Vitamin B_{12}, Folate and Folate-Binding Proteins in Dairy Products

Analysis, process retention and bioavailability

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Abstract

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Vitamin B_{12} and folate are two important B vitamins for maintaining good health, being involved in amino acid metabolism and DNA synthesis. Milk and dairy products are good sources of vitamin B_{12} and folate. Moreover, they are of interest due to their content of folate-binding proteins (FBP), since it was hypothesised that these promote folate bioavailability. Furthermore, fermentation of milk is a way to increase the content of both vitamins.

Vitamin analysis in fermented dairy products is complicated due to the complex matrix. To gain reliable data on vitamin content, retention and bioavailability, emphasis throughout the project was put on optimisation and evaluation of methods for quantification. A commercial radio protein-binding assay (RPBA), produced for clinical samples, was evaluated regarding, *e.g.* assay pH, buffer molarity and food matrix effects. The optimised RPBA was demonstrated to be suitable for quantification of vitamin B_{12} in milk and fermented dairy products, and of folate quantification in dairy samples containing mainly 5-methyl-tetrahydrofolate.

One aim of this project was to map the retention of vitamin B_{12} and folate during manufacture of six fermented dairy products. Results showed, *e.g.* that fermentation of milk followed by storage at 4 °C for 14 days decreased vitamin B_{12} concentrations in fermented milk by 40-60% compared with concentration in the milk. However, vitamin B_{12} was 4-7-fold concentrated during hard cheese making. Regarding folate, the concentrations of folate increased both during fermentation of milk to yoghurt and during manufacture of cheese.

Another aim was to study folate bioavailability from milk and fermented milks using both an *in vitro* gastrointestinal and a human model. Focus was on studying the stability and the nutritional role of bovine milk FBP, which are suggested to have a positive impact on folate bioavailability. Both studies showed FBP to partly endure *in vitro* and *in vivo* gastrointestinal passage, and further, to decrease *in vitro* and *in vivo* folate bioavailability.

Keywords: fermented dairy products, quantification, vitamin retention, folic acid, 5-methyltetrahydrofolate, folate bioavailability

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Sammanfattning

Folater och vitamin B_{12} är två viktiga B-vitaminer som bl.a. levererar byggstenar för uppbyggnad av kroppens arvsmassa (DNA) och är därför mycket viktiga vid tillstånd av kraftig cellnybildning som t.ex. vid graviditet. Folater har också rönt stor uppmärksamhet de senaste decenniet för sin förmåga att förhindra missbildningar och missfall under tidig graviditet. Båda vitaminerna krävs också i aminosyrametabolismen, för omvandlingen av homocystein till metionin. Höga halter av homocystein i blodet har både visat sig vara en riskfaktor för att drabbas av hjärt- och kärlsjukdom, och har också påvisats ha en negativ påverkan på minnesfunktioner genom att bl.a. ge en ökad risk för vissa demenssjukdomar som t.ex. Alzheimers sjukdom.

Olikt många andra vitaminer som syntetiseras i växter så produceras vitamin B_{12} endast av mikroorganismer, och återfinns därför nästan exklusivt i livsmedel med animaliskt ursprung. En speciellt rik kostkälla för vitamin B_{12} är lever, andra goda källor är t.ex. kött, fisk, ägg, mjölk, yoghurt och ost. I motsats till vitamin B_{12} återfinns folater i flera livsmedelsgrupper. Rika folatkällor är inälvsmat, bladgrönsaker, vissa frukter och bär, spannmålsprodukter samt vissa ostar. I vår svenska kost står mjölk- och mejeriprodukter för 22% av vårt vitamin B_{12}-intag och för 15% av folatintaget.

Det är svårt att ta fram tillförlitliga värden för vitamin B_{12}- och folathalter i livsmedel *p.g.a.* naturligt förekommande låga koncentrationer, förekomst av många olika vitaminformer med varierande stabilitet, samt att livsmedelsmatrisen i sig eventuellt kan leda till att vitaminkoncentrationerna över- eller underskattas. Utöver ett behov av framtagande av validerade analysmetoder för vitaminbestämning för att kritiskt kunna utvärdera hur olika betingelser under t.ex. process, odling och lagring påverkar vitamininnehållet i den mat vi äter, behöver vi också veta hur mycket av de vitaminer vi får i oss som kroppen verkligen kan tillgodogöra sig. Denna information tas fram med hjälp av biotillgänglighetsstudier. Befintliga data på biotillgänglighet av vitaminerna är ofta knapphändiga och framtagna med bristfällig metodik.

För att kunna ta fram tillförlitliga data på vitaminretention samt information om biotillgänglighet har en kommersiell radio-protein-bindar-metod (RPBA), från början framtagen för analys av kliniska prover, anpassats för analys av vitamin B_{12} och folater i mjölk- och mejeriprodukter. Efter omfattade optimerings- och utvärderingsarbete innefattande en noggrann kartläggning av hur olika faktorer såsom pH, jonstyrka på buffertlösningar, samt hur användandet av externa standardsubstanser påverkar analysresultatet, är metoden nu tillförlitlig för vitamin B_{12}-analys i mjölk- och fermenterade mejeriprodukter, samt för folatanalys i mjölk. Även övriga använda metoder för analys av FBP och biotillgängligheten har bearbetats för att uppnå större kvalitetssäkring.

Ett av syftena i detta forskningsprojekt har varit att kartlägga vitaminretentionen under framställning av utvalda fermenterade mejeriprodukter, att från mjölk till färdig produkt följa vad som händer med vitaminhalterna. Resultaten från studien visade t.ex. att fermentering av mjölk till yoghurt och filmjölk, samt efterföljande

lagring i kylskåp i 14 dagar (till bäst före-datumet) sänkte vitamin B_{12}-halterna med 40-60% jämfört med koncentrationerna i ursprungsmjölken. En betydelsefull faktor för vitamin B_{12}-halterna i fermenterade produkter som yoghurt och filmjölk är mjölksyrabakteriernas förmåga att antingen producera eller konsumera vitamin B_{12}. Jämför man koncentrationerna av vitamin B_{12} i ost med den i mjölk, har vitaminet koncentrerats 5-8 gånger i osten. För att framställa 1 kg ost åtgår ca 10 liter mjölk. Koncentrationen av folat var högre i både yoghurt, filmjölk och ost jämfört med folatkoncentrationen i mjölk. En stor andel av båda vitaminerna förloras vid avskiljning av vasslen från ostmassan under osttillverkning. Dessa förluster kan dock i vissa fall kompenseras genom mikrobiell syntes av vitaminerna under ostens mognad.

Vidare har biotillgängligheten av folater från mjölk, yoghurt och filmjölk undersökts i en humanmodell samt i en datorstyrd simulerad magtarm-modell. Fokus har legat på att studera i mjölk befintliga folat-bindande proteiners (FBP) påverkan på biotillgängligheten av folat. FBP har i några studier visat sig öka upptaget av kostens folat, och dess näringsfysiologiska roll har länge diskuterats. Resultat från detta projekt har bidragit med ny kunskap vad gäller FBPs effekter på biotillgängligheten av folat. Både studien på människa och studierna i den simulerade magtarm-modellen visade att FBP delvis ”överlever” magtarm-passagen utan att brytas ner av matsmältningsenzymer, samt att proteinet påverkar upptaget av folat i tarmen negativt, dvs sänker biotillgängligheten av folat. Effekten är större för folsyra, folatformen som används för berikningsändamål, än för den naturligt dominerande folatformen. FBP överlever pastörisering och finns i vår konsumtionsmjölk. Däremot finns den inte i yoghurt och filmjölk som värmebehandlas vid högre temperaturer. Den något bättre folatbiotillgängligheten hos yoghurt och filmjölk jämfört med mjölk kan därför förklaras av att yoghurt inte innehåller FBP. Våra studier har även visat att såväl mjölk som yoghurt och filmjölk är lämpliga livsmedel för berikning med folat. För modersmjölks-ersättningar och vällingar, som ofta berikas med folsyra, bör man se till att produkterna inte innehåller aktiv FBP. En annan roll som tillskrivits FBP är dess stabiliserande inverkan på folat under processning och lagring och kanske också i samband med passagen genom mag-tarmkanalen. Det förstnämnda har vi inte studerat, och någon positiv stabiliseringseffekt i mag-tarmkanalen kunde vi inte belägga. Däremot fann vi att FBP bildade ett stabilt komplex med den syntetiska folatformen, vilket ledde till en minskad biotillgänglighet.

Contents

Appendix

Papers I-V

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

I. Arkbåge, K., Witthöft, C., Fondén, R., and Jägerstad, M. (2003) Retention of vitamin B_{12} during manufacture of six fermented dairy products using a validated radio protein-binding assay. *International Dairy Journal 13: 101-109*

II. Strålsjö, L., Arkbåge, K., Witthöft, C., and Jägerstad, M. (2002) Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk. *Food chemistry 79(4): 525-534*

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

IV. Verwei, M., Arkbåge, K., Mocking, H., Havenaar, R., and Groten, J. The FBP binding characteristics during gastric passage are different for folic acid and 5-CH_3-H_4folate as studied in a dynamic *in vitro* gastrointestinal model. *Accepted for publication in Journal of Nutrition*

V. Witthöft, C., Arkbåge, K., Lennernäs, H., Lundin, E., and Hallmans, G. Folate bioavailability from different fortified dairy products using a human ileostomy model. *Manuscript*

Reprints are accepted papers were published by kind permission of the journals concerned.

List of abbreviations

5-CH_3-H_4folate	5-methyl-tetrahydrofolate
5-HCO-H_4folate	5-formyl-tetrahydrofolate
10-HCO-H_4folate	10-formyltetrahydrofolate
AUC	area under the (plasma concentration) curve
CRM	certified reference material
CV%	coefficient of variation (%)
ELISA	enzyme linked immunosorbent assay
ELISA	enzyme-linked immunosorbent assay
FBP	folate-binding proteins
H_4folate	tetrahydrofolate
HPLC	high performance liquid chromatography
i.m.	intramuscular
i.v.	intravenous
MS	mass spectrometry
pABG	para-aminobenzoyl-L-glutamic acid
ppm	parts per million
Pt-6-COOH	pterin-6-carboxylic acid
PteGlu	pteroyl-L-mono-glutamic acid, folic acid
$PteGlu_2$	pteroyl-L-di-glutamic acid
$PteGlu_3$	pteroyl-L-tri-glutamic acid
RPBA	radio protein-binding assay
SAX	strong anion exchange
SPE	solid-phase extraction
TIM	TNO´s gastrointestinal model
Wpc 65, 75	whey protein powders containing 65 and 75% protein

Introduction

In the post-industrial society every second person will develop folate and vitamin B_{12} deficiency during the last ten years of life (Hultdin, 2003). A combined deficiency of these two vitamins is associated with neuropsychiatric disorders among elderly, *e.g.* development of dementia and Alzheimer's disease (Seshadri et al., 2002). Moreover, deficiency of vitamin B_{12}, folate or vitamin B_6 is associated with elevated plasma homocysteine levels, a risk factor for cardiovascular diseases, the leading cause of mortality in most Western countries (Brattström et al., 1984, Boushey et al., 1995, Jacobsen, 1998). Apart from the prevention of cardiovascular diseases, several studies during the last decade focus on the protective role of folate against neural tube defects during early pregnancy (Daly et al., 1995, Berry et al., 1999, Molloy, 2002). Furthermore, there is growing evidence that a low folate status is linked to an increased cancer risk, particularly colon cancer (Giovannucci et al., 1995).

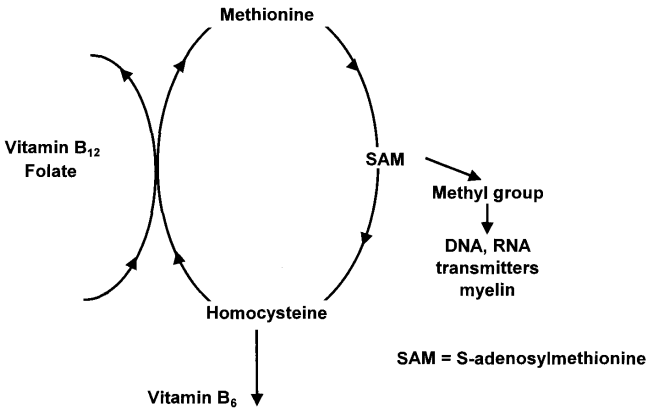

Figure 1. Linkage of folate and vitamin B_{12} in the cell metabolism.
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Taken together these health protective effects of folate and vitamin B_{12} point to a need to update and critically evaluate different foods for their potential as good sources of both these vitamins. In this perspective milk and fermented dairy products are interesting since they provide 15% of the daily intake of folate and 22% of vitamin B_{12} in the Swedish population (Livsmedelsverket, 2002).

Dairy products constitute a difficult food matrix for analyses of these vitamins. Still today, there is little reliable information about actual concentrations of vitamin B_{12} and folate in food, and about which impact food processing techniques have on vitamin retention. This is often due to use of unspecific analytical methods and insufficient methodological control. There is also a need for optimisation of samples preparation prior to analysis, which differs from one food item to another. Another important issue regarding critical evaluation of the dietary folate concerns the bioavailability, which has been estimated to range between 40-70%, based on

relatively few studies (for review see Bates & Heseker, 1994). Again, many of these studies are performed by methodology, which is today questioned.

In this thesis, efforts have therefore focussed on improved quality assurance of analytical methods, which thereafter are used to map the retention of the vitamins in typical fermented dairy products. Major emphasis is put to clarify the role, if any, of folate-binding proteins on the absorption of folate from dairy products.
The literature review following this section will begin with a brief summary on nutritional aspects of these vitamins. Thereafter the state-of-the-art concerning chemistry, analyses, occurrence and bioavailability of folate, folate-binding proteins and vitamin B_{12} are given, forming the base for the objectives and the summary of the experimental work. The last part of the thesis contains the five original papers comprising the experimental work, *i.e.* the new knowledge that came out of this project.

Literature review

Nutritional aspects

Vitamin B_{12} (cobalamin)

Two vitamin B_{12}-dependent enzymes are recognized in humans. Methylcobalamin acts as coenzyme in methionine synthetase involved in conversion of homocysteine to methionine, involving also folate. The other enzyme is methylmalonyl-CoA mutase, which requires deoxyadenosylcobalamin during conversion of methylmalonyl-CoA to succinyl-CoA in the propionate catabolism. Inadequate intake is usually not the cause of vitamin B_{12} deficiency, but instead malabsorption due to atrophy of the gastric mucosa resulting in deficient intrinsic factor (IF) production, or disease located to the terminal ileum where the vitamin is absorbed. Deficiency results in two types of clinical symptoms, anaemia and neurological dysfunction. As reviewed by Truswell & Milne (2002), the neurological symptoms are suggested to depend on accumulation of propionate due to lowered activity of methylmalonyl-CoA mutase, whereas anaemia is caused by a secondary folate deficiency, since a lack of vitamin B_{12} results in that folate is trapped as 5-methyltetrahydrofolate, and thereby a depletion of 5,10-methenyltetrahydrofolate needed for pyrimidine (DNA) synthesis occurs.

The recommended daily intake of vitamin B_{12} for adults is set to 2 μg/d for the Nordic countries (Sandström et al., 1996). In general, the population meet the recommended dietary intake by an ordinary mixed diet (Livsmedelsverket, 2002). However, certain groups, *e.g.* strict vegetarians (vegans) and elderly might suffer from inadequate vitamin B_{12} levels (Herbert, 1996, Scott, 1999). Vitamin B_{12} is exclusively produced by microorganisms and therefore higher plants lack vitamin B_{12} unless processed microbiologically. Good vitamin B_{12} food sources are e.g. liver, fish, meat, eggs, and dairy products. From only one glass of milk 40% of the recommended daily intake of vitamin B_{12} is provided.

Folate

The native forms of folate are tetrahydrofolate compounds. They play an essential role in the transfer of one-carbon units in the body. The folate derivative involved as carbon donor in purine and pyrimidine synthesis (RNA and DNA) is 5,10-methylenetetrahydrofolate, whereas 5-methyltetrahydrofolate co-operates with vitamin B_{12} in the action of methionine synthetase, which converts homocysteine to methionine (Truswell & Milne, 2002). Folate deficiency results in impaired cell division due to reduced purine and pyrimidine synthesis, resulting in megaloblastic anaemia (Scott, 1999). Folate deficiency is developed in the presence of malnutrition, due to low intake of folate-containing foods, or as a result of alcoholism. Another risk factor is malabsorption, especially for diseases affecting either gastric pH or the jejunal mucosa, *e.g.* celiac disease.

Previous recommendations concerning intakes of essential nutrients aimed to cover the needs of the consumer in order to prevent deficiency diseases. Recent knowledge has raised the question of whether recommendations should also

consider the potential that a nutrient might be important to reduce the risk of chronic disease among the middle-aged and elderly (Bailey, 1995, Bailey, 1998). In the latest edition of the Nordic Nutritional Recommendations (1996), the daily folate intake for adults was increased from 200 μg to 300 μg folate and for pregnant women a daily intake of 400 μg was recommended (Sandström et al., 1996). When publishing the dietary reference intakes (DRI) in 1998, the US Food and Nutrition Board included the concept of possible health-protective effects of folate by increasing recommendations for adults to 400 μg/d from previously 200 μg/d (Yates et al., 1998). Moreover, US Food and Nutrition Board recommends women who plan a pregnancy to consume an additional 400 μg synthetic folic acid from fortified foods or supplements. Folate is found in a variety of food products, especially vegetables, fruits, berries and cereals (reviewed by Witthöft et al., 1999). On average, milk and dairy products provide 10-15% of the daily folate intake in many Western countries.

Nomenclature, chemical structure and stability

Vitamin B_{12}

The term 'vitamin B_{12}' has two meanings. To the chemist, it means only cyanocobalamin, whereas in nutrition and pharmacology literature, vitamin B_{12} is a generic term for all corrinoids exerting biological activity in humans. In the following text the nutritional definition of vitamin B_{12} is used (Herbert, 1996). Cobalamins have a complex chemical structure based on a corrin ring system with a cobalt ion in the centre (Fig. 2). All the compounds containing this ring are designated corrinoids. The sixth position of the cobalt may be occupied by different anionic substituents, (-R). This substituent is generally one of the following: CN- (cyanocobalamin), -OH (hydroxocobalamin), H_2O- (aquacobalamin), $-NO_2$ (nitritocobalamin), $-SO_3$ (sulphitocobalamin), $-CH_3$ (methylcobalamin), or -5′-deoxyadenosyl (adenosylcobalamin). Cobalamins by definition resemble this molecular structure excluding the R-group, presented in Fig. 2.

Cyanocobalamin, the synthetic and most stable form of the vitamin, is not found in any significant amounts in nature but is an artefact from isolation procedures. This synthetic derivative of vitamin B_{12} and hydroxocobalamin are available for medical use, food fortification purposes, and nutrient supplements. The cyanide group of cyanocobalamin can be replaced by other ions. These reactions have little influence on the net vitamin B_{12} activity of foods since most cobalamin derivatives exhibit biological activity in humans. These interconversions are, however, important to consider during analysis of vitamin B_{12} (for details see the section *Methods of quantification* below). The mechanism of vitamin B_{12} degradation has not been fully explained, in part because of the complexity of the molecule and the low concentrations found in foods. Both acidic hydrolysis, exposure to alkaline conditions and reducing agents, such as ascorbic acid, are reported to induce fragmentation of the vitamin molecule (Herbert, 1996, Eitenmiller & Landen, 1999b).

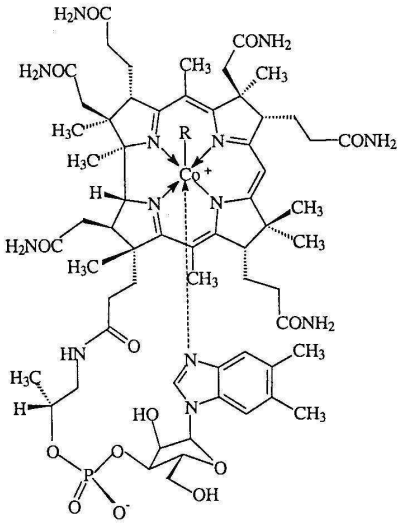

Figure 2. Chemical structure of vitamin B_{12}.
The R-position being occupied by one of the following substituents: CN- (cyanocobalamin), -OH (hydroxocobalamin), H_2O- (aquacobalamin), $-NO_2$ (nitritocobalamin), $-SO_3$ (sulphitocobalamin), $-CH_3$ (methylcobalamin), or -5´-deoxyadenosyl (adenosylcobalamin).
With kind permission from Kräutler et al., Vitamin B_{12} and B_{12}-proteins, 1998.

Folate

Folate refers to the large group of heterocyclic compounds based on the pteroic acid structure conjugated with one or more L-glutamates linked through the γ-carboxyl group of the amino acid. The generic term "folate" refers to the class of compounds having a chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid) (Blakley, 1988). In nature, the vitamin exists primarily as reduced one-carbon-substituted pteroylglutamates, varying in the presence of substituents and the number of glutamyl residues attached to the pteroyl group (Fig. 3). Five different one-carbon units can be linked at the N^5- and/or N^{10}-position of the pteroyl group: CH_3- (5-methyltetrahydrofolate), HCO- (5- or 10-formyltetrahydrofolate), CHNH- (5-formiminotetrahydrofolate), $-CH_2$- (5,10-methylenetetrahydrofolate), or –CH= (5,10-methenyltetrahydrofolate). One of the reduced folate derivatives exists without substituent and is called tetrahydrofolate (H_4folate). All together the theoretical number of all native folate compounds reaches several hundreds (Eitenmiller & Landen, 1999a). Interconversion of derivatives due to, e.g. pH shifts is reported. Synthesis of 5-HCO-H_4folate from H_2folate, and enzymatic interconversion of 5-HCO-H_4folate to other tetrahydrofolate derivatives are described (Stover & Schirch, 1991, Ndaw et al., 2001).

There are considerable differences in the stability of reduced folate compounds. In most cases, folic acid exhibits substantially greater stability than reduced forms. The order of stability of these latter forms in water is: 5-HCO-H_4folate > 5-CH_3-H_4folate > 10-HCO-H_4folate > H_4folate (Gregory, 1996). All folate compounds are

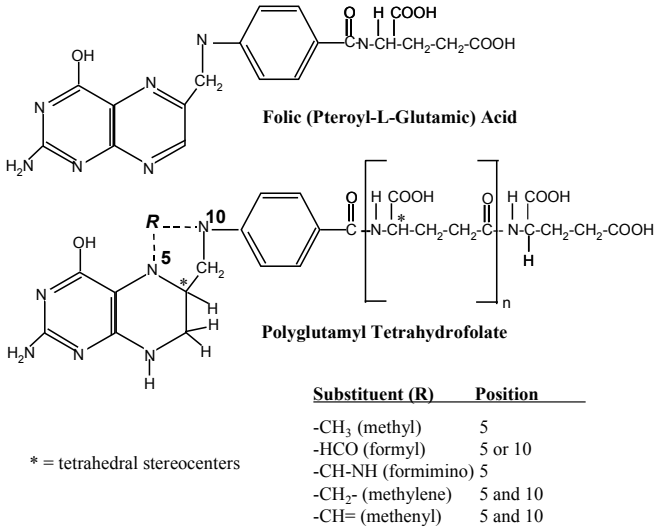

Figure 3. The structure of folate compounds.
With kind permission from Strålsjö, Folates in Berries, 2003.

endangered to oxidative degradation enhanced by light and heat, resulting in a splitting of the molecule into biologically inactive forms, of which p-aminobenzoylglutamate is a major. If present in sufficient amounts, antioxidants, e.g. ascorbic acid and thiols, protect folate from being oxidised. The rate of reaction for folate degradation in the presence of oxygen depends on the type of folate derivative and the nature of the matrix, in which the folate compound occurs, in particular with respect to pH, buffer composition, catalytic trace elements and antioxidants (Eitenmiller & Landen, 1999a).

FBP

The high affinity folate-binding proteins (FBP) in mammalians exist as a membrane-bound folate receptor as well as a soluble form in milk and other body fluids (Anthony, 1996). FBP in bovine milk exist in concentrations about 8 mg/L (Salter et al., 1972), and binds approximately 1 mol of folate per mol protein at pH 7.2 (Salter et al., 1981). The existence of a 'milk folate binder' was originally suggested by Ghitis and associates (1969). The complete amino acid sequence of this minor whey protein was determined by Svendsen et al. (1984). The molecule consists of a single polypeptide chain of 222 amino acid residues and eight disulphide bridges, and is reported to contain between 3-20% carbohydrate (Salter et al., 1981). A molecular weight of 25-40 kDa is suggested for FBP (Henderson, 1990), similar to the molecular weight of β-lactoglobulin. Bovine milk FBP and FBP purified from human milk exhibit a high degree of amino acid sequence homology (Svendsen et al., 1982). Pasteurisation of milk (at 72 °C for 15 s) is suggested to result in heat-induced alterations of the FBP molecule (Gregory, 1982). In contrast, FBP is shown to be relatively resistant to acidic environments

and are demonstrated to recover after *e.g.* gastric passage in 6-d-old goat kids (Salter & Mowlem, 1983).

Methods of quantification

Vitamin B_{12}

Vitamin B_{12} concentrations in foods are with a few exceptions only around 1 µg/100g or lower, which makes analysis of the vitamin very demanding. Almost all data available for vitamin B_{12} in foods have been obtained by the reference method for assay of cobalamins, the microbiological assay (MA) using *L. delbrueckii* ssp *lactis* as test organism. For clinical samples (blood and plasma) rapid radio protein-binding assays (RPBA) are routinely used. A recent development of the protein-binding assay is the use of a biosensor technique to quantify cobalamins in foods. Although the various forms of vitamin B_{12} can be separated chromatographically, HPLC methods are in general not sensitive enough for determination of native cobalamins in biological materials, but are useful for quantification of vitamin tablets and fortified foods.

Sample extraction

Important aspects to consider when analysing vitamin B_{12} is to stabilise the native vitamin B_{12} derivatives and to choose methods that discriminate between biologically active and inactive analogues. All methods for cobalamin analyses of food samples require a preparative step prior to analysis. Since products with an animal origin are the sole food sources of vitamin B_{12} the vitamin must be extracted from protein-rich matrices under conditions that will not lead to destruction of the vitamin. The usual steps included in sample pretreatment are homogenisation of the sample in extraction buffer at slightly acidic pH, followed by autoclaving at approximately 120 °C for 10 min. After cooling of the sample either enzyme treatment, to release vitamin B_{12} from the food matrix followed by sample clean up, or sample clean-up procedures directly, *e.g.* filtering or centrifugation, are used. Usually, cyanide, metabisulphite or nitrite is added to samples before heat extraction in order to convert the native vitamin B_{12} derivatives into one dominating form prior to quantification.

The presence of one dominating vitamin analogue in the sample is necessary during quantification of total vitamin B_{12} by *e.g.* RPBA or MA for obtaining reliable results since a deviating response for various vitamin derivatives thereby can be avoided (Muhammad et al., 1993a, Muhammad et al., 1993b). Muhammad et al. (1993a) show results from an investigation on how five common vitamin B_{12} derivatives in foods are converted into other derivatives by addition of an excess of cyanide, metabisulphite, or nitrite (Table 1). That study report cyanide to convert all but methylcobalamin into dicyanocobalamin, whereas addition of metabisulphite or nitrite only converts hydroxocobalamin into sulphitocobalamin or nitritocobalamin, respectively, leaving the remaining derivatives unaffected. In contrast, prior to characterisation of individual vitamin B_{12} derivatives by HPLC, the use of conversion substances during pre-treatment of samples is unnecessary. Extraction conditions have been shown to significantly affect the efficiency of the overall extraction process. It has therefore been suggested that the extraction

Table 1. *Conversion of cobalamin derivatives after addition of cyanide, metabisulphite or nitrite*

Conversion substances	**Hydroxo-Cbl**	**Sulphito-Cbl**	**Cyano-Cbl**	**Adenosyl-Cbl**	**Methyl-Cbl**
+ **cyanide**	diCN-Cbl	diCN-Cbl	diCN-Cbl	diCN-Cbl	CH_3-Cbl
+ **metabisulphite**	SO_3-Cbl	SO_3-Cbl	CN-Cbl	Ado-Cbl	CH_3-Cbl
+ **nitrite**	NO_2-Cbl	SO_3-Cbl	CN-Cbl	Ado-Cbl	CH_3-Cbl

diCN-Cbl, dicyanocobalamin; CH_3-Cbl, methylcobalamin; SO_3-Cbl, sulphitocobalamin; CN-Cbl, cyanocobalamin; Ado-Cbl, adenosylcobalamin; NO_2-Cbl, nitritocobalamin.
With kind permission from Muhammad et al., Food Chemistry, 1993.

conditions should be optimised for each type of food sample in order to achieve reproducible and good recoveries for the complete analysis (Richardson et al., 1978, Beck, 1979).

Microbiological assay (MA)

Over the years, growth response of various microorganisms has been used in quantification of cobalamins in foods and other biological materials. The microrganisms used have been *Lactobacillus delbrueckii* (former *L. leichmannii*), *Escherichia coli*, *Euglena gracilis,* and *Ochromonas malhamensis*. Here the AOAC official method (AOAC, 2000b) using the *L. delbrueckii* ssp *lactis* (ATCC 7830) is discussed. The other microorganisms demonstrate different disadvantages, *i.e.*, decreased sensitivity and/or lower specificity towards non-biological analogues.

The AOAC Official Method 952.20 'Cobalamin (Vitamin B_{12} activity) in vitamin preparations' uses the microbiological assay with cyanocobalamin as calibrant and addition of metabisulphite during sample pre-treatment. This MA method was originally developed for analysis of vitamin supplements, but has been extended to be valid also for food matrices. The test organism exhibits variable responses to various cobalamins. Similar growth responses are reported for cyano-, hydroxo-, sulphito-, nitrito-, and dicyanocobalamin (Muhammad et al., 1993b). However, a higher response is observed for adenosylcobalamin, whereas for methylcobalamin a lower growth response results. Using cyanocobalamin as calibrant and by addition of metabisulphite or nitrite in excess during sample preparation enables accurate determination only of hydroxo-, sulphito- and cyanocobalamin. However, the amount of adenosylcobalamin will be overestimated and the amount of methylcobalamin underestimated. If, instead, an excess of cyanide is added during the extraction procedure, accurate quantification is possible of all vitamin derivatives, except methylcobalamin that will be underestimated (Muhammad et al., 1993b). An important aspect when validating a method is the specificity. *L. delbrueckii* is reported to also utilise vitamin B_{12} analogues that are lacking biological activity, e.g. deoxyribonucleotides, and deoxyribonucleosides in addition to biologically active cobalamins (Eitenmiller & Landen, 1999b). This might result in overestimation of actual cobalamin content.

Radio Protein-Binding Assay (RPBA)

RPBA has been routinely used for vitamin B_{12} analysis in clinical samples since the 1960s. The principle of this assay is based on the competition between added isotope-labelled cyanocobalamin (^{57}Co) versus cyanocobalamin from a sample, for a limited number of cobalamin-binding proteins. Hence, the concentration of cobalamin in the unknown sample is inversely proportional to the concentration of labelled cobalamin, and calculated from a calibration curve. Early types of RPBA for quantification of vitamin B_{12} in clinical specimens and foods were often inaccurate due to the binding-protein used responding not only to active vitamin B_{12} derivatives, but also to biologically inactive analogues. The specificity of such assays has been greatly improved by use of a vitamin B_{12}-binding protein (generally hog intrinsic factor, IF) specific for the biologically active forms of the vitamin. Equivalent binding affinity of cyano-, dicyano, nitrito-, hydroxo- and methylcobalamins is reported (Muhammad et al., 1993a) for hog IF, whereas significantly deviating binding affinities were found for sulphito- and adenosylcobalamin. As for the MA, again, in order to achieve reliable results with the RPBA using cyanocobalamin as the calibrant, it is necessary to add cyanide during sample preparation to convert most native derivatives to dicyanocobalamin (see Table 1).

Studies indicate that RPBA kits including IF as vitamin B_{12}-binder, are suitable for food analysis (Richardson et al., 1978, Beck, 1979, Casey et al., 1982, Králová et al., 1982, Österdahl et al., 1986, Österdahl & Johansson, 1988, Andersson et al., 1990). Using optimised extraction procedures, RPBA and MA procedures showed agreement for most foods; however, differences were noted often enough to conclude that the two methods are not universally interchangeable for the assay of all foods. In conclusion, previous work has shown that the RPBA could be reliable for food analysis provided that careful method validation including control of selectivity has been carried out. So far, most of the published work on method validation of RPBA used for food analyses is incomplete, especially concerning control of possible interference from food matrices. Not only the food matrix itself, but also conditions introduced during extraction procedures, *e.g.* buffer strength, pH, cyanide concentration, and added enzymes, might cause non-specific binding to kit material and thereby causing misleading results.

Biomolecular Interaction Analysis (BIA)

The optical biosensor protein-binding assay is the latest methodological development for vitamin B_{12} analysis in foods (Indyk et al., 2002). It is based on biospecific recognition of the analyte, cyanocobalamin, by a vitamin B_{12}-binding protein. A special kit, QflexTM Kit Vitamin B_{12}, is commercially available using an inhibition assay for the analysis, *i.e.*, the analyte or an analogue is immobilized onto the surface of a sensor chip. A high molecular weight detecting molecule, such as a binding-protein of a defined concentration, is added to the sample. The binding-protein binds to the analyte, but at equilibrium some binding-proteins will remain free and are thereby capable of binding to the sensor surface. The detection principle relies on surface plasmon resonance (SPR), an electronic charge density wave phenomenon, which arises at the surface of a metal film when light is reflected onto the film under specific conditions.

Samples analysed by MA, by RPBA, and by the BIA method show good agreement (Indyk et al., 2002), but the BIA method demonstrates, so far, a lower sensitivity. This restricts, at the moment, its use to fortified foods, *e.g.* infant formulas and pharmaceutical vitamin mixtures.

HPLC methods

HPLC is the exclusive method enabling characterisation of different vitamin B_{12} derivatives. It has been conveniently applied to quantification of vitamin B_{12} in supplements and pharmaceutical preparations (Li et al., 2000, Markopoulou et al., 2002). For the milk matrix, a HPLC method based on UV detection with a detection limit of 0.2 µg vitamin B_{12}/L milk is described (Gauch et al., 1992). Another study report native cobalamin pattern in milk, however, owing to low levels of the various vitamin B_{12} derivatives, quantification of eluted cobalamins was performed by RPBA (Fie et al., 1994a, Fie et al., 1994b). Viñas et al. (2003) present an evaluated HPLC method suitable for quantification of four vitamin B_{12} derivatives in biological materials (beef liver, chicken blood, and human serum and urine). However, the sensitivity of the HPLC method makes it suitable only for pharmaceutical purposes.

Folate

Quantification of folate compounds is a challenge due to the instability of the vitamin and the low native amounts found in food products. Furthermore, the ability of folate derivatives to interconvert to other forms makes it even more difficult. Techniques potentially suitable for quantification of folate in foods include, as for vitamin B_{12}, microbiological and competitive-binding assay procedures, HPLC methods, and even LC-MS methods (Gregory, 1989, Gregory, 1996, Freisleben et al., 2003). The quantification of folate is complicated by the need to account for all derivatives, which could easily include several dozen compounds. In order to achieve reliable results, it should be emphasised, also here, that it is of critical importance to carefully optimise the sample preparation prior to quantification (reviewed by Martin, 1995).

Sample extraction

To prevent folate degradation, it is essential to protect samples by nitrogen, subdued light, and to cool the samples on ice throughout preparation. Pretreatment of food samples usually includes homogenisation in a buffer at close to neutral pH with added reducing agents, followed by heat extraction at 100 °C for about 10 min. A deconjugation step using γ-glutamyl hydrolase (conjugase) to cleave the glutamate chain of the folate molecule producing mono-, di- or triglutamates is most often used prior to quantification. Different sources of γ-glutamyl hydrolase exist for folate deconjugation purposes. Chicken pancreas (CP) powder used in MA analysis (AOAC, 2000a) is a crude enzyme mix including lipase, α-amylase, and γ-glutamyl hydrolase, yielding a mix of mono- and diglutamates. In HPLC analysis, conjugases which produce monoglutamates, *e.g.*, hog kidney (HK), human (HP) or rat plasma (RP), are used. Trienzyme treatment has been reported to increase the folate yield during analysis of complex food matrices (De Souza &

Eitenmiller, 1990, Martin et al., 1990, Tamura et al., 1997, Pfeiffer et al., 1997b, Lim et al., 1998, Aiso & Tamura, 1998, DeVries et al., 2001). Protease and α-amylase treatment, followed by incubation with γ-glutamyl hydrolase, has been found to increase the folate yield by 85% in human milk compared with values after γ-glutamyl hydrolase treatment alone (Lim et al., 1998). Enzyme treatment is followed by heat denaturation of enzymes, dilution and sample purification using, *e.g.* centrifugation, filtering, solid-phase extraction, or affinity chromatography.

Microbiological assay (MA)

Microbiological growth assays serve as the traditional method of folate analysis in foods. The test microrganisms used in these assays have been *Lactobacillus rhamnosus, Enterococcus hirae*, and *Pediococcus acidilatici* (Eitenmiller & Landen, 1999a). Today the only officially recognised method for folate analysis is AOAC 992.05, MA using *L. rhamnosus* (ATCC 7469). It is applicable only to quantify the 'free form of folic acid' (AOAC 992.05, 2000), which restricts its application to fortified products only. A joint collaborative study with 13 participants was recently carried out to quantify total folate by MA (*L. rhamnosus,* ATCC 7469) using trienzyme extraction for a wide range of cereal products. This method has now been recommended for Official First Action status with AOAC and First Approval status with AACC (American Association of Cereal Chemists) (DeVries et al., 2001). The response of *L. rhamnosus* (ATCC 7469) to native folate compounds decreases as the length of the glutamate chain increases (Goli & Vanderslice, 1992). Furthermore, it is discussed if the microorganism responds equally, on a molar basis, to various metabolically active forms (Newman & Tsai, 1986, Goli & Vanderslice, 1989). Although the MA show this drawback, most data on folate levels compiled in food tables and used for labelling purposes are based on this method.

Ligand-Binding Assays

Various biospecific methods are used to quantify folate in clinical samples and in foods (Wigertz & Jägerstad, 1995, Indyk et al., 2000, Arcot et al., 2002, Strålsjö et al., 2003). Finglas & Morgan (1994) categorise these methods for determination of B-vitamins into two types, 1) methods based upon antibody recognition, radio-immunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA), and 2) assays based on competitive binding of vitamins to a vitamin-binding protein, radio protein-binding assays (RPBA) and enzyme protein-binding assays (EPBA).

The radio protein-binding assay (RPBA) based on competitive binding of folate to a limited number of folate-binding proteins (FBP) is today routinely used for folate quantification in clinical samples. A study comparing several commercial RPBA kits for folate analysis of clinical samples observed problems with overestimation of folate concentrations due to improper standard calibration. Furthermore, they reported a possible small change in assay pH and matrix effects from clinical samples to contribute to a high variability found between kits and laboratories. In summary, they identified the need for further standardisation and optimisation of assay and extraction procedures (van den Berg et al., 1994). Attempts to subject the RPBA to folate analyses in foods have revealed setbacks such as low precision and variable response to different natural folate derivatives

(Givas & Gutcho, 1975, Shane et al., 1980, Gregory et al., 1982, De Souza & Eitenmiller, 1990, Finglas et al., 1993, Wigertz & Jägerstad, 1995). At prevailing assay pH of 9.2, a RPBA using FBP as vitamin-binder overestimates H_4folate compared with folic acid and 5-CH_3-H_4folate, whereas the possible presence of 5-HCO-H_4folate is not detectable (Wigertz & Jägerstad, 1995). In addition, a recent study observes a striking difference (approximately 30%) in the binding affinity of the racemic mixture (6R,S)-5-CH_3-H_4folate compared to the natural diastereoisomer (6S)-5-CH_3-H_4folate (Strålsjö et al., 2003).

Whereas the choice of calibrant and assay pH is known to be crucial for the RPBA (Givas & Gutcho, 1975, van den Berg et al., 1994), little attention has been given to disturbances caused by food matrix and buffers. Theobald et al., (1981) suggest that matrix effects might change the background radiation and unspecific binding in the assay, resulting in false results. Furthermore, sample preparation in connection with the application of RPBA for food analyses and the need of deconjugation are still being discussed (Shane et al., 1980, Finglas et al., 1993). Shane et al. (1980) report variable responses dependent on the glutamate chain length and considered the RPBA unreliable for the direct determination of polyglutamates, which again emphasises the importance of deconjugation procedures prior to RPBA quantification. Strålsjö et al. (2003) demonstrate an optimised RPBA method to be suitable for quantification of folate in a variety of berries containing mainly 5-CH_3-H_4folate.

As already mentioned with regard to vitamin B_{12}, a BIA method was recently developed for analysis of folate in milk and infant formula (Shane et al., 1980, Finglas et al., 1993, Indyk et al., 2000). An advantage is the avoidance of isotope handling. A disadvantage is expensive instruments. Moreover, another problem with this BIA method is the varying cross-reactivity of the antibody for folate derivatives besides folic acid and 5-CH_3-H_4folate, which restricts the method for quantification of fortified products, or products in which one folate form is dominating.

HPLC and LC-MS methods

Several HPLC techniques to separate folate derivatives in food and biological material have been established (Vahteristo et al., 1997, Pfeiffer et al., 1997b, Finglas et al., 1998, Konings et al., 2001, Stokes & Webb, 1999, Ndaw et al., 2001, Horne, 2001, Jastrebova et al., 2003). However, HPLC data reported on contents of various folate derivatives in certain food products are sometimes contradictory, thereby stressing the importance of further method optimisation with respect to peak identification and quantification of native folate derivatives other than 5-CH_3-H_4folate (Finglas et al., 1998). Extraction, enzyme treatment, and subsequent clean-up of samples require optimisation to permit folate analysis in various types of food and to be suitable for subsequent HPLC quantification. Only a few studies have been published on folate in fermented milk and dairy products based on HPLC analyses (Müller, 1993, Wigertz et al., 1997, Vahteristo et al., 1997, Konings et al., 2001, Lin & Young, 2000b, Lin & Young, 2000a, Young & Lin, 2000).

The most recent method for quantification of individual folate derivatives in foods and biological material is coupling HPLC with mass spectrometry (MS) (Doherty & Beecher, 2003, Pawlosky et al., 2003, Freisleben et al., 2003). LC-MS methods offer interesting possibilities. Compared with other analytical methods the use of LC-MS, and especially LC-MS-MS, enable, not only characterisation but also a superior specificity. The use of appropriate internal standards in the form of stable isotopes (*e.g.* [$^{13}C_5$]folic acid), first reported by Pawlosky and Flanagan (2001), is an important advance in LC-MS methodology allowing correction for recovery losses and variation of ionisation yields caused by matrix interferences. LC-MS quantification of folate in foods is limited due to the existence of a variety of native folate derivatives, whereas only a low number of suitable commercial internal standards are available. The dominating content of 5-CH_3-H_4folate facilitates LC-MS quantification of folate in plasma (Freisleben et al., 2003). Freisleben et al. (2003) report the presence of 5-HCO-H_4folate in wheat bread and spinach by LC-MS-MS quantification, not detectable by HPLC analysis due to weak fluorescence of this folate compound. An LC-MS method is therefore an especially interesting alternative for quantification of folate derivatives exhibiting poor fluorescence. Drawbacks for LC-MS methods used for food analysis are expensive equipment, absence of suitable commercially available internal standards, and so far, low sensitivities for some of the folate compounds.

FBP

Using an enzyme-linked immunosorbent assay (ELISA) originally developed by Høier-Madsen et al. (1986), the first data on FBP concentrations in processed and unprocessed bovine milk were presented by Wigertz et al. (1996). The ELISA principle is based on recognition of FBP by a polyclonal antibody raised against bovine milk FBP. The above-mentioned ELISA is of 'sandwich type', *i.e.*, antibodies against the analyte are coupled to the well bottom of a microtiter plate. After addition of the analyte, a second portion of biotinylated antibodies is added, followed by a substrate addition yielding a colour shift measured spectrophotometrically. The concentration of FBP in an unknown sample is calculated from a calibration curve prepared from a standard of purified bovine milk FBP (Hoier-Madsen et al., 1986). No information is available about whether the response of the ELISA antibody deviates for FBP in complex with ligand and 'free' FBP, respectively, or whether the antibody also recognises FBP that is structurally affected as a result of processing.

A recently developed method for quantification of bovine milk FBP using a BIA technique is reported by Nygren et al. (2003). The principle of the BIA method is based on binding of FBP to folic acid linked to a surface, and the quantification necessitates thereby 'free' FBP. This enables quantification of the 'free' and 'bound' FBP fractions, respectively, in a sample. First the 'free' fraction of FBP in a sample is quantified, and after adjustment of the pH below 5, followed by a cut-off filtration to get rid of the endogenous bound folate, it is possible to quantify also the fraction of 'bound FBP'. Comparison of the BIA method with the ELISA quantification of FBP demonstrates good agreement for samples in which FBP is found 'free', *i.e.*, without a bound ligand. However, a striking difference between

the methods is seen in samples in which folate is bound to FBP, *i.e.*, in which FBP:folate complexes are found.

B vitamins and FBP in dairy products

Content and occurrence

Milk and dairy products are important sources of vitamin B_{12}, especially for lacto vegetarians. The average amount of vitamin B_{12} in bovine milk is 0.4 µg/100g (range of 0.2-0.7 µg/100g) (reviewed by Renner, 1983). Only one glass of milk provides about 40% of the daily requirement of the vitamin (Livsmedelsverket, 2002). The predominant amount of vitamin B_{12} in bovine milk is biosynthesised by microorganisms in the rumen (Scott, 1999). In milk, most of the vitamin B_{12} is protein bound. The reason of this is still unknown, although a protective effect against microbial breakdown has been suggested (Ford, 1974). Hydroxocobalamin, adenosylcobalamin and methylcobalamin are the major vitamin B_{12} derivatives found in bovine milk and in hard cheese (Farquharson & Adams, 1976, Fie et al., 1994b). Scott & Bishop (1986, 1988) report vitamin B_{12} concentrations in a variety of fermented dairy products on the UK market. They find a reduced content of vitamin B_{12} in fermented milk (0.1-0.2 µg/100g) compared to pasteurised milk. Hard and blue cheeses of different types are shown to contain between 1.0 and 2.1 µg vitamin B_{12}/100g, whereas cottage cheese contains 0.7 µg/100g.

Although the folate concentration in milk is only about 5 µg/100g, an increase of the folate content usually results from fermentation of milk and whey products (Lin & Young, 2000b, Lin & Young, 2000a, Crittenden et al., 2003). Furthermore, milk is of interest due to its content of FBP. Like vitamin B_{12}, folate in bovine milk is biosynthesised by microorganisms in the rumen of the cow. The dominating folate derivative in milk is 5-CH_3-H_4folate (Gregory et al., 1984, Vahteristo et al., 1997). Lin & Young (2000a) and Wigertz et al. (1996b) demonstrate 5-CH_3-H_4folate to be the major folate derivative also in fermented milk, other forms also reported to exist in fermented milks are H_4folate and 5-HCO-H_4folate (Müller, 1993, Lin & Young, 2000b, Konings et al., 2001). However, it must be stressed that quantification of 5-HCO-H_4folate by HPLC is very difficult due to a low fluorescence of this form compared with other reduced folate derivatives. Food composition tables, based on folate quantification by microbiological assays, report total folate content of between 12 and 18 µg folate/100g for various fermented milks (Holland et al., 1991, Souci et al., 1994, Swedish National Food Administration, 1996). Cottage cheese contains between 12 µg and 27 µg folate/100g based on quantification by microbiological assays (Renner, 1989) and HPLC (Wigertz et al., 1997). Ripened soft cheese, *e.g.* Brie, Camembert and blue cheese, has been demonstrated to contain between 50 µg and 100 µg folate/100g, probably due to the synthesis of folate by microorganisms during ripening (Renner, 1989). A variety of hard cheeses, *e.g.* Edam, Gouda and Cheddar, have been reported to contain 20-40 µg of total folate/100g (Scott & Bishop, 1988, Holland et al., 1991, Swedish National Food Administration, 1996). HPLC analyses show lower folate concentrations in hard cheeses; values of 7-12 µg folate/100g, with 5-HCO-H_4folate as the dominating derivative (Müller, 1993, Konings et al., 2001), and 12-18 µg/100g of 5-CH_3-H_4folate (Wigertz et al., 1997) are reported.

However, as also valid for folate analysis in fermented milks by HPLC, the analytical methodology requires further improvement regarding complex matrices such as cheese.

Almost all naturally occurring folate in milk is bound to FBP (Ghitis et al., 1969). FBP concentrations in pasteurised bovine milk are found in the range of 160-210 nmol/L, highly equivalent to the folate concentration of 110-220 nmol/L (review by Forssén et al., 2000). The majority of FBP in bovine milk exist as the soluble form, although a small amount of particulate FBP are found. The soluble form of FBP has been suggested to play a role in folate transport in *e.g.* serum and milk, whereas the particulate form is anchored in the cell membrane and associated with membrane transport of folate (reviewed by Wagner, 1985 and Anthony, 1996). FBP is destroyed by heat treatment of milk beyond pasteurisation at 72 °C. Hence, no or very low amounts of FBP are found in fermented milks, such as yoghurt. Wigertz et al. (1996) demonstrate FBP concentrations of < 10 nmol/L in UHT milk and yoghurt.

Effects from food processing and storage

Pasteurisation and ultra-high-temperature (UHT) treatment of bovine milk results, also for vitamin B_{12}, in minor losses of only < 10% as summarised by Biesalski and Back (2002). The vitamin B_{12} content is unaltered after pasteurisation of milk and during storage of UHT milk at 7 °C for up to 18 weeks, whereas vitamin B_{12} concentration is shown to decrease after 10 weeks storage at 23°C (Andersson & Öste, 1992a, Andersson & Öste, 1992b).

Fermentation of milk to yoghurt is shown to reduce the content of vitamin B_{12} (Reddy et al., 1975, Alm, 1982, Friend et al., 1983, Rao et al., 1984, Scott & Bishop, 1986). However, early studies (Janicki & Obrusiewicz, 1970, Cerna & Hrabova, 1977) report particular strains of *Leuconostoc* and *Propionibacterium* to increase the vitamin B_{12} content substantially during fermentation of milk, and during production of Kefir and hard cheese. During cheese making 10-50% of the vitamin B_{12} content from the starting milk is recovered in cheese (Scott & Bishop, 1988, Biesalski & Back, 2002). It must be emphasised, though, that these results are based on quantification of vitamin B_{12} in retail cheeses. To our knowledge, no direct quantification of vitamin B_{12} in whey fractions has been performed. However, a large portion of the vitamin is assumed to be lost with the whey (Scott & Bishop, 1988, Biesalski & Back, 2002).

The chemical reactivity of some important folate compounds makes the vitamin one of the most vulnerable to losses during food processing. Oxidative degradation enhanced by light and heat is responsible for a major part of these losses. Pasteurisation, however, has only minor effects on the folate content of milk, causing losses of less than 10% (Renner, 1989, Andersson & Öste, 1992a, Andersson & Öste, 1992b, Wigertz et al., 1996). According to a study by Andersson (1993), folate levels in pasteurised milk were not reduced during storage beyond the expiry date. Losses of folate, however, are observed during UHT treatment of milk (Renner, 1989, Wigertz et al., 1996) and the storage of aseptically packed UHT milk (Renner, 1989, Wigertz et al., 1996, Andersson & Öste, 1992a, Andersson & Öste, 1992b). Andersson & Öste (1992) reported a 57%

retention of folate during indirect UHT treatment, with an oxygen level of 5.4 ppm in the milk. When the oxygen level was reduced to 0.6 ppm, 96% of the folate was retained. Milk in packages with an oxygen-permeable seal lost all folate within 3 weeks of storage at room temperature. A storage study performed on UHT milk containing 0.3 ppm dissolved oxygen stored at 5 °C for 4 months showed no loss of 5-CH_3-H_4folate (Wigertz et al., 1996).

Several investigators have examined various strains of lactic acid bacteria for their ability to synthesise or utilise folate during fermentation of milk (Reddy et al., 1975, Cerna & Hrabova, 1977, Alm, 1982, Friend et al., 1983, Rao et al., 1984). Crittenden et al. (2003) find that *S. thermophilus*, bifidobacteria, and *E. faecium* elevate the folate content after fermentation of skim milk, whereas fermentation with lactobacilli generally deplete the folate content. Results from a study by Lin and Young (2000a) demonstrate 2-3-fold increase of the folate content in milk after fermentation by two *B. longum* strains. In another recent study they report that addition of 2% lactose to milk increase folate synthesis during fermentation by the two strains involved in yoghurt starter cultures, *S. thermophilus* and *L. bulgaricus* (Lin & Young, 2000a). They also show that the length of the incubation period (up to 18 h) significantly affects the folate content. In the presence of added lactose, the longer incubation period result in higher folate content, whereas fermentation for 18 h in the absence of added lactose result in a substantial decrease in folate content compared with the initial milk. The latter results were in line with earlier findings from the same investigators (Lin & Young, 2000b). In accordance with results obtained previously (Reddy et al., 1975), Lin & Young (2000b) show that the folate concentrations in fermented milk decrease by up to 28% during storage at 4 ºC for two weeks.

Few studies have investigated whether storage and ripening affect the folate content of hard cheese. Reif et al. (1975) observe that the starter culture of a cottage cheese actively synthesise folate during the setting period, resulting in a significant increase in folate concentrations in the final product. Wigertz et al. (1996) report no significant change in the concentration of 5-CH_3-H_4folate in hard cheese over a two-week period after manufacture, whereas a significant decrease (about 30 %) was seen in cheese after storage for six months. Again, one must bear in mind that biosynthesis or utilisations of B vitamins by lactic acid-producing bacteria depend greatly on the strain and the manufacturing procedure used. Hence, stages of cheese-making, *e.g.*, the lowering of pH, the coagulation of milk proteins followed by drainage of whey, and the ripening procedure through the action of different enzymes or microorganisms, could result in either increased and decreased levels of folate in cheese.

Pasteurisation of milk (at 74 °C for 4 s) results in a significant reduction of the concentration of FBP from 211 ± 7 nmol/L to 168 ± 20 nmol/L (Wigertz et al., 1996). These findings are in good agreement with earlier findings by Ford (1974) who demonstrate a 10% reduction in folate-binding of milk after low-temperature-long-term pasteurisation. In a study by Gregory (1982), heat-induced alteration of the FBP molecule was observed from pasteurisation, suggesting a change in folate-binding characteristics. In contrast to this, Kohashi et al. (1985), who compared the folate-binding capacity of raw milk with pasteurised milks (at 63 °C for 30 min, at

75 °C for 15 s, and at 120 °C for 1 s), observed an almost complete reduction of the folate-binding capacity. Conflicting results from these studies could be caused by the fact that common pasteurisation temperatures are very close to those at which denaturation of FBP takes place. Thus, small fluctuations in processing conditions may have a relatively large impact on the extent of FBP denaturation. The heat treatment process at 140 °C for a few seconds explains the low levels of FBP found in UHT milk reported by Wigertz et al. (1996). During production of yoghurt the milk is subjected to heat treatment (approximately at 95 °C for 5 min) prior to inoculation, also resulting in denaturation of FBP (Wigertz et al., 1996).
In spite of the high temperatures employed in the spray-drying process, most of the FBP is retained in milk powder (Svendsen et al., 1979, Wigertz et al., 1997). During cheese-making Wigertz et al. (1997) observe half the original amount of FBP from milk recovered in the whey fraction, 97 nmol FBP/L whey compared with 160-210 nmol FBP/L milk. FBP analysis of cottage cheese and hard cheese demonstrate 540 nmol and 13 nmol FBP/kg cheese, respectively.

Folate bioavailability

Absorption and physiology

Prior to absorption, deconjugation of folate polyglutamates from foods to monoglutamates by a γ-glutamyl hydrolase (conjugase) is required (Halsted, 1990). Reisenauer et al. (1977) report the existence of two separate γ-glutamyl hydrolase activities in human jejunal mucosa: one soluble and intracellular; the other membrane-bound and concentrated in the brush border. Human brush border γ-glutamyl hydrolase is a zinc-dependent exo-peptidase with optimum activity at pH 6.5, resulting in stepwise hydrolysis of polyglutamate folate. This enzyme has been shown to play the principal role in the digestion of dietary folate.

The monoglutamate folate compounds are absorbed by an active energy-dependent, carrier-mediated process at physiological concentrations and by passive absorption at higher concentrations (Selhub et al., 1984). Absorption takes place mainly in the jejunum and is markedly influenced by pH with a maximum absorption at pH 6.3. During transport of physiological concentrations of folate over the intestinal mucosa absorbed folate derivatives are converted to 5-CH_3-H_4folate and thereafter transported to the liver, which is the storage organ of folate as polyglutamates. A portion of absorbed folate is on the first pass retained in the liver, called ‘first pass effect’ (Steinberg et al., 1979). The liver is reported to secrete folate into the bile. The significance of this process and the efficiency of reabsorption have not yet been determined in humans, as summarised by Gregory (1995). From the liver, monoglutamate 5-CH_3-H_4folate is transported in the systemic circulation to other tissues were it is metabolised into H_4folate and used as one-carbon donor, or again converted to polyglutamates for storage (reviewed by Finglas et al., 2003).

Bioavailability is commonly defined as the absorption and metabolic utilisation of a nutrient, involving processes of intestinal absorption, transport, metabolism and excretion (Gregory, 1995). Folate bioavailability can be influenced by several intrinsic or extrinsic factors. Directly linked to the organism are folate status, health and gastrointestinal function, and antagonistic effects from use of drugs and

alcohol. Extrinsic factors are many depending on the ingested folate (*e.g.* oxidation status and substituent, length of polyglutamate chain), the food matrix (*e.g.* folate interaction with fibres, and substances with antifolate activity), or processing and storage (Jackson, 1997, Gregory, 2001).

Models for assessment

Many different experimental models have been applied with the goal of assessing folate bioavailability. Initially, often bioassays were performed to determine folate bioavailability using rats (Abad & Gregory, 1987, Keagy & Oace, 1989, Swiatlo et al., 1990), pigs (Natsuhori et al., 1991), or chicken (Ristow et al., 1982). Bioassays enable determination of folate concentrations in several response tissues, such as liver, kidneys, serum and whole blood, and allow assessing growth or reproduction activity as parameters for folate bioavailability. Concerns about the appropriateness of bioassays in order to predict folate metabolism in humans have been raised, due to quantitative and qualitative physiological differences between species, and interactions such as coprophagy in rat bioassays (Gregory, 2001).

In vitro methods allow more rapid and easily performed screening of test material compared to *in vivo* studies. Furthermore, tests enable strictly controlled conditions thereby avoiding *e.g.* pathological interferences. In a recent study the bioaccessibility of folic acid and (6S)-5-CH_3-H_4folate from folate-fortified pasteurised or UHT-treated milk, both in the presence and in the absence of FBP, using a dynamic *in vitro* gastrointestinal model (TIM) was investigated (Verwei et al., 2003). Results demonstrate milk to be a suitable carrier for supplemental folate, since 60-70% of the folate is released from the milk matrix and is found available for absorption in the small intestinal lumen. Using this *in vitro* model, a significant decrease of the bioaccessibility of folic acid is seen after addition of FBP in equimolar amounts to folate, whereas the addition of FBP does not affect the bioaccessibility of 5-CH_3-H_4folate. However, *in vitro* methods can reflect the complexity of *in vivo* folate absorption, deconjugation and metabolism only to a limited extent, and validation with corresponding human *in vivo* situation is crucial. For instance, in the above-mentioned *in vitro* model, the cellular transport of folate over the intestinal mucosa is replaced by passive diffusion over a membrane with a cut-off of 5 kDa.

Studies involving human subjects provide the best means to assess folate bioavailability, by use of e.g. long-term or short-term protocols. Long-term folate kinetics are usually investigating the impact of repeated folate supplementation on folate status, *e.g.* by feeding fortified or natural foods and compare those to folic acid supplements (Sauberlich et al., 1987, Cuskelly et al., 1999, Cuskelly et al., 1996, Johansson et al., 2002). Subsequently, folate concentrations in whole blood, erythrocytes, or fastened plasma are quantified, which reflect the folate status of the past three months. These studies are intensive in respect to costs and time. Short-term folate absorption and elimination kinetics are often studied after application of a single oral folate dose, in form of a pharmaceutical preparation, or a food either folate-fortified or naturally containing folate. Comparison of the area under the plasma response curve (AUC) or urinary folate excretion allows determination of relative bioavailability of different folate derivatives, from

various doses or application techniques (Tamura & Stokstad, 1973, Pfeiffer et al., 1997a, Prinz-Langenohl et al., 1999). A drawback of some short-term protocols for the determination of folate kinetics is the fact that the application of a single folate dose of physiological magnitude only results in a small plasma and urine folate response. Therefore, often pharmacological doses (Loew et al., 1987) or preloading of body stores are used (Tamura & Stokstad, 1973), which do not reflect physiological conditions.

A model for assessment of folate bioavailability recently introduced involves human ileostomists. A major advantage with ileostomists in folate research is that interference from folate synthesis by microbes in the large intestine can be avoided. A folate absorption study using nine healthy ileostomists consuming daily either one litre of milk, one litre of fermented milk, or one litre of a carbonised soft drink, together with a standardised diet, was performed (Wigertz, 1997). That study shows that the subjects excrete less folate in their ileostomal effluents after consumption of either of the milk products compared with after consumption of the carbonised soft drink. Authors interpret results as an improved absorption efficiency of dietary folate after incorporation of milk or fermented milk into a mixed diet occurred. This study was the first using human ileostomists as a model to assess folate absorption, and it needs to be repeated for dairy products under more controlled conditions. A recent ileostomy study show folate bioavailability from spinach to be 80% calculated from AUC and compared to an oral dose of folic acid as reference dose (Konings et al., 2002). Another newly performed ileostomist study (Witthöft et al., 2003), report folate from strawberries and broccoli to be approximately 65 and 50% bioavailable, respectively, as calculated from AUC. In contrast to the spinach study, in the latter study the reference dose to which the test food AUCs where compared, was an intra-muscular injection of (6S)-5-CH_3-H_4folate.

A few years ago, Gregory and his co-workers (von den Porten et al., 1992, Stites et al., 1997, Pfeiffer et al., 1997a, Boddie et al., 2000) introduced the use of stable isotope protocols. The incorporation of isotope labelled folate compounds (from fortified food samples) into the diet provides a tool to estimate folate turnover by urinary and faecal excretion of the labelled compounds and their metabolites (Pfeiffer et al., 1997a). An advantage with stable isotopes is that labelled folate compounds from the dose, and endogenous folate compounds from body stores can be distinguished.

A food constituent of particular interest for folate bioavailability is the folate-binding proteins (FBP) in milk. The physiological role of FBP is unclear. Ford (1974) suggests that FBP in human milk may initially act in the mammary gland as a trapping agent to accumulate folate from blood plasma into the milk. It has been speculated that FBP protects folate from microbial uptake and degradation (Tani & Iwai, 1984, Jones & Nixon, 2002) and could enhance folate absorption by mucosal cells (Colman et al., 1981, Salter & Blakeborough, 1988). However, this enhancement was not found in rat studies (Tani et al., 1983, Said et al., 1986), which rather showed that FBP reduce the rate of folate absorption.

In one of these rat studies (Tani et al., 1983), it was also found that under acidic conditions in the stomach folic acid is released from FBP, but recombines after

reaching the intestine. This is in line with the observation that the dissociation of folate occurs at pH of approximately 5 and lower (Ghitis et al., 1969). However, to which extent FBP resists the gastrointestinal passage has been very little studied. Salter & Mowlem (1983) demonstrated 'survival' of FBP activity in six-day-old goat kids. The situation might though be different in adults with a more matured digestion system, but no such studies are available.

Protein-bound and free folate, respectively, are absorbed in different ways in the small intestine. Whereas free folate monoglutamates are absorbed in the jejunum, is protein-bound folate mainly absorbed in the ileum at a much slower rate (Said et al., 1986). A slower rate of absorption, coupled with protection from intestinal bacteria is suggested to improve the bioavailability of folate when bound to proteins in milk (Ford, 1974). In fact, breast-fed babies have been reported to have a better folate status than bottle-fed babies. Whereas breast-fed babies sustain their folate status on an intake of 55 μg folate/d, require bottle-fed babies 78 μg/d. It has been speculated that the discrepancy is due to the occurrence of FBP in human milk that is not present in heat-processed milk formulas (Ek & Magnus, 1979, Ek & Magnus, 1980). However, the ileostomy study by Wigertz et al. (Wigertz, 1997), mentioned above, comparing excreted amounts of dietary 5-CH_3-H_4folate, shows an equal excretion of folate after consumption of milk and fermented milk, respectively, despite that the fermented milk did not contain any FBP.

The bioavailability of naturally occurring folate compounds in most foods has not been fully determined under conditions of actual consumption, neither including the consequences of interactions between foods in a mixed diet. In conclusion, data on folate bioavailability from dairy products needs to be further elucidated using evaluated experimental models. Information about the bioavailability of various natural folate derivatives compared with folic acid, and the effect of food matrix on folate bioavailability is required. Furthermore, studies investigating the possible nutritional role of bovine FBP are warranted.

Objectives and experimental designs

Average folate intake is still below recommendations in several population groups and authorities throughout Europe are at the moment struggling to develop strategies to improve folate intake to beneficial levels. The issue whether or not to fortify food products with folic acid is widely discussed. Still today, little reliable information is available on actual concentrations of folate and vitamin B_{12} in foods, or on the impact of food processing techniques on vitamin retention. A nutritional role of the bovine milk folate-binding proteins (FBP) on folate absorption has long been speculated upon. Many of the investigations in this field were, however, performed with old and indirect quantification methods, enabling no firm conclusions to be made. Furthermore, knowledge about folate bioavailability from different food sources, both products with native folate content and folate-fortified products, is still incomplete.

Specific objectives of the present work were:

· **To optimise preparation of dairy samples prior to analysis, and to modify and evaluate a radio protein-binding assay (RPBA) method for the quantification of vitamin B_{12} and folate in dairy products.**
Aspects studied were composition of extraction buffers, choice of calibrants, absence of matrix effects, linearity, recoveries, and accuracy (Papers I-II).

· **To map the retention of vitamin B_{12} and folate during the manufacturing process of six fermented dairy products.**
The evaluated RPBA was used to study vitamin retention during one single manufacture batch of six fermented dairy products. Retention studies mainly focused on vitamin B_{12} for which samples throughout the manufacturing procedure were analysed (Paper I). Milk and "ready to consume" products from studied products were also analysed for folate, and folate retention was discussed.

· **To study folate bioavailability from folate-fortified milk products, with focus on the nutritional role of bovine milk FBP during gastrointestinal passage.**
Folate bioavailability was studied both *in vitro* and *in vivo*. In the *in vitro* studies the effects on folate bioaccessibility of; 1) dairy matrix, 2) folate form used for fortification, and 3) the effect of FBP, were investigated (Paper III). In addition, the stability and binding characteristics of FBP for 5-CH_3-H_4folate and folic acid during gastric passage were investigated (Paper IV). The human study (Paper V) focused on the bioavailability of the natural folate form, (6S)-5-CH_3-H_4folate, from folate-fortified milk and fermented milk, and the effect and stability of FBP were also studied. Priority was given to folate for bioavailability studies due to the massive interest in folate concerning health benefits, dietary recommendations and the issue raised about folate fortification of food. The human ileostomy model, the TIM model and the expertise around these models were also available exclusively for the folate application.

For an overview of the experimental designs see Table 2.

Material and methods

The following chapter gives a brief description of materials and methods used. For an overview see Table 2. More detailed information is found in Papers I-V.

Material

Standards

Cyanocobalamin used as calibrant in the RPBA, was obtained from Sigma-Aldrich (St Louis, MO, USA). (Paper I)

(6R,S)-tetrahydrofolic acid (H_4folate, trihydrochloride salt), (6R,S)-5-methyl-tetrahydrofolic acid (5-CH_3-H_4folate, calcium salt), (6R,S)-5-formyltetrahydrofolic acid (5-HCO-H_4folate, calcium salt), pteroyl-L-glutamic acid (PteGlu), pteroyl-di-γ-L-glutamic acid ($PteGlu_2$), pteroyl-tri-γ-L-glutamic acid ($PteGlu_3$), pterin-6-carboxylic acid (Pt-6-COOH) and para-aminobenzoyl-L-glutamic acid (pABG), all used as calibrants in the RPBA, were obtained from Schircks Laboratories (Jona, Switzerland). (Paper II)

Folic acid and (6S)-5-methyltetrahydrofolic acid (5-CH_3-H_4folate, sodium salt), used for fortification of dairy products for *in vitro* studies on folate bioaccessibility, were obtained from Eprova AG (Schauffhausen, Switzerland). (Paper III)

Radio labelled folate compounds, [^{3}H]-folic acid and [^{14}C]-(6R,S)-5-CH_3-H_4folate used in studies of FBP´s binding characteristics under gastric passage were obtained from Amersham Pharmacia (Buckinghamshire, England). Unlabelled folic acid and (6R,S)-5-CH_3-H_4folate (calcium salt) were obtained from Schircks Laboratories (Jona, Switzerland). (Paper IV)

(6S)-Ca-5-methyltetrahydrofolate (5-CH_3-H_4folate, calcium salt), used for *in vivo* studies on folate bioavailability was obtained from Eprova AG (Schauffhausen, Switzerland). The concentration was corrected for water and calcium contents. (Paper V)

Purity of standards were determined using the molar extinction coefficients described elsewhere (van den Berg et al., 1994, Eitenmiller & Landen, 1999b, Eitenmiller & Landen, 1999a).

The FBP calibrant used in ELISA analysis was obtained from the Central Hospital Hillerød (Hillerød, Denmark). (Papers III-V)

Dairy products

Reference dairy materials

A concentrated whey protein powder, wpc 65, (supplied by Arla Foods, Götene, Sweden) was used as an in-house reference material for vitamin B_{12} and folate analyses using the RPBA method, and for ELISA quantification of FBP. Wpc 65 was included parallel to dairy samples in every extraction and quantified in subsequent RPBA or ELISA. CRM 421 (vitamin enriched milk powder), a certified reference material obtained from the European Commission (Joint Research Centre IRMM, Geel, Belgium) was used to control method accuracy and to enable comparison of different analytical methods. CRM 421 was extracted and

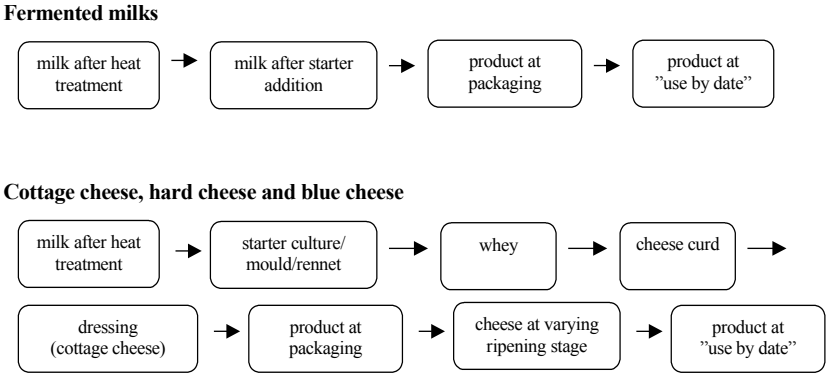

Figure 4. Sampling scheme for studies on vitamin retention during manufacture of six fermented dairy products.

quantified as a dairy sample in vitamin B_{12} and folate analysis using the RPBA method. These reference materials were subsampled and stored frozen at -18°C and -80°C, respectively, until analysis.

Dairy samples for process retention studies

Six dairy products were selected for the evaluation and optimisation of sample pre-treatment and quantification of vitamin B_{12} and folate by RPBA, and for studies of the retention of these vitamins during the manufacturing process. Samples were collected from one single manufacture batch, and during the subsequent storage period, from the dairy plants of Arla Foods in Linköping (fermented milks, Filmjölk and Yoghurt naturell), Skövde (cottage cheese, Keso®), Kalmar (hard cheeses, Herrgård® and Grevé®) and Kvibille (blue cheese, Kvibille ädel). Samples were taken according to the scheme outlined in Fig. 4. Approximately 1 kg product was collected at each point, and these portions were divided into ten subsamples and were stored at -20ºC until analysis. Milk before and after heat treatment at 76 °C for 16 s and 96 °C for 5 min, respectively, was collected at the dairy plant of Arla Foods in Stockholm. All milk samples prior to and after heat treatment originated from the same milk silo. (Paper I)

Fortified dairy products for studies of folate bioavailability and the effect of FBP

Dutch pasteurised milk and Swedish commercially available yoghurt were used to prepare eight different test products for studies of folate bioaccessibility including the effect of FBP using a dynamic *in vitro* gastrointestinal model (TIM). Fortification of yoghurt with folic acid or the natural folate diastereoisomer (6S)-5-CH_3-H_4folate and FBP, and fortification of pasteurised milk with the natural folate diastereoisomer (6S)-5-CH_3-H_4folate and FBP, were performed 1 h prior to the experiment by stirring the fortificants into the test portion. Folic acid-fortified pasteurised milk was commercially prepared and obtained from Campina (The Netherlands). A portion of 300 g test food applied into the TIM model provided 260-350 nmol folate and equimolar amounts of FBP. For details see Table 2 and Paper III.

A whey protein concentrate (wpc 75, whey powder containing 75% protein and 15% lipids) obtained from Arla Foods Innovation (Stockholm, Sweden) was used for studies on the fate and binding characteristics of FBP for folic acid and 5-CH_3-H_4folate during gastric and duodenal passage of the TIM model. The whey powder had an endogenous folate content of ca 9000 nmol/kg and an endogenous FBP content of ca 20 000 nmol/kg. Whey solutions were prepared in phosphate buffer, pH 7, with a FBP concentration of ca 160 nmol/L and fortified with a mix of radio labelled and unlabelled folate, either [^{3}H]-folic acid and folic acid, or [^{14}C]-(6R,S)-5-CH_3-H_4folate and (6R,S)-5-CH_3-H_4folate, to a final fortification concentration of approximately 160 nmol/L. (Paper IV)

The products used for studies of folate bioavailability *in vivo* were pasteurised milk fortified with the natural folate diastereomer (6S)-5-CH_3-H_4folate + FBP (aiming at equimolar amounts) and two fermented milks; one fortified with (6S)-5-CH_3-H_4folate and one fortified with (6S)-5-CH_3-H_4folate + FBP (aiming at equimolar amounts). FBP was added via the above described whey powder, wpc 75. The pasteurised milk product was produced in a single batch in a pilot plant of Arla Foods Innovation (Stockholm, Sweden). The milk was bottled and stored at -20ºC until use. For the fermented milk products a commercially available product on the Swedish market was fortified with folate and FBP 30 min prior to the start of the experiment by stirring the fortificants into the test portion of the fermented milk. A portion of 400 g of the test food was given to the human volunteers providing 241-542 nmol folate and 156-442 nmol FBP. For details see Table 2 and Paper V.

Methods

Models for evaluation of folate bioavailability

The dynamic in vitro gastrointestinal model (TIM)

TNO´s (TNO Nutrition and Food Research, the Netherlands) dynamic *in vitro* gastroIntestinal Model (TIM) was used to study the amount of folate, from folate- and FBP-fortified yoghurt and milk, available for absorption in the small intestine. The stability and binding characteristics of FBP for 5-CH_3-H_4folate and folic acid after passage through the stomach and the small intestine were also investigated in TIM. (Papers III & IV)

The TIM system (Fig. 5) has been described by Minekus et al. (1995). For details about the folate application see Verwei et al. (2003). The models used in these studies simulated the gastrointestinal conditions of a healthy adult human. The gastric small-intestinal model comprises four connected compartments that represent the stomach, duodenum, jejunum and ileum, respectively. Each compartment consists of a glass outer wall with a flexible inner wall. The pH is continuously measured in the four compartments and regulated by addition of hydrochloric acid or sodium bicarbonate. The pH values, as well as the gastric emptying and small-intestinal passage of the food, are computer-controlled events according to pre-set curves based on literature information for human *in vivo* conditions. Artificial oral fluid and gastric juice with lipase and pepsin are gradually added into the gastric compartment. Bile, pancreatic juice and

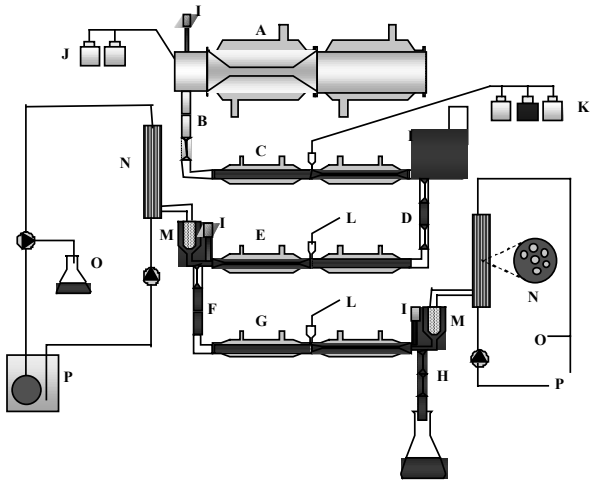

Figure 5. Schematic diagram of the dynamic in vitro gastrointestinal model (TIM).
A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileo-caecal valve; I. pH electrodes; J. gastric secretion bottles with acid and enzymes; K. duodenal secretion bottles with bile, pancreatin, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fibre semi-permeable membrane system; O. water absorption system; P. closed dialysing system.
With kind permission from Verwei et al., Journal of Nutrition, 2003.

electrolytes are gradually added into the duodenal compartment. The flexible wall is surrounded by water heated to 37 ºC. Mixing of the food with the 'secreted' enzymes is ensured by peristaltic movements in the gastrointestinal tract. The jejunal and ileal compartments are connected with semi-permeable hollow fibre membranes with a cut-off of 5 kDa, which mimic the passive absorption of digested products and water (Fig. 5, points N, O and P). During passage of the test food through TIM, total jejunal and ileal dialysate were collected during 0-1, 1-2, 2-3 and 3-5 h. The dialysate fractions contained the absorbable (bioaccessible) fraction. The non-absorbed folate fractions were collected after passage through the ileo-coecal valve as 'ileal delivery' (Fig. 5, point H). The ileal delivery was collected as one pooled sample during 0-5 h.

The human ileostomy model

A human ileostomy model (Fig. 6) described in detail by Witthöft et al. (2003) was used to study relative folate bioavailability and *in vivo* effects of FBP on folate retention after gastrointestinal passage of dairy products (Paper V). The model determines short-term folate absorption from single test food doses compared with pharmaceutical folate doses. Collection of the ileostomal effluent in individual fractions every second hour over ten hours post-dose reflected not only non-absorbed folate, but showed also a time pattern of the gastrointestinal passage.

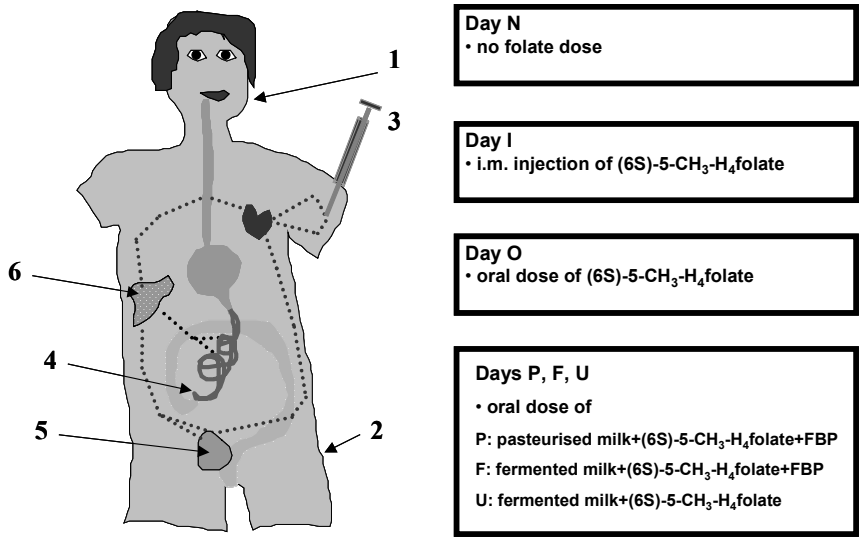

Figure 6. The human ileostomy model.
Left: Details on administration of doses and sample collection, 1) oral administration of test foods or pharmaceutical folate preparations, 2) i.m. injection of folate reference dose, 3) blood collections over 10 h post-dose (n = 11) 4) collection of ileostomal effluent every second hour over 10 h post-dose, 5) urine collection over 10 h post-dose, 6) liver = storage organ for folate, pre-saturation prior to each test day. Right: Information on test doses, all test days performed for each subject randomly 2-4 weeks apart.

Urinary excretion of folate, or the absence of increased urinary excretion, was evaluated to ensure that the ingested doses were in the physiological range.

Nine healthy subjects with pre-saturated folate body stores underwent strictly standardised independent study days (two to four weeks apart) in random order. They received, after an overnight fast, either a single oral portion of each dairy product, an oral dose of a pharmaceutical preparation of (6S)-5-CH_3-H_4folate, or an intra muscular injection of (6S)-5-CH_3-H_4folate as a reference dose. One study day with no folate dose application was carried out to account for baseline folate excretion into stomal effluent. For further details see Fig. 6 and Paper V.

Methods of quantification

Analysis of vitamin B_{12} and folate

The commercial RPBA kit, SimulTRAC-SNB Radioassay Kit, Vitamin B_{12} [^{57}Co] /Folate[^{125}I] (ICN Pharmaceuticals, Orangeburg, New York, USA), was optimised and evaluated for analysis of vitamin B_{12} and folate in milk and fermented milk products (Papers I & II). The kit principle is based on binding of the vitamins to specific binding proteins included in the kit. For vitamin B_{12} this binding protein is porcine intrinsic factor, and for folate a folate-binding protein from bovine milk is used. Since the kit is produced for clinical samples (plasma and whole blood) a crucial step in vitamin analysis in foods using this method was the pre-treatment of

samples. Simultaneous quantification of vitamin B_{12} and folate from one sample was not possible since the sample preparation procedures prior to quantification differed for the vitamins.

Optimisation of sample preparation and RPBA quantification of vitamin B_{12} and folate

Optimisation of dairy sample preparation prior to vitamin B_{12} quantification by RPBA included studies on the effect of extraction buffer molarity, and cyanide concentration on the RPBA outcome. In addition, three levels of pancreatin were tested to optimise the yield of vitamin B_{12} from the protein-rich dairy products (Paper I). Regarding folate analysis using the RPBA method, the RPBA response for various folate compounds was investigated. Two different sources of γ-glutamyl hydrolase were also tested (Paper II).

The radioprotein-binding assay procedure was carried out according to kit description apart from two important exceptions; 1) use of purity controlled external calibrants, in the same form as the dominating derivative in the food sample, and 2) dilution of all samples and standards in extraction buffer instead of buffers provided with the kit. Checks for possible matrix effects from dairy samples on the kit performance were performed for each new matrix introduced into the RPBA. This was done by introduction of matrix blank samples (dairy sample without the kit vitamin-binder) to control for unspecific binding of substances from the dairy sample to kit material. Furthermore, the effect of endogenous substances was controlled by dilution of the in-house reference material, wpc 65, into the concentration range of the calibration curve (vitamin B_{12}: 100-2000 pg/ml, and folate: 0.5-10 ng/ml). Recovery tests were used to control for vitamin losses during the entire quantification procedure. Prior to extraction of dairy samples an amount corresponding to the native vitamin content was added in form of a purity-checked calibrant. Repeatability, both within a day and between days, was controlled both for sample extraction, the RPBA step, and for the entire quantification procedure for vitamin B_{12} and folate. A certified reference material, CRM 421, was repeatedly analysed to check the method accuracy and to enable comparisons with other analytical methods (Papers I & II). The selectivity of the kit folate-binder for different folate forms was investigated (Paper I). To perform a similar study for the kit vitamin B_{12}-binder was, however, not necessary since all vitamin B_{12} derivatives were converted into the most stabile form, cyanocobalamin, during sample preparation.

Other quantification methods used for vitamin analyses

Results obtained with the RPBA were confirmed by analyses of the same samples with HPLC (only folate quantification) and microbiological assay (both vitamin B_{12} and folate) (Papers I-III).

The HPLC method used for quantification of folate in milk (Paper II) included heat extraction of samples in phosphate buffer, pH 6.1, followed by deconjugation with hog kidney conjugase and purification by solid-phase extraction using strong anion exchange (SAX) columns (Jastrebova et al., 2003). Subsequent quantification of 5-CH_3-H_4folate, H_4folate, 5-HCO-H_4folate and folic acid was

performed using a reversed-phase HPLC method with fluorescence detection (290/360 nm) and UV detection (290 nm). The analytical column was a Zorbax C8 (4.6*150 mm; 5 μm) and the mobile phase consisted of 0.03M phosphate buffer, pH 2.3, and acetonitrile as a gradient from 6-25%. The flow was 0.4 mL/min.

Extraction of samples from the folate bioaccessibility study *in vitro* (Paper III) included heat extraction in phosphate buffer, pH 6.1, and deconjugation with human plasma conjugase. Affinity chromatography columns containing FBP were used to clean and concentrate samples prior to quantification of folic acid and 5-CH_3-H_4folate. The reversed-phase HPLC method involved UV (290 nm) and fluorescence detection (290/360 nm), an inertsil 5 OD-3 column (3*100 mm; 5 μm) was used, and the mobile phase consisted of 0.03M phosphate buffer, pH 2.1, and acetonitrile 5-80%, with a flow of 0.4mL/min (Verwei et al., 2003).

Quantification of vitamin B_{12} and folate by microbiological assay (MA) was performed at the Swedish National Food Administration according to their in-house methods (AOAC, 2000b, AOAC, 2000a). In brief, for vitamin B_{12} the sample extraction involved addition of cyanide and use of α-amylase treatment, followed by quantification using *L. delbrueckii* as assay organism and cyanocobalamin as calibrant. Folate analysis included tri-enzyme treatment (α-amylase, protease and γ-glutamyl hydrolase from chicken pancreas), the microorganism used was *L. rhamnosus* and folic acid was used as calibrant.

Folate concentrations in test foods, pharmaceutical folate doses, plasma samples, and urine and ileostomal effluent samples obtained from the human experiment (Paper V) were quantified by HPLC according to Jastrebova et al. (2003). The sample pre-treatment is described in detail by Witthöft et al. (2003). In brief, with the exception of plasma samples, which were only purified by SAX prior to quantification, it included heat extraction in phosphate buffer, pH 6, followed by treatment with hog kidney conjugase. All but the urine samples were purified by solid-phase extraction using SAX columns. The urine samples were purified by use of affinity chromatography columns containing FBP.

The distribution of radioactively labelled folate derivatives within collected protein fractions after gel filtration was determined by scintillation measurements (Paper IV).

Quantification of FBP

FBP concentrations in samples from *in vitro* and *in vivo* studies on folate bioavailability (Papers III-V) were quantified using a two-site enzyme-linked immunosorbent assay (ELISA) developed by Høier-Madsen et al. (1986). The antibody against bovine milk FBP was obtained from the State Serum Institute (Copenhagen, Denmark) and the FBP calibrant from the Central Hospital (Hillerød, Denmark). The ELISA quantification procedure is described in detail by Wigertz et al. (1996). Briefly, to 1-3 g of sample 0.09 g of Triton X-100 was added. The sample was put on a shaking device, incubated for 45 min at room temperature and thereafter diluted to a concentration of approximately 0.4 nmol FBP/L and applied to a microtiter plate. An eight-point calibration curve from 0.002-1.1 nmol FBP/L was prepared and included in each ELISA run. The in-

house reference material (wpc 65) was included in every analysis to control for the between-run variation. Whey proteins (Paper IV) were fractionated by gel filtration (size-exclusion chromatography using a Sephadex column) and collected fractions analysed for FBP using ELISA. SDS-PAGE combined with immunoblotting was used to confirm ELISA results from collected protein fractions.

Results and discussion

Method optimisation

The RPBA was evaluated as a fast, relatively easily handled, method with the capacity to quantify vitamin B_{12} and folate in large series of milk and dairy samples. However, for folate quantification, it must be emphasised that knowledge about the folate pattern within the unknown sample is crucial due to a differing response of the folate-binder, provided in the kit, to individual folate compounds. This method is thereby only suitable for quantification of folate in folate-fortified food products or foods in which one folate form is dominating, such as, *e.g.* milk, where 5-CH_3-H_4folate is the main folate form (Gregory, 1984, Holt et al., 1988, Vahteristo et al., 1997). The application of food matrices into a RPBA kit produced for clinical samples required careful optimisation of sample pre-treatment and evaluation of kit performance due to introduction of new sample matrices, buffers, and calibrants. (Papers I & II). Quality control data from the entire procedure of vitamin B_{12} and folate quantification are summarised in Table 3. These and further quality data are presented and discussed in the following sections.

RPBA quantification of vitamin B_{12} (Paper I)

Performance data from the optimised quantification procedure showed the RPBA method to be suitable for the determination of vitamin B_{12} in dairy products. The overall recoveries in dairy matrices ranged between 88-96%, and the coefficients of variation between replicates both within a day and between days were below 7%. Also introduction of samples and external calibrants in extraction buffer did not affect the kit performance negatively, as seen from the calibration curve slopes of 0.97 ± 0.04 and linearities of > 0.995 (range 0-2000 pg/ml).

Table 3. *Performance data from sample preparation and quantification of vitamin B_{12} and folate in milk and dairy products using the optimised RPBA*

Parameters	**Performance**	
	Vitamin B_{12}	Folate
Quantification procedure		
Recovery (%)	88-96	94-97
Repeatability (CV%)		
- within day	< 6	< 6
- between day	< 7	< 5
CRM 421 (µg/100g DM)	2.74 ± 0.1	91 ± 5
Kit performance		
Calibration curve range (vit.B_{12} pg/ml, folate ng/ml)	100-2000	0.5-10
Slope	0.97 ± 0.04	1.09 ± 0.05
Linearity	> 0.995	> 0.995
ED-50 [1] (vit.B_{12} pg/ml, folate ng/ml)	553 ± 15	1.85 ± 0.18

[1] ED-50 is defined as the concentration where 50% of the binding proteins are occupied by tracer and the remaining 50% by vitamin from the sample

To test effects from introduction of a dairy matrix into the RPBA, seven dilutions over the concentration range of the calibration curve were performed from three independent replicates of wpc 65. These showed the same slopes as the calibration curve, and measured vitamin B_{12} concentrations were back calculated to concentrations of undiluted samples resulting in a coefficient of < 12% (Fig. 7). Control of non-specific binding of tracer to kit material, *e.g.* tube walls, was routinely included in the kit procedure (called kit blank). This was performed by addition of tracer, extraction buffer and blank reagent, to test tubes in duplicate. Resulting radiation, expressed as counts per minute, was automatically subtracted from each test tube. Non-specific binding of tracer to the food matrix was tested for each dairy product by addition of tracer, sample and blank reagent to test tubes in duplicate (called sample blank). Sample blanks from studied dairy matrices did not differ from kit blanks, showing no unspecific binding of endogenous substances within these samples to kit material other than the vitamin B_{12}-binder.
Analyses of CRM 421, a certified milk powder, resulted in a vitamin B_{12} value within the certified range. In comparison with results from studied dairy products obtained by the MA technique, RPBA results were between 6-35% lower (see Table 3 in Paper I). The microorganism used in the microbiological assay, *L. delbrueckii*, has been shown to respond also to vitamin B_{12} analogues (without vitamin activity) (Muhammad et al., 1993b, Herbert, 1996). The possible existence of such forms in dairy products may partly explain the difference in results obtained by the two methods. The vitamin B_{12}-binder used in this RPBA kit is a purified intrinsic factor from hog, which shows less than 0.01% cross-reactivity to cobinamide (inactive vitamin B_{12} analogue). Another explanation of lower values obtained with RPBA might be that pancreatin treatment of some of the products was not carried out prior to quantification (see Table 3 in Paper I). The pancreatin treatment of protein-rich dairy samples needs further optimisation, but it was shown to substantially increase the vitamin B_{12} yield.

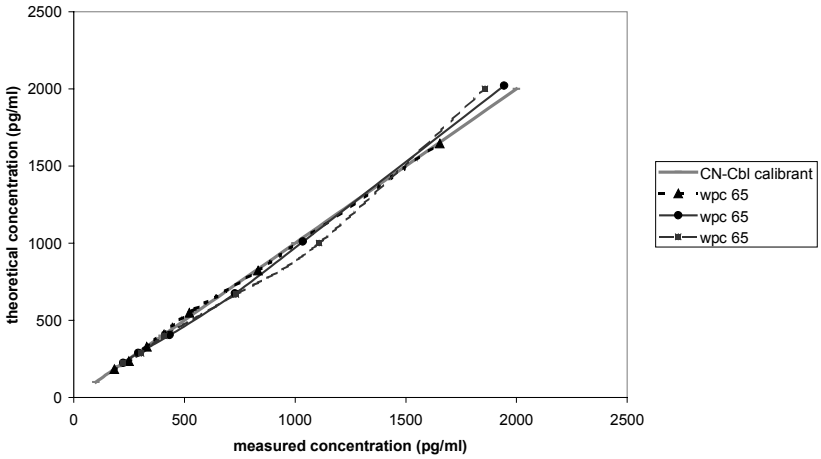

Figure 7. Comparison of a cyanocobalamin (CN-Cbl) standard curve (dilution range 100-2000 pg/ml, 5 points) with three replicate wpc 65 (whey protein concentrate) samples (dilution range 100-2000 pg/ml, 7 points).

The RPBA method has earlier been applied for vitamin B_{12} analysis in foods (Richardson et al., 1978, Králová et al., 1982, Casey et al., 1982, Österdahl et al., 1986, Andersson et al., 1990). Most studies conclude that the pre-treatment of food samples is a critical step in achieving reliable results, but few studies have systematically investigated effects on kit performance. Pre-treatment usually involves heat extraction of the food sample in a buffer, at acidic pH, containing an excess of cyanide, metabisulphite or nitrite to enable conversion of native vitamin B_{12} derivatives in the sample. Our optimised sample preparation of milk and fermented milk products included heat extraction at 121 °C for 25 min in a 0.1M acetate buffer (pH 4.5) containing an excess of cyanide, followed by pancreatin treatment (for further details see Paper I).

Interestingly, the concentration of cyanide added to the sample substantially influenced the outcome of the RPBA used in our studies. A final concentration of 0.08mM and 40mM cyanide was evaluated. The higher cyanide concentration resulted in a recovery of 121 ± 4% in cyanocobalamin spiked extraction buffer, whereas a recovery of 94 ± 2% was obtained with the lower cyanide concentration. Most previous studies use a cyanide concentration of ca 1mM (Österdahl et al., 1986, Muhammad et al., 1993a) but a 39-fold increased concentration or even higher concentrations are reported as optimal in some studies (Beck, 1979, Králová et al., 1982, Andersson et al., 1990). We do not have an explanation of this overestimation of vitamin B_{12} content due to a high cyanide concentration. Cyanide is the favourable conversion substance to add to a sample prior to quantification, in comparison to metabisulphite and nitrite. This is because cyanide converts most vitamin B_{12} forms into (di)cyanocobalamin, whereas metabisulphite converts only aquacobalamin into sulphitocobalamin and nitrite converts only hydroxocobalamin into nitritocobalamin (Muhammad et al., 1993a, for further details see Table 1 in the Introduction section). The advantage of cyanide addition is, therefore, that after extraction only one vitamin B_{12} form dominates within the sample, and this form is used for preparation of the calibration curve in the RPBA. The problem regarding a varying selectivity of the vitamin B_{12}-binder to different vitamin B_{12} forms is thereby avoided.

Excessively high extraction buffer molarity also affected the RPBA result negatively. Application of a dairy sample extracted in 0.4M acetate buffer increased the maximum binding capacity of the vitamin B_{12}-binder and, as a consequence, the competition conditions are altered for the isotope labelled vitamin B_{12} (tracer) and the vitamin B_{12} from the unknown sample for a limited number of vitamin B_{12}-binding proteins. This was most probably caused by a pH decrease after introduction of a buffer with such high molarity. Our optimised preparation of dairy products prior to quantification of vitamin B_{12} included use of 0.1M acetate buffer, which did not interfere with the binding capacity of the vitamin B_{12}-binder.

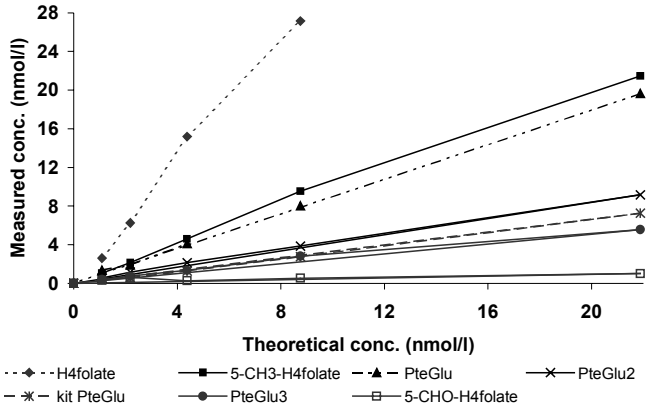

Figure 8. RPBA response to individual folate derivatives at prevailing assay pH of 9.5. H_4folate, tetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; PteGlu, Pteroyl-L-monoglutamic acid; $PteGlu_2$, Pteroyl-L-diglutamic acid; kit PteGlu, folic acid provided by the kit; $PteGlu_3$, Pteroyl-L-triglutamic acid; 5-CHO-H_4folate, 5-formyltetrahydrofolate. Concentrations are calculated against the theoretical concentrations of 5-CH_3-H_4folate. With kind permission from Strålsjö et al., Food Chemistry, 2002.

RPBA quantification of folate (Papers II & III)

In contrast to vitamin B_{12}, the endogenous mix of folate derivatives within a food sample is not actively converted during sample pre-treatment prior to RPBA quantification. To obtain reliable results with this method, knowledge about the dominating folate derivative in a sample, and use of this compound as calibrant, is crucial. The varying responses of the folate-binder provided in the kit (bovine milk folate-binding protein) for different external folate calibrants, at the prevailing assay pH of 9.5, are presented in Fig. 8 (Paper II). At this pH, the response for (6R,S)-5-CH_3-H_4folate was approximately 15% higher than for folic acid. Compared with the (6R,S)-5-CH_3-H_4folate curve, the presence of H_4folate in a sample would lead to a significant overestimation of the folate content, whereas the response for 5-HCO-H_4folate was very low, making quantification by RPBA inappropriate for this folate form. However, no measurable response was found for pABG (para-aminobenzoyl-L-glutamic acid) and Pt-6-COOH (pterin-6-carboxylic acid), two common folate degradation products, making the RPBA suitable for folate retention studies during processing of foods where degradation products from oxidation of folate can occur. The optimised sample preparation and introduction of samples and external calibrants in extraction buffer into the kit did not affect the assay performance negatively. Performance data from assays using (6R,S)-5-CH_3-H_4folate as calibrant showed the RPBA method to be reliable for milk and wpc 65 (Table 3). Overall recoveries (94-97%) and coefficients of variation both within day and between days (< 6%), as well as the absence of dairy matrix effects disturbing the assay performance were demonstrated (Paper II).

The optimised and evaluated RPBA was applied for folate analyses in yoghurt samples from studies in TIM (Paper III). Since the yoghurt products were fortified with either (6S)-5-CH_3-H_4folate or folic acid, it was obvious to use the respective

folate forms as calibrants in subsequent RPBA. Here we experienced for the first time the differing affinity of the kit binder to (6R,S)-5-CH_3-H_4folate and the newly commercially available natural diastereoisomer (6S)-5-CH_3-H_4folate, as studied in detail and reported by Strålsjö et al. (2003). The RPBA was not negatively affected by the TIM sample matrix as shown by inclusion of sample blanks prepared in jejunal and ileal dialysates and ileal delivery. Overall recoveries in jejunal and ileal dialysates and ileal delivery samples were from 90-102% for both folate forms. However, the residues from TIM, material remaining in the model compartments after ending the experiment (used for calculation of folate mass balances), denoted strong matrix effects on the RPBA, resulting in an overestimation of the folate content. This was probably caused by the high content of disturbing substances after addition of digestion enzymes and from the pH adjustment solutions. The variation between duplicate determinations of TIM samples was somewhat higher than from food samples such as milk and fermented milks. This emphasises again the need of careful evaluation of the assay performance when introducing a new matrix into the kit (Paper II).

Folate concentrations obtained by RPBA were in line with results from the same set of samples analysed by MA (Paper II) and HPLC (Papers II & III). Using HPLC, selected individual folate forms are quantified. This can result in a lower sum of folate compared with the value of total folate obtained by RPBA. This can *e.g.* be observed in Paper III when presenting folate concentrations in folic acid-fortified milk, where the native 5-CH_3-H_4folate content was not quantified by the HPLC method used, but by RPBA.

Quantification of FBP by ELISA (Papers III-V)

Method control for ELISA included recovery studies in assay buffer and milk, repeatability studies with wpc 65 (within and between assays), and analyses of blank samples (matrices free of FBP). FBP-spiked assay buffer and milk resulted in recoveries ranging from 80-110%. Coefficients of variation were <12% for six replicates of wpc 65 analysed within an assay, and the between assay variation <15% for individual wpc 65 samples quantified in separate ELISA runs (n = 20). It could be confirmed that, as expected due to the membrane cut-off of 5 kDa, no FBP was detected in TIM dialysate samples (Paper III). Results from quantification of stomal effluent samples must be considered as semi-quantitative since the extraction method used was not optimised for this matrix. However, no FBP was detected in stomal effluent samples from test days where no FBP were given (N- and U-days), proving that the matrix did not result in a false positive response (Paper V). An important aspect not previously reported is that the ELISA method does not discriminate between 'free' FBP and FBP in complexes with ligand. This was observed from studies where addition of an excess of folic acid or 5-CH_3-H_4folate to the FBP calibrant, followed by preparation of calibration curves from the original FBP calibrant (folate free) and the folate-spiked FBP calibrants, resulted in almost identical calibration curves (results not shown).

Retention studies

Vitamin B_{12} (Paper I)

Vitamin retention studies were focused on vitamin B_{12}. Varying contents of vitamin B_{12} and folate in different fermented dairy products may be expected due to disparities in manufacturing procedures. Factors such as initial heat treatment of the milk, composition of starter culture, separation of whey, and subsequent ripening periods are reported to influence the retention of these vitamins (Janicki & Obrusiewicz, 1970, Renner, 1983, Scott & Bishop, 1988, Andersson & Öste, 1992a, Andersson & Öste, 1992b). To our knowledge, this is the first study to map vitamin B_{12} retention during one and the same batch and subsequent storage period by direct quantification of vitamin B_{12} in collected milk, curd, and whey samples.
Results of the present study showed that the initial heat treatment of milk at either 76 °C for 15 s (all cheeses) or 95 °C for 5 min (fermented milks) did not alter the vitamin B_{12} concentration in the milk. Our results are in line with a previous investigation (Andersson & Öste, 1992a, Andersson & Öste, 1992b), and partly agree with previous studies reporting the heat treatment of milk to result in a decrease of vitamin B_{12} concentrations ranging from only a few percent up to 20% (Renner, 1989).

In the fermented milks the presence of viable starter cultures was most probably the major reason for the substantial (40-60%) decrease in vitamin B_{12} content seen during storage of the final product until the 'use by date' (14 days after packaging) (Fig. 9), although other degradation routes can not be ruled out. Our results support findings in previous studies, performed by MA, reporting a decrease in the vitamin B_{12} content after fermentation of milk with selected starter cultures (Reddy et al., 1975, Alm, 1982, Friend et al., 1983, Rao et al., 1984).

The separation of whey during production of cottage cheese, hard cheeses and blue cheese was the major cause of vitamin B_{12} losses in the final product (Figs.

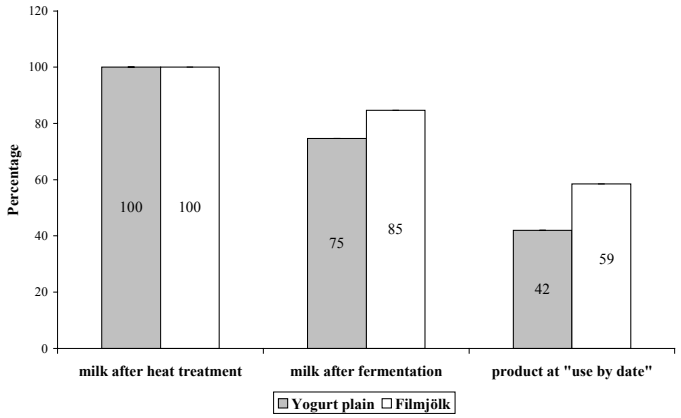

Figure 9. Retention of vitamin B_{12} during manufacture of fermented milks.
Retention is calculated and expressed as percentage of vitamin B_{12} content in starting milk, which is set to 100% (first set of bars).

10-12). During cheese-making 16-72% of the vitamin B_{12} from milk was retained in the cheese curd, whereas the rest was found in the excluded whey. The concentrations of vitamin B_{12} found in studied hard cheeses are well in line with concentrations reported previously using MA (Renner, 1983, Scott & Bishop, 1988). Mass balances of vitamin B_{12} from the entire manufacturing procedure of studied cheeses (with the exception of Herrgård®) did not deviate substantially from 100%.

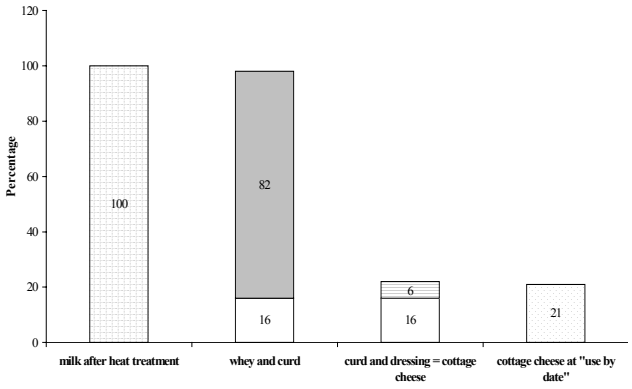

Figure 10. Retention of vitamin B_{12} during manufacture of cottage cheese.
Retention is calculated and expressed as percentage of vitamin B12 content in starting milk, which is set to 100% (first bar). The second bar represents results on distribution of vitamin B_{12} in whey and curd, after drainage following addition of starter cultures and rennet. After exclusion of the whey fraction, the third bar shows the retention of vitamin B_{12} in the rinsed curd together with the part of vitamin B_{12} originating from added dressing. The fourth bar shows the retention of vitamin B_{12} after storage of the cottage cheese until "use by date".

This may be interpreted as if negligible degradation of vitamin B_{12} occurred. Degradation, however, may not be completely excluded since losses might have been compensated for by a possible vitamin B_{12} synthesis by viable microorganisms in the starter cultures. (Paper I)

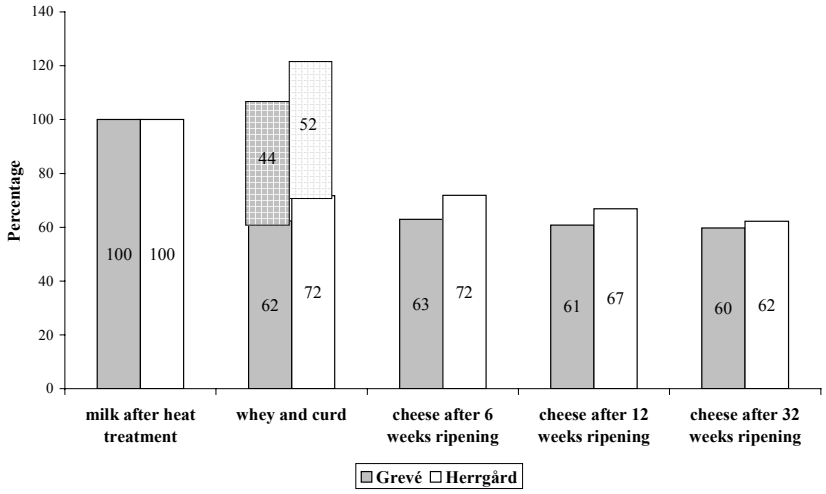

Figure 11. Retention of vitamin B_{12} during manufacture and ripening of two hard cheeses: Grevé® and Herrgård®.
Retention is calculated and expressed as percentage of vitamin B_{12} content in starting milk, which is set to 100% (first set of bars). The second set of bars show the retention of vitamin B_{12} in the whey and curd fractions, respectively. Only the curd fraction is used in the following steps, where the three following sets of bars reflect the vitamin B_{12} retention in the cheeses during ripening at 6, 12 and 32 weeks, respectively. Values are corrected for loss of water during the ripening period.

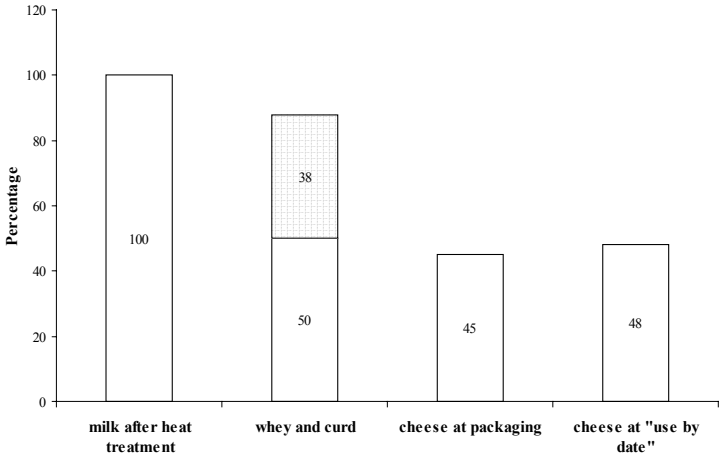

Figure 12. Retention of vitamin B_{12} during manufacture and ripening of blue mould cheese, Kvibille ädel.
Retention is calculated and expressed as percentage of vitamin B_{12} content in starting milk, which is set to 100% (first bar). The second set of bars show the retention of vitamin B_{12} in the whey and curd fraction, respectively. Only the curd fraction is used in the following steps, where the two next sets of bars reflect the vitamin B_{12} retention in cheese after packaging and at "use by date", respectively. Values are corrected for loss of water during storage.

Folate

Few investigators have reported results from characterisation of folate pattern in fermented dairy products (Müller, 1993, Vahteristo et al., 1997, Konings et al., 2001). They observed presence of H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and 10-HCO-H_4folate in yoghurt, fermented buttermilk and cheese. It must be stressed regarding these results that quantification of 5-HCO-H_4folate by HPLC is difficult due to a low sensitivity and problems with peak masking. Concentrations of 5-HCO-H_4folate are therefore easily overestimated.

Due to the possible content of 5-HCO-H_4folate combined with the varying selectivity of the kit folate-binder, we raised concerns about the reliability of the RPBA for quantification of natural folate concentrations in fermented dairy products. Folate concentrations in milk after heat treatment and in the six studied 'ready to consume' products were obtained by RPBA using the racemic commercially available (6R,S)-5-CH_3-H_4folate as calibrant, and by MA (Table 4). Results from analyses of the same set of samples with MA confirmed the RPBA results. However, even if the MA is the official method for folate analysis in food (AOAC, 2000a), it has been reported to respond differently to various folate forms (Goli & Vanderslice, 1989). Natural food folate is always present in the biologically active diastereoisomeric form, which in the case of 5-CH_3-H_4folate is the (6S)-form. The response of the kit folate-binder to the (6S)- and the racemic (6R,S)-form of 5-CH_3-H_4folate differs by approximately 30% under the prevailing assay conditions at pH 9.5 (Strålsjö et al., 2003). Therefore, results from folate

quantification with (6R,S)-5-CH_3-H_4folate as calibrant in the RPBA are underestimated by about 30%. With the above-mentioned limitations in mind, a tentative discussion on folate retention during processing follows below.

Most probably, in line with findings for vitamin B_{12}, much of the water-soluble folate is excluded with the whey fraction during manufacture of cheese. However, this can only be speculated upon since no whey fractions were analysed for folate in the present study. Folate retention calculations were based on the total amount of folate in milk and in 'ready to consume' products, respectively, multiplied by the total mass of the fraction of interest (specific data obtained during sampling from each manufacturing process).

The folate content was doubled in Filmjölk and an increase of 40% was seen in Yoghurt naturell, in comparison with milk (Table 4). These results are in line with findings from a study in which folate concentrations were determined in fermented milks and in pasteurised milk (Scott & Bishop, 1986, Scott & Bishop, 1988). In the present context the increase of folate content found in the fermented milks was attributed to the starter cultures involved. A recent study performed to examine the ability of different lactic acid bacteria strains to synthesise or utilise folate during fermentation of reconstituted milk showed a 3-4-fold increase of folate after fermentation with *L. thermophilus* species, whereas fermentation using *L. bulgaricus* species resulted in only mildly elevated or even decreased folate concentrations (Crittenden et al., 2003). Selected strains of *S. thermophilus*, *L. acidophilus*, *B. longum, and L. bulgaricus* were found by Lin & Young (2000c) to synthesise substantial amounts of folate during fermentation of reconstituted milk. Interestingly, in that study, a decrease of folate content was observed after storage of the fermented milks for up to three weeks at 4 °C, suggesting utilisation of folate by involved lactic acid bacteria.

During production of cottage cheese, rennet is added and the whey fraction is removed. The mass of the curd corresponded to 14% of the original milk amount.

Table 4. *Folate concentrations in milk and fermented dairy products, quantified by RPBA and MA*

Food	RPBA (µg/100g)	MA (µg/100g)
Milk	5 ± 0.1	5 ± 0.2
Fermented milks		
Filmjölk	9 ± 0.6	11 ± 1
Yoghurt	7 ± 0.4	10 ± 0.5
Cottage cheese		
Keso®	9 ± 0.3	na
Hard cheese		
Grevé®	16 ± 1	na
Herrgård®	18 ± 0.5	21 ± 0.6
Blue cheese		
Kvibille ädel	30 ± 2	na

Results as means ± range, n = 2. na: not analysed.

Mamma och pappa, tusen tack för allt stöd, all uppmuntran och för att ni alltid tror på mig. Åsa, Per och Johan, mina underbara syskon, tack för att ni finns, ni är en stor trygghet för mig.

Min egen lilla familj, vad vore jag utan er; Tobbe, mitt hjärta, tack för att du finns i mitt liv och tror på mig vad än jag ger mig in på, och lille Gustav, som genom sin existens gett mig helt nya perspektiv på livet.