Folates in Berries

Evaluation of an RPBA method to study the effects of cultivar, ripeness, storage and processing

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Abstract

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Folate concentrations in berries and berry products were investigated, determining folate retention from raw material to the final product. Folate content was quantified using a modified and systematically evaluated radioprotein-binding assay (RPBA).
The optimised RPBA was demonstrated to be a useful method for folate quantification in berries containing mainly 5-CH_3-H_4folate. Results after internal evaluation regarding selectivity, background radiation, unspecific binding, and accuracy are presented. To avoid unspecific binding purification of sample extracts by solid phase extraction prior to quantification was included for some berries. Microbiological assay (MA) and high performance liquid chromatography (HPLC) were used to compare obtained folate concentrations and to characterise individual folate forms in tested matrices. The predominating folate form in all tested berries was 5-CH_3-H_4folate. Therefore, the recently commercially available natural form (6S)-5-CH_3-H_4folate was chosen as calibrant.
Berries rich in folate were rosehips (100-180 µg/100g fresh weight) and strawberries (70-90 µg/100g fresh weight). These two model berries were used to study factors affecting the folate content in berries. Significant effects on folate content were observed from cultivar, grade of ripeness and year of harvest.
Folate retention in the two model berries was studied during storage, drying procedures and in commercial products. After storage of fresh strawberries in the refrigerator high folate retention was obtained, whereas storage in room temperature and at exposure to light resulted in folate losses. In rosehips folate retention during various drying procedures was improved by minimising the drying time using high temperature and cutting of berries into slices. Water content affected the stability of both folates and ascorbic acid during drying. Almost no folate losses occurred when strawberries were cooked as jam or stewed as strawberry desserts from frozen berries, with a folate retention of 79-103%. Commercial products from both berries contained 10-20 µg of folate per 100 g FW.
Berries and semi-manufactures of berries are suitable ingredients in various food products to increase their nutritional value regarding folate intake. A newly developed human ileostomy model suitable for evaluating folate bioavailability from these products is presented. Increased intake of berries and berry products could well be an alternative to folate supplements and fortification.

Keywords: folic acid, 5-methyl-tetrahydrofolate, radioprotein-binding assay, folate retention, strawberries, rosehips, folate bioavailability, method evaluation

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Swedish Summary
Svensk populärvetenskaplig sammanfattning

Svenska kvinnor i barnafödande ålder behöver fördubbla sitt dagliga intag av folat för att uppnå rekommenderade nivåer. Avhandlingen är ett led i arbetet att uppdatera metoder och kunskaper om kostens folatkällor. Den innehåller viktig information vilken kan användas för att på naturlig väg öka intaget av folat som ett alternativ till berikning. Två av våra kommersiellt viktiga bär, jordgubbar och nypon, visades innehålla betydande mängder av folat och bör därför rekommenderas för ökad konsumtion och vid framställning av nya livsmedelsprodukter.

Folat är ett B-vitamin i fokus då skyddseffekter mot missbildningar, hjärtkärlsjukdomar och cancer har rapporterats. Frukt, bär och grönsaker är goda folatkällor, speciellt gröna bladgrönsaker som t.ex. spenat. Folaterna, som förekommer i många olika former, upptäcktes på 1930-talet och fick sitt namn folat från latinets *folia*, vilket betyder blad. Den syntetiska och mer stabila formen av vitaminet kallas folsyra. De nordiska näringsrekommendationerna för det dagliga folatintaget höjdes 1996 från 200µg till 300µg per dag och en kostundersökning från 1998 ”Riksmaten” visade att svenskarnas folatintag ligger betydligt lägre än önskat. Från myndighetshåll diskuterar man därför en allmän folatberikning av vetemjöl och rågsikt.

Vitaminets viktigaste uppgift i kroppen är att leverera byggstenar till arvsmassan och det är därför nödvändigt vid all typ av celldelning, extra viktigt vid snabb tillväxt såsom fosterutveckling. Folat har uppmärksammats p.g.a. dess skyddande effekt mot fosterskador på centrala nervsystemet (ryggmärgsbråck) och mot spontana aborter. Vidare har studier visat att folat kan sänka halten av homocystein, en oberoende riskfaktor för hjärtkärlsjukdom liksom att det kan ge skydd mot vissa cancerformer och demens, t.ex. Alzheimer.

I en blandad kost kommer mer än två tredjedelar av folaterna från vegetabilier. Kosttabellernas värden utgör riktvärden där man av förklarliga skäl inte tar hänsyn till sortvariation, odlings-, skörde-, och klimatfaktorer eller förluster p.g.a. lagring och distribution. Många livsmedelsprodukter genomgår flera processteg, varvade med lagring och transport vid olika temperaturer innan de slutligen når konsumenten. Det är därför svårt att ta fram tillförlitliga värden för folathalter i den mat vi äter. Vitaminet är dessutom mycket känsliga för nedbrytning (oxidation), särskilt i närvaro av ljus och värme. Folaters vattenlöslighet leder till hög risk för urlakning med kok- och processvatten men även mekanisk bearbetning som t.ex. borttagande av skaldelar kan reducera innehållet i livsmedlet. Bland cerealieprodukter hittas exempelvis betydligt högre folathalter i fullkornsmjöl jämfört med i siktat mjöl.

För att förhindra förluster rekommenderas att skydda livsmedel mot ljus, syre och höga temperaturer. Enkla tips för hemmet är att använda lock vid kokning av grönsaker och att minimera mängden kokvatten. Det förekommer också ämnen i livsmedel, så kallade antioxidanter som skyddar folater mot nedbrytning.

Askorbinsyra (Vitamin C) är exempel på en antioxidant som finns i stora mängder i frukt och grönt.

Syftet med det här projektet har varit att studera folater i bär och se hur folathalterna påverkas under processning och lagring. En stor del av arbetet har utgjorts av optimering och validering av metoder för folatanalys i bär (**Artikel I & II**). En optimerad protein-inbindningsmetod (RPBA) har använts dels för att analysera olika sorter av vanliga svenska bär (**Artikel II**) och dels för att studera effekter av sort, mognad och odlingsbetingelser på folatinnehåll i jordgubbar och nypon (**Artikel III & IV**). Folatförluster vid lagring och kommersiell framställning av jordgubbsprodukter har också undersökts (**Artikel III**), liksom förluster vid torkning av nypon (**Artikel IV**). Slutligen har en humanmodell för att mäta upptaget av folater från livsmedel tagits fram och utvärderats (**Artikel V**). Genom att använda ileostomister, personer med bortopererad tjocktarm som försökspersoner, förhindras störande effekter från tjocktarmens mikroflora.

Bär har visat sig vara en god källa för folat och extra folatrika är jordgubbar och nypon med folathalter upp till 150µg/100g. Hallon och havtorn är medelgoda källor kring 30-50µg/100g medan blåbär, körsbär, vinbär och aronia är moderata källor med folathalter från 20µg/100g ner till 10µg/100g. Regelbundna studier av folat i jordgubbar och nypon har påvisat signifikanta variationer mellan sort, mognadsgrad och odlingsår.

Folatinnehållet har visat sig vara stabilt i färska jordgubbar under kyllagring och vid kommersiell processning till sylt och kräm medan det snabbare bryts ner vid rumstempererad lagring. I nypon har innehållet av folat och askorbinsyra följts under torkningsprocessen och visat sig vara beroende av vattenaktiviteten. Nypon bör därför torkas vid hög temperatur (>85°C) och skivas före torkningen för att sänka vattenhalten och därmed vattenaktiviteten så fort som möjligt.

För att kunna utvärdera kostens folatkällor för människans behov, krävs inte bara kunskap om folatinnehållet i den mat vi äter. Lika viktigt är hur väl vår kropp kan tillgodogöra sig folaterna som finns i olika livsmedel. Den presenterade humanmodellen utgör ett instrument för att jämföra upptaget av folat från olika livsmedel och farmaceutiska preparat.

CONTENTS

Appendix

The present thesis is a synthesis of the following papers, referred to by their Roman numerals.

Papers I-V

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

II. Strålsjö, L., Åhlin, H., Witthöft, C.M., and Jastrebova, J. Folate determination in berries by radioprotein-binding assay (RPBA) and high performance liquid chromatography (HPLC). *Accepted for publication in European Food Research and Technology*

III. Strålsjö, L., Witthöft, C.M., Sjöholm, I., and Jägerstad, M. Folate content in strawberries (*Fragaria x ananassa*) - effects of cultivar, year of harvest, ripeness, storage and commercial processing (2003). *Journal of Agricultural and Food Chemistry 51(1) 128-133*

IV. Strålsjö, L., Alklint, C., Olsson, M.E., and Sjöholm, I. Folate content and retention in rosehips (*Rosa* ssp) after drying. *Manuscript to be submitted*

V. Witthöft, C.M., Strålsjö, L., Berglund, G., and Lundin, E. A human model to determine folate bioavailability from food - a pilot study for evaluation. *Accepted for publication in Scandinavian Journal of Nutrition vol 1/2003*

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List of abbreviations

ε	Extinction coefficient
5-CH_3-H_4folate	5-methyl-tetrahydrofolic acid (6S- or 6R, S-)
5-CHO-H_4folate	5-formyl-tetrahydrofolic acid (6S- or 6R, S-)
AA	Ascorbic acid
Abs	Absorbance
AUC	Area under the curve (plasma response)
Ches	2-(N-cyclohexylamino)-ethanesulfonic acid
CP	Chicken pancreas suspension
CPM	Counts per minute
CRM	Certified reference material
cv.	Cultivar
CV%	Coefficient of variation
DM	Dry matter
ED-50%	A 50% reduction of maximum binding in the RPBA
FBP	Folate binding protein
FW	Fresh weight
H_2folate	Dihydrofolates
H_4folate	Tetrahydrofolate (6S- or 6R, S-)
Hepes	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
HK	Hog kidney suspension
HSA	Human serum albumin
HPLC	High performance liquid chromatography
k_{el}	elimination rate constant
k_{abs}	absorption rate constant
LOD	Limit of detection
MA	Microbiological assay
MCE	2-Mercaptoethanol
MW	Molecular weight
MO	Micro-organisms
p-ABG	Para-aminobenzoyl-L-glutamic acid
Pt-6-COOH	Pterin-6-carboxylic acid
PteGlu	Pteroyl -L-mono-glutamic acid
$PteGlu_2$	Pteroyl -L-di-glutamic acid
$PteGlu_3$	Pteroyl -L-tri-glutamic acid
R	Coefficient of correlation
RPBA	Radio protein binding assay
SAX	Strong anion exchange
SD	Standard deviation
SPE	Solid phase extraction
V	Volume of distribution

Introduction

Folates are a group of essential dietary compounds referring to all derivatives of tetrahydrofolic acid, a water-soluble B vitamin required for cellular replication and growth. Several health benefits related to folate have been recognised and include prevention of neural tube defects in babies, cardiovascular diseases caused by elevated plasma homocysteine and certain forms of cancer (Selhub & Rosenberg, 1996). Furthermore, a good folate status may positively affect cognitive functions (Seshadri, *et al.*, 2002) and spontaneous abortions (George, *et al.*, 2002). During developmental stages such as pregnancy, lactation and infancy, the requirement of folate is higher. The average daily intake of folate in many Western populations is often far below the recommendations (300-400 µg/day) and there is a need for a critical evaluation of all kinds of dietary folate sources (de Bree *et al.*, 1997).

Fruits and berries are rich to moderate sources of folates and contribute around 15% of the daily folate intake of the Swedish population (Becker, 2000). However, there is a lack of reliable data on folate levels in berries and a need for a critical update of the food tables. No data on the variation in folate content among berries due to different sources, growing conditions and post-harvest handling are available. Recently berries have received increased attention due to their high content of various other nutrients, especially compounds with antioxidant capacity (Häkkinen, 2000), and because losses due to processing are of a minor risk compared to vegetables, since most berries are consumed fresh or only slightly processed.

Traditionally, folates in foods are analysed by microbiological assays (MA), which quantify the total folate concentration in the food. Folates exist in foods in several forms with different substituents and varying numbers of glutamate residues, and recently an increasing number of high performance liquid chromatography (HPLC) techniques, which enable determination of various folate compounds in food, have been established (Eitenmiller & Landen, 1999; Vahteristo & Finglas, 2000). But many HPLC methods have had a limited application due to lack of selectivity and sensitivity and there is still a need for sufficient purification methods prior to quantification (Vahteristo & Finglas, 2000). Both MA and HPLC are time-consuming methods that demand high analytical skills and hence are quite expensive. Therefore, a rapid method for folate determination, which could handle many samples in one assay, for example when, studying variations in folate content in certain crops, fruits or vegetables, would be an interesting and desirable complement.

This thesis is divided into three parts. First the literature on folates is briefly reviewed, giving a background to the vitamin with emphasis on berries. In the second part our own research contributions regarding folates in berries are presented, focusing on two model crops: strawberries and rosehips. A pertinent part of the work was the evaluation and optimisation of an RPBA method for folate analysis in berries. The optimised RPBA method was used to study folate content and folate retention in berries and berry products. A human ileostomy model to determine the bioavailability of folate in food was also evaluated. The third and last part consists of the five papers, which together form the basis of the thesis.

Background – Folate review

Chemistry and stability of folates

According to recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature 1986, 'folate' should be used as the generic term for the class of compounds having similar chemical and nutritional properties as pteroyl-L-glutamic acid (folic acid, PteGlu) (Blakley, 1988). Folates are a group of essential dietary compounds consisting of a pteridine ring linked to a para-aminobenzoic acid conjugated with one or more, usually five to eight, L-glutamic acid residues (**Figure 1**). While the pteridine ring of PteGlu exists in oxidised form, native folates have either two or four additional hydrogens in their pteridine ring, forming dihydrofolates (H_2folate) or tetrahydrofolates (H_4folate) (Blakley, 1988).

Five different one-carbon units can be linked at N^5- and/or N^{10}-position of the pteroyl group: methyl (5-CH_3), formyl (5- or 10-HCO), formimino (5-CHNH), methylene (5,10-CH_2) and methenyl (5,10-CH). Taken together, the theoretical number of all native folate vitamers reaches several hundreds (Eitenmiller & Landen, 1999). To exhibit vitamin activity of the H_4folate molecule, the glutamic acid must be in the L-isomeric form and C^6 must be S-isomer (**Figure 1**, asterisk). Traditionally, commercial standards have been mixtures of C^6 as R- and S-isomers but recently, standards of pure S-isomers have been commercially available on the market. PteGlu does not occur naturally in biological systems but is produced synthetically for use in pharmaceutical and fortified food products (Eitenmiller & Landen, 1999).

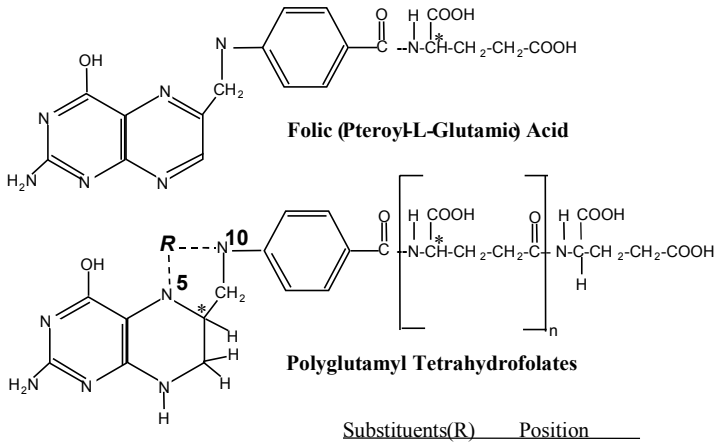


Figure 1. The folate molecules

The chemistry of folates makes the vitamin one of the most vulnerable to losses during food processing. All folates are sensitive to oxidative degradation enhanced by oxygen, light and heat resulting in a splitting of the molecule into biologically inactive forms, of which p-aminobenzoylglutamate (p-ABG) is one of the major. There are considerable differences in stability between the various folate forms with PteGlu exhibiting substantially greater stability than the reduced folate forms (H_2folate and H_4folate). The stability is dependent on the chemical composition of the pteridine ring system, with no influence from the number of glutamic acid residues. The order of stability of the H_4folates is 5-HCO-H_4folate > 5-CH_3-H_4folate > 10-HCO-H_4folate > H_4folate (Gregory, 1996). If present in adequate amounts, antioxidants, e.g. ascorbic acid and thiols, stabilise folates (Gregory, 1989; Hawkes & Villota, 1989). The rate of reaction for folate breakdown in the presence of oxygen depends on the type of folate derivative and the nature of the food matrix, in particular in respect to pH, catalytic trace elements and antioxidants (Gregory, 1989; Hawkes & Villota, 1989; Eitenmiller & Landen, 1999).

Folate Nutrition

Functions

Folate is an essential vitamin necessary for the biosynthesis of purine and pyrimidine (DNA and RNA) and with vitamin B_{12} as the cofactor for the regeneration of homocysteine into methionine. During stages of rapid cell generation and growth, like pregnancy and infancy, the folate requirement is higher. The reduced folate compounds are used as cofactors and serve as acceptors and donors of one-carbon units in a variety of biosynthetic reactions. These one-carbon unit transfers occur in two important cycles in both mammalian and plants cells namely the nucleotide biosynthesis cycle (DNA-cycle) and the methylation cycle (**Figure 2**). The reactions are mediated by attachment of one-carbon units at the N^5- and/or the N^{10}-position of pteroic acid facilitated by specific enzyme systems and co-enzymes such as $FADH_2$ and NADPH. (reviewed by Selhub & Rosenberg, 1996)

Folate deficiency can lead to impaired cell replication and accumulation of harmful metabolites, such as homocysteine. Mildly elevated plasma homocysteine is suggested to be a marker for a defect in folate metabolism and more severe elevation is considered an independent risk factor for cardiovascular disease (Graham *et al.*, 1997; Rimm, *et al.*, 1998; Brouwer, *et al.*, 1999). Impaired cell replication is observable first in fast replicating tissues such as blood, causing anaemia. Furthermore, a good folate status has an important role in the prevention of neural tube defects (Berry *et al.*, 1999; Honein, *et al.*, 2001; Richter, *et al.*, 2001) and may lower the risk for spontaneous abortion (George *et al.*, 2002). There are also indications for prevention of colon cancer (Giovannucci, *et al.*, 1995) and positive effects for cognitive functions such as Alzheimer's disease (Seshadri *et al.*, 2002).

Recently, genetic variability in several genes encoding for the enzymes in various folate-dependent reaction (**Figure 2**) have been identified and considered risk factors for a variety of clinical conditions (reviewed by Lucock, 2000). A

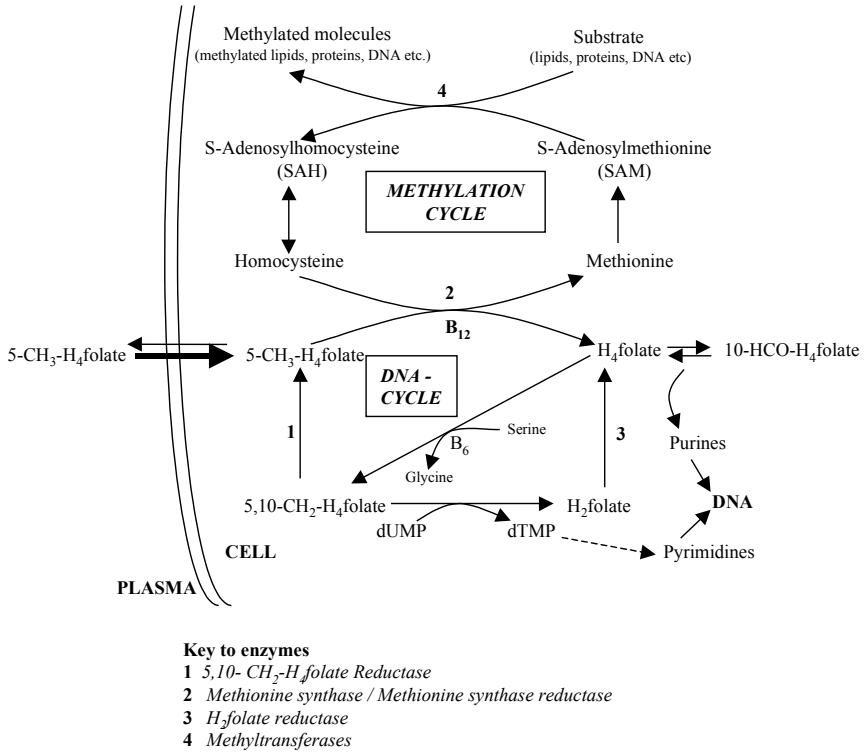


Figure 2. Folate functions in mammalian cells – the methylation and the DNA cycle

common polymorphism (677 C→T) in the gene coding for *5,10-CH_2-H_4folate reductase* affects approximately 10% of the population and is associated with elevated plasma homocysteine. Other examples are variants of the genes coding for *methionine synthase* and *methionine synthase reductase*. The development in this area is presently increasing at a rapid rate and folates are to be expected to play an important role in the concept of 'nutrigenomics'.

Absorption and bioavailability

The term 'folate bioavailability' is defined nowadays as the absorption and metabolic utilisation of folates, involving processes of intestinal absorption, transport, metabolism and excretion (Gregory, 1995). In humans, folate absorption takes place mainly in the brush border membrane of the jejunal mucosa. As described earlier, the major part of folate in the food exist as polyglutamates, which need to be enzymatically hydrolysed with pteroylpolyglutamate hydrolase (conjugase) before they can be absorbed by the complex saturable and pH dependent process (optimum at pH 6.3). Passive diffusion has also been observed at higher folate concentrations (>10μM) (Selhub, Powell & Rosenberg, 1984).

Certain factors in food, such as dietary fibre and organic acids, may inhibit the deconjugation of polyglutamates, consequently also affecting the absorption negatively (Bailey, Barton, Hiller & Cerda, 1988; Wei, Bailey, Toth & Gregory, 1996; Wei & Gregory, 1998). Prior to entering the portal circulation, the folate monoglutamates in the mucosa cells are reduced and further methylated to yield 5-CH_3-H_4folate to be transported through cell membranes (**Figure 2**). From the circulating 5-CH_3-H_4folate in blood, the liver retains one part during the first pass and the rest is distributed to other tissues. After transport into the cells the conversion of 5-CH_3-H_4folate into H_4folate by vitamin B_{12} and methionine synthase is an essential reaction. H_4folate is the preferred substrate of the synthase, which conjugates glutamate moieties to generate polyglutamate-folates, which are used for all cellular reactions. The half-life of the major body folate pool is usually estimated as 100 days (reviewed by Gregory, 1989).

The bioavailability of naturally occurring folates in the diet is variable, and an average of around 50% has been suggested (Gregory, 1997; Ball, 1998). Folate bioavailability can be influenced by several factors, intrinsic factors directly linked to the organism such as sex, age, folate status, health and gastro-intestinal functions or extrinsic factors depending on the folate form ingested or the matrix of the food. Of major importance is the form of dietary folate, type of food and composition, processing and storage of foods, aspects of physiology and effects of drugs (Gregory, 1997). A food constituent of particular interest is the FBP from milk. Folate bound to FBP is mainly absorbed in the ileum and FBP has been suggested to prevent the uptake of folate by the microflora and to promote the transport of folate through the mucosa (Selhub, Arnold, Smith & Picciano, 1984). Definitive measurements of the ability of FBP to stabilise 5-CH_3-H_4folate and H_4folate against degradation have recently been reported (Jones & Nixon, 2002). The genetic variability from a number of polymorphisms of folate-dependent enzymes may also affect the bioavailability.

Studies of folate bioavailability have been reviewed by several authors and were initially performed by bioassays using various animals to measure growth response of folate concentrations in several tissues such as liver, kidneys, serum and whole blood. However, the appropriateness of bioassays in predicting folate metabolism in humans is questionable due to physiological differences in species. The use of *in vitro* methods can to some extent reflect the complexity of *in vivo* folate absorption, deconjugation and metabolism but does not provide useful information regarding human folate bioavailability. Today, short-term- and long-term protocols or stable-isotopes techniques in humans are used to study folate bioavailability, all with their own advantages and drawbacks. Taken together, folate bioavailability is a complex area of study and although many advances in the understanding of the processes have occurred in recent years it is still difficult to be able to estimate the folate bioavailability for a given food or complete diet. (reviewed by Gregory, 1995 and Ball, 1998)

Dietary sources and human intake

In the latest edition of the Nordic Nutritional Recommendations (1996), the recommended daily intake for adults was increased from 200 µg to 300 µg folate and for pregnant women even up to 400 µg (Sandström, 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 per day from a previous 200 µg per day (Yates, Schlicker & Suitor, 1998). Moreover, in the US women who plan a pregnancy are advised to consume an additional 400 µg synthetic folic acid from fortified foods or supplements, in total 800 µg per day (Yates *et al.*, 1998).

In a review by de Bree *et al.* (1997) a European North-South gradient in dietary intake was observed with the highest intake in the Mediterranean area. Lowest folate intake was reported in the UK, Sweden and Ireland where a substantial part of the population does not meet an intake of 200 µg per day. In Sweden according to a study performed on the Swedish population 1997-98, the mean intake of folate per day is only 217 µg/day for women and 232 µg/day for men (Becker, 1999). However, due to difficulties in estimating the folate intake, with unreliable folate values in food tables and measurements of food intake, it is important to be cautious in comparing data from different studies. Miscalculations of the intake are possible as most of the food folate data derive from microbiological analysis with insufficient methodological control, and there is still today little reliable information about the folate forms and concentrations present in food and the impact of food processing techniques on folate retention.

Folate is present in a wide range of foods. A brief look into various food tables (reviewed by Witthöft, Forssén, Johannesson & Jägerstad, 1999) shows that many (leafy) vegetables, legumes, yeast and liver are particularly rich sources with folate concentrations up to 600 µg/100g. However, these foods are not necessarily the

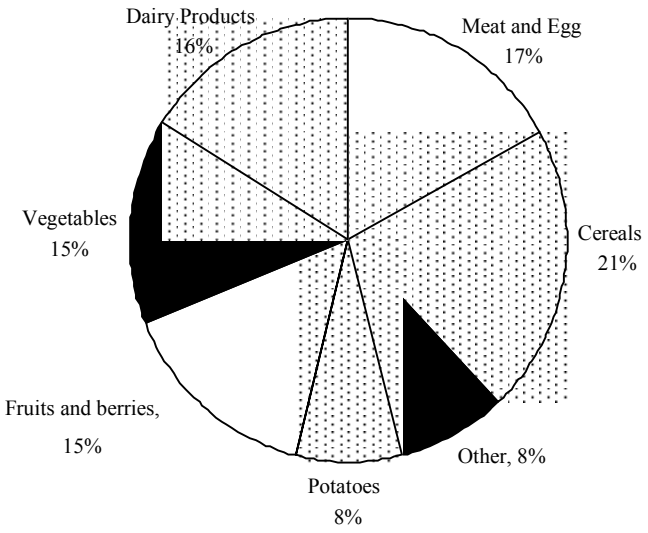


Figure 3. Folate contribution (%) in Swedish diets "Riksmaten 1997-98" (Becker, 2000).

most important in regards to consumption and in the review by de Bree *et al.* (1997) it was stated that the predominant contributors to folate intake in Europe are foods of plant origin (vegetables, fruits, berries and potatoes). Even in northern European countries, where the plant food consumption is lower than in a Mediterranean diet, these foods are estimated to contribute approximately 40% of the total folate intake of adults. Bread and cereals are also main sources, especially in countries where fortification is common. Today there are almost no folic acid fortified products in Sweden but the food and health agencies are discussing whether a fortification should be introduced in wheat flour and sifted rye flour. In the US, in 1998, the Food and Drug Administration decided on a mandatory fortification of 140 µg folic acid per 100 g of grain in all cereal grain products (Mills & England, 2001). In **Figure 3**, the food folate contribution (%) in Swedish diets according to the Swedish survey 'Riksmaten 1997-98' is presented (Becker, 2000).

Folate in fruits and berries

The Swedish survey 'Riksmaten 1997-98', showed fruits and berries combined to be one of the predominant folate contributors to the Swedish population, providing around 15% of the daily folate intake (**Figure 3**). In **Table 1**, folate concentrations for several common fruits and berries from various European food tables have been compiled, showing rich sources to be found among citrus, tropical fruits, and red berries. Contradictory values can be explained by the variation in folate content among individual sources of raw material, varieties, factors of cultivation, harvest, climate or losses due to storage and distribution as well as by methodological differences regarding folate quantification and sample pre-treatment. For some fruits and berries, however, the given folate data in various food tables has been borrowed from other countries (personal communication with the Swedish National Food Administration). This emphasises the need for a critical update of the food table data using carefully controlled methods for sample preparation and folate quantification.

Folate content in berries

Berries have recently been shown to be a good source of various nutrients, especially compounds with antioxidant capacity such as ascorbic acid, phenolics, carotenoids, tocopherols and flavonoids (Häkkinen, Heinonen, *et al.*, 1999a; Häkkinen, Kärenlampi, *et al.*, 1999b). The influences of cultivars, growing conditions, ripening and post-harvest handling on the levels of nutrients, particularly antioxidants is of increasing interest and several studies have been published (Hägg, Ylikoski & Kumpulainen, 1995; Nunes, Brecht, Morails & Sargent, 1998; Kalt, Forney, Martin & Prior, 1999; Häkkinen, Kärenlampi, Mykkänen & Törrönen, 2000; Wang & Lin, 2000). Nevertheless very little is known regarding folate content.

Strawberries (*Fragaria x ananassa*) are of particular interest due to their high content of antioxidants and as an important berry both for fresh consumption and for the food industry (Duxbury, 1992). Strawberries are cultivated in all arable regions of the globe from the Arctic to the tropics with a world production of a good 2.5 million metric tons in 1997 (Hancock, 1999), which in all likelihood is even an underestimated figure, as huge amounts of strawberries are also produced

Table 1. *Total folate content in fruits and berries compiled from four different European food tables.*

Fruits & Berries	**Folate concentration in µg/100g**			
	Sweden[a]	United Kingdom[b]	Germany[c]	Denmark[d]
Apple	2	1	12	3
Apricot	9	5	4	9
Avocado	62	8	30	91
Banana	19	14	17*	28
Bilberry	6	6	6	6
Blackberry	34	34	34	nr
Black currant	23	+	16*	8
Cherry	3	5	75*	nr
Cherry (sweet)	6	5	52*	8
Cloudberry	30	nr	nr	nr
Cranberry	2	2	nr	2
Date	13	13	21	21
Elderberry	17	17	17	12
Fig (dried)	8	9	14	9
Gooseberry	12	8	19*	12
Grape	4	2	43*	5
Grape (dried)	3	10	4	4
Grapefruit	10	26	11	39
Kiwifruit	42	+	nr	42
Lemon	11	+	6	32
Lime	8	8	nr	8
Lingonberry	2	6	3	2
Mango	36	+	36	71
Melon, water	2	2	5	3
Melon, netted	17	nr	nr	30
Melon, honeydew	30	2	30	15
Nectarine	4	+	nr	4
Orange	30	31	42*	46
Papaya	10	1	nr	58
Peach	4	3	3	4
Pear	4	2	14	5
Pineapple	11	5	4	12
Plum	1	3	2	3
Raspberry	26	33	30*	44
Red currant	12	+	11	26
Rose hip (dried)	2	nr	nr	210
Sea buckthorn	nr	nr	10	10
Strawberry	99	20	65*	63

[a] Sweden (Livsmedelsverket, 2002), [b]United Kingdom (Holland, Unwin & Buss, 1991), [c]Germany (Souci, Fachmann & Kraut, 1994), [d] Denmark (Levnedsmiddelstyrelsen, 1996),
* = analysed by HPLC, + = folate present in the food sample, but not reliable value available, nr = no value reported

on the private market. The ever-increasing productivity of the strawberry industry has made frozen strawberries of the same quality readily available all year around. Berries are quickly chilled within 24h after harvesting for shipping as either fresh or frozen directly to the consumer markets or foodservice operations (Duxbury, 1992). In Sweden, the demand of strawberries for fresh consumption and industrial applications is much larger than the domestic cultivation. Much emphasis in strawberries is placed on quality, which is affected by cultivar, climate, plant nutrition, cultural systems, harvesting and handling systems. However, parameters for good quality are often drawn up with a view to suitability and adaptability to growing conditions, disease resistance, harvesting methods, and market types and yields, not to nutritional quality (Hancock, 1999). There are today many published studies showing that strawberries contain a high content of ascorbic acid, anthocyanins and phenolic compounds (Hägg *et al.*, 1995; Kalt *et al.*, 1999; Häkkinen & Törrönen, 2000) and possess a high antioxidative capacity (Wang & Lin, 2000), as well as a few that discuss folate content (Müller, 1993; Vahteristo, Lehikoinen, Ollilainen & Varo, 1997; Konings, *et al.*, 2001). The European food tables present a range from 20 µg/100g to 99 µg/100g for reported folate content in strawberries (**Table 1**).

Rosehips (*Rosa* ssp) are traditionally used in Sweden for sweet dessert soups. Health authorities conscientiously promoted the use of wild rosehips among the Swedish population before and during the Second World War, an effort to increase the consumption of foods containing ascorbic acid (Alklint, Viberg, Sjöholm & Wienna, 1997). In rosehips, the level of ascorbic acid can be as high as 15-20 mg /g fresh fruit (1.5-2%) (Ziegler, Meier & Sticher, 1986). Recent studies have also shown rosehips to be rich in other antioxidants such as carotenoids and phenolics (Razungles, Osamianski & Sapis, 1989; Mikanagi, Yokoi, Ueda & Saito, 1995; Hodisan, Socaciu, Ropan & Neamtu, 1997) and also that they possess antioxidative capacity (Gao, Björk, Trajkovski & Uggla, 2000) and antimutagenic effect (Karakaya & Kavas, 1999). Industrial rosehip products were successfully supplied to the Swedish households, effectively replacing home-made products. Today most of the rosehips used in the industry are imported dried rosehips from Chile (Alklint *et al.*, 1997). As shown in **Table 1**, not much is known about the folate content in rosehips. Only two food tables reported folate values for dried rosehips, 2 and 210 µg/100g, respectively.

Folate retention after post-harvest handling and processing of berries

Folate's sensitivity to oxidative degradation enhanced by oxygen, light and heat, splitting the molecule into biologically inactive breakdown products, makes them vulnerable to losses during food handling. The very limited information available in this area has been comprehensively reviewed by Hawkes & Villota (1989) and carefully supplemented with more recently published papers by Scott, Rébeille & Fletcher (2000). In these reviews it is suggested that large processing losses in vegetables and legumes occur simply through the leaching of folates into the surrounding water used for washing, blanching, canning or cooking, and not only by oxidation. After various procedures of thermal processing of vegetables such as blanching (De Souza & Eitenmiller, 1986) steaming (Petersen, 1993) and quick soaking and cooking (Hoppner & Lampi, 1993), it was shown that both leakage and oxidative degradation can cause folate losses up to 70-80%.

Practically no information at all is available on processing and storage stability of folates in fruits and berries. But, since these products are often consumed fresh or slightly processed, folate losses due to leaching or any adverse effects from heat and oxidation should be of minor risk. Furthermore, for berry products such as jam, marmalade, and desserts and canned products usually the whole product is consumed including the accompanying medium. Nonetheless, reported folate data for various common berry products are very low (< 5 µg/100g FW) according to the Swedish food table (Livsmedelsverket, 2002). No study has been found focussing on folate retention in processed berries but the folate content in strawberries was shown to be unaffected after six months of domestic frozen storage (Vahteristo, Lehikoinen, Ollilainen, Koivistoinen & Varo, 1998).

Berries are of great importance as raw material for the fruit processing industry, which still follows the same kind of procedures as traditional domestic production in regard to both products and techniques, in contrast to other food industries, for example the dairy industry, where technical development has raised the quality of industrial products so that they are now superior to home-made alternatives.

Most berries are non-climacteric fruit with high respiration rates, which are harvested when the ripening process is at optimum eating quality. As a group they are probably the most perishable of all fruits, with some only having a few days of shelf life even under optimal conditions (Wills, McGlasson, Graham & Joyce, 1998). Bulk-harvested strawberries have a shelf life of six to eight days if they are forced-air cooled and stored at 2 °C (Deuel, 1996). The soft texture of berries makes them highly susceptible to physical damage, easily leading to general senescence and rotting. Berry processing starts immediately when the fruit is harvested and the quality benefits from rapid cooling (Morris & Sistrunk, 1991). Recommended storage temperature for most berries is –1 °C to 0 °C at 90-95% relative humidity (Wills *et al.*, 1998). The shelf life is dependent on temperature and the degree of infection from fungi, as well as to other factors such as weather conditions, field conditions, humidity, and packing container (Deuel, 1996). Most berries are harvested manually as their soft texture is easily damaged by mechanical treatment and as all berries in the field do not ripen at the same time. However, in areas where there is a shortage of harvest labour, mechanical harvesting of strawberries may be a necessity for the survival of the strawberry industry (Morris & Sistrunk, 1991). The type of handling between harvest and freezing has a great influence on the quality of the berries.

Freezing is probably the best way to preserve berries. Colour and aroma are generally well maintained, while the texture tends to become softer after thawing. Unfortunately, thawing makes the berries loose their shape and volume due to cell wall collapse resulting in juice loss to the surrounding medium. Viberg (1998) showed that the use of a liquid thawing medium, in the form of various sugar solutions is superior to more common thawing performed in air medium.

Folate determination in foods

The most commonly used techniques for food folate determination include microbiological, HPLC and protein-binding methods, all with several modifications. Today the only officially recognised method for folate analysis is the microbiological assay (MA) using *L. rhamnosus* ATCC 7469 as the test organism (AOAC, 2000). This method is only applicable to quantify the 'free form of folic acid', which restricts its application to fortified products only. Today, no method has official status as a reference method for natural folate in food, and most folate values published in food tables were established by the MA. The protein-binding assays (RPBA) are based on binding of the folate molecule to a folate binding protein (FBP) and are today routinely used for clinical diagnostics (plasma, serum or whole blood) (van den Berg, Finglas & Bates, 1994; Raiten & Fisher, 1995). HPLC methods allow a more detailed characterisation of the individual folate forms and both the intact folate polymers as well as the various monomer forms in samples after conjugase treatment can be quantified (reviewed by Vahteristo & Finglas, 2000). Sample pre-treatment is of critical importance to all methods of quantification and needs to be carefully validated prior to folate quantification in foods. In food, folates are present in low concentrations and in different chemical forms, both as monoglutamates and as polyglutamates. Considering folate's susceptibility to oxidation by heat, pH and oxygen, food folate analysis becomes a real challenge and the need for validated methods becomes obvious.

Sample pre-treatment

The preparation of food samples prior to folate quantification varies in respect to purpose, food and method of quantification. Sample preparation aims to extract native food folates from the matrix while avoiding losses by oxidation and interconversion. Therefore, the choice of extraction method, time, buffer pH, antioxidants and conjugases are factors that can have an impact on the results, as reviewed by Eitenmiller & Landen (1999).

Folates are usually extracted from the food matrix using heat treatment diluted in buffer with added antioxidants. Folate stabilisation prior and during the analysis is essential and the most of the buffered solutions have a pH relatively close to neutral. Optimisation of stabilisation procedures is difficult, as individual folate forms possess different pH-optima for maximum stability. Using antioxidants throughout the whole sample preparation, folates are protected from inter-conversion and oxidative degradation. Ascorbic acid is the most commonly used antioxidant, and is often recommended in combinations with thiols, such as 2-mercaptoethanol (Wilson & Horne, 1984; Gregory, Engelhardt, Bhandari, Sartain & Gustafson, 1990; Pfeiffer, Rogers & Gregory, 1997b; Vahteristo, 1998). Exclusion of oxygen by overlay with nitrogen (Gounelle, Ladjimi & Prognon, 1989; Viberg, Jägerstad, Öste & Sjöholm, 1997) as well as the use of low temperatures and shelter from light should be applied prior to homogenising the samples. Homogenisation, together with heat treatment, disrupts the cellular structures and the folates are released into the buffer by leakage. Lately, the use of proteolytic and amylolytic enzymes during extraction, especially for cereal based foods, has brought about an increase in the total folate concentrations, and

procedures for this so called trienzyme extraction are becoming more and more established (De Souza & Eitenmiller, 1990; Martin, Landen & Soliman, 1990; Pfeiffer *et al.*, 1997b; DeVries, Keagy, Hudson & Rader, 2001; Tamura, Mizuno, Johnston & Jacob, 1997). (reviewed by Tamura, 1998)

The majority of food folates are present as polyglutamates and most methods for quantification require a deconjugation into mono- or diglutamates (Gregory, 1997; Ball, 1998). As these enzymes are not commercially available, they have to be prepared by the investigator. Common sources of conjugases (pteroyl-polyglutamate hydrolase, EC 3.4.12.10) are chicken pancreas (CP), hog kidneys (HK), human and rat plasma (HP, RP) but also endogenous enzymes from the plant food material have been used (Leichter, Landymore & Krumdieck, 1979). Procedures for folate deconjugation have to be optimised in respect to time, temperature, pH, folate stabilisation and substrate-enzyme ratio depending on the characteristics of the sample matrix and enzyme source. The type of end product varies depending on the enzyme source and to some extent also on conditions used (Engelhardt & Gregory, 1990; Goli & Vanderslice, 1992). Principally CP produces a mixture of mono- and diglutamates, while HK and plasma sources produce exclusively monoglutamates (Lakshmaiah & Ramasastri, 1975; Gregory, 1984; Wilson & Horne, 1984; Day & Gregory, 1985). The choice of enzyme treatment depends on the requirements of the analytical method but also on the food sample. It is important to carefully evaluate the use of different enzyme treatments for every new food matrix, including consideration of the trienzyme treatment.

Purification of food extracts is often necessary, especially when using HPLC determination. Several solid phase extraction procedures with commercial disposable cartridges were reported (Vahteristo, Ollilainen, Koivistoinen & Varo, 1996; Wigertz & Jägerstad, 1995; Witthöft, 1998), and the use of affinity chromatography with FBP attached to agarose as established by Selhub, Ahmad & Rosenberg, (1980) is also common (Seyoum & Selhub, 1993; Pfeiffer *et al.*, 1997b; Konings, 1999; Kariluoto, Vahteristo & Piironen, 2001).

Quantification

Microbiological assay

Traditionally, MA is the most commonly used method and is based on the nutritionally folate requirements of the micro-organism *Lactobacillus rhamnosus*, (ATCC 7469). In accurate pH the organism responds equally to most native folates after deconjugation to mono-, di- or triglutamates (Tamura, Shin, Williams & Stokstad, 1972), an assumption subjected to discussion (Eitenmiller & Landen, 1999). Differences in growth response to the common folate forms found in foods have been reported (Goli & Vanderslice, 1989) and since standard curves for the assay are established usually using PteGlu unequal response can significantly affect accuracy (Phillips & Wright, 1982). Microbiological methods are considered to be extremely laborious and time-consuming. Despite the development of microtiter plate format assays and procedures using cryoprotected *L. rhamnosus*-strains as reviewed by Tamura (1990), the MA still requires extensive analytical experience to be suitable for routine folate analysis. In a BCR collaborative study Finglas, van den Berg & de Froidmont-Görtz, (1996) reported

several pitfalls, such as the type of organism, assay pH and quantification of total folate which can interfere with accurate measurements.

However, MA is still considered to be one of the best and most versatile methods and recently, a joint collaborative study with 13 participating laboratories was carried out quantifying total folate by MA (*L. rhamnosus*) with trienzyme extraction in a wide range of cereal products. It has been recommended that this method be given Official First Action status with AOAC and First Approval status with AACC (DeVries *et al.*, 2001).

Protein binding assays

Both competitive and non-competitive ligand-binding assays are used for folate determination. The principle of the competitive-binding assay is based on competition between the folates in the sample or standard, and a known amount of labelled folate, for the limited binding sites on a folate binding-protein (FBP). In non-competitive assays the folates in the sample are incubated with FBP and the remaining sites on the protein are quantified by titration with labelled folates (Martin, 1995). The folates in the assays are labelled with radioisotopes (radio protein-binding assay, RPBA) or enzymes (enzyme protein-binding assay, EPBA). A number of factors are known to affect the performance of the assay. In particular the affinity of the FBP can be influenced by temperature, time, and incubation medium and dramatically by pH (Givas & Gutcho, 1975; Gregory, Day & Ristow, 1982; De Souza & Eitenmiller, 1990). Further problems are caused by varying affinity to different folate forms and the number of glutamate residues (Shane, Tamura & Stokstad, 1980). In particular, the affinity to formyl forms of H_4folate is very low (Shane *et al.*, 1980; Wigertz & Jägerstad, 1995; Arcot, Shrestha & Gusanov, 2002).

Today, competitive RPBA methods are routinely used for folate analysis of clinical samples (van den Berg *et al.*, 1994; Raiten & Fisher, 1995). The most common standard is PteGlu because of its increased stability compared to native folates. At pH 9.3 the affinity of the FBP is shown to be equal for PteGlu and 5-CH_3-H_4folate (Givas & Gutcho, 1975), the dominant native folate form in plasma. However, in a study by van den Berg *et al.* (1994) several commercial RPBA kits for folate quantification in clinical samples were compared and the need for further standardisation and optimisation of the RPBA assays and extraction procedures was underlined. Calibration of the kits is crucial (Raiten & Fisher, 1995) and more attention needs to be given to interference from food matrix and buffers. Theobald, Batchelder & Sturgeon (1981) suggested that matrix effects might change the background radiation and unspecific binding in the assay, resulting in false results. Sample preparation in connection with application of RPBA for food folate quantification and the need of deconjugation are still being discussed (Finglas, Faure & Southgate, 1993; Shane *et al.*, 1980). Shane *et al.* (1980) observed variable folate response depending on the length of the glutamate chain, prompting them to consider the RPBA as unreliable for the direct determination of polyglutamates.

Not long ago, a new biospecific technique in the form of an immuno-binding assay performed in an optical biosensor system was presented (Boström Caselunghe & Lindeberg, 2000; Indyk, *et al.*, 2000). The method utilises the

phenomenon of surface plasmon resonance (SPR) for detection and ready-to-use-kits are now commercially available from Biacore AB (Uppsala, Sweden). The assay is based on monoclonal antibodies against PteGlu and was introduced on the market as an easy and simple method for the determination of PteGlu in fortified food (Boström *et al*., 2000).

High performance liquid chromatography

High performance liquid chromatography (HPLC) methods allow quantification of individual folate forms and also offer the opportunity to detect the length of the polyglutamate chain. The water-soluble nature of the folates, together with differences in ionic properties and hydrophobicity, makes them well suited for ion exchange- or reversed-phase HPLC and many separation systems have been developed (reviewed by Ball 1998; Eitenmiller & Landen, 1999 and Vahteristo & Finglas, 2000). The pH, ionic strength, and polarity of solvents are used to optimise separation. Usually low pH with or without gradient of the organic solvent is used with C_{18} or phenyl columns (Vahteristo & Finglas, 2000). Determination of food folates can be performed on enzyme-treated extracts in the form of folate monoglutamates or as intact polyglutamate folates. Most HPLC quantification procedures require sample purification prior to quantification e.g. solid phase extraction (SPE) reversed phase chromatography or affinity chromatography. Lack of commercially available calibrants, especially of reduced folate polyglutamates, hampers identification and can result in an underestimation of the folate content, expressed as the sum of individual folates.

Objectives

The folate intake in many Western populations today is far below the daily recommendations. Therefore, the food industry, the health and food authorities and the general population alike all have a need for and interest in a critical evaluation of dietary folate sources. According to the Swedish study, 'Riksmaten 1997-98', almost all types of foods contribute to the daily intake of folates. Fruits and berries, with their 15% contribution, constitute one of the main sources. However, unspecific quantification methods with insufficient quality control are used to establish folate concentrations reported in food tables. Presently, no knowledge is available surrounding the effects of folate content in fruits and berries due to cultivar, growing conditions and post-harvest handling. Even the folates are vulnerable to losses during food handling, only a few studies on folate retention after storage and processing are to be found for other foods, while none focus on fruits and berries. An evaluation of dietary folate sources requires not only knowledge about the folate content. Equally important is to what extent folates in various foods are available for absorption in the body.

The main objectives of the present study were:

- To optimise and evaluate a radio protein-binding assay (RPBA) for total folate quantification in berries, including method comparison with HPLC and MA (**Paper I & II**).

- To obtain reliable data on total folate concentrations in berries and to characterise the individual folate forms present (**Paper II**).

- To study folate variation due to cultivar, ripeness and year of harvest in the model berries strawberries and rosehips (**Paper III & IV**).

- To study folate retention after post-harvest handling and processing in the model berries strawberries and rosehips (**Paper III & IV**).

- To evaluate a human ileostomy model for the determination of folate bioavailability from foods (**Paper V**).

Materials and methods

Study design and sampling

Evaluation of the modified RPBA for food folate analysis

A commercial RPBA kit (SimulTRAC-SNB Radioassay kit. Vitamin B12 [^{57}Co] / Folate [^{125}I] (ICN Pharmaceuticals Inc. Costa Mesa, CA) was modified using external calibration (5-CH_3-H_4folate), to quantify total folate in berries (**Paper I & II**). The method has been evaluated and optimised for reliable folate quantification in some foods (e.g. berries, dairy products). The binding affinity of the FBP in the modified RPBA kit was controlled for six folate derivatives (**Paper I**) and the different diastereomers (6S)- and (6R,S)-5-CH_3-H_4folate (**Paper II**). When the biologically active (6S)-5-CH_3-H_4folate became commercial available as a standard, the method was improved further (**Paper II**).

Method evaluation included tests on linearity, recovery, precision (intra- and inter-assay) and selectivity. Special emphasis was given to control background radiation and unspecific binding caused by chosen buffers or tested sample matrices. For each new food matrix, the additional need of sample purification, for example solid phase extraction (SPE), to avoid unspecific binding has to be tested (**Paper II**). Certified reference material from the European Commission was used for validation purposes (Finglas, Scott, Witthöft, van den Berg & de Froidmont-Görtz, 1999). The modified RPBA was compared with other methods such as HPLC (Jastrebova, Witthöft, Grahn, Svensson & Jägerstad, 2003; Vahteristo *et al.*, 1996) and MA using *L. rhamnosus* (AOAC, 2000) for quantifying the folate content in selected berries (**Paper II**). Several methods for sample pre-treatment, including deconjugation with CP or HK conjugases, prior to RPBA quantification were also tested (**Paper I**).

Folate content in berries

A study quantifying the folate content in nine different Swedish berries (bilberry, black currant, blueberry, chokeberry, raspberry, rosehip, sea buckthorn, strawberries, and sweet cherry) was performed (**Paper II**). All berries were harvested and collected from SLU Balsgård, Kristianstad, in 2001. Strawberries and rosehips, two interesting berries for the Swedish food industry with a high content of folates, were used as model foods for further studies on folate variation in the raw material of berries.

To check the variation in folate content between different cultivars, folate was analysed in eight different strawberry cultivars, cv. Lina, Honeoye, Melody, Mimek, Rhapsody, Sara, 916401(white) and Vit Åke Troedsson (white), all collected from SLU Balsgård as above (**Paper II**).

In **Paper III** the folate content in strawberries was studied further, investigating effects of harvest year, cultivar and ripeness during a period of three harvest seasons (1999 - 2001). All strawberries, cv. Honeoye, Senga Sengana, BFr 77 111 Balsgård, Eros, Polka, Bounty, Kent, Elvira, Emily, Dania and Elsanta were

collected and cultivated from O. Torstensson farm (Bromölla, Sweden). An already frozen strawberry cultivar (Senga Sengana) was imported from Poland.

In both studies (**Paper II & III**), all samples were frozen immediately after harvest on trays (-20 °C) and thereafter stored subsampled (200 g) in vacuum in plastic bags at -20 °C until analysis. In order to be able to compare results from the different studies all figures given in the thesis have been re-calculated using (6S)-5-CH_3-H_4folate standard calibration after the final improvement of the RPBA according to **Paper II**.

The folate content in two different cultivars of rosehips (*R. Dumalis* and *R. Rubiginosa*) also obtained from O. Torstensson farm (Bromölla, Sweden) was studied in **Paper IV.** All samples were freeze-dried prior to analysis.

Folate retention during post-harvest handling and processing

Studies of folate retention during post-harvest handling and processing were carried out on the two model berries, strawberries and rosehips (**Paper III & IV**).

Folate retention during storage of fresh strawberries was investigated (**Paper III**). Two strawberry cultivars, cv. Honeoye (Grödby, Bromölla), cultivated for industrial processing and cv. Zefyr (Fredrikslund, Uppsala), cultivated for fresh consumption were used. The freshly harvested strawberries were stored subsampled in cardboard boxes at different temperatures for between 0 and 9 days.

Folate retention during the drying of berries was investigated in rosehips (**Paper IV**). Whole and sliced rosehips (*R. Dumalis* and *R. Rubiginosa*) were dried in a laboratory-convective dryer at constant temperatures, 70 to 90 ºC. For the chosen optimal drying method folate retention and water activity was checked throughout the drying process, from 0 to 100 min. The content of ascorbic acid was also quantified as a parameter related to folate stability. Freeze-drying was used as reference drying method.

Folate content in 7 commercial berry products was checked by analysing Swedish common rosehip and strawberry products such as jam, stewed sauce desserts and syrup kindly donated by Procordia Foods, Eslöv Sweden (**Paper III & IV**). To be able to calculate folate retention, the folate content in corresponding raw material for each strawberry product was also investigated.

Evaluation of an ileostomy model to determine folate bioavailability

A human ileostomy model using short-term plasma kinetics to determine folate bioavailability from single test food doses versus pharmaceutical folate doses was developed and evaluated (**Paper V**). Using healthy ileostomist volunteers, who lack colon with associated microflora, allowed direct quantification of non-absorbed folate in faeces without effect of micro-organisms. Relative folate absorption was estimated using the area under the curve of plasma folate after an oral portion of test food as compared to a pharmaceutical folate dose. In addition, urinary folate excretion was evaluated as a possible parameter for folate absorption. Collection of ileostomal effluent in individual fractions every 2 hours

post-dose reflects not only non-absorbed folate, but also a time pattern of the intestinal passage.

After an overnight fast, volunteers underwent trials during four independent study days (each at least two weeks apart) in random order, receiving either a single oral portion of each test food (broccoli and strawberry), an oral dose of a pharmaceutical preparation of (6S)-5-CH_3-H_4folate or an i.m. injection of the pharmaceutical preparation. For one volunteer a fifth day was included without any folate application, to receive baseline data.

Folate determination

Table 2. *Overview of methods and analysed samples used in the thesis*

Paper	Sample	Sample preparation	Quantification
Paper I	Strawberries Rosehips Milk CRM 421[8] CRM 485[8]	CP & HK deconjugation SPE purification[1]	Total folate, RPBA[5] Total folate, MA[6] Folate characterisation, HPLC[2]
Paper II	Swedish berries Strawberries	HK deconjugation SPE purification[2]	Total folate, RPBA[5] Folate characterisation, HPLC[2]
Paper III	Strawberries Strawberry products[9]	CP deconjugation	Total folate, RPBA[5]
Paper IV	Rosehips Rosehip products[9]	CP deconjugation	Total folate, RPBA[5] Ascorbic acid, HPLC[7]
Paper V	Plasma Stomal effluent Urine Strawberry Broccoli	HK deconjugation SPE purification[2] FBP affinity chromatography[3,4]	Folate characterisation, HPLC[2]

[1] Vahteristo *et al.* (1996), with modifications
[2] Jastrebova *et al.* (2003)
[3] Konings (1999)
[4] Kariluoto *et al.* (2001)
[5] ICN (1998), with modifications
[6] AOAC, (2000)
[7] Wimalasiri & Wills (1983) and Estevé *et al.* (1997)
[8] EU certified reference materials, (Finglas *et al.*, 1999)
[9] Commercial berry products kindly provided by Procordia Food AB (Eslöv, Sweden)

Standards

All standard substances (H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and $PteGlu_{1-3}$) were purchased from Dr Schircks Laboratories (Jona, Switzerland) or kindly provided by MERCK Eprova AG (Schaffhausen, Switzerland). In **Papers I & III,** mixtures of C^6-diastereomers (R and S) of each standard were used but in **Papers II, IV & V** only S-isomers. The purity of all standard substances was checked as described by van den Berg et al. (van den Berg *et al.*, 1994) using molar extinction coefficients reported by Blakley (1969) (**Papers I & III**) or Eitenmiller & Landen (1999) (**Papers II, IV and V**). Standard stock solutions for calibration purpose (~200 µg/ml, purity corrected) were stored at –80 °C in phosphate buffer pH 6.1 containing 1% AA and 0.1% MCE.

Sample pre-treatment

A flow chart describing sample preparation, including extraction and enzyme treatment procedures prior to folate quantification in different foods, is shown in **Figure 4**. Amounts of food sample, buffer and conjugases are given in the figure capture. The extraction buffer was phosphate buffer (0.1 M, pH 6.1), 1.0% (w/v) AA and 0.1% (v/v) MCE. To prevent folate oxidation, samples were protected by nitrogen and subdued light and cooled on ice throughout sample preparation. When calculating folate concentrations in food, corrections were made for the folate content in the conjugase suspensions added to the food sample during preparation.

Prior to quantification by HPLC (**Papers I, II, V**) or the modified RPBA (**Paper II)**, food extracts were purified with solid phase extraction (SPE) on strong-anion-exchange (SAX) (Isolute cartridges 500 mg, 3 ml, International Sorbent Technology, UK or LiChrolut 200 mg, MERCK, Germany) according to Jastrebova *et al.* (2003). Aliquots of extract were applied to the preconditioned cartridges and eluted with 0.1M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) AA and 0.1% (v/v) MCE. In **Paper V** affinity chromatography was used for purification of urine samples. Columns containing 0.5 mg FBP (Scripps, USA) per 2 ml affi gel were prepared according to Konings (1999). The purification procedure was carried out with minor modifications according to Kariluoto (2001).

Quantification

In **Papers I-IV**, total folate content of food extracts was determined using the modified commercial RPBA kit, SimulTRAC-SNB Radioassay kit, Vitamin B_{12}[57Co] / Folate[^{125}I] from ICN Pharmaceuticals (Orangeburg, USA). Food extracts were quantified according to the kit description, with some modifications. External calibration with 5-CH_3-H_4folate in the concentration range of 0.5 to 10 ng/ml was used, instead of the kit calibrant (PteGlu) at concentrations from 1 to 20 ng/ml. The external standard was diluted in 0.1 M phosphate buffer pH 6.1 containing 1% (w/v) AA (**Paper I**). Moreover, 0.1M sodium acetate buffer containing 10% (w/v) sodium chloride and 1% (w/v) AA was used for dilution of external standard when quantifying samples after SPE purification (**Paper II**).

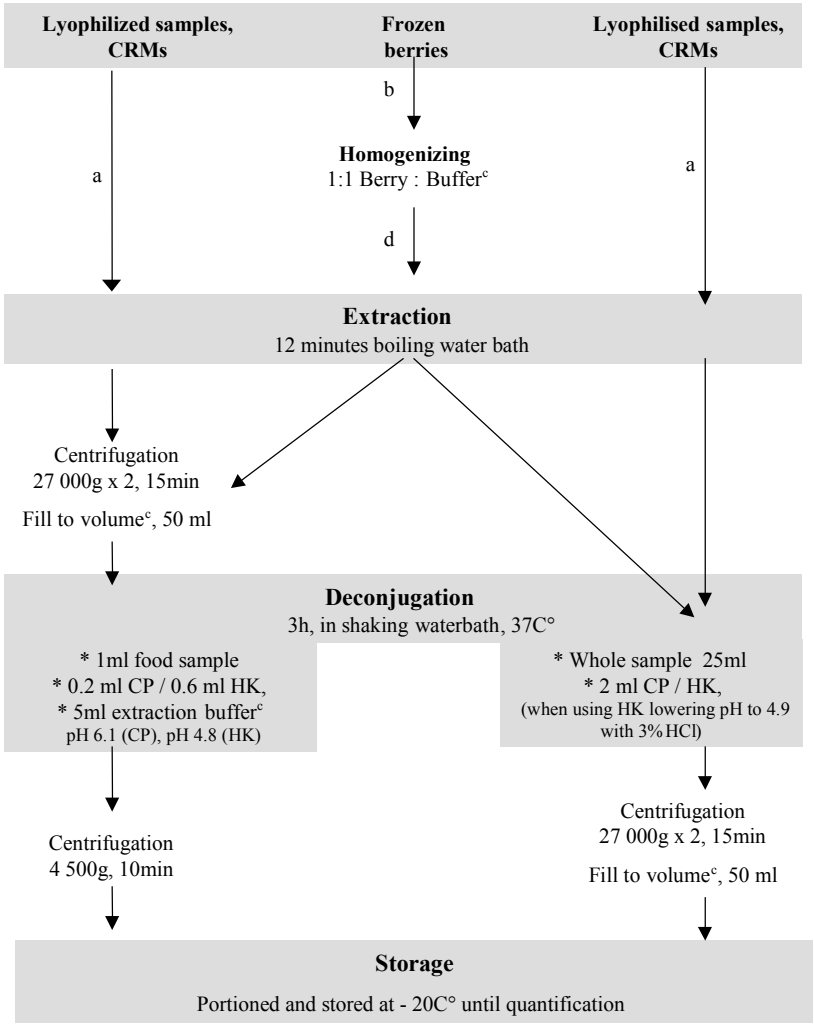


Figure 4. Flow chart describing standard procedures for extraction and deconjugation prior to folate quantification in berries. To prevent folate oxidation, samples were protected by nitrogen atmosphere and subdued light and cooled on ice throughout sample preparation. (a) 0.2-0.5 g of lyophilised samples and 20ml extraction buffer; (b) frozen berry samples were cut into pieces and homogenised in extraction buffer; (c) extraction buffer: phosphate buffer (0.1 M, pH 6.1), 1.0% (w/v) AA and 0.1% (v/v) MCE; (d) 4 g of berry homogenate and 16 ml extraction buffer.

Using RPBA for food folate quantification requires dilution of the standard in the same buffer as food samples.

In **Papers I, II & V**, quantification of individual folate forms, (6S)-H_4folate, (6S)-5-CH_3-H_4folate, (6S)-5-HCO-H_4folate, by HPLC was carried out according

to Jastrebova *et al.* (2003). The analytical column was a Zorbax SB C8, 150x4.6, 5 µm (Agilent Technologies, USA) and sample peaks were verified using retention time and relative peak area using both fluorescence and DAD detectors. Limits of quantification were 0.5 ng/ml for H_4folate, 0.3 ng/ml for 5-CH_3-H_4folate and 4 ng/ml for 5-HCO-H_4folate.

Statistical analysis

Average concentrations, standard deviations (SD) and coefficients of variation (CV %) were calculated for all samples, generally triplicates, using the computer program Excel 97 from Microsoft. Distribution ranges are given for duplicate samples. To check significant variations, one-way analysis of variance with general linear modelling and Tukey's pairwise comparison ($\alpha = 0.05$) using the software Minitab release 13 (Minitab Inc., State College, PA) was performed. Variations from $p < 0.05$ were considered significant.

Results and discussion

Evaluation of the modified RPBA for food folate analysis

In **Table 3**, parameters describing the RPBA assay performance using external calibration with (6S)-5-CH_3-H_4folate are presented. The adjustments of assay procedures using a different buffer and external calibration in a lower calibration range than the original kit did not affect the assay negatively. The internal evaluation proved the modified RPBA to be a useful method for folate

Table 3. *Quality parameters for folate quantification in berries using the modified RPBA*

Parameter			Performance
Assay performance			
ED-50%			7.2 nmol/l
LOD (ED-90%)			0.7 nmol/l
Slope			0.9
Linearity (1-20 nmol/l)			> 0.995
Internal evaluation			
Recovery[a]	-	(6S)-5CH_3-H_4folate	90-106%
	-	(6R,S)-5CH_3-H_4folate	94-113%
Selectivity[b]	-	different folate forms	no (see Figure 5)
	-	degradation products	yes
Matrix effects[c]	-	background radiation	94-107%
	-	unspecific binding to FBP	
		original kit buffer	15%
		folate free berry extracts	3-56%
Precision[d]	-	intra-assay	< 4%
	-	inter-assay	< 8%
Reference material[e]	-	CRM 485	258 ± 4 µg/100g
Method comparison[f]	-	HPLC	6.7 ± 4.8 µg/100g, y = 0.97x + 8.15, r = 0.980
	-	MA	1.1 ± 9.3 µg/100g y = 1.04x - 0.26, r = 0.899

[a] Recovery (%) = ($C_{spiked\ sample} - C_{sample}$)/$C_{spike}$ x 100 (calculated according to **Paper I**)
[b] Selectivity = Response of different folate forms (**Figure 5**) and degradation products (**Papers I & II**)
[c] Matrix effects = Disturbing effects of background radiation without addition of FBP (**Paper I)** and unspecific binding to FBP (**Paper II**)
[d] Precision = Coefficients of variation (CV%) for RPBA quantification including sample-pre-treatment expressed as intra- and inter-assay (**Paper I**)
[e] EU certified reference material CRM 485 (315 ± 28 µg/100g) quantified by MA (Finglas *et al.*, 1999) (**Paper II**)
[f] Method comparisons expressed as mean difference ± std dev and equation of correlation (**Figure 7**)

quantification in berries. In this section improvements during the optimisation will be discussed, regarding selectivity, background radiation, unspecific binding, accuracy and method comparisons.

Methods of in-house sample pre-treatment prior to folate quantification with CP or HK conjugase treatment (**Figure 4**) have proved to be suitable for berries. The optimal food-to-buffer ratio during extraction was found to be 0.2-0.5 g DM sample to approximately 20 ml buffer (**Paper I**). Other tested parameters included the use of different buffer systems: 0.1M phosphate pH 6.1 (Vahteristo *et al.*, 1996) or Hepes/Ches pH 7.85 (Wilson & Horne, 1984), length of heat treatment (5-15 min) and deconjugation (1-5h). No significant variations of folate content were observed for tested berries, e.g. raspberries, rosehips and strawberries. The crucial question of whether enzyme treatment of foods is required prior to RPBA quantification could not be answered definitively by our studies. However, as Shane *et al.* (1980) observed variable responses in the RPBA depending on the length of the glutamate chains, we recommend an enzyme treatment.

Selectivity

The modified RPBA was selective to only biologically active folate forms as no measurable response was found for the main folate degradation products Pt-6-COOH and p-ABG, also observed by Gregory *et al.* (1982). However, the binding affinity of FBP varied for individual folate forms (**Figure 5**). Compared to 5-CH_3-H_4folate the curves can be interpreted as a total overestimation for H_4folate, overestimation for PteGlu and underestimation for the kit PteGlu. 5-HCO-H_4folate

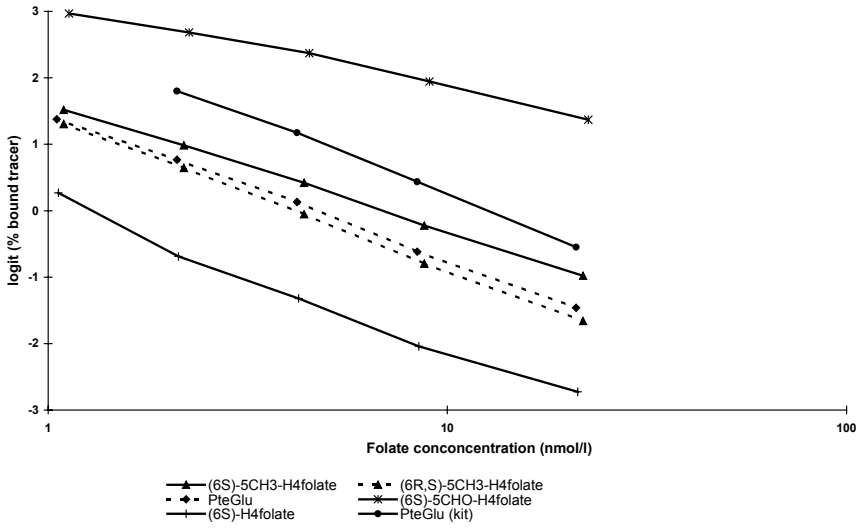


Figure 5. RPBA calibration curves with ED-50% in brackets of (6S)-5-CH_3-H_4folate (7.2 nmol/l), (6R,S)-5-CH_3-H_4folate (4.9 nmol/l), (6S)-H_4folate (1.3 nmol/l), (6S)-5-HCO-H_4folate (148 nmol/l), PteGlu (4.9 nmol/l) and PteGlu kit (12.1 nmol/l).

resulted in almost no response. Similar observations have been made by others (Shane *et al.*, 1980; Wigertz & Jägerstad, 1995; Arcot *et al.*, 2002). This demonstrates that the RPBA is only useful for matrices containing almost 100% of one folate form, which should be used as calibrant.

Furthermore a pronounced difference in the response of the two diastereomers (6R,S)- and (6S)-5-CH_3-H_4folate, was observed (**Figure 5**). The (6R,S)-5-CH_3-H_4folate along with PteGlu, had an approximately 30% stronger response in the RPBA assay compared to biological active (6S)-5-CH_3-H_4folate. This finding is exceedingly important when using commercial kits with PteGlu calibration for clinical analysis of natural folates. Earlier studies, reporting similar response for PteGlu and 5-CH_3-H_4folate at pH around 9.3 were performed with the diastereomer mixture (6R,S)-5-CH_3-H_4folate (Givas & Gutcho, 1975; Theobald, *et al.*, 1981; Wigertz & Jägerstad, 1995; Arcot *et al.*, 2002).

Background radiation and unspecific binding

Buffer concentration, reducing agents and absence or presence of tested food matrices did not affect the background radiation in the assay when no binding protein was added (**Paper I**). In contrast, the background radiation of the original kit buffer with human serum albumin (HSA), when tested with plasma samples, was 25% higher. Furthermore, unspecific binding of the folate binder was also 15% higher for the kit buffer with HSA compared to 0.1M phosphate buffer (**Paper I**). This is one explanation as to why the use of the original RPBA without adjustments may result in misleading values for folate concentrations in foods, underlining the need to use the same buffer for standard and sample dilutions. This important aspect has been overlooked in previous studies using commercial RPBA kits for quantification of food folates (Andersson & Öste, 1992; Finglas *et al.*, 1993; Wigertz & Jägerstad, 1995).

The absence of unspecific binding due to matrix effects of food samples in the modified RPBA was initially confirmed by dilution curves of tested foods (berries and milk) in the calibration range between 0.5 and 5 ng/ml 5-CH_3-H_4folate (**Paper I**). However, when the new calibrant (6S)-5-CH_3-H_4folate was introduced, extremely high folate values for some berry samples were found. A further investigation of matrix effects was performed using folate-free berry extracts obtained by removing the folates with the help of SAX (**Paper II**). For berries with a high folate content such as strawberries and rose hips, the response of the folate-free berry extracts was only 3-5% of the total response, whereas for berries containing low folate concentrations such as black currants and blueberries, it ranged up to 50% (**Paper II**, **Table 1**). HPLC chromatograms of berry extracts confirmed the presence of more endogenous compounds in non-purified extracts than in purified extracts (**Figure 6a-d**). As the impact of matrix effects varies, a test for unspecific binding is recommended for every new food matrix prior to RPBA quantification.

It is important when purifying samples by SPE with elution in 0.1M sodium acetate buffer (10% NaCl, 1% AA) (Jastrebova *et al.*, 2003) to also use the same buffer during external calibration of the RPBA assay (**Paper II**).

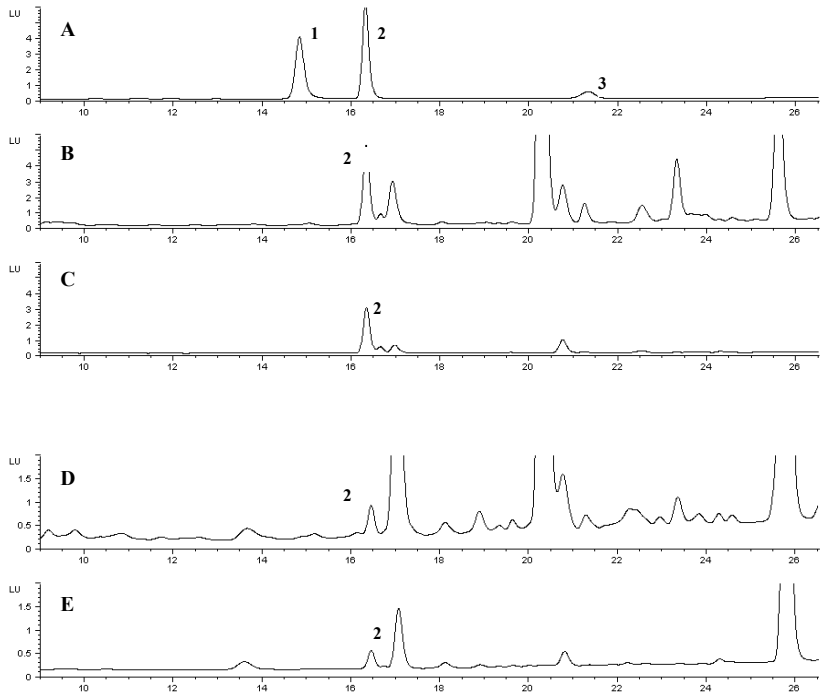


Figure 6a-d. Chromatograms of reduced folate monoglutamates detected by fluorescence (λex = 290nm, λem = 360nm). **Samples:** (**A**) standard mixture containing (6S)-H_4folate 40 ng/ml, (6S)-5-CH_3-H_4folate 35 ng/ml and (6S)-5-HCO-H_4folate 150 ng/ml in 0.1M sodium acetate buffer (10% NaCl, 1% AA); (**B**) strawberry extract (non-purified); (**C**) strawberry extract (purified); (**D**) black currant extract (non-purified); (**E**) black currant (purified). **Peaks:** (**1**) H_4folate, (**2**) 5-CH_3-H_4folate, (**3**) 5-HCO-H_4folate. **Column:** Zorbax SB C8 (150 x 4.6mm, 5µm). Mobile phase: acetonitrile-phosphate buffer (pH 2.3) with acetonitrile gradient form 6% to 25%. Flow: 0.4ml/min, injection volume 20µl.

Accuracy

The accuracy of the optimised RPBA was determined by analysis of lyophilised certified reference materials, e.g. CRM 421 (fortified milk powder) and CRM 485 (mixed vegetables). After initial modifications of the RPBA method, folate concentrations quantified in CRM 421 and CRM 485 after CP conjugase treatment were found to be 91 ± 5 µg/100g and 204 ± 15 µg/100g, respectively (**Paper I**). These results are lower than the reported values quantified by MA of 142 ± 14 µg/100g and 315 ± 28 µg/100g, respectively (Finglas *et al.*, 1999). However, after final optimisation using the (6S)-5-CH_3-H_4folate calibrant and SPE purification the obtained concentration for CRM 485 was 82% of the reported value (258 ± 4 µg/100g) (**Paper II**).

Method comparison

Folate concentrations obtained in selected berries by the optimised RPBA show high correlation for both HPLC and MA results (**Table 4** and **Figure 7**). For the comparison with HPLC eight different strawberry samples were also included (**Paper II**). The slope (0.97) can be interpreted as the existence of an error of systematic character and is constant for the whole investigated concentration range. This means that the bias expressed as a mean difference of 6.7 ± 4.8 µg/100g is almost negligible when analysing samples with high folate concentrations but appears to be a problem in low concentration ranges. Had the HPLC method been an official reference method, this consistent bias could be adjusted for by subtracting all values with the mean difference (Bland & Altman, 1986). When comparing with MA the mean difference was low (1.1 ± 9.3 µg/100g), calculated as presented in **Paper II**. However, this calculation was based on a small number of observations.

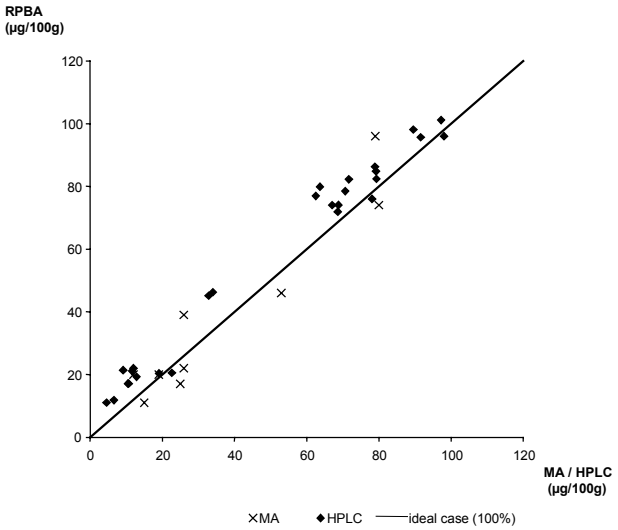


Figure 7. Comparison of folate concentrations in berries (µg/100g FW). Data obtained by HPLC and MA are plotted on the x-axis against the RPBA data on the y-axis. Samples quantified by RPBA and HPLC were pre-treated according to **Paper II** using HK deconjugase and SPE purification. Eight strawberry samples were also included. Correlation y = 0.97x + 8.15, r = 0.980, mean difference 6.7 ± 4.8 µg/100g. Samples quantified by MA were pre-treated by trienzyme extraction according to DeVries *et al.* (2001). Correlation MA and RPBA: y = 1.04x – 0.26, r = 0.899, mean difference 1.1 ± 9.3 µg/100g.

Table 4. *Folate concentrations in selected Swedish berries obtained by different quantification methods.*

Berries	**Scientific name, variety**	**Folate Concentration (µg/100g FW)**			
		RPBA[a]	**HPLC**[b]	**MA**[c]	**Food table**[d]
Rosehip	*Rosa Dumalis*, -	**96 (93.6 – 99.4)**	91 (89.6 – 91.6)	79 (74.9 – 82.7)	2
Strawberry	*Fragaria x ananassa*, Honeoye	**74 (72.3 – 75.8)**	67 (65.9 – 68.3)	80 (77.5 – 82.7)	99
Raspberry	*Rubus idaeus*, Diana	**46 (45.2 – 46.3)**	33 (32.8 – 34.0)	53 (52.7 – 53.3)	26
Sea buckthorn	*Hippophae rhamnoides*, Aromatnaja	**39 (37.0 – 41.9)**	50 (48.0 – 52.8)	26 (25.0 – 26.1)	10[e]
Cherry, sweet	*Prunus avium*, Kaiser Frans	**22 (21.4 – 22.0)**	11 (9.2 – 12.0)	26 (24.5 – 27.9)	6
Bilberry	*Vaccinium myrtillus*, Swedish wild	**20 (19.3 – 21.1)**	12 (11.6 – 12.9)	19 (17.8 – 19.7)	6
Black chokeberry	*Aronia melanocarpa*, -	**20 (20.4 – 20.6)**	21 (19.1 – 22.7)	12 (12.2 – 12.7)	nr
Black currant	*Ribes nigrum*, , 9508-3B	**17 (17.1 – 17.1)**	11 (10.5 – 10.7)	25 (23.6 – 25.5)	23
Blueberry	*Vaccinium corymbosum*, -	**11 (11.1 – 11.9)**	6 (4.5 – 6.6)	15 (13.0 – 16.4)	nr

All results are mean values of duplicates with single values in brackets (except food table data). Samples quantified by RPBA and HPLC were pre-treated according to **Paper II**. Samples quantified by MA were pre-treated with trienzyme treatment at the Swedish Food Administration according to DeVries et al (2002). nr = no reported value

[a] RPBA quantification after SPE purification according to **Paper II** expressed as total folate concentrations after calibration with (6S)-5-CH_3-H_4folate

[b] HPLC quantification according to Jastrebova et al (2003) expressed as (6S)-5-CH_3-H_4folate

[c] MA quantification according to AOAC expressed as total folate concentrations after calibration with PteGlu

[d] total folate concentrations according to the Swedish food table

[e] German food table according to Müller (1993)

Folate content in berries

Our studies (**Paper II**) have shown 5-CH_3-H_4folate to be the predominant folate form in all tested berries and some representative chromatograms were shown in **Figure 6**. Only traces of H_4folate could be detected in some berries, far below the detection limit (< 3 µg/100g). Vahteristo *et al.* (1997) reported the same observation determining folate concentrations in strawberries, bilberries and black currants whereas Konings *et al.* (2001) reported strawberries containing only around 75% of 5-CH_3-H_4folate. However, in the study by Konings and co-workers (2001) strawberry constituted the only berry samples analysed and the reported content of 5-HCO-H_4folate (14 ± 10 µg/100g) seems uncertain and may be overestimated.

The total folate content in nine selected Swedish berries harvested in 2001 indicated high variations in folate content from berry to berry (**Table 4**). Rich sources were the two model berries, rosehip and strawberry, with 96 µg/100g and 74 µg/100g, respectively. Moderate folate sources were raspberries (46 µg/100g) and sea buckthorn (39 µg/100g), whereas sweet cherries, bilberries, chokeberries, black currants and blueberries all contained about 20 µg/100g folate or less. Presented values are based on RPBA quantification but, as discussed above, results for both HPLC and MA were in the same range (**Table 4**).

Compared to data reported from European food tables (**Table 1**) our studies showed higher folate concentrations for most berries. However, agreement between the different food tables is low, which makes them unsuitable for confirmation of results. Furthermore, results reported in food tables are obtained from pooled samples in order to estimate a mean value for each food item. Also folate concentrations in Finnish berries reported from Vahteristo *et al.* (1997) were derived from analyses of composite samples of strawberries (36 µg/100g), bilberries (11 µg/100g) and black currants (8 µg/100g). In our study the folate content was controlled for each individual variety of berry. Moreover, berries were treated optimally in our study by immediate freezing after harvest, compared to the study of Vahteristo *et al.* (1997) where the berries were purchased in different public supermarkets.

Model berries – strawberries and rosehips

Our studies using the model berries strawberries and rosehips have shown that folate concentrations in berries are affected by such factors as cultivar, ripeness and year of harvest (**Papers II, III & IV**). In **Table 5**, folate concentrations (µg/100g DM) in four strawberry cultivars and two rosehip varieties were compared for harvests in 1999, 2000 and 2001. Using cv. Honeoye different grades of ripeness were also compared (**Papers III**).

The fact that folate concentrations in berries are significantly affected by cultivar and year of harvest is not surprising, as similar studies regarding other nutrients, such as ascorbic acid, anthocyanins and other phenolic compounds, have also reported significant variations (Häkkinen & Törrönen, 2000; Wang & Lin, 2000). In respect to rosehips, the variety *R. Dumalis* yielded higher folate concentrations than the *R. Rubiginosa* variety during all three years, and berries

from both varieties contained more folate in 2000 and 2001 compared to 1999. Pronounced variations in folate content have also been observed within different cultivars of raspberries, cherries, sea buckthorn and red and white currants grown at the same farm in 2001 (Åhlin, 2002). On the other hand, four cultivars of black currant showed no significant variation in folate content.

The mean folate concentration in all strawberry cultivars studied in this thesis was 75 ± 4 µg/100g FW (**Table 6**). Previously published studies report folate contents (FW) in strawberries from 36 µg/100g (Vahteristo *et al.*, 1997) to around 65 µg/100g (Müller, 1993; Konings *et al.*, 2001) and the four European food tables from 20 µg/100g to 99 µg/100g (**Table 1**). However, as water content has proved to be of significance, folate contents should be presented as dry matter (DM) when comparing berries for comparison purposes (**Paper III**). In **Paper II** the folate content was presented as µg/100g FW. After correction with a DM of 10 g/100g for frozen strawberries a rough estimation of the DM mean folate concentration for all strawberry samples could be made (787 ± 40 µg/100g DM) (**Table 6)**. Rosehip samples were freeze-dried prior to analyses and therefore directly compared based on DM (**Paper IV**). We have observed freeze-drying to be necessary prior to folate analysis in rosehips, to obtain results with a good repeatability and to get satisfactory recovery during extraction.

The results regarding effect of ripeness on the folate content in strawberries were not consistent (**Table 5**). Wang & Lin (2000) studied the content of anthocyanins and other phenolic compounds in strawberries at different grades of ripeness and showed that individual nutrients had different optimal states in relationship to ripeness in the berry. Unfortunately, Wang & Lin (2000) reported only results from one harvest year and did not confirm their results. Our study has

Table 5. *Folate concentrations in the two model berries harvested in 1999, 2000 and 2001*

Samples	**Ripeness**[a]	**Folate Concentrations**[bc] (µg/100g DM)		
		1999	*2000*	*2001*
Strawberries				
Honeoye	Unripe	845 ± 32	726 ± 6	933 ± 41
	Ripe	731 ± 32	771 ± 25	921 ± 51
	Fully ripe	772 ± 57	826 ± 13	990 ± 96
S. Sengana (Swe)	Ripe	699 ± 53	622 ± 12	
BFr 77111	Ripe	778 ± 61	702 ± 16	
Elsanta	Ripe	940 ± 82	663 ± 45	
Rosehips				
R. Dumalis	Ripe	542 ± 40	600 ± 64	599 ± 49
R. Rubiginosa	Ripe	427 ± 20	nd	531 ± 24

[a] Unripe (orange), Ripe (perfect ripe), Fully ripe (dark red)

[b] All results are means of triplicates ± standard deviation quantified by RPBA and recalculated using (6S)-5-CH_3-H_4folate calibration

[c] For significant differences of strawberry folate content, see **Paper III, Tables 2 & 3**
nd = not detected

shown that it is necessary to continue observations for subsequent years.

Table 6. *Total folate concentrations in 20 strawberry cultivars harvested in 1999, 2000 and 2001*

Cultivar	**Cultivated**	**DM (g/100g)**	**Folate content[a] (µg/100g)**	
			DM	*FW*
1999				
Elsanta	Grödby	10.7	940 ± 82	101 ± 9
S. Sengana (Pol)	Poland	8.5	931 ± 41	79 ± 3
Honeoye	Grödby	7.1	863 ± 58	61 ± 4
BFr 77111	Grödby	7.7	778 ± 61	60 ± 4
S. Sengana (Swe)	Grödby	7.7	699 ± 77	54 ± 4
Mean ± SD		**8.3 ± 1.4**	**842 ± 103**	**71 ± 19**
2000				
Honeoye	Grödby	10.0	771 ± 25	77 ± 3
Bounty	Grödby	9.1	767 ± 18	70 ± 2
Emily	Grödby	9.7	761 ± 9	74 ± 2
Eros	Grödby	9.2	731 ± 18	67 ± 2
Zefyr	Grödby	10.3	726 ± 19	74 ± 6
BFr 77111	Grödby	9.6	702 ± 16	67 ± 2
Dania	Grödby	10.7	667 ± 23	72 ± 3
Elsanta	Grödby	11.7	663 ± 45	77 ± 6
S. Sengana (Swe)	Grödby	7.0	622 ± 12	64 ± 3
Elvira	Grödby	11.4	619 ± 15	70 ± 2
Kent	Grödby	10.6	597 ± 23	63 ± 3
Polka	Grödby	11.8	515 ± 28	61 ± 3
S. Sengana (Pol)	Poland	10.7	489 ± 35	53 ± 4
Mean ± SD		**10.1 ± 1.3**	**664 ± 92**	**68 ± 7**
2001				
Melody	Balsgård	nd	990	99 ± 3
Rhapsody	Balsgård	nd	970	97 ± 5
Honeoye	Grödby	9.8	921 ± 35	91 ± 3
916401 (White)	Balsgård	nd	860	86 ± 6
Sara	Balsgård	nd	800	80 ± 2
Mimek	Balsgård	nd	790	79 ± 1
Vit (White)	Balsgård	nd	780	78 ± 4
Lina	Balsgård	nd	730	73 ± 2
Mean ± SD			**855 ± 96**	**85 ± 9**
Mean ± SD			**760 ± 132**	**74 ± 13**

[a] Results are means of triplicates (1999, 2000) and duplicates (2001) ± standard deviation quantified by RPBA and recalculated using (6S)-5-CH_3-H_4folate calibration, nd = not detected (DM of 10g/100g used for mean estimation)

Folate retention during post-harvest handling and processing

Our studies on folate retention during post-harvest handling and processing have shown the folate stability to be affected by storage as well as drying and commercial processing (**Papers III & IV**).

Storage of fresh berries

Folate retention (%) was studied (**Paper III**) in two different strawberry cultivars, one cultivated for fresh consumption (cv. Zefyr) and one for industrial processing (cv. Honeoye). To account for evaporation during storage the retention was calculated on folate content expressed as µg/100g DM (**Table 7**). Results indicated high folate stability in intact berries during storage at 4 °C but lower stability when strawberries were stored at room temperature. Berries are also rich sources of ascorbic acid (Häkkinen *et al.*, 1999b), an important antioxidant that can stabilise folates (Gregory, 1989). Studies monitoring ascorbic acid in fresh berries during storage show the ascorbic acid retention to be in the same range as folate retention (Hägg *et al.*, 1995; Nunes *et al.*, 1998; Kalt *et al.*, 1999; Häkkinen & Törrönen, 2000).

Table 7. *Folate retention (%) during storage of fresh strawberries, cv. Zefyr at 4 °C and 20 °C and cv. Honeoye at 4 °C.*

Cultivar	**Temperature (°C)**	**Folate retention during different days of storage[a] (%)**			
		0	*3*	*6*	*9*
Honeoye	4	100	99	nd	nd
Zephyre	4	100	84	73	71
Zephyre	20	100	62	nd	nd

[a] Folate retention (%) = Folate $_{(\text{storage day})}$ / Folate $_{(\text{fresh berries})}$ x 100 calculated from means of triplicates folate concentrations (µg/100g DM)
nd = not detected

It is important to present retention studies on a DM basis as apparent increase in nutrient content expressed as FW is often due to water loss rather than to an actual increase of the vitamin (Nunes *et al.*, 1998). Kalt *et al.* (1999) reported ascorbic acid losses in various berries after eight days of storage to be minimal but did not account for water loss in the samples. Nunes *et al.* (1998) showed a moderate ascorbic acid retention (25-90%) in strawberries depending on the type of packaging and storage temperature. Another study reported 10-20% lower levels of flavonols and ascorbic acid in strawberries and black currants stored for 24h at room temperature compared to those stored at 5°C (Häkkinen & Törrönen, 2000).

Drying

No significant variations in folate or ascorbic acid content were observed when drying sliced rosehips, var. *R. Dumalis* and var. *R. Rubiginosa*, at various temperatures (70-90 °C, **Paper IV**). Folate stability was shown to be dependent on drying time and water activity influenced by the cutting of rosehips into slices and to some extent the temperature (**Paper IV, Tables 2 & 3**). In both varieties higher folate retention was observed after cutting rosehips into slices to decrease the required drying time. The same observation has been reported earlier when studying ascorbic acid retention in rosehips during drying (Alklint, 1994).

To study the kinetics of folate degradation during drying, samples of sliced rosehips were analysed throughout the drying procedure (**Figure 8**). After just 25 min, folate retention decreased to 60%. At this state the water activity had dropped from around 0.95 to 0.75 (**Paper IV, Figure II**). Thereafter folate retention was relative stable, remaining at 54% after 100 min when the drying procedure was interrupted at a water activity of 0.37 and water content of 3%.

Commercial products

Folate content in several commercial Swedish berry products made from strawberries and rosehips was studied (**Table 8**). All products contained approximately 10-20 µg/100g FW folate, with the highest concentrations in strawberry jam. However, expressed as folate content per serving portion (approximately 250 g), both strawberry and rosehip desserts provide about 10% of the recommended daily intake (25-30 µg).

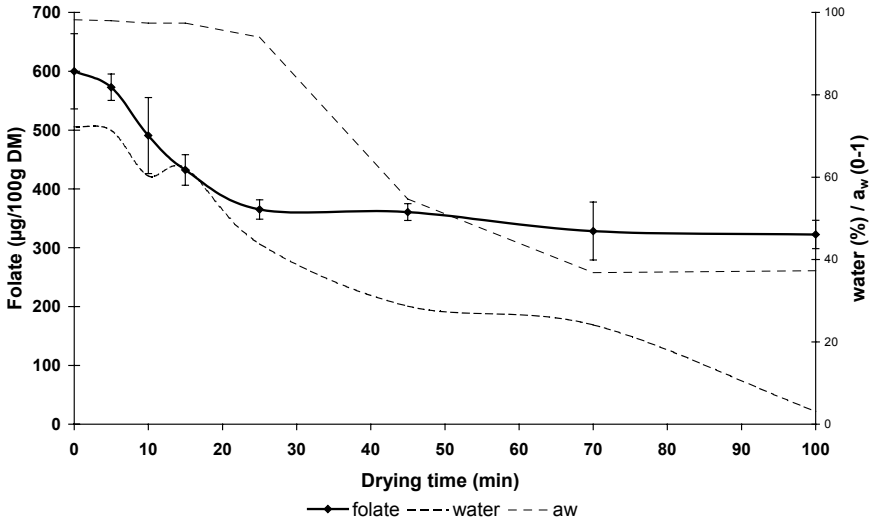


Figure 8. Folate concentrations, water content and water activity (a_w) in sliced rosehips *R. Dumalis* during air-drying (85 °C) for 100min.

Table 8. *Folate content in Swedish commercial rosehip and strawberry products*

Product[a]	**Product**[b]			**Folate**[c]
	Berry content (%)	Calories (kcal/100g)	Sugar (%)	(µ/100g FW)
Rosehip				
dessert soup	15	65	16	16 ± 2
dessert soup	9	50	13	11 ± 1
Strawberry				
Jam	52	180	46	22 ± 3
Jam	35	170	41	13 ± 1
stewed dessert	20	90	23	9 ± 1
stewed dessert	15	100	21	10 ± 1
Syrup	36 (juice)	200	46	-

[a] All products are kindly provided by Procordia Foods, Eslöv, Sweden.
[b] Figures from manufactures given on food lables (**Papers III & IV**)
[c] Results are means of triplicates ± standard deviation quantified by RPBA and recalculated using (6S)-5-CH_3-H_4folate calibration

The corresponding raw material of strawberry products was analysed to determine folate retention. Interestingly, almost no folate losses occurred when strawberries were cooked as jam or stewed as strawberry desserts from frozen berries, with folate retention of 79-103% (**Paper III, Table 4**).

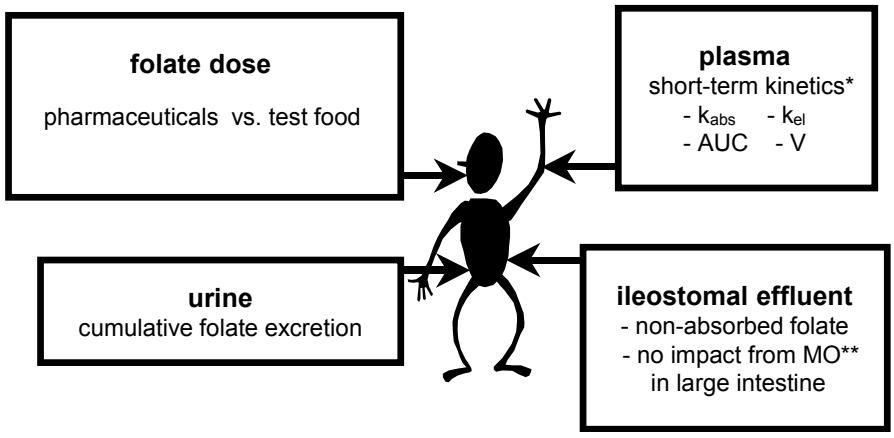
This finding is contradictory to data in food tables, which indicate that strawberry products such as jam and marmalade contain less than 10 µg/100g folates (Holland *et al.*, 1991; Levnedsmiddelstyrelsen, 1996; Livsmedelsverket, 2002). Viberg (1998) estimated water evaporation during the cooking process of strawberry jam to be around 5%, but that was not considered for our results. However, the high folate retention found in the present study cannot only be explained by water loss.

Ileostomy model to evaluate folate bioavailability

The presented human model is considered a suitable tool for studying folate absorption from foods using stomal folate excretion and plasma AUC to determine absolute and relative folate absorption (**Figure 9**). The study design allows correction for inter-individual variation by inclusion of a study day with no folate application accounting for basic plasma and stomal effluent data (N-day). The two volunteers in the pilot study complied well with the strictly standardised study design and the procedures for sample-taking as well as chosen sizes of test food portions aimed at a folate dose of ca. 200 µg were appropriate (**Paper V**).

In contrast to other studies, where an oral dose of a folic acid pharmaceutical is often given as the reference dose (Prinz-Langenohl, Brönstrup, Thorand, Hages & Pietrzik, 1999; Konings, *et al.*, 2002) we have chosen the biological folate form (6S)-5-CH_3-H_4folate to ensure the same handling in the body. In dual label stable isotopes trials, usually a bolus i.v. injection of folic acid is used as the reference dose (Pfeiffer, Rogers, Bailey & Gregory, 1997a; Gregory, 2001; Finglas, *et al.*, 2002). We have chosen to apply the reference dose by i.m. injection, because with given number and time points of blood sampling, the assessment of the plasma concentration curve is more accurate after an i.m. than i.v. injection due to a slower folate invasion into the plasma compartment. We standardised volunteers' body stores by saturation prior to each test day in order to minimise the hepatic first-pass uptake affecting the plasma response. The total folate excretion after 10h into stomal effluent was used as a parameter to assess absolute folate absorption from given doses, while the individual 2h sampling gave information on the gut passage times of the test foods.

Relative folate absorption for test foods and pharmaceutical preparations, calculated by extrapolated AUCs, ranged from 47-64% and 53-67% for volunteer 1 and 2, respectively and the non-absorbed 5-CH_3-H_4folate excreted with stomal effluent 10 h post-dose corresponded to about 20-45% of the oral doses (**Figure 10**). For both volunteers only small quantities of urinary folate were determined as 5-CH_3-H_4folate and no dose relation was observed.



* k_{abs} = absorption rate constant, k_{el} = elimination rate constant, AUC = area under the plasma response curve, V = volume of distribution
** MO = micro-organisms

Figure 9. Human ileostomy model

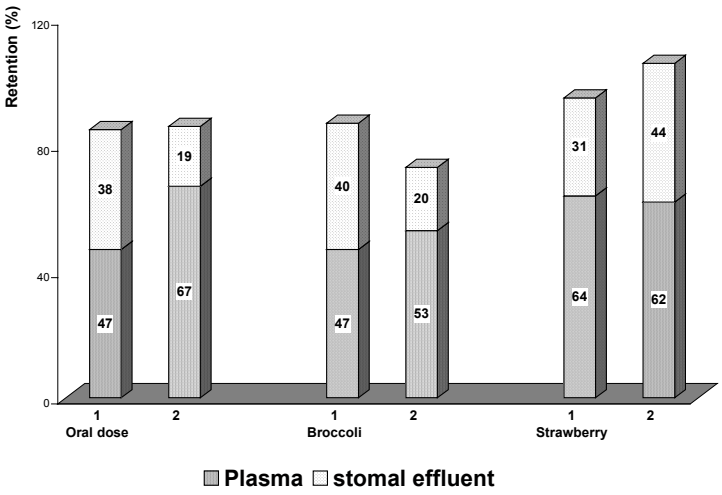


Figure 10. Relative and absolute folate absorption for volunteers 1 & 2.

Agreement between findings on relative folate absorption and non-absorbed folate can be assessed by calculation of the mean folate 'recovery' of the human model, based on data from extrapolated plasma AUCs and folate excretion into stomal effluent 10h post-dose (**Figure 10**). The small amounts of urine excretion were not considered (1-3% of the dose over 24h).

The ileostomy model for studying nutrient digestion and absorption was introduced by Sandberg, Andersson, B., Hasselblad, Isaksson & Hultén (1981). It offers a method for direct and quantitative determination of small intestine excretion, providing direct assessment of non-absorbed folate in faeces unaffected by micro-organisms. Recently, a first folate ileostomy trial has been published (Konings *et al.*, 2002) indicating a high mean folate absorption of 73-91% from two spinach meals compared to an oral dose of folic acid based on 24h post dose stomal folate excretion. Because only two volunteers completed our study, the results of folate bioavailability from strawberries are not representative.

However, the results imply that our presented model provides a suitable standardised tool for comparing folate absorption from foods before and after food processing. The model will be used to determine folate absorption from various foods and pharmaceutical folate preparations using a greater number of volunteers to enable statistical and biokinetical interpretation of data.

General discussion - conclusions and future research

In the introduction mention was made of the fact that the average daily intake of folate in many Western populations is often below the recommended level and that there is a need for a critical evaluation of dietary folate sources. This thesis, investigating the folate content in berries, was a step into that direction. The main conclusions were:

- The optimised RPBA was demonstrated to be reliable for folate quantification in berries containing mainly 5-CH_3-H_4folate. It is a useful complement to more expensive HPLC methods when screening folate content in many samples of the same kind, for example when studying variations of folate content in a certain crop.
- The recently commercially available biologic (6S)-5-CH_3-H_4folate is the folate form to use for calibration when quantifying folate content in berries by RPBA. For some berries purification of sample extracts prior to quantification is required to avoid disturbing matrix effects.
- The predominant folate form in all tested berries was 5-CH_3-H_4folate. However, the folate concentrations among different berries varied. Rich sources were the two model berries, rosehip (100-180 µg/100g FW) and strawberries (70-90 µg/100g FW). Commercial products of both berries contained 10-20 µg of folate per 100 g FW and were also considered good folate sources.
- Cultivar, ripeness and year of harvest were all found to significantly affect the folate content in model berries. These results indicate similar effects to be expected in other berries as well. As high variations in water content were observed it is necessary to present folate results as dry matter (DM).
- High folate retention in fresh strawberries was obtained during storage in refrigerators at 4 °C, whereas storage under exposure of light at room temperature should be avoided. In rosehips, water activity affected the folate degradation during drying procedures. Therefore, folate retention could be increased by minimising the drying time using high temperatures and slicing of berries.
- A new human ileostomy model enables to determine absolute and relative folate absorption from foods using stomal folate excretion and plasma AUC for comparison of dietary folate sources. Application of a reference dose of the biologic form (6S)-5-CH_3-H_4folate by i.m. injection ensures same handling by the body and an exact estimation of the reference dose.

We recommend fresh and frozen berries as well as processed berry products as rich folate sources. For instance, 150-200 g of rosehips or 250-350 g of strawberries (~200 µg folate) supplies 60-100% of the daily European intake recommendations (200-300 µg/day) or approximately 50% of the US

recommendations (400 µg/day). This is relevant as folate is among the most limited of all vitamins as regards, the nutritional requirements in humans (Gregory, 1996). Our studies, which show folate content in berries to be pronouncedly higher than reported food table data, indicate that fruits and berries are folate sources that contribute more to the total folate intake than previously assumed. Probably, also unreliable folate data exist for other foods.

In studying folate content in strawberries and rosehips, significant effects due to cultivar, year of harvest and ripeness were observed. By choosing cultivars high in folate and optimising growing and storage conditions, folate-rich berries can be obtained. The high to moderate retention of folate in berries during storage and processing is probably related to the antioxidative milieu, with high contents of organic acids and phenolic compounds. Using mild drying procedures dried berry products are potentially rich folate sources (~ 500 µg/100g DM). Our observations are interesting to the food industry using berries and semi-manufactures of berries as ingredients in food products with increased nutritional value (functional foods). Furthermore, the newly developed ileostomy model can provide a tool for evaluating the folate bioavailability from these products. Increased intake of berries and berry products could be an alternative to folate supplements and fortification.

In this thesis we have focussed on berries but similar variations in folate content can also be expected in other crops. Further studies regarding growing conditions and post-harvest handling affecting folate content are needed for all kind of crops. Of equal importance is to gain more information about folate retention during processing, especially for crops that require extensive heat treatment during industrial processing and household procedures prior to consumption, such as cereals and pulses.

Most of our data were obtained using RPBA. After systematic evaluation on berries and certified reference material, the optimised RPBA with external (6S)-5-CH_3-H_4folate calibration was found to be reliable for folate quantification in foods containing mainly 5-CH_3-H_4folate. The need for purification of sample extracts to avoid unspecific binding has to be tested for each new food matrix. Earlier attempts by others to use the RPBA for folate quantification in foods were unsuccessful due to problems with low precision, poor agreement with other methods and differing response of various folate forms (Ruddick, Vanderstoep & Richard, 1978; Shane *et al.*, 1980; Gregory *et al.*, 1982; De Souza & Eitenmiller, 1990; Finglas *et al.*, 1993). In this thesis solutions to many of these drawbacks are presented. However, we agree that folate content in foods containing many folate forms cannot be reliably estimated by RPBA. Characterisation of folate forms in food samples is therefore essential when considering the RPBA for folate quantification.

The RPBA with PteGlu calibration is currently used for folate quantification in clinical studies and for routine diagnosis of folate deficiency (van den Berg *et al.*, 1994; Raiten & Fisher, 1995). Therefore, the discrepancy (~30%) between the RPBA response of PteGlu standard and the endogenous plasma folate (6S)-5-CH_3-H_4folate can result in misinterpretation of folate status. Further investigation is necessary.

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