folate, compared to single use of either of these cartridges. The combined use of cartridges was also shown to be quantitatively reliable (Nilsson *et al.*, 2004).

An alternative sample purification method is affinity chromatography, based on selective folate-binding proteins (FBP) from bovine milk. It was used for biological extracts first by Selhub and coworkers (Selhub *et al.*, 1988). Although they claimed all folate derivatives were quanitatively bonded to FBP regardless of affinities, later studies showed that recovery of 5-HCO-H_4folate was a major

obstacle due to its low affinity. There was 10 and 20% loss of 5-HCO-H_4folate if the column was loaded with 25 and 50% of column capacity respectively (Pfeiffer *et al.*, 1997), which was in agreement with other investigations (Kariluoto *et al.*, 2001; Selhub, 1989). Therefore, it is of critical importance not to overload column and to check capacity regularly because the binding capacity decreases after application of several extracts. For instance, it decreased by 58% after 18 extracts (Konings, 1999) and 26 and 47% after 12 and 22 samples respectively (Freisleben *et al.*, 2003a). The situation gets even more complicated if internal standards are used because the useful capacity of folate binding sites is reduced (Freisleben *et al.*, 2003a). Further limitations are that FBP columns are not commercially available, which complicates their routine use. They are also much more expensive than SPE. The main advantage of affinity chromatography is that folate binding protein is highly specific towards folate (Pfeiffer *et al.*, 1997) and gives therefore much cleaner chromatograms, resulting in 10-fold lower LOD and LOQ as compared to SAX (Freisleben *et al.*, 2003a). However, some researchers report unsatisfied specificity when using affinity chromatography (Kariluoto *et al.*, 2001; Ruggeri *et al.*, 1999).

1.7 Detection systems

1.7.1 UV and fluorescence spectrophotometry

Folates have different UV absorption maxima which depend on buffer pH (Eitenmiller & Landen, 1999; Temple Jr & Montgomery, 1984) and have maximum absorbances at 270-300 nm for different derivatives at pH 3.5, except from 5,10-CH^+=H_4folate (355 nm) (Lucock *et al.*, 1995). Using fluorescence detection is more selective, and sometimes more sensitive, than UV detection (Gregory *et al.*, 1984b). 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate have similar wavelength maxima around 295-300 nm (excitation) and 356-360 nm (emission) (Gounelle *et al.*, 1989; Gregory *et al.*, 1984b). The native fluorescence intensity differs in the proportion of 10:5:1 for 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate respectively (Gounelle *et al.*, 1989). However, the fluorescence intensity is often not adequate to quantify 5-HCO-H_4folate naturally present in food (Gregory *et al.*, 1984b). Furthermore, H_2folate, 10-HCO-H_4folate and 10-HCO-folic acid have also very low fluorescence intensity and folic acid does not fluoresce, which also rules out the use of fluorescence detection for these derivatives (Eitenmiller & Landen, 1999). Fluorescence intensity is strongly influenced by pH and has its optimum at pH 2.5 for 5-CH_3-H_4folate, 3.0 for H_4folate and 2.0 for 5-HCO-H_4folate (Gounelle *et al.*, 1989). From pH 3.0 to 7.0 the fluorescence is constant for 5-HCO-H_4folate whereas it gradually decreases to zero at pH between 3-7 for H_4folate and 5-CH_3-H_4folate (Gounelle *et al.*, 1989). A practical implication of this was the need to do a postcolumn mobile phase adjustment of pH from 6.8 to 2.7-3.0 to obtain optimum fluorescence for 5-CH_3-H_4folate (Holt *et al.*, 1988; Osseyi *et al.*, 2001).

1.7.2 Mass spectrometry

The introduction of mass spectrometric methods, MS, coupled to HPLC or GC, has provided a powerful tool to increase specificity and sensitivity during analysis

and is now well established in food research (Careri *et al.*, 2002). With use of MS-MS technique an apparent high selectivity can be obtained without sample purification or adequate separations of analytes or both. However, there is still a need to optimise separation and purification in real samples since ionisation of analytes can be strongly influenced by other co-eluting matrix compounds; so-called ion suppression effects or matrix effects (Careri *et al.*, 2002). The use of non-volatile mobile phase during HPLC analysis with UV and fluorescence detectors is not compatible with MS detection because salts negatively influence the ionisation process of analytes. Therefore other volatile buffers need to be used, but these negatively influence chromatographic parameters such as retention and peak shapes.

The reason for the increased interest in mass spectrometric methods was the limited sensitivity and specificity of other detection systems (UV and fluorescence), working in compliance with HPLC. Due to the hydrophilic properties of folate, reversed-phase chromatography coupled to electrospray ionisation (ESI) is more suitable than APCI (Nelson *et al.*, 2001; Stokes & Webb, 1999). The first report on use of LC-MS for folate analysis was based on reversed-phase chromatography with negative ion mode electrospray. The application of method to real samples was however problematic due to matrix effects (Stokes & Webb, 1999).

In 2001, an LC-MS-MS method with polar HILIC-column and normal-phase chromatography was reported (Garbis *et al.*, 2001). The advantage of that method was the possibility to use higher organic content in mobile phase to improve the MS signal. Moreover, a buffer with neutral pH without any loss of folate retention could be used, in contrast to reversed-phase chromatography where the loss of folate retention is usual at neutral pH due to ionisation of the carboxylic groups of folates. Negative ion mode was used because it provided cleaner mass spectra than positive ion mode. However, most mass spectrometry methods published since have still used RP-LC-MS with good results.

Later research showed electrospray with positive ion mode provided the highest signals for 5-CH_3-H_4folate and H_4folate whereas negative ion mode was the best for 5-HCO-H_4folate, H_2folate and folic acid in aqueous solution (Nelson *et al.*, 2001). These findings were later supported by Pfeiffer et al. (2004), but there are other reports of higher LC-MS intensities in positive ion mode also for folic acid, 5-HCO-H_4folate and 10-HCO-folic acid (Freisleben *et al.*, 2003b; Zhang *et al.*, 2005). Use of acetic acid in mobile phase was reported to enhance the MS signal of folate derivatives compared to formic acid (Pfeiffer *et al.*, 2004) but the opposite was reported by Zhang et al. (2005).

Use of different kinds of stable isotope internal standards to correct for losses during sample pre-treatment and ion suppression is a valuable tool. The use of stable isotope of $^{13}C_5$-folic acid was first introduced in an LC-MS method to determine folic acid in fortified food using negative ion mode (Pawlosky & Flanagan, 2001). This method was later used to determine folic acid in fortified breads from Chile (Pawlosky *et al.*, 2003b). Quantification of 5-CH_3-H_4folate in

human plasma by use of $^{13}C_5$-5-CH_3-H_4folate as internal standard was also introduced in 2001 (Nelson *et al.*, 2001) and some years later also the stable isotope of $^{13}C_5$-5-CH_3-H_4folate was introduced as well as $^{13}C_5$-folic acid internal standards for LC-MS analysis of folate in citrus juices (Thomas *et al.*, 2003) and other foods (Pawlosky *et al.*, 2003a). Here ESI was operated in positive mode for 5-CH_3-H_4folate and switched to negative mode for folic acid. An alternative to use of $^{13}C_5$-labelled folate was introduced when $^{2}H_4$-folic acid was synthesised, from which basic structure labelled 5-CH_3-H_4folate, H_4folate, 5-HCO-H_4folate and 10-HCO-folic acid were prepared (Freisleben *et al.*, 2002). They were then applied as internal standards for food folate analysis by use of LC-MS-MS (Freisleben *et al.*, 2003b). Use of LC-MS-MS technique gives much higher specificity and sensitivity than LC-MS due to possibility of performing multiple reaction monitoring (MRM) by selecting precursor ion(s) and then fragmenting it further by use of collision-induced dissociation (CID). In this way a specific spectrum of the fragmented precursor ion(s) can be obtained, and quantification be based on one of the product ions. Analysis of some food using stable isotope assay LC-MS-MS method revealed detection of some folate forms not detectable by HPLC/FD (Freisleben *et al.*, 2003a). Furthermore, isotopically labelled standards were shown to correct for folate losses during sample pre-treatment.

Even other kinds of internal standards have been used, for example methotrexate (MTX) was used to determine folates in plasma (Garbis *et al.*, 2001). However, when it was applied to spinach extract, the matrix suppressed the ionisation of MTX by 50% compared to other folate derivatives (Zhang *et al.*, 2005). Therefore MTX was regarded unsuitable as internal standard, and instead unlabelled folic acid was used as internal standard, with the justification that this form is not present itself in spinach. However, the usefulness of MTX and folic acid is limited since they cannot compensate for ion suppression effects or degradation of folates.

1.8 Yeast as rich folate source

Yeast has for thousands of years been used during fermentation processes for food productions, but industrial and commercial use started first at the end of the 19th century (Bekatorou *et al.*, 2006). Yeast is e.g. used during manufacturing of wine, cheese, bread, dairy products, beer and sourdoughs. Although there is a large diversity of yeasts, only a few are commonly associated with the production of fermented and microbial foods. They are all either ascomycetous yeast or members of the imperfect genus *Candida* (Adams & Moss, 2000). The most common food grade yeast worldwide is baker's yeast (*Saccharomyces cerevisi*ae) which is used for bread and baking products (Bekatorou *et al.*, 2006). *Saccharomyces cerevisiae* is also the most frequently encountered yeast in fermented beverages and foods based on fruits and vegetables (Adams & Moss, 2000). In kefir, species of *Saccharomyces*, *Candida, Kluyveromyces, zygosaccharomyces* and *Torulaspora* have often been identified (Angulo *et al.*, 1993; Simova *et al.*, 2002; Witthuhn *et al.*, 2005).

For industrial applications yeast is usually cultured in a molasses-based medium, which is a waste product from the sugar industry. This medium yields a large

biomass of yeast to a low cost (Michels, 2002). However, in the laboratory, yeasts are cultured in either a defined minimal medium, when conditions need to be strict, or a rich medium when rapid growth and high biomass concentrations are desired (Madigan *et al.*, 2000).

Food grade yeasts are used as sources of high nutritional value of proteins, enzymes and vitamins with application in the health food industry as nutritional supplements, food additives, conditioners and flavouring agents, to produce microbiologic media as well as livestock feeds (Bekatorou *et al.*, 2006). Particularly interesting is their ability to synthesise folate. Yeast is regarded as a rich folate source, and different national food tables report a content of 716-1000 µg folate/100 g compressed baker's yeast (Witthöft *et al.*, 1999). However, little is known about what metabolic processes and conditions regulate the folate synthesis. It is hence interesting to investigate these folate promoting factors to determine under what circumstances folate synthesis is optimal, so that in future we can develop food products with increased naturally folate content derived from yeast metabolism. This is desirable because there is a need to increase folate intake via consumption of foods with naturally high folate content, since most EU member states currently do not permit food fortification with synthetic folic acid (Wolfe *et al.*, 2004).

In the light of a general limited intake of folate in respect to RDI, efforts have been made to circumvent this phenomenon. Mandatory folic acid fortification of food has been implemented in USA, Canada, Chile (Hacohen & Lerman-Sagie, 2006) and most recently in Australia and Ireland. In Europe several countries have practiced voluntary fortification for several years, e.g. UK, Spain, Portugal, Switzerland and Austria, but it is not allowed, or is restricted, in Denmark, Finland and Sweden (Eichholzer *et al.*, 2006). Not making it mandatory was due to several reasons; e.g. the public's "freedom of choice" issue, risk of masking of vitamin B12 deficiency by folic acid, cancer promotion and increased risk of twinning rate (Hacohen & Lerman-Sagie, 2006). Most recently the fortification with folic acid has been strongly questioned from an ethical point of view and because of lack of knowledge about the possible negative effect of too high folic acid intake on population from different sectors and ages (Eichholzer *et al.*, 2006; Smith, 2007; Ulrich & Potter, 2006). However, as recently stated, countries deciding against mandatory fortification need to promote and fund research on additional effective means to improve folate intake (Eichholzer *et al.*, 2006).

In recent years there has been an interest in trying to increase the natural folate content in foods by biofortification i.e. by use of folate producing micro-organisms, or plants, during food productions (Basset *et al.*, 2005; Crittenden *et al.*, 2003; Drewek & Czarnocka-Roczniakowa, 1986; Holasova *et al.*, 2004; Holasova *et al.*, 2005; Jagerstad *et al.*, 2005; Kariluoto *et al.*, 2006a; Kariluoto *et al.*, 2006b; Kariluoto *et al.*, 2004; Lin & Young, 2000; Pompei *et al.*, 2007; Sanna *et al.*, 2005; Sybesma *et al.*, 2003a, Sybesma *et al.*, 2003b). There is clear evidence that baker's yeast contributes significantly to the final folate content in bread (Kariluoto *et al.*, 2006a; Kariluoto *et al.*, 2004). In bread dough, 65-72 % of the folates were found to be of yeast origin (Keagy *et al.*, 1975; Osseyi *et al.*,

2001). However, due to degradation of folates during the baking process, the final folate content in bread was only 16 % higher than that of flour (Osseyi *et al.*, 2001). It was also found that the loss of endogenous folate, about 30%, due to baking was in the same range as for folic acid (Osseyi *et al.*, 2001). This implies that added and native folates have similar sensitivity to baking heat treatment. It is believed that folate content in food can be further improved by using strains of baker's yeast with better ability to synthesise folate compared to traditional strains (Gujska & Majewska, 2005; Kariluoto *et al.*, 2004).

Limited data are available about folate content in different yeast strains and folate retention, i.e. during storage of yeast and food processing, except for bread. Furthermore, little is known about the retention of folate changes during yeast production and the impact of different cultivation conditions. Some studies have been done on folates in dry yeast with reported folate values of 55.5 nmol/g (Seyoum & Selhub, 1998), 50 nmol/g (2330 µg folate/100 g dry yeast) (Ndaw *et al.*, 2001) and 35.2 nmol/g (1625 µg folate/100 g dry yeast) (McKillop *et al.*, 2003). There are also limited and contradictory data on the folate derivatives present in yeast. In one study, these were identified as H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate and/or 10-HCO-H_4folate (Seyoum & Selhub, 1998) but in another study they were 5-CH_3-H_4folate, 5-HCO-H_4folate, 10-HCO-H_4folate, H_4folate, H_2folate and 5-CH^+=H_4folate (McKillop *et al.*, 2003).

Knowledge about the constitution of folate glutamatic residues is more consistent. Yeast folate exists mainly in polyglutamatic forms as hexa- (12-16%), hepta- (67-71%) and octopolyglutamtes (10-13%) throughout the growth cycle of yeast (Bassett *et al.*, 1976), which is in good agreement with the findings of other studies (Ndaw *et al.*, 2001; Seyoum & Selhub, 1998). Others found that polyglutamates constituted 98% of the total folate pool (McKillop *et al.*, 2003).

From the above mentioned it is obvious that knowledge about yeast folates is limited. Hence, there is much work that needs to be done to increase the knowledge about yeast folates before yeast can be used appropriately as a biofortificant in foods.

1.9 Determination of folates in yeast

The analytical methods used so far have mostly not been optimised for yeast folate analysis or were unspecific. The analysis of yeast folates has been a side issue that promoted other areas of interest, e.g. production of concentrated food folate extracts (McKillop *et al.*, 2003), study of food folate bioavailability (Seyoum & Selhub, 1998), evaluation of a special method with precolumn conversion of folate derivatives to 5-CH_3-H_4folates (Ndaw *et al.*, 2001), and metabolic engineering of yeasts to study inhibition effect of S-adenosylmethionine for methylenetetrahydrofolate reductase (Roje *et al.*, 2002). The methods used have been HPLC coupled to microbiological assay or HPLC with fluorescence detection. Therefore the data of yeast folates are uncertain and more specific methods need to be developed, both regarding sample pre-treatment and instrumentation.

Folate analysis of yeast as such has several difficulties that need to be overcome. One such problem is the oxidative degradation of folates during sample pre-treatment and storage. This is especially important because H_4folate, one of the most labile folate derivatives, exists in large amount in yeast. Furthermore, care must be taken to use appropriate buffers and solution due to possible conversion of 5-HCO-H_4folate to 5,10-CH^+=H_4folate. This is important because 5-HCO-H_4folate also exists in high amount in yeast.

Another problem is how to extract folate from yeast and whether tri-enzyme or mono-enzyme method should be used. Many studies report the use of α-amylase and protease to enhance folate extraction by breaking folate bindings to carbohydrates and proteins. However, in a comparison of tri-enzyme and mono-enzyme treatment of yeast extract, no differences in folate recovery could be found between these enzyme treatments (Ndaw *et al.*, 2001), which implies that heat treatment followed by mono-enzyme treatment with conjugase are enough.

Yet another problem is deconjugation of yeast folates.Yeast is known to contain many substances, e.g. nucleic acids, that act as conjugase inhibitors to different extent depending on the conjugase source (Mims, Swendseid & Bird, 1947). Therefore, there is clearly a risk of incomplete deconjugation. Yeast probably contains substances with inhibitory effects for rat serum or rat plasma conjugase or both. This is supported by the finding of significantly decreased activity of rat plasma when it was applied to yeast compared to pteroylpolyglutamate folate standards. Hence it was concluded that use of rat plasma might lead to considerable underestimation of folate contents in food (Ndaw *et al.*, 2001). Due to a wide range of possible conjugase inhibitors in different foods, it may therefore be important to evaluate optimal conjugase amount and deconjugation time, as was done for deconjugation of folate in extracts of spinach (Kirsch & Chen, 1984). A factor known to overcome the inhibitory effect is addition of reducing agents (Mims, Swendseid & Bird, 1947). High conjugase activity in bile and rat plasma was also shown to require the presence of reduced sulfhydryl group (Horne, Krumdieck & Wagner, 1981), and these groups are present in both MCE and BAL.

Furthermore, using chromatography to separate the many different folate derivatives in yeast and its optimisation is a challenge. Moreover, the detection step for so many folates is challenging. Some derivatives have low UV and fluorescence absorbances and are difficult to analyse in the actual concentration present in yeast. Development of specific and sensitive detection methods is necessary to quantify even these folate derivatives.

1.10 Objectives

The main objectives of this study were first to improve/develop analytical methods for quantifying and characterising folate derivatives in yeast by:

- Developing a simple and rapid method for screening of main folate derivatives in yeast strains **(Paper I).**
- Studying stability of folates in yeast extracts prior to analysis **(Paper II)**.
- Studying chromatographic behaviour of folates by use of HPLC with different columns and buffers **(Paper III)**.
- Developing a sensitive and specific LC-MS method for quantifying different folate derivatives in yeast **(Paper IV)**.

Secondly, possibilities for enhancing folate production by yeast were investigated by:

- Screening of folate producing capacity in different yeast strains **(Paper V & VI).**
- Studying effect of cultivation conditions on folate producing capacity of yeast **(Paper VII).**

2 Materials and methods

The following section gives a brief overview of the materials and methods used. A more detailed description is given in the papers of this thesis.

2.1 General method description

Folate standards used were folic acid, (6S)-5,6,7,8-tetrahydrofolate, sodium salt (H_4folate), (6S)-5-formyl-5,6,7,8-tetrahydrofolate, sodium salt (5-HCO-H_4folate) and (6S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH_3-H_4folate). All were a kind gift from Merck Eprova AG, Schaffhausen, Switzerland. Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-formylfolic acid, sodium salt (10-HCO-folic acid) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). The folate standards were stored at −80 °C until use. The purity of all standards was checked according to the procedure of van den Berg et al. (1994) using molar extinction coefficients reported by Eitenmiller and Landen (1999), but for 10-HCO-folic acid the molar extinction coefficient reported by Baggot and Johanning (1999) was used.

The rat serum was obtained from Scanbur, Sollentuna, Sweden. Dialysis tubing was prepared by cutting the desired pieces of cellulose tubing (obtained from Sigma), which retain proteins with MW > 12,000. It was put into a beaker with Milli-Q water, boiled twice (change of water in between) and then stored in beaker (in the second boiling water) covered with parafilm at 2-8 °C up to 6 months. Prior to dialysis the dialysis tubing(s) to be used was dried with a paper, rat serum was transferred and Spectra/Por® dialysis tubing closures was used to cap tubes. Rat serum was dialysed in three steps at 4 °C by stirring the tube(s) using 1000 ml of 50 mM phosphate buffer, pH 6.1 containing 0.1% 2,3-dimercaptopropanol in each step. Dialysed rat serum was kept in small portions (1.0 ml) at -80 °C for a maximum of 6 months. The rat serum was never vortexed nor was the dialysis tubes touched without gloves.

HPLC analyses were performed using an HPLC system (Agilent 1100) consisting of a gradient quaternary pump, a thermostated autosampler (8 °C) and a thermostated column compartment (23 °C). The systems were controlled by a personal computer running Agilent Chemstation software. Diode array detector (DAD) and a fluorescence detector were coupled to the HPLC system. For the detection and quantification of H_4folate, 5-CH_3-H_4folate and 5-CHO-H_4folate a fluorescence detector was used (excitation at 290 nm and emission at 360 nm) and for 10-CHO-folic acid and folic acid a DAD detector was used (the DAD channel was set at 290, 340 and 269 nm).

LC-MS analyses were performed using an HPLC system (Agilent 1100) consisting of a gradient quaternary pump, a thermostated autosampler (8 °C) and a thermostated column compartment (20 °C). The system was controlled by a personal computer running Agilent Chemstation software. To the LC system was

connected a variable UV detector (UV absorbance was read at 290 nm) and a single quadrupole mass analyzer (G1946D).

2.2 Methods

2.2.1 HPLC analysis of folate in yeasts and folate stability

The column Aquasil C_{18}, which was found to provide good chromatographic separation of folates in a previous study (Johansson *et al.*, 2005) was applied in **Paper I-III** and **V-VII**. The column was 150 x 4.6 mm; 3 µm (Thermo Electron Corporation, USA) with a guard column Opti-guard C_{18}, 1 mm (Optimize Technologies, INC, USA) at 23 °C. The flow rate was 0.4 ml/min; the injection volume 20 µl; the temperature in the thermostated autosampler 8 °C. The mobile phase used was acetonitrile 30 mM phosphate buffer (pH 2.3) under linear gradient elution conditions. The gradient started at 6% (v/v) acetonitrile with a lag of 5 min, then the gradient was raised linearly to 25% acetonitrile during 20 min and was kept constant for 2 min; thereafter it was decreased linearly to 6% acetonitrile during 1 min and was applied for 14 min to re-equilibrate the column.

In **Paper I** different tests were performed to evaluate suitable sample pre-treatment conditions. Dry baker's yeast, *Saccharomyces cerevisae*, (trademark: original kronjäst) was used as a model matrix. It was a gift from Jästbolaget, a Swedish yeast company in Rotebro, Sweden. As extraction solution phosphate buffer (0.1 M, pH 6.1) was used with 2% sodium ascorbate (w/v) and 0.1% 2-mercaptoethanol (v/v) (used in **Paper I-II** and **V-VI**) or 0.1% (v/v) 2,3-dimercapto-1-propanol or both (used in **Paper II-IV & VII**). Optimisation involved test of recovery when different sample-to-buffer ratios were used (25, 50, 70, 100, 200, 300 and 400 mg to 20 ml extraction buffer). It also involved investigation of deconjugation efficiency by addition of different volumes of rat serum (25, 50 and 80 µl) to provide complete deconjugation. Heat treatment to extract folates (boiling water bath for 12 min) was also compared with a traditional method (use of bio beads) for destruction of yeast cell wall for extraction of different cell components. To increase throughput of samples, it was investigated whether solid phase extraction, SPE, was necessary prior to HPLC analysis by comparing purified samples on SAX with non-purified samples.

The SPE procedure was performed according to the description of Jastrebova et al. (2003). Briefly, a Visiprep SPE Vacuum Manifold (Supelco, USA) was used for elution under reduced pressure. Aliquots of yeast extracts (0.5 ml **(Paper I)** or 10 ml **(Paper IV)** were applied on SAX cartridges preconditioned with methanol (2×2.5 ml) and water (2×2.5 ml). Cartridges were washed with water (2×2.5 ml) to remove matrix interfering components. The retained folates were eluted slowly (flow rate not exceeding 1 drop/s) with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 2-mercaptoethanol 0.1% (v/v) **(Paper I)** or 0.1% (v/v) 2,3-dimercapto-1-propanol **(Paper IV)**. The first portion (0.7 ml) of eluate was discarded and the second portion 3.8 ml **(Paper I)** or 4.5 ml **(Paper IV)** was collected and weighed.

In **Paper II** a study was performed to evaluate the stability of yeast folates (H_4folate and 5-CH_3-H_4folate) during sample preparation and storage of extracts. Different antioxidants 2-mercaptoethanol (MCE), dithiothreitol (DTT), 2,3-dimercapto-1-propanol (BAL), and 2-thiobarbituric acid (TBA) in combination with ascorbate was tested in commonly used extraction buffers for folate analysis (acetate, phosphate, and HEPES/CHES buffers) to see if protecting efficiencies varied.

Five different sample treatments were also tested for real yeast extracts with use of BAL and MCE **(Table 1)**. Treatment A included extraction and deconjugation of folates on the same day. Treatment B involved extraction of folates with an intermediate freezing step (18 h) before the deconjugation. Treatment C and D were done exactly as treatment A and B, respectively, but the same single yeast extract was thawed/frozen every seventh day to evaluate folates' sensitivity to repeated freezing/thawing. For treatment E, extracted yeast samples were stored non-deconjugated in freezer (−22 °C) for 4 weeks and were then thawed and immediately deconjugated and analyzed on HPLC. The obtained folate concentrations were compared to those obtained on the first sample pre-treatment day (treatment A–D, unstored) to evaluate an eventual degradation.

Table 1. Description of the Different Sample Pre-treatments of Yeast Extracts

A	C	B	D	E
Extraction		Extraction		Extraction
↓		↓		↓
Deconjugation		Freezing (18 h)		Freezing
(Analysis of unstored samples)		↓		(4 weeks)
↓		Thawing (once)		↓
Freezing (1, 2, 3, or 4 weeks)		↓		Thawing (once)
↓	↓	Deconjugation		↓
Thawing (once)	Repeated thawing/freezing *	(Analysis of unstored samples)		Deconjugation
↓	(up to 4 times)	↓		↓
Analysis	↓	Freezing (1, 2, 3, or 4 weeks)		Analysis
	Analysis	↓	↓	
		Thawing (once)	Repeated thawing/freezing	
		↓	(up to 4 times)	
		Analysis	↓	
			Analysis	

* The same single yeast extract was thawed/frozen every seventh day during four weeks.

2.2.2 Chromatographic separation of folate derivatives

The chromatographic properties of folate derivatives were studied by comparing peak shapes after gradient elution on different alkyl-bonded columns **(Paper III)**. These columns can be divided into two different groups: 1) columns suitable for use in highly aqueous mobile phases, i.e. Atlantis dC18 (***Atl***), Aquasil C18 (***AqC18***) HyPurity Aquastar C18 (***HyAqS***), HyPurity Advance (***HyPAc***) and

Zorbax SB Aq (***ZoAqc***) and 2) columns with classical alkyl-bonded phases, i.e. Zorbax SB C8 (***ZoC8***), Zorbax SB C18 (***ZoC18***), Zorbax C18 (***Rx ZoRx***), Synergi Max (SyC12), Genesis C18 (***GeC18***), Ace C18 (***Ace***), XBridge C18 (***XBr***) Hypersil Gold C18 (***Gold***) and SunFire C18 (***SunF***).

The mobile phase used was a binary gradient mixture of aqueous buffer and acetonitrile. Different initial concentrations of acetonitrile at the start of programme were applied ranging from 3 to 10%. The lag phase (5 min) as well as the linear gradient raise (19% in 20 min) was the same for all programmes. After 25 min run time the acetonitrile concentration was kept constant for 2 min and thereafter linearly decreased during 1 min to its initial concentration that was applied for 14 min to re-equilibrate the column.

Two buffers were used for preparation of mobile phases: 30 mM potassium phosphate buffer (PhB) pH 2.3 and 5 mM acetic acid (HAc) pH 3.5. For columns HyAqS, XBr and Ace, three additional buffers were used: 30 mM PhB pH 3.5, 5 mM PhB pH 3.5 and 6 mM H_3PO_4 pH 2.3.

Retention times (t_R) of five folate derivatives were used to compare column retention performance. Peak area was used to estimate recovery. Peak widths ($w_{0.5}$) were calculated at half-heights. Peak asymmetry (A_s) was estimated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak. To compare the selectivity of different columns, the apparent resolution factor (R_s) for two adjacent peaks with retention times $t_2 > t_1$ was calculated according to the formula commonly used for isocratic elution.

To check the applicability of standard test procedures for column selection, retention times of folates and column characterisation parameters were compared by using the Tanaka protocol (Kimata *et al.*, 1989).

2.2.3 LC-MS analysis of folates

The column used in **Paper IV** was a reversed-phase column; Ace C_{18}, 150 mm × 4.6 mm i.d.; 3µm (Advanced Chromatography Technologies, Aberdeen, UK) with a guard column Opti-guard C_{18}, 1mm (Optimize Technologies, Inc., USA) at 20 °C. The flow rate was 0.3 ml/min and the injection volume 20 µl.

The mobile phase was either a binary gradient mixture of acetonitrile and aqueous buffer or a tertiary mixture of acetonitrile (gradient), methanol (constant content) and aqueous buffer. The same linear acetonitrile gradient from 6% to 20% (as described in the final method below) was applied in all cases. The content of methanol in the tertiary mixture was ranged from 2% to 8%. Two volatile aqueous buffers were used to prepare mobile phases: 10 mM acetic acid (HAc), pH 3.4 or 10 mM formic acid (HFo), pH 2.1.

Retention times (t_R) of five folate derivatives were used to calculate apparent resolution, R_s, as well as obtained peak area and peak heights to compare effect of methanol addition in mobile phase.

In the final method used to analyse folate derivatives in yeast, the mobile phase was tertiary mixture of acetonitrile–methanol (6%) and 10 mM aqueous acetic acid, pH 3.4, under linear acetonitrile gradient elution. The gradient started at 6% (v/v) acetonitrile with a lag of 8 min, then the gradient was raised linearly to 20% acetonitrile during 12 min and was kept constant for 4 min; thereafter, it was decreased linearly during 1 min to 6% acetonitrile, which was applied for 11 min to re-equilibrate the column. The methanol content was kept constant at 6%. To quantify folates, selected ion monitoring (SIM) of protonated molecular ions $[M+H]^+$ was used (with *m*/*z*-values for H_4folate: 446; 5-CH3-H_4folate: 460; 10-HCO-folic acid: 470; 5-HCO-H_4folate: 474 and folic acid: 442).

2.2.4 Screening of folates in different yeast strains

Different yeasts strains were selected to compare folate producing capacity (**Paper V)**. The selected strains were divided into different groups, i.e. one group of yeast strains belonging to *S. cerevisiae* and one group of diverse yeasts. This was done to get a picture of strain dependent differences within species and differences among a wide range of yeasts in nature. Moreover some yeast strains were isolated and selected from kefir granules to evaluate their folate producing potential during kefir manufacturing **(Paper VI)**. All strains were grown on an orbital shaker (30 °C, 220 rpm) in a similar way. Yeasts showing only respirative catabolism were cultivated until late respiratory phase (usually OD_{610} 5–10, 12–24 h). For strain comparison at comparable physiological state, yeasts showing fermentative catabolism were grown until all glucose was consumed (coinciding with the end of the respiro-fermentative phase) and the population clearly had entered the respiratory phase (usually OD_{610} 8–10, 12–24 h). At this stage cells were harvested, centrifuged and then lyophilised and stored at -20 °C until sample pre-treatment and folate analysis.

2.2.5 Cultivation condition and folate content in yeast

During this study a diploid yeast strain, SKQ2n, belonging to S. cerevisiae, was used **(Paper VII)**. A synthetic nutrient poor medium, CBS, and two nutrient-rich media, YPD and molasses were used to investigate folate production during controlled batch fermentation. Samples were collected every second hour during 48 hours and thereafter centrifuged, lyophilised and stored at -80 °C until sample pre-treatment and folate analysis.

To study folate production at different growth rates, continuous chemostat cultivation was used. It was therefore possible to control the metabolism and growth rate of the culture by altering the dilution rate (i.e. the growth rate). In addition, a continuous culture permits precise studies of the relation between cell composition and growth rate – without changes in the environment, such as accumulation of waste products. Samples were withdrawn after reaching steady state conditions, centrifuged, lyophilised and stored at -80 °C until sample pre-treatment and folate analysis.

3 Results and discussion

3.1 Development and validation of analytical methods

*3.1.1 HPLC analysis of yeast folates and their stability **(Paper I and II)***

Because folates exist mainly as polyglutamates in yeast, the addition of a deconjugation step is necessary. In **Paper I,** 50 µl of rat serum to 1 ml yeast extract was required for complete deconjugation of folates. In contrast, use of 25 µl rat serum resulted in incomplete deconjugation **(Figure 4)**. The result illustrates the importance of evaluating the appropriate amount of conjugase to be applied to a sample. As discussed above, this optimal amount of conjugase depends not only on folate concentration in sample, but also on concentration of inhibitors, which makes the prediction of conjugase amount difficult.

Heat extraction of H_4folate was shown to depend on the sample weight in extraction buffer (20 ml) during heat treatment **(Paper I)**. Use of optimal ratio of sample weight to extraction buffer volume might be crucial for breaking all the possible folate bindings to the matrix. To date, studies in this area that compare a wide range of foods are few; however, some studies have improved final extraction yields (de Quiros *et al.*, 2004; Zhang *et al.*, 2005).

The possibility of excluding SPE treatment prior to HPLC analysis **(Figure 4)** was valuable because the number of samples was high in later works **(Papers V & VII)**; thus considerable savings were made of resources in terms of time and money. The reason for the success may be due to a matrix that is folate rich and therefore the sample amount can be restricted, which limits the amount of disturbing substances. Nevertheless, some disturbances in the UV chromatogram at the retention time occurred, which corresponded to 5-HCO-H_4folate and 10-HCO-folic acid. In combination with their low UV and fluorescence absorbances, this method had limitations with respect to sensitivity and specificity for these folate derivatives.

Little is known about folate stability during analysis and the degradation pathways. To correctly analyse and interprete results, however, it is crucial to know how to treat the sample to avoid degradation; what can and what cannot be done. In **Paper II** it was shown that stabilisation of H_4folate did not depend on buffer systems used, but rather on the type of antioxidant applied to different buffers. This means that when comparing extraction efficiency of different buffers, not only buffer pH and composition are important but also type of antioxidant. It is difficult to evaluate whether difference in recovery depends on extraction or degradation mechanisms.

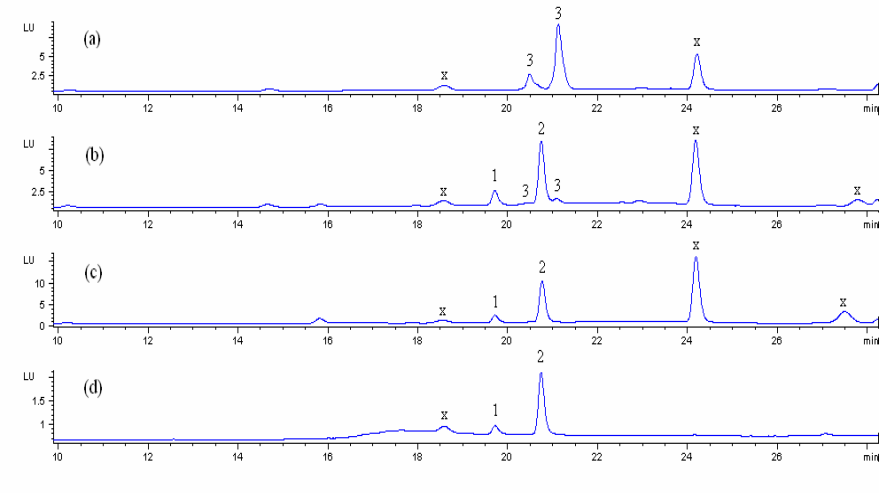


Figure 4. Chromatograms of yeast extracts. Peaks: 1 = H_4folate, 2 = 5-CH3-H_4folate, 3 = folate polyglutamates, x = interfering compounds from yeast matrix. (a) = yeast extract which had no addition of rat serum during deconjugation step, (b) = partially deconjugated yeast extract with 50 µl rat serum to 3 ml yeast extract, (c) = completely deconjugated yeast extract with 50 µl rat serum to 1 ml yeast extract, (d) = yeast extract purified by SPE (0.5 ml yeast extract was applied to SAX cartridge and eluated with 3.8 ml elution buffer).

In the stability study of yeast extracts during heat treatment, no differences between MCE and BAL could be found for either 5-CH_3-H_4folate or H_4folate **(Paper II)**. However, during storage in freezer for several weeks, extensive degradation of H_4folate was found with use of MCE **(Figure 5)**. The same pattern, but to a much less extent, was also seen by use of BAL **(Figure 5)**. Furthermore, repetitive freezing/thawing of samples had even more adverse effect of H_4folate stability, but even here BAL exhibited a significantly better stabilising effect **(Figure 5)**. The superiority of BAL compared to MCE is in good agreement with the findings of Blakley (1960); nonetheless, MCE has been the most frequently used antioxidant over the years.

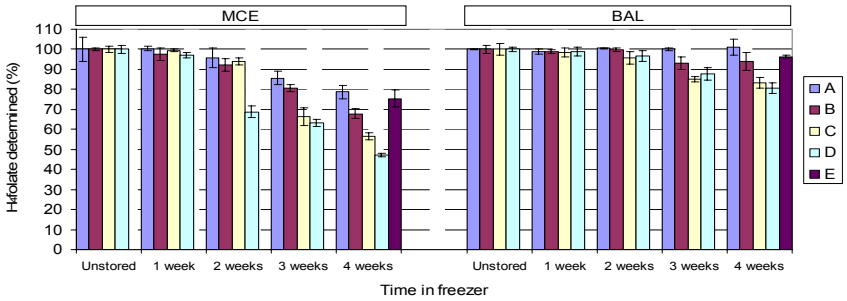


Figure 5. The stability of H_4folate in yeast extracts expressed as percentage H_4folate remaining after treatment as compared to the unstored samples. For extraction 0.1 M phosphate buffer pH 6.1 containing 2% sodium ascorbate (w/v) was used with either 0.1% MCE (v/v) or 0.1% BAL (v/v). All values are means of triplicates; for the explanation of treatments (A-E), see Table 1.

In 1945, BAL was used as the first medical chelating agent and worked as an antidote to the arsenical vesicant war gas lewisite during WWII (Walshe, 2006). Later it was also found to be a powerful chelating agent of copper and was applied as a medical chelating agent of "Wilson's disease", a disease in which the concentration of cupper is increased both in the brain and the liver. Bearing in mind the strong chelating properties of BAL for metal ions in therapeutic use, and also for Zn^{2+} in buffer systems (Rohm, 1985) and Bi^{3+} (Domenico *et al.*, 1997), BAL may also exhibit the same effect in food extracts since food extracts naturally contain metal cations.

Because foods are complex matrices, prediction of the folate stability is indeed difficult. Foods can contain substances that influence folate stability by acting as both oxygen consumer and oxidative agents (Gregory, 1996). Moreover the same component can act differently during different sample pre-treatment steps. In contrast to the inhibitory effect of citrate for conjugase during deconjugation step, citrate acts as an antioxidant against folate oxidation during extraction step (Gregory, 1996).

3.1.2 Optimisation of separation of dietary folates ***(Paper III)***

The low sensitivity and specificity of some folate forms underlines the need for more sophisticated detection methods. During recent years application of LC-MS methods for folate analysis have gained attention. However, the information is limited about the effects of different columns on the final peak performance and shape when applied to volatile buffers or phosphate buffers or both. The choice of buffer was therefore a subjective matter. In **Paper III**, an attempt was initiated to study the chromatographic behaviour of folates both by use of conventional and volatile buffers.

The differences in protonation and polarity of different folate forms were discussed thoroughly. These properties determine the elution order of folate derivatives in RP-HPLC at pH < 3: H_4folate < 5-CH_3–H_4folate < 10-HCO-folic acid< 5-HCO–H_4folate < folic acid; that is, the more protonated H_4folate and 5-CH_3–H_4folate elute first (early-eluting folates) whereas less protonated 10-HCO-folic acid, 5-HCO-H_4folate and folic acid elute much later (late-eluting folates).

At higher pH (pH = 3–4) the chromatographic behaviour of folates becomes more complicated due to the multifunctional nature of folates with both acidic and basic sites. In this connection, early- and late-eluting folates may exhibit widely different chromatographic behaviour at pH >3 when using phases with polar ionisable groups on the silica surface.

Use of polar ionisable groups on the silica surface is a way to improve retention of polar ionisable compounds in highly aqueous solution due to increased wettability of silica surface. This phenomenon was confirmed when comparing columns with increased polarity with classical columns. However, whether retention is improved or decreased depends on the charge of polar group on silica

surface. Repulsive inteactions between like-charged groups and analytes may result in loss of folate retention instead.

Moreover mobile phase composition is important. It was found that increased acetonitrile content lead to dramatic changes of retention of 5-CH_3-H_4folate and H_4folate in classical columns, which may be due to decrease in "effective" pore size and higher solvation of alkyl layer at the silica surface. On the other hand, with use of columns with increased polarity, good pore accessibility and less solvation effects were still obtained even though acetonitrile content increased.

The most difficult folate forms to obtain adequate separation between are 5-HCO-H_4folate and 10-HCO-folic acid, as also described by Johansson et al. (2005). Use of alternative columns with enhanced polarity was shown in **Paper III** to give the best result obtaining an $R_s \geq 1.5$. This indicated that secondary interactions with polar group on the silica surface might be important in this respect. On the other hand separation between 5-CH_3-H_4folate and H_4folate was similar between classical and alternative columns, which indicates that hydrophobic interactions might be more important for these forms.

Increasing pH from 2.3 to 3.5 with use of phosphate buffer was found to improve the baseline separation of late-eluting folates. However, increase of pH lead to decrease in retention on classical columns, probably due to decreased hydrophobic interactions of the glutamate part of the folate molecule with the alkyl chain on the silica surface. In contrast, the increase of pH barely affected retention by use of alternative columns with polar phases, which indicated that polar interactions could play an important role in retention mechanisms on polar-endcapped columns. When using volatile acetic acid buffer (pH 3.5) adverse peak deterioration was found for all folate derivatives on all the columns tested. Less shielding of silanol polar groups on silica surface due to decreased competition of buffer ions may lead to increase in ionic interactions between analyte molecules and these groups. With use of volatile buffers, it was found that alternative columns with polar groups on silica surface gave the most dramatic change in retention time and peak deteriorations. The changes in retention depended on the charge of the folate derivative and on the charge of the polar group on silica surface of the column. The very good result in the use of alternative columns with enhanced polarity applying phosphate buffer at low pH (2.3) became a disadvantage when volatile buffers of low ionic strength were used. This is probably due to the strong ionic interactions between analytes and polar groups of silica surface. Therefore use of classical columns separating folates mainly by hydrophobic interactions might be a better alternative for LC-MS applications.

*3.1.3 Optimisation of LC-MS analysis of folates **(Paper IV)***

On the basis of the findings in **Paper III**, the column Ace C_{18} was found to be among the most promising for LC-MS application with use of buffers of low ionic strength **(Paper IV)**. However, the resolution of 10-HCO-folic acid and 5-HCO–H_4folate still needed to be improved. Therefore a new gradient programme was tested with use of methanol, applied isocratically, in connection to an acetonitrile

gradient elution profile with 10 mM acetic acid, pH 3.4. It was found that increased methanol content improved the resolution of 10-HCO-folic acid and 5-HCO–H_4folate but adversely affected peak heights of all folate derivatives. However, use of 6% methanol concentration provided satisfactory separation between 10-HCO-folic acid and 5-HCO–H_4folate and good peak shapes for all folate forms, and therefore that programme was chosen.

To optimise signal response the ionisation mechanisms and responses were studied extensively with use of acetic acid (pH 3.4) and formic acid (pH 2.8). It was found that acetic acid gave the highest MS response in both positive and negative mode compared to use of formic acid. The higher response in positive mode was contrary to expectations since folate should have been more protonated in formic acid buffer solution. This may be because gas phase basicity of folates, as well as ion evaporation mechanisms, may be more important than acidic-basic properties in the bulk solution. Indeed, it was also found that basic-acidic properties of folates only slightly affected the intensity of MS signal in positive mode.

In contrast, by use of negative mode, the MS response of folates could be explained in terms of their different acidic-basic properties. Intensities of 5-CH_3-H_4folate and H_4folate were much lower than for the other forms due to easier formation of zwitter ions. Lower MS signal could be expected for all folate derivatives with use of formic acid instead of acetic acid due to suppressed ionisation of carboxylic groups and enhanced protonation of pteridine ring due to lower pH.

Both positive and negative modes provided comparable intensity of MS signals for 10-HCO-folic acid, 5-HCO–H_4folate and folic acid, whereas the intensity for 5-CH_3-H_4folate and H_4folate were much higher in positive mode. Therefore positive mode was chosen in the final LC-MS method.

Due to the multifunctional character of folate, there can be many different products formed in LC-MS interface, and the zwitter-ion character of the molecule may result in suppression of the MS signal of main fragment/adduct. One of the most interesting findings was the formation of double charged adducts and fragments of folate derivatives, found primarily in positive mode but also in negative mode. This was not expected since a pH < 1.5 is needed to provide protonation of the second basic site in the pteridine moiety, which indicated that the pH of electrospray droplets could be much lower than of bulk solution – so-called “wrong-way around” results for ESI.

The LC-MS method developed in **Paper IV** provided a more sensitive detection mode of 10-HCO-folic acid, 5-HCO-H_4folate and folic acid compared to UV and fluorescence detection. The possibility of concentrating the yeast extracts during the SPE step made also the method more sensitive for analysis of yeast folates. No internal standards was used to correct for possible differences in MS responses during sequence, but the folate responses were found to be stable after several

injections of yeast extracts, indicating absence of matrix effects. Moreover recoveries were satisfactory (89-106%), as were intra-day and inter-day variations.

The obtained content of 5-CH_3-H_4folate and H_4folate in dry baker's yeast was in good agreement when comparing both the method in **Paper I** and in **Paper IV**. However, by use of LC-MS two more folate derivatives, 10-HCO-folic acid and 5-HCO-H_4folate, could be quantified **(Figure 6)**. This means that the previous reported total folate content, 2800 µg/100g dry yeast (63 nmol/g) **(Paper I),** underestimated the real content due to inability to detect some folate forms. The total folate content in baker's yeast was found to be 3520 µg/100 g dry yeast (80 nmol/g) by use of LC-MS. This amount of folate is much higher than reported earlier, e.g. 55.5 nmol/g (Seyoum & Selhub, 1998), 50 nmol/g (Ndaw *et al.*, 2001) and 35.2 nmol/g (McKillop *et al.*, 2003). These lower folate contents may be explained by higher losses of folates during sample pre-treatment and purification. The use of affinity chromatography for instance (Seyoum & Selhub, 1998) may result in possible loss of 5-HCO-H_4folate, whereas use of only one antioxidant (ascorbic acid) (McKillop *et al.*, 2003; Ndaw *et al.*, 2001) may result in losses of folates due to degradation. Furthermore, the long incubation time (42 h) of plates at 37 °C may also result in folate degradation (McKillop *et al.*, 2003).

The degradation of folates during storage does not seem extensive, except for H_4folate in compressed yeast. This form is also regarded as the most labile of the folate derivatives quantified in this study. However, compared to compressed baker's yeast, dry baker's yeast had, regardless of storage time, lower content of 10-HCO-folic acid but a higher content of 5-HCO-H_4folate, whereas content of 5-CH_3-H_4folate differed only slightly when calculating on dry weight basis. Prior to storage the content of H_4folate was considerably higher in compressed baker's yeast than dry baker's yeast, but this difference reduced progressively with storage time.

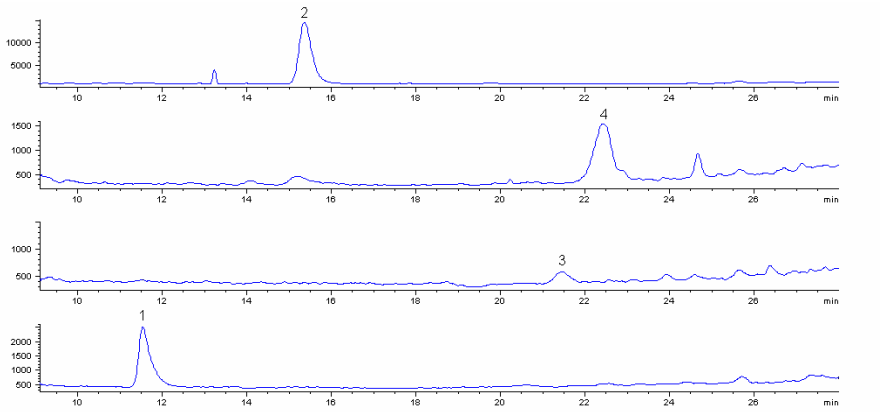


Figure 6. SIM chromatograms of dry baker's yeast extract. 1 = H_4folate 35.7 ng/ml); 2 = 5-CH_3-H_4folate (107 ng/ml); 3 = 10-HCO-folic acid (3.7 ng/ml), 4 = 5-HCO-H_4folate (30.1 ng/ml).

3.2 Inherent biodiversity of folate content in yeasts (Paper V & VI)

Cultivation of 44 yeast strains (**Paper V)** at comparable physiological state revealed highly strain-dependent differences in the range of 4,000-14,500 µg total folate content/100 g dry weight. It seems that yeast strains of *S. cerevisiae* and closely related species (group 1) **(Figure 7)** were better at producing folate than the heterogeneous yeast species (group 2) from diverse environments. It is difficult to compare this result with literature data since differences in folate production between yeasts are an almost unexplored area. The main folate derivatives were 5-CH_3-H_4folate and H_4folate whereas 5-HCO-H_4folate was detected only in trace amounts. For group 1, H_4folate content was fairly consistent for all the yeast strains, but 5-CH_3-H_4folate content differed. In contrast, for group 2 considerable variations were observed for both H_4folate and 5-CH_3-H_4folate. The obtained results mean that strains can also be selected for desired folate composition and not only for high folate producing capacity.

In **Paper VI**, however, even 5-HCO-H_4folate could be quantified when kefir yeast strains were assayed for their folate content. The comparison of ratio of sample peak heights and areas from fluorescence detector and diode array detectors to ratio of standard peak heights was found to be a valuable tool for identification of 5-HCO-H_4folate. The total folate content was quite homogenous ranging from 10,010 to 11,700 µg/100 g of dry yeast.

These results indicate that choosing the appropriate starter culture may considerably increase folate content in foods containing yeast. Furthermore, the folate content in media was low, which indicates that folates exist mainly as polyglutamates inside the yeast cell.

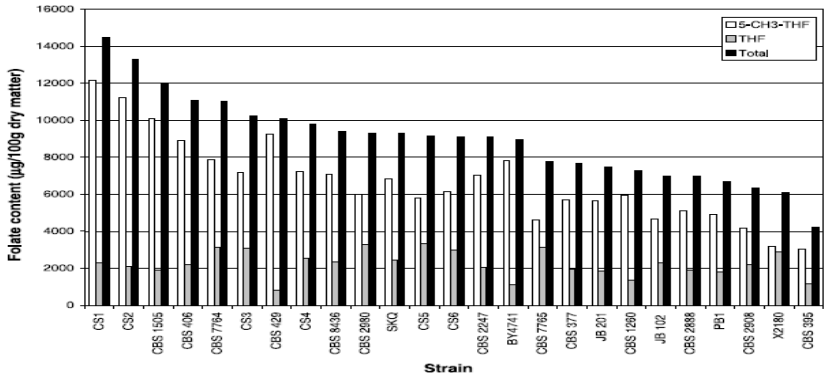


Figure 7. Folate content and distribution of main forms in group 1. Group 1 consists of strains belonging to *Saccharomyces cerevisiae* and closely related species.

3.3 Effect of cultivation conditions on folate content in yeast (Paper VII)

The purpose with the work in **Paper VII** was to obtain an overview of folate content and composition in a typical laboratory strain of *S. cerevisiae* in relation to the culturing medium and physiological state of the cells. The folate content and composition were found to differ considerably, depending on the actual growth phase of yeast **(Figure 8)**. The highest folate content, (about 12,000 µg/100 g dry weights) was found in the respiro-fermentative phase but during the whole respiratory and stationary phases folate content, especially 5-CH_3-H_4folate, declined.

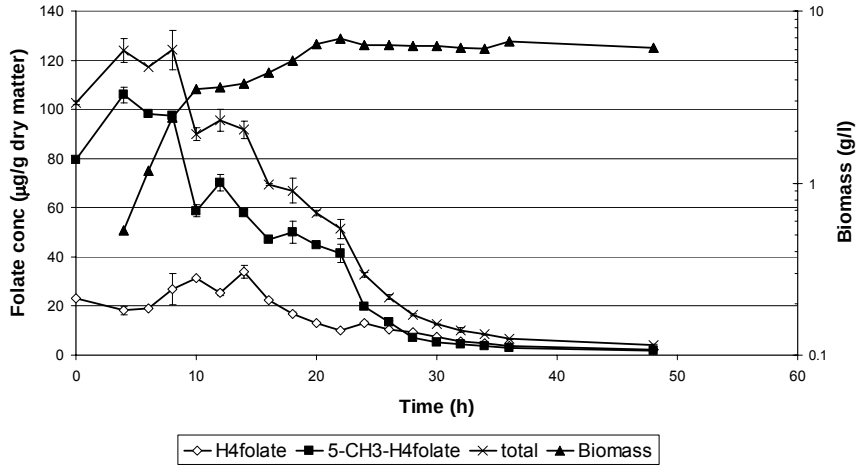


Figure 8. Folate content and composition during controlled batch fermentation of SKQ2n in synthetic medium. Error bars indicate maximum and minimum values from duplicate samples, independently extracted and analysed by HPLC, from one fermentation.

It was found that during rapid growth rate, the folate content was at a maximum level. During this phase there is a high requirement for nucleotides for DNA replication and mRNA, as well as for methionine and proteins synthesis, and consequently the needs of folate are also high. In fact, requirement of folate seemed to be highest when yeast was fermentatively degrading glucose. During this phase the growth rate was high (µmax = 0.34). Throughout respiratory growth, folate content per cell decreased. Growth rate was lower in this phase and consequently the need for folate seemed to decrease.

The cultivation during continuous culture confirmed this picture that fast growing cells have higher folate needs compared to slow growing cultures. During that trial, 5-CH_3-H_4folate and total folate content increased linearly with increasing growth rate, whereas H_4folate was moderate and independent of growth **(Figure 9)**, which confirms the results from the batch experiment. In other words, by controlling growth rate it is possible to regulate the 5-CH_3-H_4folate content of yeast cultures.

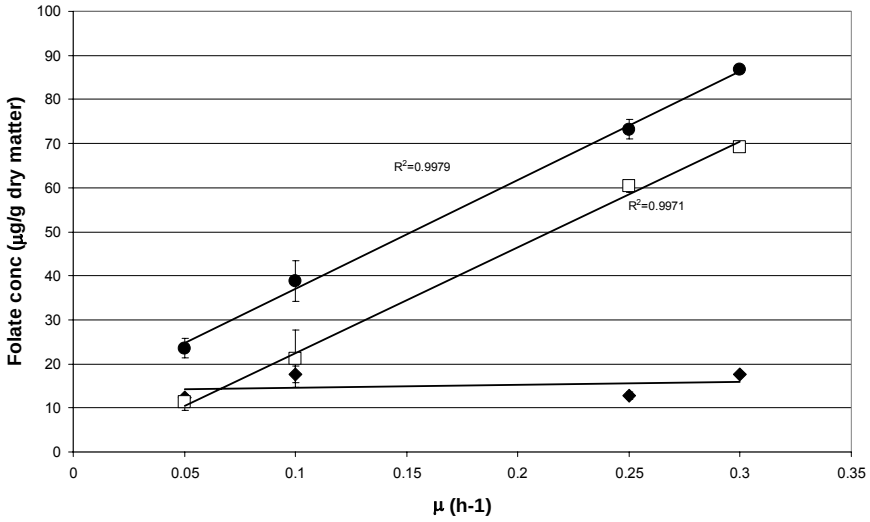


Figure 9. Folate content and composition at different growth rates obtained by changing the dilution rate in a continuous chemostat cultivation. 5-CH3-H_4folate (□), H_4folate (♦) and total folate content (●). Samples were withdrawn after steady state conditions were reached, as controlled by a stable oxygen level after at least three residence times. Error bars indicate maximum and minimum values of duplicate samples, independently extracted and analysed by HPLC, from one steady state per growth rate. Regression analysis showed a strong positive relation between folate concentration and growth rate for 5-CH3-H_4folate r2=0.997 and for total folate content r^2=0.998.

In contrast to the proportional increase in folate content with increased growth of yeast, an opposite relationship during growth of lactic acid bacteria (*S. thermophilus*) occurred for unknown reasons (Sybesma *et al.*, 2003b). Folate production was there stimulated when growth rate was decreased.

In **Paper VII** it was also found that nutrient-poor medium promoted folate production in yeast significantly compared to nutrient-rich medium. These differences may be because YPD and molasses are rich media containing amino acids and nucleotides that can be taken up by cells and used, and therefore less needs for biosynthesis of endogenous folates are signalled and sensed.

4 Main findings and future perspectives

- ✓ A rapid and simple HPLC method for screening of yeast folates was successfully developed. Quantification was possible without prior sample purification of yeast extracts.

- ✓ Ratio of buffer volume: sample amount was found important for complete extraction of yeast folates.

- ✓ Folate stability, especially H_4folate, was found to depend on sample treatment and storage conditions. It was shown that BAL is a much more efficient antioxidant in protecting folates compared to the most commonly used antioxidant, MCE.

- ✓ It was found that folates exhibited a mixed retention mechanism on reversed-phase silica phases including both hydrophobic and secondary polar interactions. Best selectivity was achived on polar-endcapped silica phases when phosphate buffered mobile phases were used. The use of LC-MS compatible buffer resulted in peak deterioration, poorer selectivity and decrease in recovery on all phases tested. Classical phases were found to be more suitable than alternative phases under these conditions.

- ✓ Use of aqueous acetic acid as buffer for LC-MS analysis of folates was found to give higher MS responses as compared to formic acid.

- ✓ Ionisation patterns were extensively studied in both positive and negative ion mode. The acidic-basic properties of folates were successfully used to predict the ionisation patterns, but they were not sufficient to predict the intensity of MS signal and the proportion of different ionisation products. For the first time double charged folate ions were detected in both positive and negative ion mode.

- ✓ Chromatographic separation of folates in LC-MS could be optimised by use of a tertiary mixture of acetonitrile (linear gradient), methanol (6%) and aqueous acetic acid as mobile phase.

- ✓ Yeast was confirmed to be a rich folate source. Dry baker's yeast was found to contain 3520 µg folates/100 g by use of the optimised LC-MS method. Four folate derivatives in yeast could be quantified: H_4folate, 5-CH_3-H_4folate, 10-HCO-folic acid and 5-HCO-H_4folate. H_4folate was found to be the most labile folate form in baker's yeast.

- ✓ Folate derivatives detected in yeast strains by the simplified HPLC method were H_4folate and 5-CH_3-H_4folate. There were highly strain dependent differences in total folate content, which ranged from 4,000 to 14,500 µg/100 g dry yeast.

- ✓ Total folate content in kefir yeast strains was found to vary from 10,010 to 11,700 μg/100 g dry yeast. The folate derivatives found were H_4folate, 5-CH_3-H_4folate, and 5-HCO–H_4folate.

- ✓ Large variations in yeast folate content and composition at different stages of growth were observed. 5-CH_3-H_4folate and total folate concentrations increased linearly with increasing growth rate, whereas the content of H_4folate was moderate and constant. Fast growing cells seem to have higher folate demands compared to slow growing cultures.

- ✓ Yeast folate synthesis was higher in minimal culture media than in rich media.

- ✓ There are clear possibilities for increasing folate content in yeast-fermented foods, e.g. bread and dairy products, if a proper yeast strain is used under optimal growth conditions in optimal culturing media.

The methods developed in this thesis were applied for folate analysis in yeast. The purpose was to study the potential use of yeast as a biofortificant of folates in food. Even though many advances in the understanding of folate analysis and yeast folate metabolism were made, there is still more reseach that needs to be done:

- ➢ More knowledge is needed about yeast folates, e.g. studies of substance(s) that promote/inhibit folate synthesis by yeast. Before it can be used as a biofortificant, yeast needs to be applied to food under realistic food manufacturing processes. The final food products need to be analysed for their folate content with reliable analytical tools. In this respect further method development for the different food sample matrixes is needed because all foods differ in matrix composition and may therefore need different solutions for sample pre-treatments and analysis. Especially important is the sample pre-treatment step.

- ➢ Little is known about degradation, degradation products and interconversions for folate derivatives at different pH, temperatures and buffers. With such knowledge, actions can be taken to prevent degradation by choosing appropriate conditions and sample handling. In this respect more stability studies with use of different antioxidants and folate derivatives need to be performed.

- ➢ Moreover the extraction of folate is cumbersome. Some argue for the necessity of tri-enzyme treatment whereas others are not convinced of that. In fact, reported results are contradictory and the optimal way of performance is unknown. Therefore more research is needed about this. It would be valuable if an alternative extraction method could be evaluated.

- ➢ Choice of analytical method is also another aspect where opinion diverge; some prefer microbiological methods, others chromatographic methods. Mass spectrometric methods for folate analysis will probably be more frequently

used in the future because of the advantage in studying individual folate forms and their specificity and sensitivity. The possibilities with mass spectrometry are overwhelming, but the application to folate analysis is relatively novel. It was less than ten years ago that the first application was reported and therefore more needs to be learnt about folate analysis with LC-MS.

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