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Analysis of intact Folate and Folate Catabolites in Bioavailability and Stability Studies using Stable Isotope, Ileostomy and Breast Milk Designs

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ZUSAMMENFASSUNG

Die hohe Anzahl natürlich vorkommender Vitamere sowie deren chemische Labilität und geringe Konzentrationen in Proben sind eine große Herausforderung für die Folatanalytik. Der Einsatz von unterschiedlich markiertem Folat erleichtert die Bestimmung der Folatkonzentration in Lebensmitteln und klinischen Proben und somit die Bestimmung der Folatbioverfügbarkeit. Wegen ihres raschen Wachstums haben Säuglinge einen besonders hohen Folatbedarf, der über eine ausreichende Zufuhr mit der Muttermilch gedeckt werden muss. Aus hygienischen Gründen wird Spendermilch in Milchbanken wärmebehandelt, was jedoch zu Folatverlusten führt. In dieser Arbeit wurden Stabilisotopenverdünnungsassays für die gleichzeitige Quantifizierung von unmarkiertem und [$^{13}C_5$]-markiertem intaktem Folat und von zwei Folatmetaboliten in klinischen Proben entwickelt. Als interne Standards wurden zunächst die Metabolitenisotopologen, [$^{2}H_4$]-p-Aminobenzoylglutamat und [$^{2}H_4$]-p-Acetamidobenzoylglutamat, synthetisiert. In einer "Area under the curve/ ileostomy"-Humanstudie wurde im Anschluß die Bioverfügbarkeit von reduziertem Folat und Folsäure aus Vollkornbrot untersucht. In einer weiteren Studie wurden mittels Simulation gebräuchlicher Verfahren Folatkonzentration und Vitamerenverteilung in Muttermilch sowie der Effekt einer Wärmebehandlung bei 57 °C, 62,5 °C und 73 °C auf den Folatgehalt von Muttermilch untersucht.

Die entwickelten Stabilisotopenverdünnungsassays ermöglichten die selektive Analyse von unmarkiertem und [$^{13}C_5$]-markiertem Folat. Die Nachweisgrenze lag in Plasma unter 0,6 nmol/L, in Ileostomieexkret und Urin unter 0,7 μg/100 g. Die Bioverfügbarkeit von Folat aus Vollkornbrot war vergleichbar für die getesteten Substanzen, bezüglich der Plasmakinetik unterschieden sich reduziertes Folat und Folsäure dagegen signifikant. 5-Methyltetrahydrofolat war das Hauptvitamer in Muttermilch. Die Folatkonzentration in Muttermilch betrug 150,4 ± 46,4 nmol/L, die Folatverluste nach Wärmebehandlung 23,9 – 39,2 nmol/L.

Basierend auf den Ergebnissen der neuen Assays wurde die Bioverfügbarkeit von Folat aus Vollkornbrot bestimmt (ca. 90%). Die Folatverluste in Muttermilch nach Wärmebehandlung waren akzeptabel, alle untersuchten Verfahren eigneten sich gleichermaßen zur Zubereitung eines sicheren Nahrungsmittels für Säuglinge.

ABSTRACT

Folate analysis poses a great challenge because of the high variety of vitamers present in nature, their chemical lability and generally low sample folate contents. Application of differently labelled folate facilitates the precise determination of folate concentrations in food and clinical samples and allows for reliable results on folate bioavailability. Due to rapid growth, infants have particularly high folate requirements. These have to be met by the folate content in their mother's own breast milk or donated breast milk. Donated breast milk is heat treated in milk banks, yet, this leads to folate losses.

In this thesis, new stable isotope dilution assays were developed for the simultaneous quantification of unlabelled and [$^{13}C_5$]-labelled intact folate and two folate catabolites in clinical samples. Initially, for the application of the [$^2H_4$]-labelled folate analogues as internal standards, the synthesis of the catabolite isotopologues, [$^2H_4$]-p-aminobenzoyl glutamate and [$^2H_4$]-p-acetamidobenzoyl glutamate was required. Thereafter, in a novel human "Area under the curve/ileostomy" model, the bioavailability of reduced folate and folic acid from wholemeal bread was investigated. In a second trial, folate concentration and pattern in breast milk and the effect of heat treatment at 57 °C, 62.5 °C and 73 °C on breast milk folate content were investigated by simulation of generally applied procedures.

Unlabelled and [$^{13}C_5$]-labelled folate was determined selectively by the new stable isotope dilution assays. Limits of detection were below 0.6 nmol/L in plasma and below 0.7 μg/100 g in ileostomal effluent and urine. Folate bioavailability from wholemeal bread was similarly high for the tested fortificants. Yet, plasma kinetics revealed differences between reduced folate and folic acid. 5-Methyltetrahydrofolate was identified as the predominant vitamer in breast milk. The folate content of untreated breast milk was 150.4 ± 46.4 nmol/L. Folate losses after heat treatment ranged between 23.9 and 39.2 nmol/L.

Based on the sensitive determination of folate concentrations in food and clinical samples by the developed assays, folate bioavailability from bread was determined (ca. 90%). Folate losses in breast milk after heat treatment were acceptable, which proved all tested procedures suitable to provide a safe aliment for infants.

Für all die Menschen,
die mich bisher begleitet und mein Leben geprägt haben.

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Publications

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

I Barbara E. Büttner, Veronica E. Öhrvik, Cornelia M. Witthöft and Michael Rychlik (2011). Quantification of isotope-labelled and unlabelled folates in plasma, ileostomy and food samples. *Analytical and Bioanalytical Chemistry 399: 429—439.*

II Barbara E. Büttner, Veronica E. Öhrvik, Peter Köhler, Cornelia M. Witthöft and Michael Rychlik. Quantification of isotope-labelled and unlabelled folates and folate catabolites in urine samples by stable isotope dilution assay. *Revised manuscript submitted to the International Journal for Vitamin and Nutrition Research.*

III Veronica E. Öhrvik, Barbara E. Büttner, Michael Rychlik, Eva Lundin and Cornelia M. Witthöft (2010). Folate bioavailability from breads and a meal assessed with a human stable-isotope area under the curve and ileostomy model. *The American Journal of Clinical Nutrition 92: 532—538.*

IV Barbara E. Büttner, Cornelia M. Witthöft, Magnus Domellöf, Olle Hernell and Inger Öhlund (2013). Effect of type of heat treatment of breastmilk on folate content and pattern. *Breastfeeding Medicine 8: DOI: 10.1089/bfm.2013.0008.*

Barbara Büttner's contribution to the single publications

The contribution to the single publications is described as follows:

I Planned, performed and evaluated all experiments for the method development and validation; shared responsibility for writing and revising the publication with the main supervisor.

II Planned and performed all reactions for folate catabolite synthesis; planned, performed and evaluated all nuclear magnetic resonance spectroscopy and mass spectrometry experiments; had main responsibility for writing the publication; shared responsibility for revising the publication with the main supervisor.

III Participated in the human trial together with the co-author and the secondary supervisor; participated in preparation of the clinical samples for analysis; performed and supervised the analysis of food and human samples by stable isotope dilution assay; contributed to the publication.

IV Planned the trial with the secondary supervisor; organized the breast milk samples together with the co-author; set up the analytical method; performed the adjustment of the heat treatment procedures and all laboratory sample analysis; evaluated the results; shared the responsibility for the statistical evaluation of the results with the co-author; had the main responsibility for writing and revising the publication.

List of Abbreviations

10-HCO-PteGlu	10-Formylfolic acid
10-HCO-H_4folate	10-Formyltetrahydrofolate
$^{13}C_5$	Fivefold [^{13}C]-labelled
$^{2}H_4$	Fourfold deuterium-labelled
5,10-CH^+-H_4folate	5,10-Methenyltetrahydrofolate
5,10-CH_2-H_4folate	5,10-Methylenetetrahydrofolate
5-CH_3-H_4folate	5-Methyltetrahydrofolate
5-HCO-H_4folate	5-Formyltetrahydrofolate
A	Peak area
AC	Affinity chromatography
ACN	Acetonitrile
Ap-ABA	para-Acetamidobenzoic acid
Ap-ABG	para-Acetamidobenzoyl glutamate
AUC	Area under the curve
BAL	2,3-Dimercaptopropanol
BM	Bread fortified with (6S)-[$^{13}C_5$]-5-CH_3-H_4folate ***(test meal)***
BP	Bread fortified with [$^{13}C_5$]-PteGlu ***(test meal)***
CID	Collision induced dissociation
c_{max}	Maximum concentration (Concentration of folate at maximum)
CMV	Cytomegalovirus
CP	Chicken pancreas
CV	Coefficient of variation
DFE	Dietary folate equivalent
DM	Dry matter
DNA	Desoxyribonucleic acid

EC	End capped
e.g.	*exempli gratia* (for example)
EMBA	European Milk Bank Association
ESI	Electrospray ionisation
FBP	Folate binding protein
FD	Fluorescence detector
FP	Breakfast meal including bread fortified with [$^{13}C_5$]-PteGlu ***(test meal)***
H_2folate	Dihydrofolate
H_4folate	Tetrahydrofolate
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
i.e.	*id est* (this means)
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
L-Glu-di-methylester	L-Glutamic acid-di-methylester
LOD	Limit of detection
MA	Microbiological assay
MCE	β-/ 2-Mercaptoethanol
MES	2-(N-morpholino)ethanesulfonic acid
MQ-water	Millipore-water
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
n.d.	Not detected
NMR	Nuclear magnetic resonance
NTD	Neural tube defect
p-ABA	para-Aminobenzoic acid
p-ABG	para-Aminobenzoyl glutamate

PH	Phenyl
PteGlu	Pteroylglutamic acid (Folic acid)
RC	Regenerated cellulose
RDA	Recommended daily allowance
R_f	Response factor
RHT	Rapid high temperature
RNA	Ribonucleic acid
RP	Reversed phase
RS	Rat serum
RT	Retention time
SAX	Strong anion exchange
SIDA	Stable isotope dilution assay
SPE	Solid phase extraction
Std	Standard
Stdev	Standard deviation
t_{max}	Maximum time (time at maximum folate concentration)
UER	Urinary excretion ratio
UV	Ultraviolet
UVD	Ultraviolet detector
vs.	versus

1. Introduction

1.1. Folate

Roughly 80 years ago, Lucy Wills discovered a factor in yeast, that ameliorated macrocytic anemia prevalent in Indian women (Hoffbrand 2001). Later, this factor was found to be especially abundant in green leaves of many plants and, therefore, the name "folic acid" (lat. folium = leaf) was suggested (Mitchell *et al.* 1941).
The generic term "folate" describes a group of water-soluble B-vitamins deduced from folic acid (PteGlu; s. Figure 1.1) by chemical structure and nutritional properties of the vitamers (Eitenmiller and Landen jr. 1999).

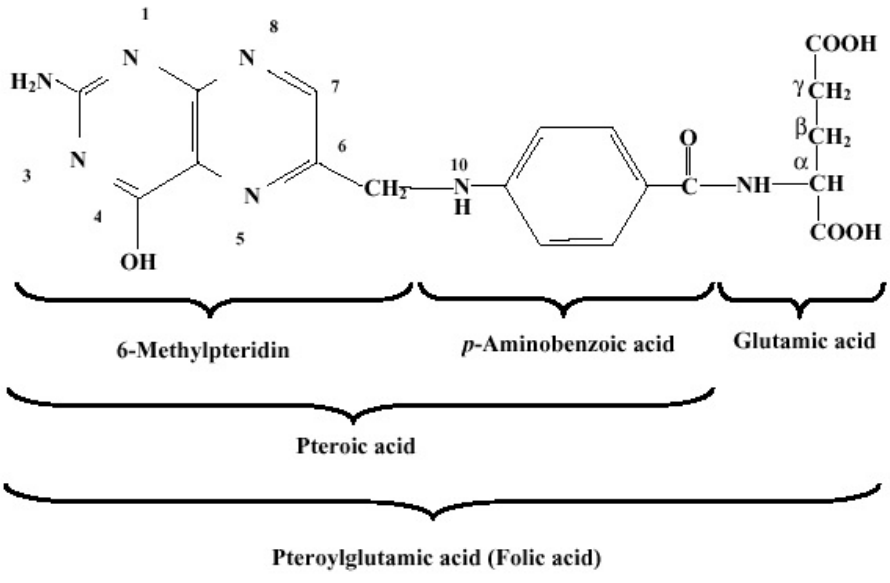

Figure 1.1.: Structure of folic acid (modified from Pölönen 2000).

The folate molecule is made up of a pteridine ring connected to a p-aminobenzoate moiety, which is linked to L-glutamate. Variations in the oxidation state of the

pteridine ring, the substituent at N^5- and/or N^{10}-position (s. Table 1.1) and the glutamyl chain length result in different folate vitamers.

Table 1.1.: One-carbon substitution of different folate vitamers.

Folate vitamer	**Substituent at N^5**	**Substituent at N^{10}**
H_4folate	H	H
5-CH_3-H_4folate	CH_3	H
5-HCO-H_4folate	HCO	H
10-HCO-H_4folate	H	HCO
10-HCO-PteGlu	—	HCO
5,10-CH_2-H_4folate	-CH_2-	
5,10-CH^+-H_4folate	-CH^+-	

In contrast to PteGlu, which is the fully oxidized vitamer not present in significant quantities in nature, natural folate occurs mainly in reduced and one-carbon substituted forms (Pfeiffer *et al.* 2010).

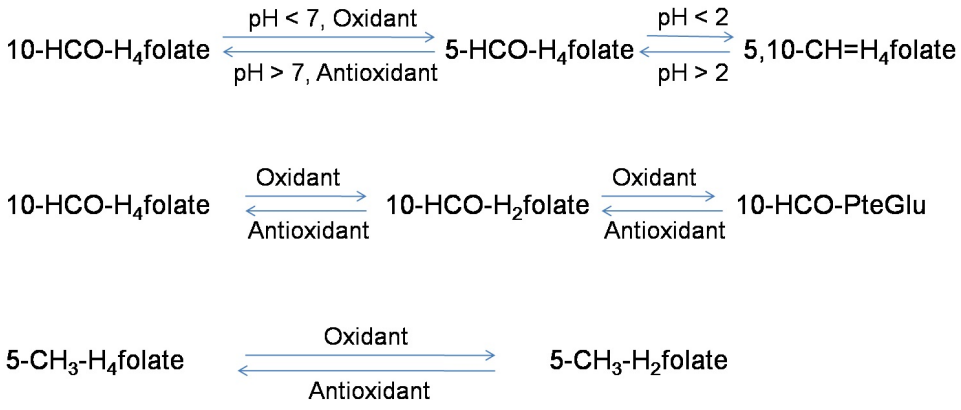

Figure 1.2.: Summary of interconversion reactions of folate (Pfeiffer *et al.* 1994 and 2010, Bagott 2000, de Brouwer *et al.* 2007).

Folate is susceptible to interconversion (s. Figure 1.2) and degradation by light, oxygen, extreme pH values or high temperatures. The folate molecule is degraded

by cleavage of the C^9-N^{10}-bond into compounds without any vitamin activity, *e.g.* p-aminobenzoyl glutamate (p-ABG), pteroic compounds and oxidation products hereof (Pfeiffer *et al.* 1994).
PteGlu is the most stable vitamer. Synthetic PteGlu is used widely for fortification purposes (Pfeiffer *et al.* 2010). In contrast, reduced folate is more susceptible, especially in solution (Pfeiffer *et al.* 1994). Substitution at N^5- and/or N^{10}-position has been shown to increase oxidative stability while the number of glutamyl-residues does not have any influence (Eitenmiller and Landen jr. 1999).

1.2. Human folate metabolism

In contrast to plants or bacteria, mammals can not synthesize folate and depend on intake through food or supplements (Pfeiffer *et al.* 1994). Knowledge on the complex human folate physiology and metabolism is essential to the understanding of nutritional requirements (Gregory III and Quinlivan 2002).

1.2.1. Folate absorption

Folate absorption occurs in the jejunum in a saturable process in two steps (Gregory III and Quinlivan 2002). First, dietary folate polyglutamate is hydrolyzed by pteroylpolyglutamyl-hydrolase (EC 3.4.12.10; Gregory III 1997). Subsequently, monoglutamate is absorbed into the enterocyte (Pfeiffer *et al.* 1994).
At physiological concentrations, transport of monoglutamate across the luminal membrane is mediated by receptors and carriers (Gregory III and Quinlivan 2002). In the enterocyte, folate is converted to 5-methyltetrahydrofolate (5-CH_3-H_4folate) by dihydrofolate-reductase and methyltetrahydrofolate-reductase (Chanarin and Perry 1969) prior to transport across the basolateral membrane into the plasma. In contrast, higher intakes of PteGlu (> 200 μg/dose) exceed the metabolic capacity of the intestinal mucosa (Gregory III and Quinlivan 2002). Diffusion-mediated transport dominates (Pfeiffer *et al.* 1994) and vitamers other than 5-CH_3-H_4folate appear in the systemic circulation (Gregory III and Quinlivan 2002).

1.2.2. Folate distribution and physiological functions

Newly absorbed folate monoglutamate is transported to the liver (Wright *et al.* 2007), the major site of one-carbon metabolism and folate storage (Gregory III and Quinlivan 2002). On the first pass through the liver, an extensive amount of folate is retained ("First pass effect"; Rogers *et al.* 1997). Due to lower hepatic affinity towards reduced, substituted folate than towards PteGlu, reduced folate is released quickly from the liver into bile (Steinberg *et al.* 1979). Distribution to peripheral tissues occurs via the "Enterohepatic cycle" (Steinberg 1984), *i.e.* the bile folate is re-absorbed from the small intestine and delivered to the tissues via systemic circulating plasma (Steinberg 1984, Wright *et al.* 2007). Additionally, this pathway is essential for folate homoeostasis as temporary fluctuations in folate availability, *e.g.* between meals, are balanced (Steinberg 1984, Lin *et al.* 2004).
For systemic transport, plasma folate is bound to low affinity protein binders, *e.g.* albumin, or to high affinity folate binding protein (FBP; Shane 2001).
Monoglutamate transport into body cells is accomplished by membrane carriers and FBPs (Shane 2001). Folate is retained in the cells by (i) conjugation to polyglutamate and (ii) association with specific proteins, mainly enzymes (Shane 2001). Tetrahydrofolate (H_4folate) is the active coenzyme species in one-carbon metabolism (Gregory III and Quinlivan 2002), *i.e.* the transfer of one-carbon units in both methylation cycle and nucleotide synthesis (s. Figure 1.3).
In the methylation cycle, methionine is built from homocysteine, which prevents accumulation of homocysteine and maintains the methionine supply of the organism. Methionine is further conjugated to S-adenosylmethionine, the most important methylating coenzyme in the human metabolism. During the synthesis of purines, H_4folate reacts with formiate donators, *e.g.* histidine, to 5-formyltetrahydrofolate (5-HCO-H_4folate) and 10-formyltetrahydrofolate (10-HCO-H_4folate). The reaction products provide the C^4- and C^8-atom and the C^2- and C^8-atom of the purine core, respectively. The synthesis of pyrimidines is based on demethylation of 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) and formation of dihydrofolate (H_2folate) and desoxy-thymidinphosphate. In addition, H_4folate is crucial for amino acid metabolism, *e.g.* of serine or histidine. (Löffler *et al.* 2007)

As folate is essential to the biosynthesis of desoxyribonucleic acid (DNA), ribonucleic acid (RNA) and amino acids, folate deficiency interferes with cell division and growth. Amongst the first signs is macrocytic anemia because the cell division rate of the blood building system is high (Löffler *et al.* 2007). During pregnancy, a period of rapid growth, folate deficiency is associated with negative foetal outcomes, *e.g.* low birth weight, spontaneous abortion, stillbirth and neural tube defects (NTD; O'Connor *et al.* 1997, Hoffbrand 2001). Additionally, folate deficiency leads to elevated plasma homocysteine levels (> 10 μmol/L; Cuskelly *et al.* 2001), an independent risk factor for vascular and brain diseases, *e.g.* dementia, Alzheimer's or Parkinson's disease (Hoffbrand 2001). In contrast, at normal vitamin B_{12} status, high serum folate levels protect from cognitive impairment (Morris *et al.* 2007).

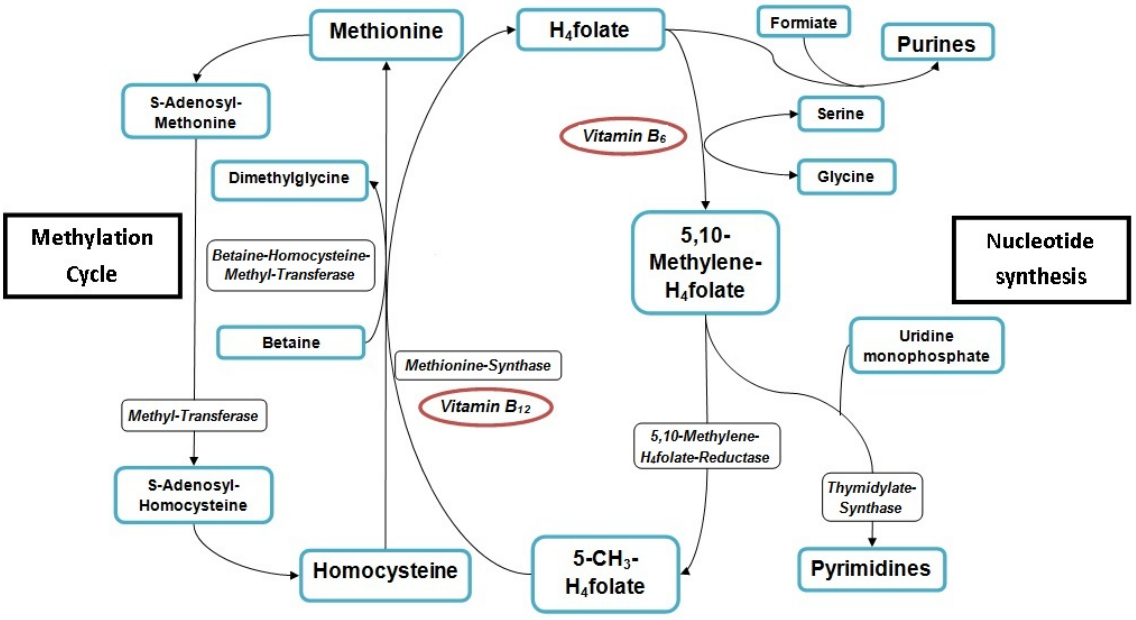

Figure 1.3.: Folate in one-carbon metabolism (modified from Löffler *et al.* 2007).

The impact of folate status on carcinogenesis is discussed controversially (Ulrich 2007). Low folate status may be related to cancer development (Hoffbrand 2001, Ulrich 2007). Recent findings suggest that higher folate intake decreases the risk of initiation or early development of colorectal cancer (Lee *et al.* 2011). Yet, at the same time, it is speculated that high concentrations of systemically-circulating

unmetabolized PteGlu may increase cell's capacity for division, which may become relevant for cancer development and tumor growth (Wright *et al.* 2007).

1.2.3. Folate turnover and excretion

Every day, about 0.8% of whole-body folate is excreted predominantly in catabolized forms (Gregory III and Quinlivan 2002). During catabolic folate degradation, the C^9-N^{10} bond is cleaved and polyglutamate is deconjugated yielding pterin and p-ABG (Shane 2001). A high proportion of p-ABG is N-acetylated in the liver prior to excretion (Gregory III and Quinlivan 2002).
Folate is excreted via urine and bile. Besides 6% of active folate vitamers, urine folate consists mainly of catabolites, *e.g.* p-ABG and p-acetamidobenzoyl glutamate (Ap-ABG; 56% of urinary folate; Lin *et al.* 2004). Bile folate leaves the body via faeces (Lin *et al.* 2004). Faecal folate containing 38% pteroylmonoglutamate and its oxidation products (Lin *et al.* 2004) originates from (i) unabsorbed dietary folate, (ii) folate from bile or other gastrointestinal secretions, (iii) sloughed mucosa cells or (iv) microbial synthesis (Gregory III and Quinlivan 2002).

1.3. Folate bioavailability and intake

Besides food folate content and nutritional folate requirements, folate bioavailability is a major criterion for sufficient intake (Pfeiffer *et al.* 1994, Gregory III *et al.* 2005).

1.3.1. Bioavailability

The term "bioavailability" was originally used in experimental pharmacology (Pfeiffer *et al.* 1994). Nutritional science defines "bioavailability" as extent and velocity of absorption and metabolic utilisation of a nutrient (Pfeiffer *et al.* 1994, Rychlik 2009), including processes of intestinal absorption, transport, metabolism and excretion. Both intrinsic and extrinsic factors influence nutrient bioavailability (s. Table 1.2).

Table 1.2.: Factors influencing folate bioavailability.

Type	**Factor**	**Examples**	**References**
Intrinsic	Health status	Age, genetic polymorphism, illness	Frosst *et al.* 1995, de Meer *et al.* 2005, Zhao and Goldman 2007
	Nutritional status	Vitamin B_{12} deficiency ("Methyl-trap")	Gregory III *et al.* 2005, Löffler *et al.* 2007
	Gastrointestinal function	pH value, activity of intestinal enzymes, maldigestion	Pfeiffer *et al.* 1994, Gregory III *et al.* 2005
Extrinsic	Type of folate	Oxidation status, substituents, stability, polyglutamate chain length	Pfeiffer *et al.* 1994, Gregory III *et al.* 2005, Rychlik 2009
	Food folate content	Saturation of intestinal folate absorption	Gregory III and Quinlivan 2002
	Food matrix	Enzyme inhibitors, folate binding protein, conjugase, fibre, ascorbic acid	Pfeiffer *et al.* 1997, Finglas *et al.* 2002, Witthöft *et al.* 2003
	Antagonists	Alcohol, drugs, antifolates	Gregory III 1997, Mason and Choi 2005, Löffler *et al.* 2007

1.3.2. Methods to determine bioavailability

Since the first definitive studies, several models have been developed to determine folate bioavailability, including both long-term and short-term protocols. Long-term intervention trials assess the physiological effects of repeated ingestion of *e.g.* foods fortified with folate on serum folate, erythrocyte folate and/or plasma homocysteine status. Short-term protocols are designed to examine the response of plasma, faeces and/or urine folate concentration to a single test dose (Witthöft *et al.* 2003, Gregory III *et al.* 2005), *i.e.* the kinetics of absorption, metabolism and transport of folate (Wright *et al.* 2007). Several approaches for the investigation of short-term folate kinetics have been described, *e.g.*:

- "AUC-technique": This method compares the plasma area under the curve (AUC) after ingestion of a test dose and after administration of a reference dose. The bioavailability of the oral, intravenous or intramuscular reference dose is set to 100% and the results are presented as relative folate absorption (Finglas *et al.* 2002, Rychlik *et al.* 2003, Witthöft *et al.* 2003, Wright *et al.* 2007). This approach is only applicable if absorption and metabolization mechanisms of folate in test and reference dose are equal, which has been challenged for reduced folate relative to PteGlu (Wright *et al.* 2003) and different routes of administration (Rogers *et al.* 1997).

- "UER-technique": The percentage of intact folate of a [^{13}C]-labelled oral dose excreted in urine is compared to that of a [^{2}H]-labelled intravenous dose and relative absorption is assessed (Finglas *et al.* 2002). It is essential to consider the different handling of orally *vs.* intravenously administred PteGlu (Pfeiffer *et al.* 1997, Rogers *et al.* 1997), *e.g.* by the use of (6S)-5-CH_3-H_4folate as intravenous reference dose (Finglas *et al.* 2002).
  The absorption of folate administered with food relative to that of a PteGlu supplement can be determined by a comparison of the respective urinary excretion ratio (UER) values, if the post-dose collection time allows the relative ratio to reach a constant (Finglas *et al.* 2002).

- "Oral-faecal balance-technique": This method compares consumed to excreted

folate and assesses the absolute folate absorption (Witthöft *et al.* 2003). Ileostomal and faecal folate does not solely derive from non-absorbed folate (Gregory III and Quinlivan 2002). Assessment of baseline excretion (Konings *et al.* 2002, Witthöft *et al.* 2003) or use of stable-isotope labelled tracers (*Paper III*) help to account for non-dose folate. Samples of ileostomal effluent are preferred over stool samples to minimize interferences with microbial folate synthesis in the large intestine (Asrar and O'Connor 2005, Aufreiter *et al.* 2009).

The mentioned techniques have been applied successfully to the investigation of folate bioavailability from foods, *e.g.* fortified bread, rice or pasta (Pfeiffer *et al.* 1997), spinach (Konings *et al.* 2002) and yeast, fortified bread and fermented milk (Witthöft *et al.* 2006), or supplements (Rychlik *et al.* 2003).

1.3.3. Folate status and intake

The nutritional status is a function of quantity and bioavailability of ingested folate (Gregory III *et al.* 2005). For an adequate folate status, either satisfactory amounts of highly available folate or increased amounts of folate with lower bioavailability have to be consumed.

Dietary recommendations intend to support sufficient folate intake in the majority of a healthy population (de Bree *et al.* 1997). To account for differences in bioavailability of natural folate and added PteGlu, recommended dietary allowance (RDA) values for folate are expressed in terms of dietary folate equivalents (DFE). DFE are defined as the quantity of natural food folate plus 1.7 times the quantity of PteGlu consumed with fortified foods or supplements (Gregory III *et al.* 2005).

Insufficient folate intake is common among European populations (de Bree *et al.* 1997), *e.g.* 79% of the male and 86% of the female German population do not reach the RDA of 400 μg/day (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz 2008). In the United States, mandatory folic acid fortification has increased folate status significantly (Kalmbach *et al.* 2008). Consumption of PteGlu fortified milk (Green *et al.* 2005) and biofortified eggs from chickens receiving PteGlu fortified feed (Roth-Maier and Böhmer 2007) are suitable to raise

folate intake. Supplementation with (6S)-5-CH_3-H_4folate has been shown to be more effective than PteGlu-supplementation (Lamers *et al.* 2006). Mandatory fortification (DGE 2007) and/or supplement intake are considered possibilities to raise folate intake and status in European populations (SLV 2007a, BfR 2008).

1.4. Determination of folate in different samples

Accurate folate determination has to account for the analytes' labile nature and low concentration and the high variety of vitamers present in different samples (Nelson 2007). This requires, *e.g.* working under subdued light (gold fluorescent), gassing with inert gases, *e.g.* N_2 or Ar, or application of antioxidants, *e.g.* β-/ 2-mercaptoethanol (MCE) or 2,3-dimercaptopropanol (BAL), ascorbic acid and its salts or dithiothreitol (Lucock *et al.* 1993, Pfeiffer *et al.* 2010). Folate determination is accomplished by microbiological assay (MA), protein-binding or chromatographic assay. In contrast to before mentioned assays, chromatographic methods are capable of separating and measuring individual folate vitamers, which is of great importance as the vitamers differ in stability and bioavailability. Depending on the type of assay and the type of sample, analysis is generally composed of several steps: Folate extraction, cleanup and concentration of the extract, separation and detection as reviewed in detail elsewhere (Pfeiffer *et al.* 2010).

1.4.1. Sample preparation for chromatographic assays

Sample preparation for chromatographic determination of folate pattern and concentration starts by heat extraction to liberate the folate analytes from the sample matrix and to precipitate protein and inactivate matrix enzymes catalysing folate oxidation or interconversion. Subsequent enzyme treatment ensures complete release of bound folate. Depending on the sample matrix, enzyme digestion includes α-amylase and/or protease treatment followed by conjugase treatment to cleave folate polyglutamate to monoglutamate (Eitenmiller and Landen jr. 1999). Generally, tri-enzyme treatment, *i.e.* use of α-amylase and protease in addition to conjugase, is preferred over mono-enzyme treatment, *i.e.* use of conjugase

alone (Pfeiffer *et al.* 2010), as it has been shown to increase measurable folate concentration, *e.g.* in human milk (Lim *et al.* 1998) and food samples (Tamura *et al.* 1997). Sample cleanup and extract concentration are conducted by affinity chromatography (AC) or solid phase extraction (SPE). Despite AC's high suitability for determination of trace amounts, its inherent limitations, *e.g.* decreasing binding capacity after few runs or binding disparities between different vitamers and natural and racemic folate standards (Freisleben *et al.* 2003a), made SPE a commonly applied purification method. For folate determination, strong anion exchange (SAX) materials based on trimethylaminopropylsilica are used widely (Nilsson *et al.* 2004), *e.g.* during analysis of plasma and urine samples (Mönch *et al.* 2010). Non-polar reversed phase (RP) sorbents, *e.g.* phenyl-end capped (PH-EC), or a combination of SAX and PH-EC are suitable alternatives (Nilsson *et al.* 2004).
Many different buffers have been described for preparation of biological and food samples. One criterion important for a buffer's suitability for folate determination is the pH value. It has to provide optimal conditions for the enzyme treatment and (in combination with antioxidants) to stabilize the folate vitamers throughout the preparation procedure. Phosphate buffers (pH 4.0 – 8.0, 1% ascorbic acid, 0.1 – 2.0% MCE) are well suited for extraction of food samples (Eitenmiller and Landen jr. 1999, de Brouwer *et al.* 2007). 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) buffer (pH 5.0, 2% ascorbic acid, 0.2 mol/L MCE) has protected folate well from degradation during preparation of clinical samples (Mönch *et al.* 2010).

1.4.2. LC separation

RP high performance liquid chromatography (HPLC) is used widely due to its simplicity, sensitivity and compatibility with multiple detection methods. Compared with ion-pair chromatography, methods based on ion suppression are adopted more often (Pfeiffer *et al.* 2010). There, the pH value of the mobile phase is kept below four and the charges of the folate molecule are masked, which, together with the pteridine moiety's lipophily, is sufficient for retention on RPs (Pfeiffer *et al.* 1994). Alkyl-, *e.g.* octadecyl- (C_{18}) or octyl- (C_8), or PH-bonded stationary phases are most promising for separation of folate monoglutamates (Johansson *et al.* 2005, Pfeiffer

et al. 2010). Phosphate, formiate or acetate buffers (pH 2.0 – 3.5) are used as aqueous component of the commonly binary mobile phase. Due to the suppression of the ionisation of the carboxylic groups in the folate molecule, the amount of organic solvent required for good separation can be decreased. Acetonitrile (ACN) or methanol are commonly used organic solvents (Johansson *et al.* 2005). For detection by (tandem) mass spectrometry (MS(/MS)), the content of salts and compounds with poor volatility in the mobile phase is kept low (Pfeiffer *et al.* 2010). Amongst volatile buffers, aqueous acetic acid provides the best sensitivity for LC-MS analysis in positive electrospray ionisation (ESI) mode (Patring and Jastrebova 2007).

1.4.3. Detection

HPLC methods are combined with selective and sensitive detectors, *e.g.* ultraviolet detector (UVD)/diode array detector, fluorescence detector (FD), electrochemical detector or mass spectrometer (Pfeiffer *et al.* 2010). The type of detection depends on (i) the amount of analyte present (sensitivity), (ii) the sample matrix (selectivity) and (iii) the vitamer(s) of interest (Lucock *et al.* 1995).
UV detection is applicable to all folate vitamers. Due to susceptibility to matrix interferences (Pfeiffer *et al.* 1994), the use of an UVD is often restricted to detection of oxidized folate or verification of peak identity and purity by comparison of the fluorescence and the UV absorbance of one folate vitamer (Vahteristo *et al.* 1996, Gujska and Kuncewicz 2005).
The FD is used commonly in folate determination as it is highly specific, more sensitive than the UVD (Pfeiffer *et al.* 1994) and cheaper than a mass spectrometer. The application is limited to the fluorescence intensity of the analytes. Only fully reduced and/or substituted folate fluoresces naturally depending on pH and/or buffer composition. Native fluorescence of H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate at pH 2.3 allows detection at low picomolar concentrations (Eitenmiller and Landen jr. 1999). The fluorescence detection of oxidized folate requires derivatisation, *e.g.* cleavage into pteridine compounds (Pfeiffer *et al.* 1994) or acid hydrolysis into p-aminobenzoic acid (p-ABA) and subsequent derivatisation with fluorescamine (Zhang *et al.* 2003).

A mass spectrometer is a very specific detector for folate determination. Single-stage systems still require intensive cleanup for selective performance especially in complex matrices, *e.g.* bread (Freisleben *et al.* 2003a). In contrast, MS/MS offers maximum selectivity, sensitivity and specificity (Nelson 2007). The triple quadrupol mass spectrometers are very valuable as they enable a high number of transitions per time, reveal a wide linearity range and are scarcely susceptible to interfering ions (Freisleben *et al.* 2003a).

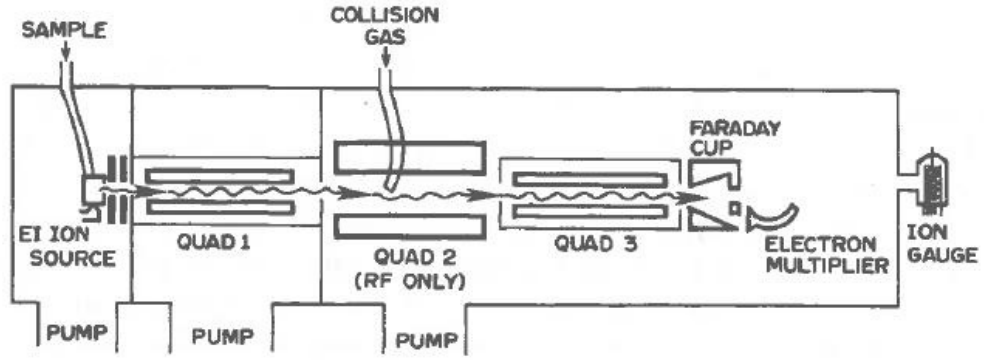

Figure 1.4.: A triple quadrupol mass spectrometer operated in MS/MS-mode (reproduced from Yost *et al.* 1979 with permission. ©Elsevier Science 1979); EI: Electron ionisation, QUAD: Quadrupol, RF: Radio frequency.

When operating triple quadrupol mass spectrometers in the MS/MS mode (s. Figure 1.4), the first quadrupol (QUAD 1) selects precursor ions. The second quadrupol (QUAD 2) acts as "field-free region" for metastable dissociation or as collision cell where precursor ions dissociate due to energy transfer during collision with neutral gas molecules (collision induced dissociation (CID)). Characteristic product ions are detected in the third quadrupol (QUAD 3; Gross 2004). Fragmentation reactions of one or more selected precursor ion(s) are followed specifically (selected reaction monitoring or multiple reaction monitoring (MRM)) and interference with unspecific matrix compounds becomes negligible (Gross 2004). Coupled to a LC system, MS/MS provides high selectivity because of (i) chromatographic separation, (ii) selection of one or more defined precursor ion(s) by the respective mass-to-charge ratio (m/z) and (iii) detection of a characteristic transition (Pfeiffer *et al.* 2010).

Several ionisation techniques have been applied to the structural analysis of folate (Nelson 2007). ESI is considered one of the softest ionisation types and therefore most suitable for folate quantification (Nelson 2007, Pfeiffer *et al.* 2010). Compared to negative mode ESI, positive mode ESI has been shown to provide more intense signals for 5-CH_3-H_4folate and H_4folate, the most common vitamers in food (Patring and Jastrebova 2007). Hence, positive mode ESI is adopted widely (Pfeiffer *et al.* 2010) with the neutral loss of the glutamic acid-moiety being predominantly used for folate detection (Pfeiffer *et al.* 2004, Patring and Jastrebova 2007).

1.4.4. Stable isotope dilution assay (SIDA)

In general, the use of a suitable internal standard (IS) helps to account for procedural losses. In MS, the use of an IS is required further to account for variations in ionisation yields (Pfeiffer *et al.* 2010). Due to identical properties and ionisation efficiency, stable-isotope labelled ISs are most suitable for MS (Gross 2004). Common ISs for intact folate and folate catabolites are either [^{13}C]-labelled (Maunder *et al.* 1999, Mönch *et al.* 2010) or [^{2}H]-labelled (Maunder *et al.* 1999, Freisleben *et al.* 2002). Isotopologic standards for SIDA need to fulfill several prerequisites: (i) stable labeling, (ii) minimal isotope effects and (iii) absence of spectral overlap (Rychlik and Asam 2008).

In addition to the advantages of MS/MS detection, isotope dilution leads to a more accurate and comfortable folate quantification (Freisleben *et al.* 2003a). Absolute recoveries are no longer required (Nelson *et al.* 2003), peak assignment is facilitated and satisfactory chromatographic separation becomes less essential (Freisleben *et al.* 2003a). The principle of the SIDA is depicted in Figure 1.5. Following addition of the IS and its equilibration with the analyte, the ratio of the isotopologues is stable throughout the procedure. Final MS separates analyte and IS by different m/z of precursor and/or product ion. Based on the known amount of IS, the amount of analyte in the sample is calculated.

The SIDA has been proven suitable for analysis of both food and clinical samples. Folate contents in spinach, meat, wheat bread and orange juice determined by SIDA were in good agreement with literature data (Freisleben *et al.* 2003b). Contrary,

a comparison of legume folate contents determined by SIDA and by HPLC-FD or MA revealed differences between the methods for certain legumes (Rychlik *et al.* 2007). 5-CH_3-H_4folate concentration in plasma was determined accurately by SIDA (Nelson *et al.* 2003). Recently, intact folate and folate catabolites were quantified successfully by SIDA in plasma, red blood cells and urine (Mönch *et al.* 2010).

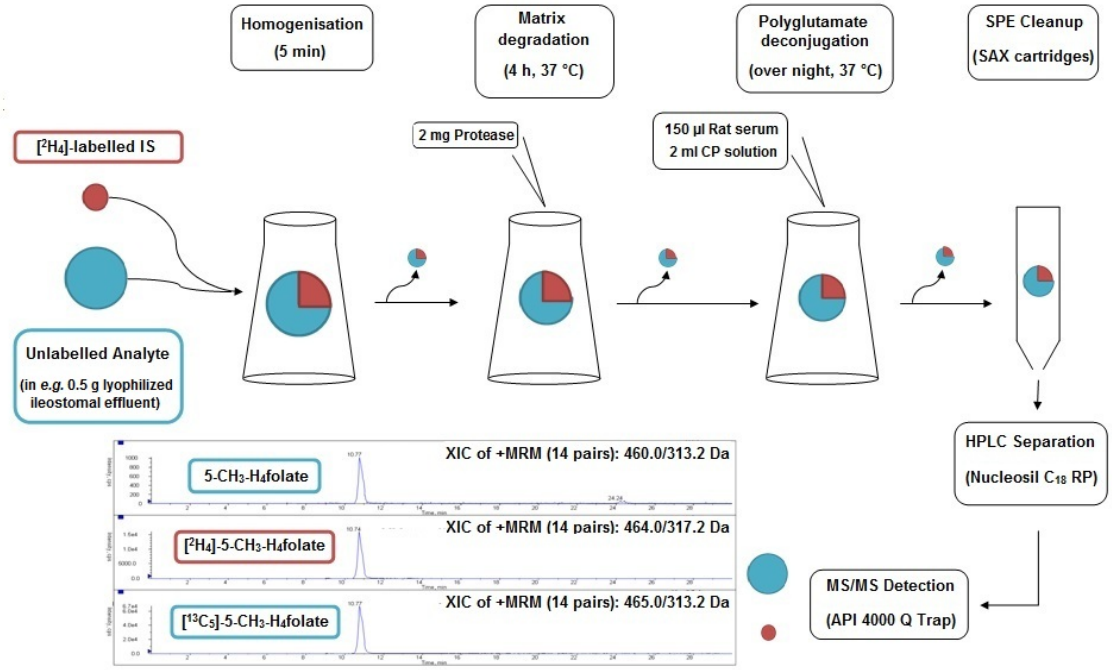

Figure 1.5.: Principle of the stable isotope dilution assay (for 5-CH_3-H_4folate); CP: Chicken pancreas, HPLC: High performance liquid chromatography, IS: Internal standard, MRM: Multiple reaction monitoring, MS/MS: Tandem mass spectro- metry, RP: Reversed phase, SAX: Strong anion exchange, SPE: Solid phase extraction, XIC: Extracted ion chromatogram.

Bioavailability studies benefit from the possibility to differentiate between differently labelled and unlabelled folate: (i) differently labelled isotopologues in test dose and as IS, respectively, allow both differentiation of dose folate and endogenous folate and accurate folate quantification in body compartments and fluids (Hart *et al.* 2002, *Paper III*) and (ii) in dual-label stable isotope protocols the bioavailability can be determined relative to a reference dose, replacing practices of subject saturation or the application of high doses (Melse-Boonstra *et al.* 2006).

1.5. Folate in breast milk

Breast milk is the first and exclusive aliment for a new born infant. It provides the nutrients required for growth and progression during the first months in life. In a recent survey on infant feeding practices, 61% of US American mothers stated to breast feed their infants (Bank and West 1985). In Croatia, ca. 66% of the mothers breast fed their term infant at three months of age (Zakarija-Grković *et al.* 2012). Comparing different ethnic groups living in the Netherlands, 98% and 92% of the Turkish and the Moroccan women, respectively, initiated breast feeding while the initiation rate was 84% for Dutch women (de Hoog *et al.* 2011).

1.5.1. Folate supply of infants

Due to its essential role in DNA, RNA and protein biosynthesis (Löffler *et al.* 2007), folate is of major importance for infant growth and development. Amongst other nutrients, folate affects brain development substantially (Georgieff 2007).
The folate supply of infants is assessed diversely: In preterm infants, folate is believed to be commonly deficient, which is critical as the brain is particularly vulnerable to nutrient deficiency between 24 and 42 weeks of gestation (Georgieff 2007). Contrary, the nursing term infant is believed to be well protected from folate deficiency because milk folate concentration is maintained at the expense of maternal folate reserves (O'Connor *et al.* 1997). Except from severe folate deficiency, maternal folate status has minimal influence on milk folate concentration (O'Connor *et al.* 1997, Villalpando *et al.* 2003). Supplementation with 416 μg (6S)-5-CH_3-H_4folate or 400 μg PteGlu/day for 16 weeks did not affect milk folate concentration of women with adequate folate status (Houghton *et al.* 2009). Besides breast milk, casein- and soy-based formulas have been shown to provide term infants with sufficient amounts of folate (Han *et al.* 2009).
Dietary recommendations for both preterm infants and lactating women are established to ensure sufficient folate intake of child and mother. The recommended daily folate intake for preterm infants up to 1.8 kg body weight is 35 – 100 μg PteGlu/kg body weight/day (Agostoni *et al* 2010). Generally, there are two feeding

options to meet the preterm infant's nutritional requirements: (i) human milk, which may require the use of a fortifier or (ii) preterm formula (Shah and Shah 2009, Agostoni *et al.* 2010). The establishment of dietary recommendations for lactating women is hampered by (i) the high variation between published human milk folate concentration values (s. Table 1.3) and (ii) the need to address the presumably low folate status at birth (high folate requirements during pregnancy) by the additional intake of a certain amount of folate (O'Connor *et al.* 1997).
Besides methodological differences and/or difficulties (O'Connor *et al.* 1997), individual factors account for the high variation between literature values for breast milk folate concentration, *e.g.*:

- A relation between breast milk folate concentration and dietary iron intake and maternal iron and vitamin B_{12} status is discussed (Villalpando *et al.* 2003, Khambalia *et al.* 2006).
- Non-nutritive factors, *e.g.* smoking, maternal age or maternal socioeconomic status, influence breast milk folate content (O'Connor *et al.* 1997).
- The time of breast milk collection affects folate concentration. It varies during the lactation period, a day and a nursing session (Udipi *et al.* 1987, Agostoni *et al.* 2010).
- Differences in race or dietary habits between Asia and Europe or America may influence folate secretion into human milk (Han *et al.* 2009).

1.5.2. Significance of breast milk consumption for infants

Breast feeding outclasses formula feeding in terms of positive health effects, *e.g.* protection against overweight and obesity development (Rzehak *et al.* 2009), and authentic nutrient composition (Wight 2001). Breast milk contains numerous bioactive compounds, *e.g.* digestive enzymes, immunologic and growth factors or hormones (Wight 2001, Shah and Shah 2009), and nutrients, *e.g.* essential long-chain polyunsaturated fatty acids (Tully *et al.* 2001), which are required for normal development but may not be present in formula (Lucas *et al.* 1992, Wight 2001).

Table 1.3.: Survey of world wide published breast milk folate concentrations (mean ± Stdev). [nmol/L]

Country	**Sampling period post partum**	**Concentration**	**Analytical Method**	**Reference**
USA	3 and 6 months	180±5[1]	MA (5-HCO-H_4folate[2])	Lim *et al.* 1998
USA	[3]	110[4]	[3]	USDA
Mexico	22±13 days	108±32	MA (5-HCO-H_4folate[2])	Villalpando *et al.* 2003
Korea	until 4 months	$\geq$ 300	MA (5-HCO-H_4folate[2])	Han *et al.* 2009
Japan	until 1 year	141±66[5]	HPLC	Sakurai *et al.* 2005
Sweden	until 3 months	110[5]	MA (PteGlu[2])	SLV
Finland	[3]	110[5]	HPLC	NIfHW
UK	colostrum($<$ 2 days)	45[5]	MA (after deconjugation)	Food Standards Agency
Germany	[3]	180[5]	[3]	Souci Fachmann Kraut

[1] Concentration given in μg/L, calculated to nmol/L ($*$ 2,1 nmol/ng).
[2] Folate calibrant.
[3] No information available.
[4] Expressed as dietary folate equivalents.
[5] Concentration given in μg/100 g, calculated to nmol/L ($*$ 22,7 nmol $*$ g/ng $*$ L).

HPLC: High performance liquid chromatography, MA: Microbiological assay, NIfHW: National Institute for Health and Wellfare, SLV: Sveriges Livsmedelsverket, Stdev: Standard deviation, USDA: U.S. Department of Agriculture.

Especially for preterm infants, fortified breast milk is recommended as standard food (Shah and Shah 2009, Agostoni *et al.* 2010) because of its beneficial properties, *e.g.* stimulation of gastrointestinal motility and maturation (Wight 2001). Compared to feeding formula, feeding breast milk has been associated with higher intelligence quotient at school age in preterm infants (Lucas *et al* 1992).
Unfortunately, as complications during labor and delivery and/or poor infant characteristics, *e.g.* reduced sucking strength, can influence breast feeding negatively (Dewey *et al.* 2000), premature born infants are at greater risk for insufficient supply with breast milk than term infants. If mother's own milk is not available, donor milk provides a suitable alternative for the health care of preterm infants. Feeding donor milk has been associated with a reduced incidence of necrotizing enterocolitis and fewer episodes of diarrhea (Boyd *et al.* 2007). Donated preterm milk is of increased value because its nutrient composition is especially suited for premature born infants (Tully *et al.* 2001, Wight 2001).

1.5.3. Banking of breast milk

Milk banking, *i.e.* collection (mostly until 3 months post partum), preparation, treatment, storage and distribution of donated human milk , is common all over the globe. Currently, 186 milk banks exist in Europe, another 13 are in the planning stage (Weaver 2013). The European milk bank association (EMBA) promotes breast milk donation and European milk bank cooperation and establishes uniform guidelines to ensure quality control of breast milk banking (Weaver 2013).
For hygienic reasons and to protect infants from health risks, donor milk is collected, stored frozen, (mostly) heat treated and then stored refrigerated until feeding. In Swedish milk banks, the commonly applied heat treatment procedure is Holder pasteurization, *i.e.* heating to 62.5 °C for 30 minutes (Omarsdottir *et al.* 2008). Figure 1.6 shows a pasteurizer commonly used for heat treatment of donated human milk in Swedish milk banks.
In order to (i) optimize inactivation of virus and bacteria species and, at the same time, (ii) to preserve breast milk's health beneficial properties, three different heat treatment procedures for donor milk were discussed at the annual EMBA meeting

in Vienna in 2009 (personal communication with I. Öhlund, classification according to Krämer 2002):

- Low temperature/ Duration heating pasteurization (57 °C, 30 minutes),
- Holder pasteurization (62.5 °C, 30 minutes),
- Short time heating procedure, *e.g.* rapid high temperature (RHT; 72 °C, 15 seconds; Goldblum *et al.* 1984) or flash-heat treatment (to 72.9 °C, temperature > 56 °C, ca. 6 minutes; Israel-Ballard *et al.* 2005 and 2008).

Temperatures between 55 °C and 65 °C are sufficient to devitalize vegetative bacteria, yeast or mould fungi and their spores (Krämer 2002). Pasteurization of breast milk at 57 °C and Holder pasteurization inactivate human immunodeficiency virus (HIV), cytomegalovirus (CMV) and human T-lymphotrophic virus effectively (Tully *et al.* 2001, Wight 2001, Hamprecht *et al.* 2004). RHT treatment procedures (72 °C, several seconds) profoundly reduce the number of bacteria and CMV in breast milk (Goldblum *et al.* 1984, Hamprecht *et al.* 2004).

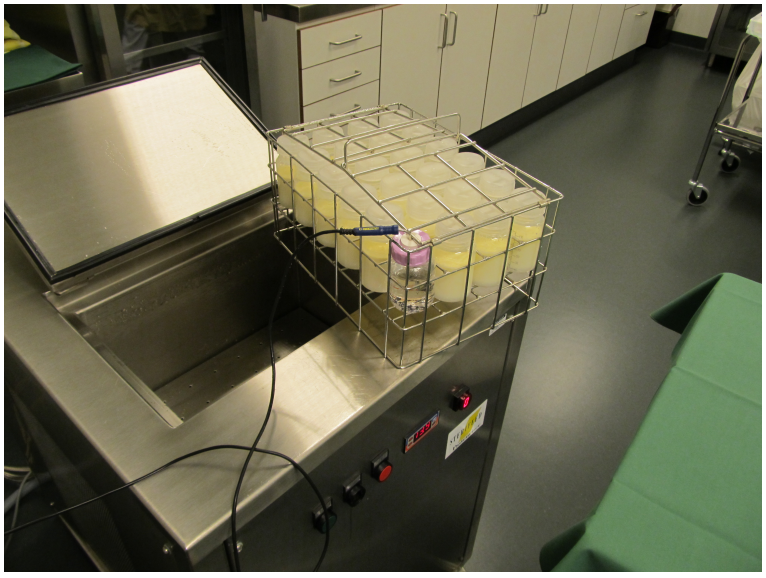

Figure 1.6.: Pasteurizer commonly applied in Swedish milk banks for heat treatment of donated breast milk (I. Öhlund, Umeå, Sweden).

Feeding RHT treated milk has been associated with a decreased risk for HIV transmission from infected mothers to their infants (Mbuya *et al.* 2010).

1.5.4. Effect of heat treatment on breast milk folate content

Besides the effect of heat treatment on microorganisms in breast milk, the changes in nutrient content, especially folate content, during heat treatment have been studied widely (Goldsmith *et al.* 1983, Goldblum *et al.* 1984, van Zoeren-Grobben *et al.* 1987, Donnelly-Vanderloo *et al.* 1994, Hamprecht *et al.* 2004, Israel-Ballard *et al.* 2005 and 2008). Preceding results revealed folate losses after pasteurization (van Zoeren-Grobben *et al.* 1987, Donnelly-Vanderloo *et al.* 1994) while breast milk folate concentration was stable (Goldblum *et al.* 1984, Hamprecht *et al.* 2004) or increased (Israel-Ballard *et al.* 2005 and 2008) after short time heating procedures. The latter findings are surprising as significant losses should be expected due to folate's instability.

In Sweden, banked breast milk is fortified either based on nutritional analysis or blindly (Omarsdottir *et al.* 2008). In case of folate degradation during heat treatment, enrichment ensures sufficient folate supply of (premature born) infants. Contrary, in case of stable breast milk folate concentration, enrichment may lead to the intake of unreasonably high amounts of folate. For instance, if folate supports normal brain development at a given concentration, it may be toxic at another (Georgieff, 2007). There is increasing concern about high folate intake due to blind fortification of banked breast milk (personal communication with I. Öhlund).

The development of a standardized preparation procedure for donated breast milk with defined effects on folate stability during storage and heat treatment could (i) facilitate adequate fortification of donor milk, (ii) help to avoid "over-fortification" and (iii) protect premature born infants, a population in critical health conditions, from exposure to further health risks.

2. Objectives

The main objectives of this thesis were the development and adjustment of methods for the determination of folate vitamers and catabolites in human samples and the application of the new methods in studies on bioavailability and processing stability of folate. These methods comprised folate extraction including enzyme treatment, SPE cleanup, LC separation and detection by MS as well as FD and UVD. Folate quantification was based on the use of ISs and external calibration. Specific objectives were:

- To develop, adjust and validate SIDAs for the simultaneous determination of differently labelled intact folate in plasma, urine and ileostomal effluent.
- To synthesize fourfold deuterium ([$^2H_4$])-labelled p-ABG and Ap-ABG as ISs in the SIDA for the determination of folate catabolite concentrations in urine.
- To apply these SIDAs in a novel human stable-isotope AUC/ileostomy model to determine
  - Folate bioavailability from a single dose of wholemeal bread fortified with either fivefold [^{13}C]-labelled ([$^{13}C_5$])-5-CH_3-H_4folate or [$^{13}C_5$]-PteGlu,
  - Bioavailability of [$^{13}C_5$]-PteGlu ingested with wholemeal bread alone or as part of a complete breakfast meal.
- To examine the effect of three different heat treatment procedures on folate concentrations in breast milk. The investigated procedures were
  - Low temperature pasteurization (57 °C, 30 minutes),
  - Holder pasteurization (62.5 °C, 30 minutes),
  - RHT treatment (72 °C, 15 seconds).

3. Materials and Methods

3.1. Chemicals and materials

3.1.1. Technische Universität München

H_4folate, 5-CH_3-H_4folate, 10-formylfolic acid (10-HCO-PteGlu), 5-HCO-H_4folate, Schirks, Jona, Switzerland; PteGlu, p-ABG, Sigma-Aldrich, Steinheim, Germany; Ap-ABG (*Paper II*, Mönch *et al.* 2010);
[$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu (Freisleben *et al.* 2002); [$^2H_4$]-p-ABG, [$^2H_4$]-Ap-ABG (*Paper II*);
[$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, [$^{13}C_5$]-PteGlu, Merck & Cie, Schaffhausen, Switzerland; [$^{13}C_5$]-p-ABG, [$^{13}C_5$]-Ap-ABG (Mönch *et al.* 2010).
The following chemicals were purchased from the sources given in parentheses: Acetic acid (glacial acetic acid) p.a., Acetonitrile LiChrosolv, Formic acid (98 – 100%) p.a., n-Hexane LiChrosolv, Hydrochloric acid fuming 37% LiChrosolv, Methanol LiChrosolv, Potassiumdihydrogenphosphate p.a., Potassiumhydroxide, di-Sodiumhydrogenphosphate p.a., Sodiumhydroxide p.a., Water LiChrosolv (Merck KGaA, Darmstadt, Germany), Acetic anhydride, α-Amylase, Type II-A, from Bacillus species, p-Aminobenzoic acid potassium salt, Deuteriumoxide, Dicyclohexylcarbodiimid, Diisopropylethylamine, L-Glutamic acid-di-methylester (L-Glu-di-methylester), 1-Hydroxybenzotriazole, 2-(*N*-morpholino)ethanesulfonic acid monohydrate minimum 99.5% titration, Palladium on activated charcoal (30%), Protease, Type XIV, from *streptomyces griseus*, Sodiumacetate waterfree, Sodiumphosphate dibasic dihydrate, Sodiumsulfate, Tetrahydrofuran waterfree, Trifluoracetic anhydride (Sigma-Aldrich, Steinheim, Germany), L(+)-Ascorbic acid (VWR, Darmstadt, Germany), Chicken pancreas (CP; Difco, Becton Dickinson,

Sparks, USA), Diethylether GPR Rectapur, Sodiumascorbate (VWR, Leuven, Belgium), Ethylacetate technical (VWR Prolabo, Fontenay-sous-Bois, France), Dichlormethane Picograde (Promochem, LGC Standards GmbH, Wesel, Germany), β-Mercaptoethanol (AppliChem, Darmstadt, Germany), Rat serum (RS) azidefree (Biozol, Eching, Germany), Sodiumchloride (Mallinckrodt Baker, Deventer, Netherlands).
Corning® Syringefilter (Regenerated cellulose (RC), 0.20 μm; Corning BV, Amsterdam, Netherlands), Rotilabo Spritzenfilter (RC, 0.20 μm; Carl Roth GmbH & Co KG, Karlsruhe, Germany).
Discovery® DSC-SAX SPE tube (100 mg 1 mL), Discovery® DSC-SAX SPE tube (500 mg 3 mL; Supelco, PA, USA), Strata SAX SPE tube (100 mg 1 mL), Strata SAX SPE tube (500 mg, 3 mL; Phenomenex, Aschaffenburg, Germany), HybridSPE^{TM}-Precipitation-Cartridge (30 mg, 1 mL; Sigma-Aldrich, MO, USA).

3.1.2. Swedish University of Agricultural Sciences

10-HCO-PteGlu, Schirks, Jona, Switzerland; (6S)-H_4folate, (6S)-5-CH_3-H_4folate, (6S)-5-HCO-H_4folate, PteGlu, [$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, [$^{13}C_5$]-PteGlu, Merck & Cie, Schaffhausen, Switzerland.
The following chemicals were purchased from the sources given in parentheses: Acetonitrile LiChrosolv, Hydrochloric acid fuming 37% LiChrosolv, Methanol LiChrosolv, Phosphoric acid, Potassiumdihydrogenphosphate p.a., di-Potassiumhydrogenphosphate, Potassiumhydroxide, Sodiumacetate, Sodiumchloride, Sodiumhydroxide p.a. (Merck KGaA, Darmstadt, Germany), 2-Mercaptoethanol p.a. (Merck Eurolab AB, Stockholm, Sweden), L(+)-Ascorbic acid (VWR International Fontenay-sous-Bois, France), 2,3-Dimercaptopropanol, Protease, Type XIV, from *streptomyces griseus* (Sigma-Aldrich, Steinheim, Germany), Sodiumascorbate (VWR International, Leuven, Belgium), α-Amylase (Megazyme International, Bray, Co. Wicklow, Ireland), Rat serum (Scanbur AB, Sollentuna, Sweden).
Isolute PH(EC) SPE column (500 mg, 3 mL), Isolute SAX SPE column (500 mg, 3 mL; Sorbent AB, Västra Frölunda, Sweden).

Table 3.1.: The analytical methods developed in this thesis.

Parameter	**Plasma**	**Ileostomal effluent**	**Urine**	**Breast milk**
Principle	SIDA	SIDA	SIDA	External calibration
Standard concentration	0.05–0.12 μg/mL	1.1–6.2 μg/mL	1.1–6.2 μg/mL	3.0–4.6 μg/mL
Extraction buffer	MES buffer	MES buffer	MES buffer	Phosphate buffer
Enzyme treatment	-	Di-enzyme	-	Di-enzyme
Cleanup	SPE (SAX)	SPE (SAX)	SPE (SAX)	SPE (SAX)
Quantification	LC-MS/MS	LC-MS/MS	LC-MS/MS	HPLC-UV/FD
References	*Paper I,* Mönch *et al.* 2010	*Paper I,* Mönch and Rychlik 2012	*Paper II,* Mönch *et al.* 2010	*Paper IV*

Di-enzyme: Treatment with protease and conjugase, HPLC: High performance liquid chromatography, LC-MS/MS: Liquid chromatography-tandem mass spectrometry, MES: 2-(*N*-morpholino)ethanesulfonic acid monohydrate, SAX: Strong anion exchange, SIDA: Stable isotope dilution assay, SPE: Solid phase extraction, UV/FD: UV and fluorescence detection.

3.2. Methods

Table 3.1 gives an overview over the methods developed for folate determination in different human samples in this thesis. A more detailed description follows below.

3.2.1. Folate extraction from plasma, ileostomal effluent and urine

The following buffers and enzyme preparations were used for analysis of plasma, ileostomal effluent and urine samples (*Paper I*, *Paper II*):

- 0.1 mol/L Phosphate buffer (for CP solution): 0.1 mol/L Na_2HPO_4 * 2 H_2O solution was adjusted to pH 7.4 by 0.1 mol/L KH_2PO_4 solution. The buffer was prepared on day of use.
- CP solution: 5 mg CP were solved in 30 mL 0.1 mol/L phosphate buffer containing 1% ascorbic acid at pH 7.0 and stirred for 15 minutes. The solution was filled into 2 mL Eppendorftubes and kept at $-20\,°C$ until day of use.
- Folate conjugase from RS (Biozol): RS was applied without any clean up step. Aliquots of RS were filled into Eppendorftubes and kept at $-20\,°C$ until day of use.
- MES buffer (Extraction buffer): 4.3 g MES (0.2 mol/L), 2.0 g ascorbic acid (2.0%) were solved in MQ-water. pH was adjusted to 5.0 by 1.0 mol/L NaOH. After addition of 1.4 mL MCE volume was adjusted to 100 mL by MQ-water.
- Equilibration buffer (for SPE of plasma and urine samples): 0.4 g KH_2PO_4 were solved in MQ-water and volume was adjusted to 250 mL (Part A). 0.9 g Na_2HPO_4 were solved in MQ-water and volume was adjusted to 500 mL (Part B). pH was adjusted to 7.0 by addition of part A to part B. Per 500 mL equilibration buffer 1 mL MCE (0.2%) was added.
- Equilibration buffer (for SPE of ileostomy samples): 140 mL KH_2PO_4 (3 mmol/L) were mixed with 310 mL Na_2HPO_4 (6 mmol/L). pH was adjusted to 7.5 by 1.0 mol/L NaOH. 1 mL MCE (0.2%) was added per 500 mL buffer.

- Elution buffer (for SPE): 2.5 g sodium chloride (5%), 0.5 g ascorbic acid (1%) and 0.6 g sodium acetate trihydrate (0.1 mol/L) were solved in MQ-water, 50 μL MCE (0.1%) were added and volume was adjusted to 50 mL by MQ-water.
- 0.1 mol/L Phosphate buffer (for response solution): 1.8 g Na_2HPO_4 * 2 H_2O were solved in MQ-water and volume was adjusted to 100 mL by MQ-water (Part A). 0.7 g KH_2PO_4 were solved in MQ-water and volume was adjusted to 50 mL by MQ-water (Part B). pH was adjusted to 7.0 by addition of part B to part A. For solutions of intact folate, 2 mL MCE (0.2 mol/L) were added per 150 mL buffer.

These buffers were prepared on day of use or on day before use and kept chilled below 8 °C overnight.

Table 3.2.: Concentrations of [$^2H_4$]-labelled folate standard solutions in MES buffer (pH 5.0). [μg/mL]

Folate vitamer	**Stock solution[1]**	**Working solution[2]**	
		Plasma	**Ileostomal effluent, urine**
[$^2H_4$]-H_4folate	50	0.12	1.5
[$^2H_4$]-5-CH_3-H_4folate	65	0.05	1.1
[$^2H_4$]-10-HCO-PteGlu	30		1.1
[$^2H_4$]-5-HCO-H_4folate	450	0.09	6.2
[$^2H_4$]-PteGlu	6	0.04	1.1
[$^2H_4$]-p-ABG	13		1.7
[$^2H_4$]-Ap-ABG	10		1.3

[1] The stock solution was prepared by dilution of an aliquot of a highly concentrated solution of the standard with MES buffer (pH 5.0). This dilution was not prepared in a volumetric flask, but by pipetting. Thus, no exact concentration can be given for the standard stock solutions.

[2] Likewise, standard working solutions were prepared by dilution of the stock solutions by pipetting. The exact concentration was determined by LC-MS/MS. Aliquots of the working solutions in the respective concentration were added to plasma, ileostomal effluent or urine.

[$^2H_4$]-labelled folate isotopologues were used as ISs for the determination of intact folate and folate catabolites in human samples. The synthesis of [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG followed described pathways (*Paper II*). IS stock solutions were prepared by dilution of an aliquot of a highly concentrated solution of each [$^2H_4$]-labelled standard (prepared by former co-workers) with MES buffer. For IS working solutions, IS stock solutions were diluted with MES buffer (s. Table 3.2). Folate extraction from plasma, ileostomal effluent and urine samples and extract cleanup have been described previously (*Paper I*, *Paper II*). To determine the endogenous folate content of the enzyme preparations used, blank samples were extracted and cleaned up accordingly. The purification procedures on a 12-port vacuum manifold (Visiprep, Supelco, Sigma Aldrich, Steinheim, Germany) are summarized in Table 3.3.

Table 3.3.: Summary of solid phase extraction cleanup on strong anion exchange cartridges for human samples (*Paper I*, *Paper II*).

Material, Solvent	**Plasma**	**Ileostomal effluent**	**Urine**
Cartridge size	100 mg, 1 mL	500 mg, 3 mL	500 mg, 3 mL
Elution order:			
n-Hexane[1,2]	2	2	2
Methanol[1,2]	2	2	2
Equilibration buffer[1,2]	2	2	3
		Sample application	
Equilibration buffer[1,3]	2	5	5
Elution buffer [mL]	0.5	1.5	1.0

[1] In numbers of cartridge volumes.

[2] Pre-conditioning of the cartridge.

[3] Washing of the cartridge.

Equilibration buffer for plasma and urine analysis: Phosphate buffer (pH 7.0, 0.2% β-mercaptoethanol (MCE)), Equilibration buffer for ileostomal effluent analysis: Phosphate buffer (pH 7.5, 0.2% MCE), Elution buffer: 5% sodium chloride, 1% ascorbic acid, 0.1 mol/L sodium acetate trihydrate, 0.1% MCE.

In addition to SIDA, unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine were analyzed by a simplified "Standard addition" procedure to test the suitability of standard addition as alternative for SIDA when isotopically labelled ISs are not available. First, the samples were analyzed without addition of [$^{13}C_5$]-labelled ISs for folate catabolites. Subsequently, the samples were analyzed following addition of a known amount of the [$^{13}C_5$]-labelled isotopologues of the folate catabolites. It was aimed to decrease the peak area ratio, A(unlabelled)/A([$^{13}C_5$]-labelled), obtained in the first analysis by 50%, which was considered reasonable for the calculation of the catabolite concentrations in urine. For the simplified "Standard addition" procedure, 10 g urine (pooled over 12 hours), [$^{2}H_4$]-labelled ISs (H_4folate, 5-CH_3-H_4folate and PteGlu) in amounts commensurate to the expected sample folate concentration (target peak area ratio 0.5 – 3 for standard and analyte), [$^{13}C_5$]-labelled ISs (p-ABG and Ap-ABG; only during the second extraction as described above) and 4 mL MES buffer were filled into a Pyrex glass flask. Folate extraction was accomplished as described for the SIDA for urine samples.
During development of the presented SIDAs, several modifications of preceding procedures (Mönch *et al.* 2010, Mönch and Rychlik 2012) were examined:

- Precipitation of plasma protein by chemical reagents (*Paper I*) or by cleanup on a novel HybridSPE^{TM}-Precipitation-Cartridge.
- Di-enzyme treatment for ileostomal effluent samples: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C; *Paper I*, Mönch and Rychlik 2012).
- Elution of ileostomal folate from the SPE cartridge with 1.5 mL, 2.0 mL or 1 mL+1 mL elution buffer (*Paper I*, Mönch and Rychlik 2012).
- Extraction of increased urine amounts for folate determination (*Paper II*).

3.2.2. Folate quantification in plasma, ileostomal effluent and urine

For the quantification of folate in plasma, ileostomal effluent and urine by SIDA, sample extracts and blank samples were analyzed by LC-MS/MS. Separation of the

different folate vitamers was achieved by RP-HPLC, detection by a triple quadrupol mass spectrometer in MRM mode (*Paper I*, *Paper II*). MS peaks were integrated, peak area ratios for [$^2H_4$]-labelled IS and respective analyte (A(Std)/A(Ana)) were calculated and converted to molar ratios (n(Std)/n(Ana)). Sample folate content was determined based the amount of [$^2H_4$]-labelled IS added to the sample in the beginning of the extraction. Results for unlabelled folate vitamers were corrected for the endogenous folate content of the enzyme preparations used.

For the conversion of peak area ratios into molar ratios, response factors (R_f)/ response curves were determined for intact folate and catabolites (s. Tables 3.4 and 3.5). Unlabelled and [$^{13}C_5$]-labelled analytes and respective ISs were mixed to different molar ratios from 10:1 to 1:10. These mixtures were analyzed by LC-MS/MS. Response curves were established by plotting molar ratios against peak area ratios.

Table 3.4.: Ranges of response curve linearity for analysis of plasma and urine samples (*Paper II*).

Folate vitamer (Analyte)	**n**	**Standard surplus**	**Analyte surplus**	**R_f**
H_4folate	8	20	4	0.311
5-CH_3-H_4folate	8	13	5	1.076
[$^{13}C_5$]-5-CH_3-H_4folate	9	14	7	1.054
10-HCO-PteGlu	7	57	2	0.142
5-HCO-H_4folate	8	10	9	0.573
[$^{13}C_5$]-5-HCO-H_4folate	7	7	7	0.556
PteGlu	8	20	5	0.362
[$^{13}C_5$]-PteGlu	9	20	5	0.403
p-ABG	8	5	17	0.653
[$^{13}C_5$]-p-ABG	8	13	7	0.642
Ap-ABG	8	4	25	0.709
[$^{13}C_5$]-Ap-ABG	8	4	21	0.935

n: Number of molar ratios between 10:1 and 1:10 analyzed, R_f: Response factor.

Response curve establishment was repeated after two years and R_f values were compared for each investigated vitamer (s. Table 3.5) to examine the response curve stability of the used mass spectrometer over time.
As apparent from Table 3.5, variation in R_f values over the time of two years ranged from 5% to 29%. With the exception from [$^{13}C_5$]-5-CH_3-H_4folate (change in R_f value of 29%), the change in R_f values over time did not exceed 15%, which can be considered acceptable. Nevertheless, it is preferable to repeat response curve establishment more often to (i) ensure precise results and (ii) maintain comparability of not successively determined results.

Table 3.5.: Ranges of response curve linearity for analysis of ileostomal effluent.

Folate vitamer (Analyte)	**n**	**Standard surplus**	**Analyte surplus**	**R_f**	**Change [%]**[1]
H_4folate	8	20	4	0.313	8.2
5-CH_3-H_4folate	8	14	5	1.009	8.5
[$^{13}C_5$]-5-CH_3-H_4folate	9	14	7	1.005	28.9
10-HCO-PteGlu	8	58	2	0.139	10.9
5-HCO-H_4folate	8	7	13	0.511	11.9
[$^{13}C_5$]-5-HCO-H_4folate	8	15	7	0.494	14.8
PteGlu	8	21	5	0.337	13.1
[$^{13}C_5$]-PteGlu	8	20	5	0.387	5.1

[1] Response curve stability over time was investigated by repeated response curve establishment and comparison of R_f values. Data in this column show the change in R_f in reference to the R_f determined two years earlier (*Paper I*).

n: Number of molar ratios between 10:1 and 1:10 analyzed, R_f: Response factor.

To account for moderate degradation of the [$^2H_4$]-labelled ISs due to continuous thawing and freezing of the IS working solutions, the exact concentration of each [$^2H_4$]-labelled IS working solution (s. Table 3.2) added to the sample in the beginning of the extraction was determined prior to quantification of sample folate content.
Each working solution was mixed with a solution of known concentration of the corresponding unlabelled folate isotopologue. For the solution of the corresponding

unlabelled isotopologue, it was dissolved (s. Table 3.6) and the solution (10 μg/mL) was filled into a silica glass cell. UV absorption at a specific wavelength (s. Table 3.6) was measured against the blank solvent by a spectrophotometer (SPECORD 50, Analytik Jena, Jena, Germany) to determine the concentration of the solution and the purity of the unlabelled folate.

Table 3.6.: Parameters for the determination of standard concentration and purity by spectrophotometer (Mönch *et al.* 2010, Mönch and Rychlik 2012, *Paper I*, *Paper II*).

Folate vitamer	**Solvent**	**Wavelength [nm]**	**Molecular weight [g/mol]**	ϵ **[L/(μmol$*$dm)]**
H_4folate	PM	299	445.4	27.71
5-CH_3-H_4folate	PM	290	459.4	23.71
[$^{13}C_5$]-5-CH_3-H_4f.	PM	290	464.4	23.71
10-HCO-PteGlu	0.1 M HCl	252	469.5	24.87
5-HCO-H_4folate	PM	288	473.5	23.14
[$^{13}C_5$]-5-HCO-H_4f.	PM	288	478.5	23.14
PteGlu	PM	282	441.4	27.61
[$^{13}C_5$]-PteGlu	PM	282	446.4	27.61
p-ABG	PB	275	266.1	11.4
[$^{13}C_5$]-p-ABG	PB	275	271.1	11.4
Ap-ABG	PB	266	308.1	17.1
[$^{13}C_5$]-Ap-ABG	PB	266	313.1	17.1

ϵ: Coefficient of extinction, PB: 0.1 mol/L Phosphate buffer (pH 7.0), PM: 0.1 mol/L Phosphate buffer (pH 7.0, 0.2 mol/L mercaptoethanol).

ϵ values were adopted from literature (Mönch *et al.* 2010, Mönch and Rychlik 2012).

The (measured) concentration was calculated according to equation 3.1, the purity according to equation 3.2. Based on the determined purity, the nominal concentration of the unlabelled standard solutions, *i.e.* the concentration calculated from the amount dissolved in a certain volume, was corrected.

$$Concentration\ [\mu g/mL] = \frac{Abs * MW}{\epsilon * d} \tag{3.1}$$

Abs: Measured absorption.
ϵ: Coefficient of extinction (s. Table 3.6).
MW: Molecular weight (s. Table 3.6).
d: Cell length (always 1 cm).

$$Purity[\%] = \frac{nominal\ concentration}{measured\ concentration} * 100 \tag{3.2}$$

nominal concentration: Calculated based on weighed and diluted amount.

measured concentration: Calculated based on measured UV absorption.

Then, a defined volume of the [$^2H_4$]-labelled IS working solution was mixed with a defined volume of the solution of the corresponding unlabelled folate vitamer. The mixture was diluted to the concentration expected in the sample and analyzed by LC-MS/MS. MS peaks were integrated, peak area ratios for [$^2H_4$]-labelled IS and respective unlabelled folate (A(Std)/A(unlabelled)) were calculated and converted to molar ratios (n(Std)/n(unlabelled)) based on the response curve for the analyzed vitamer in the respective sample type (s. Tables 3.4 and 3.5). The concentration of the [$^2H_4$]-labelled IS was determined based on the amount of unlabelled folate added to the mixture prior to LC-MS/MS analysis. This concentration was then used to determine the exact amount of IS added to the sample, which was required for the determination of sample folate content by SIDA.

Quality parameters, *i.e.* intra-assay coefficient of variation (CV), inter-assay CV and limit of detection (LOD) for SIDAs for intact folate and folate catabolites were determined in folate-free surrogate matrices for plasma, ileostomal effluent (Mönch *et al.* 2010, *Paper I*) and urine (Mönch *et al.* 2010, *Paper II*). LOD was assessed according to Hädrich and Vogelgesang (1996) for intact folate and based on the "Signal-to-Noise-Ratio" for folate catabolites.

Additionally, the content of unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine was determined by a simplyfied "Standard addition" procedure. Sample extracts were analyzed by LC-MS/MS. Separation was achieved by RP-HPLC, detection by a triple quadrupol mass spectrometer in MRM mode. Afterwards, all MS peaks were integrated and two different peak area ratios were calculated:

(i) ratio Y (A(unlabelled)/A([$^{13}C_5$]-labelled)) in the samples before the addition of [$^{13}C_5$]-labelled catabolite and (ii) ratio Z (A(unlabelled)/A([$^{13}C_5$]-labelled + x)) in the samples after the addition of the amount x of [$^{13}C_5$]-labelled catabolite. Under the assumption that the detected peak areas truly represent the analyte content in the sample (s. equations 3.3 and 3.5), the two ratios Y and Z were combined for the calculation of the amount of [$^{13}C_5$]-labelled catabolite per injection volume as described in equations 3.3 – 3.7. Subsequently, the content in the complete urine samples was calculated.

$$Y = \frac{A(unlabelled)}{A([^{13}C_5])} \equiv R_f * \frac{m(unlabelled)}{m([^{13}C_5])} \tag{3.3}$$

$$m(unlabelled) = \frac{Y}{R_f} * m([^{13}C_5]) \tag{3.4}$$

$$Z = \frac{A(unlabelled)}{A([^{13}C_5] + x)} \equiv R_f * \frac{m(unlabelled)}{m([^{13}C_5] + x)} \tag{3.5}$$

$$m(unlabelled) = \frac{Z}{R_f} * m([^{13}C_5] + x) \tag{3.6}$$

$$\begin{aligned} \frac{Y}{R_f} * m([^{13}C_5]) &= \frac{Z}{R_f} * m(x) + \frac{Z}{R_f} * m([^{13}C_5]) \\ \frac{m([^{13}C_5])}{R_f} * (Y - Z) &= \frac{Z}{R_f} * m(x) \\ m([^{13}C_5]) &= \frac{Z * m(x)}{Y - Z} \end{aligned} \tag{3.7}$$

Similarly, the two ratios Y and Z were combined for the calculation of the amount of unlabelled catabolite per injection volume as described in equation 3.8. Subsequently, the content in the complete urine samples was calculated.

$$\begin{aligned} \frac{m(unlabelled)}{Y} * R_f &= \frac{m(unlabelled)}{Z} * R_f - m(x) \\ \frac{m(unlabelled)}{Z} * R_f - \frac{m(unlabelled)}{Y} * R_f &= m(x) \\ m(unlabelled) &= \frac{m(x)}{\frac{R_f}{Z} - \frac{R_f}{Y}} \end{aligned} \tag{3.8}$$

3.2.3. Folate extraction from breast milk

In the buffers for extraction of folate (Patring *et al.* 2005) from breast milk, MCE was replaced by BAL (*Paper IV*). Likewise, buffers for SPE cleanup of breast milk extracts were prepared as described (Nilsson *et al.* 2004), but MCE was replaced by BAL (*Paper IV*). For the dilution of standard solutions of the investigated folate vitamers, the following buffers were prepared on day of use:

- Buffer C: 18 mL 0.6 mol/L KH_2PO_4 and 46 mL 0.3 mol/L K_2HPO_4 were mixed. The pH was checked and if necessary adjusted to 7.0 by KOH. 20 μL MCE were added and volume was made up to 500 mL by Millipore-Water (MQ-water).
- Buffer D: 18 mL 0.6 mol/L KH_2PO_4 and 46 mL 0.3 mol/L K_2HPO_4 were mixed. 5 g sodium ascorbate and 0.5 mL MCE were added. The pH was checked and if necessary adjusted to 6.1 by KOH. Volume was made to 500 mL by MQ-water.
- Buffer E: 1 g ascorbic acid was solved in 0.1 mol/L sodium acetate containing 10% sodium chloride. The pH was checked and if necessary adjusted to 4.5 by KOH. 100 μL MCE were added and volume was made up to 100 mL by 0.1 mol/L sodium acetate containing 10% sodium chloride.

Initial standard solutions (conc. 1 mg/mL) were prepared with buffer C. Standard stock and working solutions were prepared by dilution with buffers D and E (s. Table 3.7). Aliquots of the stock solutions were stored at $-80\,^{\circ}C$ for up to three months, thawed and diluted to working solutions used for calibration when needed. Determination of standard purity and concentration was one important prerequisite for the calculation of the folate concentration in the standard stock and working solutions and the correct calibration of the HPLC system used for quantification. The initial standard solutions were diluted to 10 μg/mL with buffer C and UV absorption was measured against the blank solvent as blind value by a spectrophotometer (Shimadzu UV-2101-PC, UV-VIS Scanning Spectrophotometer, Bergman Labora AB, Danderyd, Sweden). Calculations were done according to equations 3.2 and 3.1.

Table 3.7.: Concentrations of standard stock and working solutions used for calibration of the HPLC system for breast milk analysis. [μg/mL]

Folate vitamer	**Stock solution**		**Working solution**	
	Buffer for dilution	**Concentration**	**Buffer for dilution**	**Concentration**
H_4folate	E	154.0	E	4.6
5-CH_3-H_4folate	D	151.3	E	3.2
10-HCO-PteGlu	D	158.2	E	4.0
5-HCO-H_4folate	D	148.2	E	4.4
PteGlu	D	150.7	E	3.0

D: Buffer D (0.1 mol/L phosphate buffer, pH 6.1, 2% sodium ascorbate, 0.1% 2,3-dimercapto-propanol (BAL)), E: Buffer E (0.1 mol/L sodium acetate, 10% sodium chloride, 1% ascorbic acid, 0.1% BAL).

Folate extraction from breast milk and SPE cleanup followed validated procedures (Nilsson *et al.* 2004, Patring *et al.* 2005) with modifications (*Paper IV*).
The following enzyme preparations were used during the investigation of the optimal enzyme treatment for folate extraction from breast milk:

- α-Amylase: α-Amylase was applied without any cleanup step. Aliquots were filled into 15 mL-Eppendorf tubes and kept refrigerated under argon.
- Protease: Protease suspension (5 mg/mL in 0.05 mol/L phosphate buffer) was treated as described for RS (Patring *et al.* 2005).
- Rat serum: RS was treated as described previously (Patring *et al.* 2005).

Surprisingly, tri-enzyme treatment was recommended for folate extraction from breast milk (Lim *et al.* 1998) although breast milk does not contain starch. Different enzyme treatments were investigated to (i) reassess the necessity of tri-enzyme treatment, (ii) optimize protease incubation during folate extraction from breast milk and (iii) investigate whether protease treatment releases carbohydrate bound folate that can be released completely by α-amylase treatment following incubation

with protease (Öhrvik *et al.* 2010b). During setup of the presented method, the following modifications were examined:

- Tri-enzyme treatment: α-Amylase (60 μL) treatment during heat extraction (12 minutes, boiling water bath), protease treatment (2.5 mg protease, 3 hours 37 °C), deconjugation with RS (50 μL RS, 3 hours 37 °C).
- Tri-enzyme treatment including twofold treatment with α-amylase (Tri+α): During heat extraction (60 μL) and during deconjugation (40 μL).
- Incubation time and protease amount for sufficient matrix breakdown, *i.e.* 2.5 mg protease, 2 hours; 5.0 mg protease, 2 hours and 3 hours; 10.0 mg protease, 2 hours and 3 hours.
- Combined SPE cleanup on SAX and PH(EC) cartridges: Purification of the eluate from the SAX cartridge on a PH(EC) cartridge (Nilsson *et al.* 2004).

3.2.4. Folate quantification in breast milk

For the quantification of folate in breast milk, all extracts were analyzed by HPLC-UV/FD. Peak identification was achieved by retention time (RT), peak symmetry and peak area ratio (s. Table 3.8).

Table 3.8.: Identification criteria for folate vitamers investigated in breast milk (n = 7; peak symmetry determined by the ChemStation® software).

Folate vitamer	**RT [min]**	**Peak symmetry**	**A_{FD}/A_{UVD290}, A_{UVD290}/A_{UVD280} (PteGlu)**
H_4folate	18.2	0.79	15–17
5-CH_3-H_4folate	19.3	0.85	20–21
10-HCO-PteGlu	22.5	1.01	4–6
PteGlu	24.1	1.00	1.0–1.1

A_{UVD280}: Peak area in UV detector at 280 nm, A_{UVD290}: Peak area in UV detector at 290 nm, A_{FD}: Peak area in fluorescence detector, RT: Retention time.

For H_4folate, 5-CH_3-H_4folate and 10-HCO-PteGlu, peak identification was based on the ratio between the peak areas in FD and in UVD at 290 nm. For PteGlu, peak identification was based on the ratio between the peak areas in UVD at 290 nm and in UVD at 280 nm.

Quantification of H_4folate, 5-CH_3-H_4folate and 10-HCO-PteGlu was based on the signal in the FD, quantification of PteGlu on the signal at 290 nm in the UVD. Peaks were integrated and peak areas were transformed into concentrations by an external calibration curve of each vitamer.

External calibration curves were established for the examination of the relevant folate vitamers. Standard solutions (n = 8) containing the vitamers in increasing concentrations were prepared by dilution of the stock solutions with buffer E and analyzed by HPLC-UV/FD. Calibration curves were determined by plotting substance concentration (c(folate)) against resulting peak area (A(folate); s. Table 3.9).

Table 3.9.: Calibration parameters for folate quantification by HPLC-Fluorescence detection (n = 8; PteGlu: UV detection; *Paper IV*).

Folate vitamer	**Slope[1]**	**y-axis intercept**	**Linear range**	
			[ng/mL]	**[nmol/L]**
H_4folate	0.95	-0.39	1.9–92.4	4.2–207.6
5-CH_3-H_4folate	1.91	1.54	0.4–124.1	0.8–270.2
10-HCO-PteGlu	0.21	0.11	39.6–158.2	84.3–337.0
PteGlu	0.15	0.21	6.0–120.6	13.7–273.2

[1] x-Axis: Concentration [ng/mL], y-Axis: Peak area, Slope = y/x.

Quality parameters, *i.e.* linear range of the calibration, intra-assay CV, inter-assay CV and recovery from a sample spiked with H_4folate and 5-CH_3-H_4folate at 50% and 100% of the native folate content, were determined for HPLC analysis of breast milk samples (*Paper IV*). To ensure optimal method characteristics, the certified reference material CRM 421 (milk powder) was analyzed according to the final extraction and cleanup procedure described above.

3.3. Instrumental data

3.3.1. LC-MS/MS systems

The LC-MS/MS system for analysis of plasma and urine consisted of a Finnigan Surveyor Plus HPLC System (Thermo Electron, Waltham, MA, USA), which was connected to a diode array detector and a triple quadrupol TSQ Quantum Discovery mass spectrometer (Thermo Electron, Waltham, MA, USA; Mönch *et al.* 2010, *Paper II*).
The LC-MS/MS system for analysis of plasma and ileostomal effluent consisted of a Shimadzu High Performance Liquid Chromatograph (s. Table 3.10; Shimadzu Corporation, Kyoto, Japan) linked to an API 4000 Q-Trap mass spectrometer (Applied Biosystems, MDS SCIEX, CA, USA; *Paper I*).

Table 3.10.: Single devices of the Shimadzu HPLC system for analysis of plasma and ileostomal effluent.

Device	**Name**
Controller	CBM-20A Prominence
Online-Degasser	DGU-20A_3 Prominence
Solvent Delivery Module (Pump)	LC-20AD Prominence
Autosampler	SIL-20A HT Prominence
Column oven	CTO-20AC Prominence

The LC (s. Table 3.11) and MS parameters for the adopted systems have been described (Mönch *et al.* 2010, *Paper I*).

3.3.2. HPLC-UV/FD system

The HPLC system consisted of a gradient quaternary pump, a thermostated autosampler and a thermostated column compartment, a multiwavelength detector (280 nm, 290 nm) and a FD (ex/em at 290/360 nm; Agilent 1100, Agilent Technologies, USA; Patring *et al.* 2005). The LC parameters have been described previously (*Paper IV*).

Table 3.11.: Parameters for HPLC separation of folate from plasma, ileostomal effluent and urine samples in this thesis.

Parameter	**Ileostomal effluent[1]**	**Urine[2], Plasma[2]**	**Plasma[1]**
HPLC System	Shimadzu (s. Table 3.10)	Finnigan Surveyor Plus	Shimadzu (s. Table 3.10)
Column	Nucleosil C_{18} RP (250x3.0 mm, 5 μ, 100 Å)	Nucleosil C_{18} RP (250x3.0 mm, 5 μ, 100 Å)	Nucleosil C_{18} RP (150x2.0 mm, 3 μ, 100 Å)
Precolumn	C_{18} (Macherey-Nagel, Düren, Germany)	C_{18} (Phenomenex, Aschaffenburg, Germany)	C_{18} (Phenomenex, Aschaffenburg, Germany)
Mobile Phase A	1.0% Acetic acid	0.1% Formic acid	1.0% Acetic acid
Mobile Phase B	0.1% Formic acid in acetonitrile	0.1% Formic acid in acetonitrile	0.1% Formic acid in acetonitrile
Gradient	0 min 0% B, 2 min 10% B, 25 min 25% B, 27 min 100% B, 30 min 100% B, 32 min 0% B	0 min 0% B, 2 min 10% B, 25 min 25% B, 27 min 100% B, 30 min 100% B, 32 min 0% B	0 min 0% B, 2 min 10% B, 17 min 30% B, 19 min 100% B, 22 min 0% B, 23 min 0% B
Flowrate	0.3 mL/min	0.3 mL/min	0.2 mL/min
Injection volume	20 μL	10 μL	20 μL

[1] According to *Paper I.*

[2] According to Mönch *et al.* 2010, *Paper II.*

3.3.3. Preparative HPLC system

Intermediate and final products of the synthesis of $[^2H_4]$-labelled and unlabelled folate catabolites were purified by preparative HPLC (*Paper II*). The different gradients used for purification of the different substances are depicted in Figure 3.1. The organic solvents were removed from the collected fractions in the vacuum, water by lyophilisation (*Paper II*).

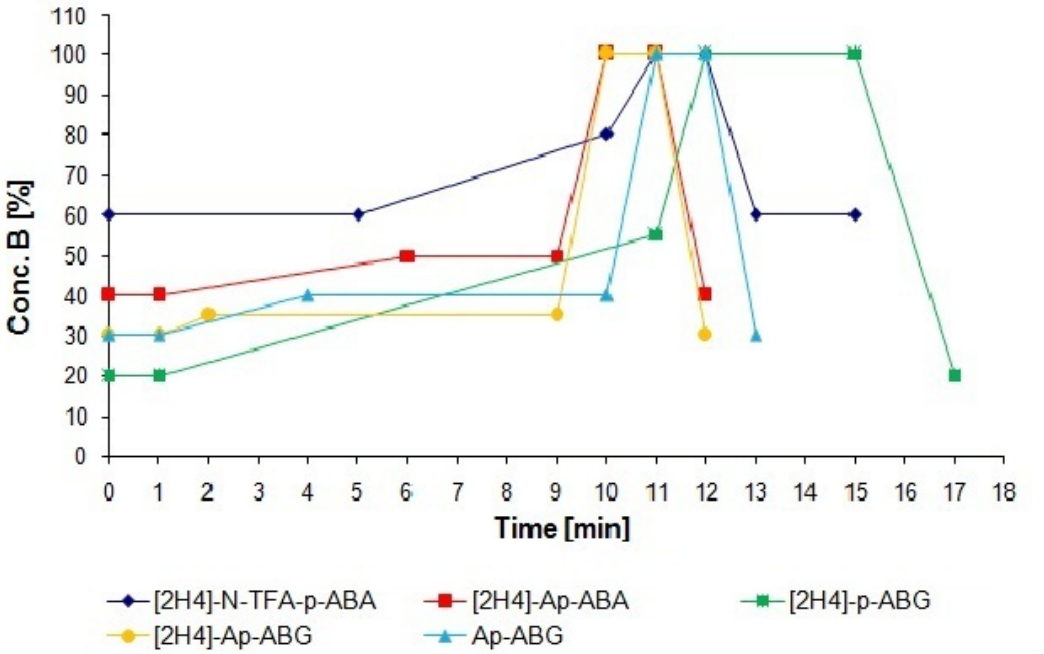

Figure 3.1.: Gradients for preparative HPLC; solvent A: 0.1% Formic acid, solvent B: Methanol; $[^2H_4]$-N-TFA-p-ABA: $[^2H_4]$-N-Trifluoracetyl-p-aminobenzoic acid, $[^2H_4]$-Ap-ABG: $[^2H_4]$-p-Acetamidobenzoyl glutamate, $[^2H_4]$-Ap-ABA: $[^2H_4]$-p-Acetamidobenzoic acid, Ap-ABG: p-Acetamidobenzoyl glutamate, $[^2H_4]$-p-ABG: $[^2H_4]$-p-Aminobenzoyl glutamate.

3.4. Study design

3.4.1. Human stable-isotope AUC/ileostomy model (*Paper III*)

This randomised cross-over study combined standardised procedures for collection of plasma, ileostomal effluent and urine samples (Witthöft *et al.* 2003 and 2006)

and methods for folate determination in human and food samples by SIDA (Rychlik *et al.* 2003, Mönch *et al.* 2010, Mönch and Rychlik 2012) to determine folate bioavailability from bread.

Wholemeal bread fortified with (6S)-[$^{13}C_5$]-5-CH_3-H_4folate or [$^{13}C_5$]-PteGlu was baked (Öhrvik *et al.* 2010b), frozen immediately and stored at -20 °C until served to the subjects. Folate content in the bread was quantified before and after the trial. Ileostomal effluent, urine and plasma were obtained from eight healthy ileostomists. Ileostomal effluent samples were frozen (-20 °C), thawed, pooled and re-frozen prior to analysis by SIDA. Urine and plasma samples were frozen and stored at -20 °C until folate extraction The study protocol was approved by the Regional Ethical Review Board in Uppsala, Sweden.

3.4.2. Folate stability during heat treatment of breast milk (*Paper IV*)

In this study, a method for folate determination in human milk, including enzyme extraction, SPE cleanup and quantification by HPLC-UV/FD, was set up. The effects of different enzyme treatment procedures on breast milk folate concentrations were compared by the "2 sample t-test" and ANOVA (Minitab® 15.1.0.0, Minitab Ltd., Coventry, UK). A two-sided $p < 0.05$ was always considered significant.

Three heat treatments were simulated for 25 mL samples (usual volume: 120 mL). For pasteurization at 57 °C and 62.5 °C at Uppsala University Hospital, Uppsala, Sweden, the treatment time (usually 30 minutes) was adjusted to the smaller sample volume in a "Down Scaling experiment". Briefly, 25 mL samples were pasteurized for 5, 12 and 22 minutes at 57 °C and 62.5 °C, respectively. The folate concentration was determined and plotted *vs.* the pasteurization time. In reference to a standard sample (120 mL, treated for 30 minutes at 57 °C or 62.5 °C), the required treatment time for the 25 mL samples was elucidated. For the simulation of the RHT treatment in the boiling water bath in the laboratory, the time required to heat breast milk from 17 °C to 73 °C was determined in two preliminary experiments (*Paper IV*). First, nine plastic extraction tubes (25 mL pooled breast milk per tube) closed by screw caps and one tube containing a thermometer were

heated to 73 °C. Second, to confirm the observed heating time of 4 minutes and 20 seconds, all tubes were sealed, heated in the boiling water bath for 4 minutes and 20 seconds and the temperature was measured immediately after removal from the water bath.

Samples of donated breast milk (n = 38; 100 mL) were transported frozen to SLU, Uppsala, Sweden and stored at -20 °C. Then, they were thawed in a refrigerator over night and divided into four subsamples (25 mL): (i) Untreated breast milk, (ii) low temperature pasteurization (57 °C, 23 minutes), (iii) Holder pasteurization (62.5 °C, 12 minutes) and (iv) RHT treatment (boiling water, 4 minutes 20 seconds). Additionally, for a secondary experiment on temporal variations in breast milk folate content, milk from one mother expressed on different days and at different times of the day was obtained (information provided anonymously). All samples were stored at -20 °C until folate determination.

4. Results and Discussion

4.1. Synthesis of folate standards

4.1.1. Relative abundance P

One criterion for the suitability of an isotopically labelled IS for the investigation of the respective unlabelled or differently labelled isotopologue (analyte) is the unequivocal spectral differentiation of IS and analyte (Rychlik and Asam 2008). The mass increment between IS and analyte has to be sufficient to preclude interferences caused by the abundance of naturally occuring isotopes.

Compared to the molecules of intact folate, the molecules of the folate catabolites p-ABG and Ap-ABG contain a lower number of C-, H-, N- and O-atoms. Therefore, the minimum mass increment for unequivocal mass spectral separation of IS and analyte is lower for folate catabolites than intact folate. For intact folate, a label resulting in a mass increment of at least three has been considered sufficient to prevent spectral overlap in LC-MS analysis (Freisleben *et al.* 2002). Hence, the mass increment of four between intended ISs and unlabelled folate catabolites or procuct ions of [$^{13}C_5$]-labelled folate catabolites introduced by a fourfold [^{2}H]-label was considered sufficient to prevent interference with the naturally occuring M+4 isotopes of the folate catabolites.

Additionally, if the mass increment of four is sufficient for unequivocal separation in one-dimensional MS, this mass increment will, together with the increased specificity in two-dimensional MS, allow for highly selective detection.

4.1.2. Synthesis pathways and yields

For the development of dual-label SIDAs for folate catabolites in urine, all target analytes along with their respective ISs have to be available. p-ABG is available commercially, the [$^{13}C_5$]-labelled catabolites have been synthesized previously (Mönch *et al.* 2010).

Contrary, Ap-ABG is not available. In a simple one-step synthesis, Ap-ABG was obtained by acetylation from p-ABG (s. Figures A.1 and A.2). The yield was satisfactory (70% after preparative cleanup) when the pH value was adjusted carefully (*Paper II*).

For the synthesis of [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG, p-ABA was deuterated (s. Figure A.4), [$^2H_4$]-p-ABA was protected (s. Figures A.5 and A.6) and coupled to L-Glu-di-methylester (s. Figures A.7 and A.8), prior to cleavage of the protection groups (s. Figures A.9 – A.12; Maunder *et al.* 1999, Freisleben *et al.* 2002, Mönch *et al.* 2010, *Paper II*). In agreement with Maunder *et al.* (1999), who found the yield for coupling of protected p-ABA to L-Glu-di-methylester and removal of protection groups to be 60 – 70% and 50%, respectively, the total yield for coupling and cleavage of the protection groups was as low as 30% (after preparative cleanup; *Paper II*). Several explanations are possible: (i) the reaction product was trapped in a precipitate built during coupling, (ii) the extraction of the product from the synthesis suspension was incomplete and (iii) some product was lost during preparative purification.

4.1.3. Studies by MS and NMR spectroscopy

The mass shift from *m/z* 138 to *m/z* 142 (s. Figure A.4) clearly revealed the incorporation of four [^{2}H]-atoms into the p-ABA molecule. But the rate of fourfold incorporation was incomplete. The isotopologic distribution in the mass spectrum indicated 58% of fourfold labelled, 32% of threefold labelled and 10% of at least onefold labelled p-ABA (*Paper II*). The exact rate of incorporation of four [^{2}H]-atoms was determined as 75% by NMR (nuclear magnetic resonance) spectroscopy (*Paper II*). Despite a higher number of deuteration cycles, the rate

of incorporation was lower than described (Freisleben *et al.* 2002). In contrast to synthesis in an autoclave at 200 °C (Freisleben *et al.* 2002), we used Pyrex® glass tubes at 140 °C, which meant lower pressure and temperature and may account for the lower rate of incorporation observed.

A comparison of chemical shift, shape and integrals of the signals in the obtained ^{1}H-NMR spectra with literature (Maunder *et al.* 1999, Mönch *et al.* 2010; spectra: s. *Paper II*; s. Table 4.1) proofed the synthesized compounds to be Ap-ABG, [$^{2}H_4$]-p-ABG and [$^{2}H_4$]-Ap-ABG. Due to incomplete deuteration, one singlet remained between 7.6 and 7.9 ppm in the ^{1}H-NMR spectra of the [$^{2}H_4$]-labelled catabolites.

Table 4.1.: Signals in NMR and mass spectra of the synthesized folate catabolites.

	Ap-ABG	**[$^{2}H_4$]-p-ABG**	**[$^{2}H_4$]-Ap-ABG**
	Signals in NMR spectra (integral)		
Glutamyl protons	d*	d*	d*
Aromatic protons	d*	u	u
Acetyl proton	d*	n*	d*
	Base peaks in mass spectra (positive ESI mode)		
One-dimensional MS ($[M+H]^+$)	309.1	271.1	313.1
MS using $[M+H]^+$ as the precursor ion ($[M+H-147]^+$)	162.2	124.2	166.2

d: Signals detected, n: Signals not detected, u: Unexpected detection of a singlet due to incomplete deuteration, *: In agreement with literature (Maunder *et al.* 1999, Mönch *et al.* 2010).

ESI: Elektrospray ionisation, $[M+H]^+$: Protonated molecular ion, $[M+H-147]^+$: Protonated fragment ion (loss of the glutamyl-moiety), MS: Mass spectrometry, NMR: Nuclear magnetic resonance.

The base peaks detected in the mass spectra of the synthesized folate catabolites (s. Table 4.1) are in good agreement with reported observations (Freisleben *et al.* 2002, Patring and Jastrebova 2007, Mönch *et al.* 2010).

Precursor and product ions of [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG contained the [$^2H_4$]-label constantly (s. Figures A.10, A.12 – A.14; *Paper II*). Unequivocal spectral separation was possible for (i) precursor and product ions of unlabelled and respective [$^2H_4$]-labelled catabolites and (ii) product ions of [$^{13}C_5$]-labelled and respective [$^2H_4$]-labelled catabolites (*Paper II*).
In summary, the folate catabolites Ap-ABG, [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG were synthezised successfully and were suitable ISs for the simultaneous quantification of differently labelled folate by LC-MS/MS in MRM mode.

4.2. Development of SIDAs for analysis of human samples

4.2.1. Precipitation of plasma protein

Precipitation of plasma protein during folate extraction is achieved by heat (Hart *et al.* 2002, Wright *et al.* 2003) or chemical reagents (Nelson *et al.* 2004) to prevent difficulties in analysis, *e.g.* clotting on the HPLC column.
Besides the described chemical precipitation reagents (s. Table 2 in *Paper I*), a novel SPE technology especially developed for plasma analysis was tested. HybridSPE^{TM}-Precipitation-Cartridges utilise zirconia-coated particles and exhibit selective affinity towards phospholipids, which possibly cause ion suppression (Sigma-Aldrich, Steinheim, Germany).
Reagents containing formic acid were not suitable because they clogged the SPE cartridges thus prolonging the duration of the cleanup procedure. The greater exposure to light, oxygen and room temperature led to increased folate losses (*Paper I*). Additionally, a modified elution buffer caused folate degradation as indicated by a double peak in the chromatogram (s. Figure B.1; *Paper I*). Among the other tested reagents, the use of MeOH resulted in the highest peak intensity (18000 cps; s. Figure B.1). After cleanup on a novel HybridSPE^{TM}-Precipitation-Cartridge, peak intensity was poor. For 5-CH_3-H_4folate, the main folate in plasma, peak height was 350 cps compared to 14000 – 18000 cps after protein precipitation by a chemical reagent and cleanup on SAX cartridges (s. Figures B.1 and B.2). It was concluded

that protein precipitation was achieved most efficiently when using 100% MeOH as precipitation reagent to give a final MeOH concentration of 60% (*Paper I*).
HPLC column lifetime was improved from two months (300 runs) to one year (1200 runs) by precipitation of plasma protein during folate extraction. In contrast to preceding methods (Rychlik *et al.* 2003, Mönch et al. 2010), the use of a smaller column (diameter: 2.00 mm; particle size: 3 μ) which may be more susceptible to clogging required precipitation of plasma protein during folate extraction for (i) optimal LC analysis and (ii) to maintain a high throughput of samples, an essential prerequisite for comparable results in large trials.
In summary, protein precipitation was easy to achieve and sensitivity remained high, as reported before (Nelson *et al.* 2001).

4.2.2. Preparation of ileostomal effluent samples

The effect of mono- and di-enzyme treatment on folate extraction from ileostomal effluent was examined by a comparison of absolute peak areas (s. Table B.1), peak area ratios (s. Table B.2) and baseline interferences in the single mass traces.
As indicated by higher peak intensity (s. Figures B.3— B.5), peak area increased after di-enzyme treatment (s. Table 4.2). There were some exceptions from this trend (s. Table 4.2), which were spread randomly over the different folate vitamers and samples, but did not show a distinct tendency. Hence, a systemic error in the extraction procedure or a negative effect of di-enzyme treatment on a single folate vitamer could be precluded.
Except from sample 3 (s. Table 4.2), peak area ratios remained constant within a range of 20%. The peak areas of analytes and ISs changed similarly after protease treatment. Compared to mono-enzyme treatment, less matrix peaks were detected in the baseline of the single mass traces after di-enzyme treatment (s. Figures B.3— B.5).
As peak area ratios remained constant, folate release from ileostomal effluent was not higher after matrix break down by protease. An improved extraction should affect ISs only marginally and cause alterations in the peak area ratio. Higher peak intensity and area for both analytes and ISs, were mainly attributable to reduction

of matrix interferences, which is in good agreement with reported effects of protease treatment in food (Mönch and Rychlik 2012). The stable peak area ratios proofed the time for equilibration of analyte and IS prior to enzyme treatment to be sufficient. Additionally, systematic errors, *e.g.* discrimination of analyte or added standard, in the method could be precluded with respect to protease treatment.
There were no restrictions to the comparison in this experiment (s. Table 4.2) because the observed changes were completely attributable to protease treatment. As it improved assay sensitivity, protease treatment (4 hours, 37 °C) was included into the extraction procedure of folate from ileostomal effluent.

Table 4.2.: Changes in peak area and peak area ratio (A(Std)/A(Ana)) after di-enzyme treatment of three ileostomal effluent samples (number 1–3) in relation to mono-enzyme treatment. [%]

Folate vitamer	**Peak area**			**Peak area ratio**		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
H_4folate[1]	22	51	385	n.c.	n.c.	n.c.
5-CH_3-H_4folate	11	**-4**	**-49**	0	-5	-89
[$^2H_4$]-5-CH_3-H_4folate	13	1	400	#	#	#
[$^{13}C_5$]-5-CH_3-H_4folate	0	n.d.	900	0	n.d.	100
10-HCO-PteGlu	39	**-40**	3900	-14	6	-11
[$^2H_4$]-10-HCO-PteGlu	29	**-45**	4300	#	#	#
5-HCO-H_4folate	**-8**	31	7200	0	14	2900
[$^2H_4$]-5-HCO-H_4folate	4	18	120	#	#	#
PteGlu	5	29	**-15**	-13	15	-37
[$^2H_4$]-PteGlu	17	8	33	#	#	#
[$^{13}C_5$]-PteGlu	14	27	n.d.	0	18	n.c.

[1] [$^2H_4$]-H_4folate was degraded. No peak area ratios were calculated.

Standard for the calculation of the respective unlabelled and [$^{13}C_5$]-labelled isotopologues.

n.c.: Not calculated, n.d.: Not detected.

The bold face typed numbers indicate where decreased peak areas were detected after di-enzyme treatment.

Additional enzyme treatment steps increase the risk for folate losses because of the prolonged exposure to light, oxygen and elevated temperatures. High folate degradation may diminish the positive effects of matrix break down, *e.g.* enhanced extraction. Buffers with protective properties are used to avoid folate losses. MES buffer has been shown to protect folate well during deconjugation (Mönch and Rychlik 2012) and SPE (Mönch *et al.* 2010). In the present experiment, MES buffer protected folate well from degradation during protease treatment. This can be seen from the peak areas of the [$^2H_4$]-labelled ISs which remained stable within the range of 20% during the prolonged extraction procedure (s. Table B.1).

In food (Tamura *et al.* 1997) and breast milk (Lim *et al.* 1998), folate extraction was most complete after tri-enzyme treatment. Treatment with α-amylase seemed unreasonable for ileostomal effluent as the break down of carbohydrates in the human body starts in the mouth and is almost complete after passage of the stomach (Löffler *et al.* 2007). Samples of ileostomal effluent are taken later in human digestion.

The difference between two elution procedures from the SPE cartridge (1.5 mL or 1 mL+1 mL) was examined by a comparison of absolute peak areas (s. Table B.3) and peak area ratios (s. Table B.4).

Eluting with 1 mL+1 mL elution buffer, the largest peak areas were detected in the first mL (I) for naturally occurring folate whereas the peak areas for PteGlu reached a maximum in the second mL (II). Peak areas for naturally occurring folate in the extracts eluted with 1.5 mL elution buffer were similar to those detected in I. In contrast, peak areas for PteGlu in the extracts eluted with 1.5 mL elution buffer were higher than those in II. Peak area ratios remained constant within the range of 20% in all experiments (s. Table B.4). The peak areas of analytes and ISs were affected similarly by the different elution procedures. Again, systematic errors, *e.g.* discrimination of analyte or added standard, in the method could be precluded with respect to SPE.

Based on the observation of reverse trends for natural folate and PteGlu after the 1 mL+1mL procedure and the results after elution with 1.5 mL buffer, it was concluded that the latter procedure was optimal for SPE cleanup of ileostomal effluent samples because it combined the optima for both naturally occuring folate and PteGlu. The reduction of the elution volume during the SPE sample cleanup

from 2.0 mL (Mönch and Rychlik 2012) to 1.5 mL increased peak intensity and, thus, sensitivity of the analysis (*Paper I*).
Besides evaluation of peak areas, results were based on calculation of peak heights (s. Tables B.5 and B.6) supporting the outcome as clearly as peak areas. Hence, peak heights can be considered a useful measure for rough estimations. Evaluation based on peak areas should still be preferred as the peak area represents the whole amount of analyte in the sample while the peak height only represents the amount at a single moment and is not as precise.
In summary, protease incubation and reduction of the elution volume during SPE during folate extraction from ileostomal effluent increased the signal intensity and improved assay sensitivity clearly.

4.2.3. Determination of folate catabolites in urine

Prior to analysis of urine samples from a human folate bioavailability trial, the suitability of a previously published extraction method (Mönch *et al.* 2010) for this purpose was checked. Minor modifications, *i.e.* extraction of increased amounts of urine (10 g) and bigger SPE cartridges (500 mg, 3 mL; *Paper II*), increased the sensitivity of the method considerably (s. Table B.7). Especially detection of Ap-ABG, the main folate catabolite in urine, was improved.
Following the adjustment of the extraction procedure, research focused on the simultaneous quantification of unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine. As differently labelled ISs were not available in the beginning of this doctorate, [$^{13}C_5$]-p-ABG and [$^{13}C_5$]-Ap-ABG could not be determined in urine by SIDA according to the preceding method (Mönch *et al.* 2010).
Therefore, [$^{13}C_5$]-labelled folate catabolites were initially quantified by a simplified "Standard addition" procedure. Addition of [$^{13}C_5$]-p-ABG and [$^{13}C_5$]-Ap-ABG prior to extraction, decreased the peak area ratio, A(unlabelled)/A([$^{13}C_5$]-labelled), by 47.6% (median, range: 4.0 – 76.7%, n = 32). This met the pursued decrease of 50% (s. page 53) well and the catabolite content in urine was calculated according to equations 3.7 and 3.8. But, this procedure was (i) tedious, (ii) time consuming and (iii) the equations were complicated and prone to errors.

Following the successful synthesis of the [$^2H_4$]-labelled folate catabolites, SIDAs for the simultaneous determination of unlabelled and [$^{13}C_5$]-labelled p-ABG and Ap-ABG in urine were developed (*Paper II*). It was then possible to overcome the shortcomings of the simplified "Standard addition" procedure.
In a preliminary study, unlabelled and [$^{13}C_5$]-labelled catabolites were quantified in urine (n = 3) by both procedures (s. Table B.8) to (i) compare the methods (s. Table 4.3) and (ii) test the new SIDA's suitability for human trials.

Table 4.3.: Difference between the contents of unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine determined by simplified "Standard addition" procedure and stable isotope dilution assay. [%]

Sample	**p-ABG**	**[$^{13}C_5$]-p-ABG**	**Ap-ABG**	**[$^{13}C_5$]-Ap-ABG**
A	283	500	390	260
B	150	100	338	2550
C	575	600	365	244

Folate content in urine quantified by both methods is summarized in Table B.8. The differences are presented in relation to the catabolite content determined by stable isotope dilution assay.

p-ABG content determined by SIDA (ca. 20 nmol/day) was in good agreement with reported results (Mönch *et al.* 2010) while Ap-ABG content (ca. 120 nmol/day) was lower than reported. Mönch *et al.* (2010) collected the urine samples after ingestion of unlabelled folate affecting the content of unlabelled catabolites in urine. The samples examined here were collected after ingestion of [$^{13}C_5$]-labelled folate, which does not affect the content of unlabelled catabolites but leads to the appearance of [$^{13}C_5$]-labelled catabolites instead. In the former study, urine was collected for 24 hours, while in the latter study, urine collection stopped after twelve hours. Therefore, it seems logical that the latter results for Ap-ABG were lower. Additionally, Ap-ABG content in the former study was reported to be high (Mönch *et al.* 2010). The SIDA results for p-ABG and [$^{13}C_5$]-p-ABG were around the LOD estimated based on the "Signal-to-Noise-Ratio" in the chromatograms of successively diluted spiked blank samples (0.5 μg/100 g and 0.09 μg/100 g, respectively). As statistically determined LOD values are generally below estimated values, the SIDA

results were considered reliable. The low results for p-ABG and [$^{13}C_5$]-p-ABG did not compromise the validity of the comparison between "Standard addition" and SIDA. This assumption should be verified by the statistical determination of the LOD (Hädrich and Vogelgesang 1996).
Results by SIDA were 77.0% (median, range: 57.5 – 96.9%, n = 12) lower than results by the simplified "Standard addition" procedure. The simplification of the "Standard addition" procedure may account for the observed discrepancy. Usually, the sample is spiked with increasing amounts of standard, the peak area ratio decreases stepwise and a calibration curve is established by plotting amount of added standard to decrease of peak area ratio. Using several samples increases the precision of the calibration as an incorrect result can be detected and excluded. Contrary, in this study, the quantification was based on the peak area ratio alteration after a single addition of standard because the amount of urine available did not allow for the extended procedure. Furthermore, determination of the method's range of linearity reveals the decrease in peak area ratio to pursue by standard addition. In this study, it was blindly aimed for a 50% decrease, which may be unsuitable.
For future use of the "Standard addition" procedure, further investigations are required, *e.g.* minimum number of calibration samples or optimum decrease in peak area ratio. If the observed discrepancy can be explained, the "Standard addition" procedure should be preferred over non-isotopic methods for mass spectral folate determination as it enables correction for inter-sample variations in ionization yield (Bailey 2010).
In summary, of the two methods for simultaneous quantification of unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine, SIDA was more sensitive, required less work load and produced reliable results. The new SIDA's sensitivity is suitable for the determination of folate catabolites in urine derived from human studies.

4.2.4. LC-MS/MS analysis

Acetic acid (1% in MQ-water) in the mobile phase led to remarkably increased (i) intensity of the MS signals and, thus, (ii) sensitivity of the LC-MS/MS system for analysis of plasma and ileostomal effluent. The range of sensitivity of the preceding

method using a different mass spectrometer (Mönch *et al.* 2010) was reached. The superiority of acetic acid over formic acid has been described previously (Patring and Jastrebova 2007).

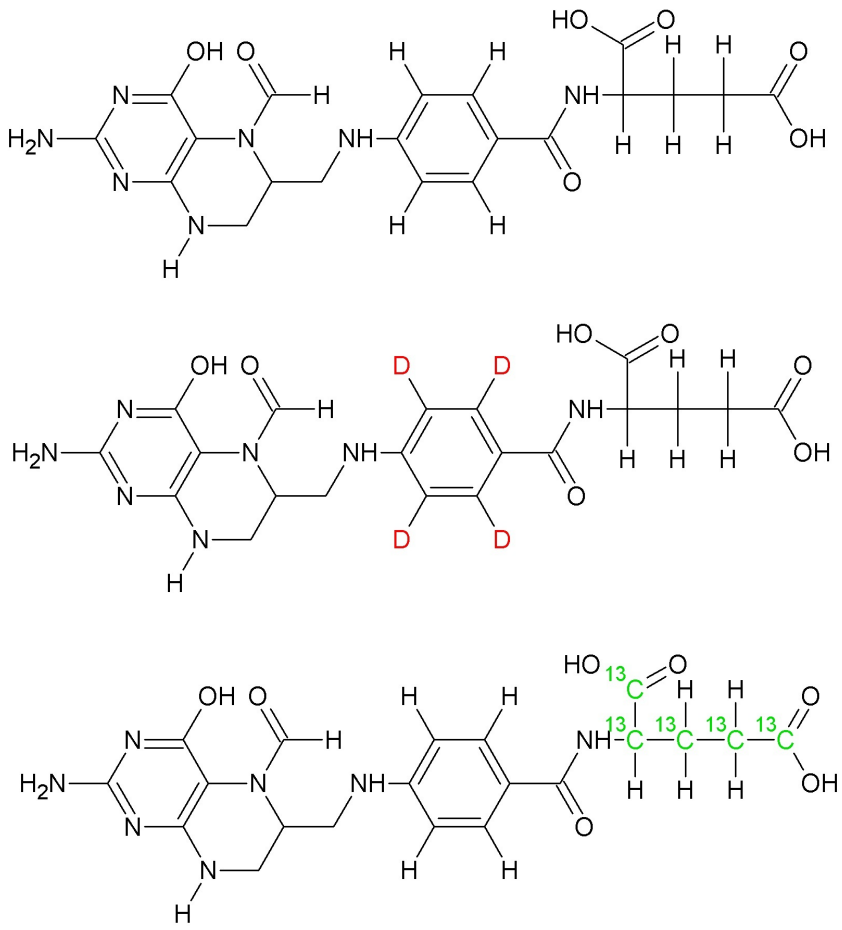

Figure 4.1.: Structures of 5-HCO-H_4folate, [$^2H_4$]-5-HCO-H_4folate and [$^{13}C_5$]-5-HCO-H_4folate (from top to bottom; D: [^{2}H]).

MS detection in MRM mode enabled unequivocal differentiation between precursor and product ions of [$^2H_4$]-labelled ISs and unlabelled and [$^{13}C_5$]-labelled analytes even when the mass increment for either the precursor ions or the product ions was not sufficient for clear separation in single stage MS: (i) Vitamers that form product ions of the same *m/z*, *e.g.* 5-HCO-H_4folate and [$^{13}C_5$]-5-HCO-H_4folate (*m/z* 327.2), were separated by their different precursor ions (*m/z* 474.1 and 479.1, respectively; s. Figure 4.1); (ii) vitamers with precursor ions of the same *m/z*, *e.g.* 5-HCO-H_4folate

and [$^2H_4$]-10-HCO-PteGlu (*m/z* 474.1) were separated by their different product ions (*m/z* 327.2 and 299.2, respectively); (iii) vitamers with an insufficient mass increment between their precursor ions, *e.g.* [$^2H_4$]- and [$^{13}C_5$]-labelled isotopologues (one mass unit), were separated by their product ions (four mass units; s. Figure 4.1; *Paper I*, *Paper II*).
Additionally, detection of characteristic cleavage reactions minimizes interferences with matrix compounds and increases method accuracy. This was confirmed by the quality parameters of the presented methods (*Paper I*, *Paper II*).

4.3. Bioavailability of folate from wholemeal bread

Using a novel stable-isotope AUC/ileostomy model, (i) the bioavailability of [$^{13}C_5$]-5-CH_3-H_4folate and [$^{13}C_5$]-PteGlu from wholemeal bread and (ii) the influence of a breakfast matrix on the bioavailabilty of [$^{13}C_5$]-PteGlu from wholemeal bread were investigated (*Paper III*).

4.3.1. Folate content in plasma and ileostomal effluent

The newly advanced SIDAs were applied successfully to the simultaneous quantification of unlabelled endogenous folate and [$^{13}C_5$]-labelled dose folate in the obtained plasma and ileostomal effluent samples.
Independent of the [$^{13}C_5$]-labelled folate ingested, only [$^{13}C_5$]-5-CH_3-H_4folate was detected in plasma. Bioconversion of [$^{13}C_5$]-PteGlu (450 nmol; s. Table 4.4) into [$^{13}C_5$]-5-CH_3-H_4folate appeared complete (*Paper III*). The [$^{13}C_5$]-5-CH_3-H_4folate response was described by a plasma response curve (s. Figure C.1). Besides [$^{13}C_5$]-5-CH_3-H_4folate, unlabelled 5-CH_3-H_4folate, PteGlu and H_4folate were detected in plasma (92%, 7% and 2% of unlabelled folate, respectively; *Paper III*). After ingestion of [$^{13}C_5$]-5-CH_3-H_4folate-fortified bread (BM study day), the median plasma $AUC_{0\rightarrow12}$ of [$^{13}C_5$]-5-CH_3-H_4folate was about twice as high as the median plasma $AUC_{0\rightarrow12}$ after ingestion of [$^{13}C_5$]-PteGlu-fortified bread alone (BP study day) or with a breakfast meal (FP study day; s. Figure C.1). Also, the dose-

normalized AUC supported this trend (s. Table 4.4). Likewise, [$^{13}C_5$]-c_{max} and [$^{13}C_5$]-t_{max} differed significantly after ingestion of reduced and oxidized folate (s. Table 4.4; *Paper III*). The observed discrepancies between [$^{13}C_5$]-5-CH_3-H_4folate and [$^{13}C_5$]-PteGlu might partly be explained by a less precise estimate of the kinetic parameters after ingestion of [$^{13}C_5$]-PteGlu: Sampling was less frequent at t_{max}, which may have led to underestimation of c_{max} and AUC (*Paper III*).

Table 4.4.: Kinetic plasma parameters of [$^{13}C_5$]-5-CH_3-H_4folate after a single oral dose (median (range); n = 8; *Paper III*).

Parameter	**BM**	**BP**	**FP**
[$^{13}C_5$]-dose [nmol]	464 (450–467)	451 (448–472)	462 (448–471)
[$^{13}C_5$]-c_{max}[1] [nmol/L]	10.6 (6.3–16.5)	3.1 (1.7–4.5)	3.3 (2.1–5.7)
[$^{13}C_5$]-t_{max}[2] [min]	40 (40–90)	150 (90–240)	240 (120–240)
[$^{13}C_5$]-$AUC_{0\rightarrow12}$ [nmol∗h/L] per nmol[3]	0.15 (0.07–0.54)	0.07 (0.05–0.17)	0.06 (0.03–0.20)

[1] Maximum plasma concentration.

[2] Time after ingestion of the test dose when the maximum concentration was reached.

[3] Dose normalized.

$AUC_{0\rightarrow12}$: Area under the plasma response curve from 0 to 12 hours post dose, BM: Wholemeal bread fortified with [$^{13}C_5$]-5-CH_3-H_4folate ingested with pear sauce and a glass of water, BP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with pear sauce and a glass of water, FP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with a breakfast meal consisting of liver pâté, orange juice, cornflakes and fermented milk.

The ileostomal effluent contained [$^{13}C_5$]-labelled folate (5-CH_3-H_4folate and PteGlu from the test dose) and unlabelled H_4folate, 5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate and PteGlu (s. Figures C.2 – C.4).

The content of [$^{13}C_5$]-labelled folate in the ileostomal effluent collected during 24 hours post dose ranged from 2% to 9% of the dose (18.7 (1.0 – 42.2) nmol)

and did not differ between ingested folate vitamer or food matrix, *i.e.* bread or breakfast meal (*Paper III*). The majority of [$^{13}C_5$]-labelled folate was detected in the ileostomal effluent collected during twelve hours post dose (s. Figure C.2). After ingestion of [$^{13}C_5$]-PteGlu, trace amounts of [$^{13}C_5$]-5-CH_3-H_4folate (0.1% of the dose) were detected, mainly in the ileostomal effluent collected during twelve hours post dose (s. Figure C.2). As LC-MS/MS analysis had confirmed the absence of [$^{13}C_5$]-5-CH_3-H_4folate in the [$^{13}C_5$]-PteGlu fortified bread, ileostomal [$^{13}C_5$]-5-CH_3-H_4folate was suggested to originate from bioconvertion of [$^{13}C_5$]-PteGlu (*Paper III*), which may derive from (i) bile or (ii) lysed enterocytes (Gregory III and Quinlivan 2002).

The content of unlabelled folate in ileostomal effluent collected during twelve hours post dose (103 (43 – 178) nmol) did not differ significantly between the three study days (s. Figures C.3, C.4). During this period, subjects were served standardized snacks to controll folate intake (BM, BP: 120 nmol, FP: 340 nmol).

While about 70% of the folate ingested during twelve hours post dose carried the [$^{13}C_5$]-label, only $< 20\%$ of the folate in ileostomal effluent collected during twelve hours post dose carried the [$^{13}C_5$]-label. This suggests that only one third of the ileostomal folate derived from recently ingested food (*Paper III*).

4.3.2. Determination of folate bioavailability by a human stable-isotope AUC/ileostomy model

In agreement with others (Konings *et al.* 2002, Wright *et al.* 2003), we observed differences in absorption kinetics between [$^{13}C_5$]-5-CH_3-H_4folate and [$^{13}C_5$]-PteGlu. Bioconversion of PteGlu prior to systemic transportation (Chanarin and Perry 1969) required more time than the immediate transport of 5-CH_3-H_4folate into the systemic circulation, which may account partly for the significant delay in t_{max} after ingestion of [$^{13}C_5$]-PteGlu. Additionally, the liver has been suggested to be the main site for bioconversion of PteGlu (Wright *et al.* 2007). As liver accumulation is greater for oxidized folate than reduced folate (Steinberg *et al.* 1979), more PteGlu would be retained and less PteGlu than reduced folate would appear in the systemic circulation.

[$^{13}C_5$]-labelled folate content in ileostomal effluent was low indicating a high absorption of the test dose (*Paper III*). This finding is in agreement with other reports on high relative, fractional or true absorption of orally administered folate (Konings *et al.* 2002, Lin *et al.* 2004, de Meer *et al.* 2005). Contrary, the endogenous folate from the bread ingredients, *e.g.* flour or yeast, may be trapped in the bread matrix while the added [$^{13}C_5$]-labelled folate is absorped to a higher extent. If this is true, the absorption of [$^{13}C_5$]-labelled folate determined based on the low content of [$^{13}C_5$]-labelled folate in the ileostomal effluent does not represent the absorption of natural food folate correctly. When assessing folate absorption from food, possible differences between endogenous folate and added folate need to be considered.
Several factors affect folate bioavailability from food, *e.g.* ingested vitamer or food matrix (s. Table 1.2). The present study revealed (i) significant differences in absorption between reduced and oxidized folate and (ii) similar effects on bioavailability of [$^{13}C_5$]-PteGlu from wholemeal bread when the bread was ingested alone or as part of a breakfast meal (*Paper III*). Folate bioavailability was estimated as about 90% (*Paper III*).
The plasma response to oral folate doses has been studied widely (Konings *et al.* 2002, Rychlik *et al.* 2003, Witthöft *et al.* 2006).Trials using unlabelled dose folate that can not be differentiated from endogenous folate may face difficulties in interpretation (Wright *et al.* 2003). The new SIDAs allowed application of [$^{13}C_5$]-labelled test doses and differentiation of dose folate from endogenous folate, which enabled (i) to gain further insight in human plasma folate kinetics, (ii) to follow [$^{13}C_5$]-labelled folate during 24 hours, despite additional intake of unlabelled folate twelve hours post dose and (iii) to investigate folate bioavailability from bread.

4.4. Folate stability during heat treatment of breast milk

In the presented trial, (i) the folate content and pattern in Swedish milk banking samples and (ii) the effect of different heat treatment procedures on breast milk folate content were investigated. For this purpose, the in-house method for folate extraction at SLU (Patring *et al.* 2005) was adapted to the analysis of breast milk.

4.4.1. Preparation of breast milk samples

Pooled unpasteurized breast milk was used for method adaptation. PteGlu was not detected in this sample. Because of a co-eluting compound, 5-HCO-H_4folate was not detectable. 5-HCO-H_4folate was not considered to contribute significantly to the folate content in breast milk. Therefore, the interfering compound was not investigated further and 5-HCO-H_4folate was excluded from all investigations.
First, the conditions for enzyme treatment of breast milk samples were optimized. In contrast to Lim *et al.* (1998), who quantified folate by MA, tri-enzyme treatment including α-amylase treatment did not increase analyzed folate contents (sum of folates) in breast milk compared to di-enzyme treatment (s. Table 4.5). However, higher 5-CH_3-H_4folate contents were detected after treatment with protease and α-amylase ($p < 0.05$). As the finding that di-enzyme treatment was sufficient for complete folate extraction from breast milk could be confirmed, treatment with protease and RS only was chosen for preparation of breast milk samples.

Table 4.5.: Folate concentrations in a pooled breast milk sample after different types of enzyme treatment (mean ± Stdev; n = 3). [nmol/L]

Folate vitamer	**Type of enzyme treatment**			
	Mono[1]	**Di[2]**	**Tri[3]**	**Tri+α[4]**
H_4folate	29.9±2.5[a]	32.1±2.5[a]	30.9±4.6[a]	33.1±0.4[a]
5-CH_3-H_4folate	55.5±3.6[a]	65.9±5.1[b]	72.2±5.4[b]	73.4±1.2[b]
10-HCO-PteGlu	4.1±0.9[a]	4.3±0.1[a]	4.9±0.3[a]	5.3±1.5[a]
Sum of folates	89.6±3.6[a]	102.3±8.3[b]	108.0±10.2[b]	111.8±2.3[b]

[1] 50 μL rat serum (RS) 3 hours 37 °C.

[2] 2.5 mg protease 3 hours 37 °C, 50 μL RS 3 hours 37 °C.

[3] 60μL α-amylase during heat extraction, 2.5 mg protease 3 hours 37 °C, 50 μL RS 3 hours 37 °C.

[4] 60μL α-amylase during heat extraction, 2.5 mg protease 3 hours 37 °C, 50 μL RS + 40 μL α-amylase 3 hours 37 °C.

Sum of folates: Sum of H_4folate, 5-CH_3-H_4folate and 10-HCO-PteGlu content.

Folate concentrations were corrected for endogenous folate content in enzyme preparations.

Values with different letters superscript in the same row were significantly different ($p < 0.05$).

The current study results do not suggest the need for intensive enzyme treatment prior to quantification. The discrepancy between previous results (Lim *et al.* 1998) and results presented in this thesis may be explained by application of (i) enzymes from different companies (different sources, different extraction techniques, different activity; inter-batch variation) and (ii) different folate quantification methods (MA *vs.* HPLC-UV/FD). The growth response of the testorganism used in MA is affected by all folate vitamers, various antifolates and other factors in the sample extract (Lim *et al.* 1998, Pfeiffer *et al.* 2010) whereas HPLC-UV/FD methods quantify a limited number of individual folate vitamers.

For optimal protease treatment, incubation time and enzyme amount were investigated (s. Tables D.1 and D.2). Incubation with 5.0 mg protease per 7 g breast milk resulted in significantly higher folate concentrations (sum of folates) than incubation with 2.5 mg protease per 7 g sample (s. Table D.1). Treatment with 10.0 mg enzyme per 7 g breast milk did not result in further increased folate concentrations (s. Table D.2). Folate concentration (sum of folates) increased with incubation time (s. Table D.1). But, there were no significant differences between di- and tri-enzyme treatment (s. Table D.1). For optimal protease treatment, 7 g of breast milk were incubated with 5.0 mg enzyme for 3 hours at 37 °C.

Second, variations in the SPE cleanup procedure were tested to increase the method's sensitivity. The chromatograms after SPE cleanup on SAX cartridges showed a high number of matrix peaks. After combined cleanup on SAX and PH(EC) cartridges (Nilsson *et al.* 2004), chromatogram clarity was improved slightly as determined by visual examination (s. Figures D.1 and D.2). Identification of the peaks of interest was marginally easier as some (matrix) peaks close to the peaks of interest were removed. Yet, folate concentration in the eluat after combined cleanup was too low for reliable quantification (s. Figure D.3). Therefore, SPE cleanup on SAX columns only was chosen. Improvement of assay sensitivity by the combination of SAX and PH(EC) cartridges did not vindicate the high financial and time requirements of the procedure.

Based on these experiments, folate extraction from breast milk was accomplished by heat extraction, protease treatment, conjugase treatment and SPE cleanup on SAX cartridges (*Paper IV*).

4.4.2. Adjustment of the heat treatment procedures

For financial and ethical reasons, this trial was based on the simulation of the treatments with smaller than usual volumes of breast milk. Considering the high value of donor milk in the health care of premature born infants, it was not arguable to work with the regular volume (100 – 120 mL) when folate determination only required a few millilitres while the rest would remain unused. To keep the trial results transferable to usual conditions, the treatment time for the investigated procedures was adjusted to smaller volumes (25 mL; *Paper IV*).
In a "Down Scaling experiment", a folate concentration-pasteurization time-curve was established at 57 °C (s. Figure 4.2) and 62.5 °C (y = -0.39 x + 53.95, linear approximation), respectively (*Paper IV*).

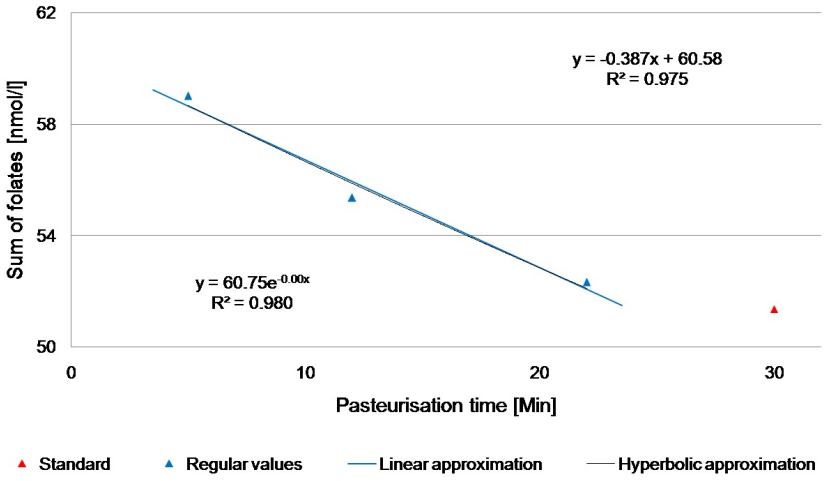

Figure 4.2.: Folate concentration-pasteurization time-curve at 57 °C; Sum of folates: Sum of H_4folate and 5-CH_3-H_4folate content.

A hyperbolic relation between folate concentration and pasteurization time was to expect as thermal degradation of 5-CH_3-H_4folate, the main vitamer in breast milk, is adequately described by a first-order kinetic model (Morgan *et al.* 1986, Viberg *et al.* 1997, Indrawati *et al.* 2004, Verlinde *et al.* 2010). By the determined equations, the treatment time for the 25 mL samples was calculated in reference to the folate concentration (sum of folates) in the standard sample as 23 minutes and 12 minutes at 57 °C and 62.5 °C, respectively (*Paper IV*).

Additionally, a "one point calibration" procedure was adopted to confirm the findings of the "Down Scaling experiment". The treatment time required for the 25 mL breast milk samples was calculated to 22.4 minutes at 57 °C according to the rule of proportion based on the folate concentration of 51.4 nmol/L in the standard sample (57 °C, 30 minutes, 120 mL, n = 2) and 52.3 nmol/L in the small sample (57 °C, 22 minutes, 25 mL, n = 2). Similarly, the required treatment time at 62.5 °C was calculated to 12.1 minutes based on the folate concentration of 49.0 nmol/L in the standard sample (62.5 °C, 30 minutes, 120 mL, n = 2) and 49.3 nmol/L in the small sample (62.5 °C, 12 minutes, 25 mL, n = 2).

The two preliminary experiments for the simulation of the RHT treatment in the laboratory showed that heating breast milk (initial temperature: 17 °C) for 4 minutes and 20 seconds in the boiling water bath was sufficient to heat the breast milk to 73 °C for 15 seconds (*Paper IV*). This procedure was considered a suitable simulation of RHT treatments as RHT methods are home pasteurization methods that use a pot with boiling water on the stove (Israel-Ballard *et al.* 2008, Mbuya *et al.* 2010). In summary, the pasteurization time was set to 23 minutes and 12 minutes at 57 °C and 62.5 °C, respectively (s. Figure D.4) and breast milk was heated for 4 minutes and 20 seconds in the boiling water bath (*Paper IV*).

As the conditions for heat treatment during the described procedures were kept as close to reality as possible, this trial was suitable to gain knowledge on (i) folate content and pattern in breast milk and (ii) folate stability during heat treatment of breast milk. Still, simulation bears the risk of falsification and/or errors. Therefore, the results should be considered indicative and transferred carefully to usual conditions. Particularly, these shortcomings have to be taken into account: (i) Higher impact of heat and light on the folate content of the 25 mL-sample as the surface:volume-ratio was higher, (ii) more space for air and microorganisms in the bottle (the same bottles as for the usual samples were used) and (iii) imprecisions during the adjustment of the treatment time, *e.g.* due to the specific composition of the individual mother's breast milk, folate losses after heat treatment may vary between mothers and the pooled breast milk sample used in the "Down Scaling experiment" may not have been representative.

4.4.3. Heat treatment of breast milk

Power calculation revealed a minimum sample size of 34 samples (s. Figure D.5; *Paper IV*). For 40 samples, the statistical power was well above 0.8 (s. Figure D.6). The sample size in the present trial (n = 38) provided sufficient statistical power for the planned investigations.

The adapted method for folate extraction from breast milk and the adjusted heat treatment procedures were applied successfully in this trial to study breast milk folate content, pattern and stability during heat treatment.

Folate content in untreated breast milk (until 3 months post partum) varied widely (s. Table 4.6). The predominant vitamer was 5-CH_3-H_4folate. H_4folate was detected in 37 samples. 10-HCO-PteGlu was detected in a few samples in trace amounts, *i.e.* < 2 nmol/L, while PteGlu was not detected at all.

Table 4.6.: Folate content in untreated and heat treated breast milk (mean ± Stdev; *Paper IV*). [nmol/L]

Folate vitamer	**Low T (n = 38)**	**Holder P (n = 37)**	**RHT (n = 38)**	**Untreated (n = 38)**
H_4folate	22.4±12.3^a	13.6±9.1^b	7.6±2.9^c	33.1±14.6^d
5-CH_3-H_4folate	104.8±33.6^a	102.0±32.6^a	103.7±31.0^a	117.4±37.0^a
Sum of folates	126.6±40.4^a	115.6±37.3^a	111.3±31.6^a	150.4±46.4^b

Holder P: Holder pasteurization (62.5 °C, 12 minutes), Low T: Low temperature treatment (57 °C, 22 minutes), RHT: Rapid high temperature treatment (4 minutes 20 seconds in the boiling water bath; initial temperature: 17 °C, final temperature: 73 °C), Sum of folates: Sum of H_4folate and 5-CH_3-H_4folate content.

All treatments were applied to 25 mL breast milk samples.

Folate concentrations were corrected for endogenous folate content in enzyme preparations.

Values with different letters superscript in the same row were significantly different ($p < 0.05$).

Despite high variation, folate content of untreated breast milk was well in the range of literature values (s. Table 1.3). The observed variations can partly be explained by variability in breast milk folate content during one nursing session, one day and

the complete lactation period (Udipi *et al.* 1987, Han *et al.* 2009, Agostoni *et al.* 2010). Congruently, in a secondary experiment, breast milk from one mother indicated high variations within a day and during lactation: The sum of folates increased from 115.6 nmol/L in the morning to 174.2 nmol/L in the afternoon and varied between 74.8 nmol/L, 163.2 nmol/L, 142.6 nmol/L and 176.9 nmol/L on days 13, 25, 26 and 51 post partum, respectively. The concentrations in untreated breast milk were sufficient to meet the RDA for term infants up to eleven months of age (110 nmol folate/day; SLV 2007b; milk consumption 800 mL/day).

Compared to untreated breast milk, H_4folate concentration was 11.2 – 25.4 nmol/L lower after heat treatment ($p < 0.001$). The effects of heat treatment increased in the order of low temperature treatment, Holder pasteurization and RHT treatment. Contrary, 5-CH_3-H_4folate concentration was not affected by the three treatments ($p = 0.179$; s. Figure D.7; *Paper IV*) and decreased by 13.0 – 13.7 nmol/L. Heat treatment decreased the sum of folates by 23.9 – 39.2 nmol/L ($p < 0.01$). But, there were no differences between the investigated heat treatment procedures with respect to the sum of folates.

The effects on breast milk folate content (sum of folates) after the examined heat treatment procedures were comparable. In agreement with previous publications (van Zoeren-Grobben *et al.* 1987, Donnelly-Vanderloo *et al.* 1994), the results in Table 4.6 suggested significant folate losses after heat treatment. Losses have also been observed for vitamin A and riboflavin content after RHT treatment (Israel-Ballard *et al.* 2008). Contrary, others reported stable breast milk folate content after Holder pasteurization (Hamprecht *et al.* 2004) and RHT treatment (Goldblum *et al.* 1984) or increased breast milk folate content after RHT treatment (Israel-Ballard *et al.* 2005 and 2008). Besides potentially enhanced adverse effects of heat treatment on the small sample volume, differences in analytical methodology may account for this discrepancy. The determination of the total folate content in heat treated breast milk may be hampered by non-folate substances released from the matrix during heat treatment triggering the chemoluminescent assay applied in the former studies (Hamprecht *et al.* 2004, Israel-Ballard *et al.* 2005 and 2008) and leading to an increased response. In contrast, in this trial, single vitamers were quantified and studied specifically.

The observed similarity of the examined heat treatment procedures supports breast milk handling all over the world considering two aspects: (i) as folate losses (sum of folates) were similar after all treatments, folate supply of infants remains similar (if folate contents in untreated breast milk are similar) and (ii) while western countries achieve inactivation of pathogenic microorganisms by treatment of donor milk in highly advanced pasteurizers (s. Figure 1.6; Hamprecht *et al.* 2004, Omarsdottir *et al.* 2008), RHT methods (Israel-Ballard *et al.* 2005) can be adopted alternatively in resource restricted areas. Hence, all procedures can be considered alternatives that can be chosen depending on available resource.

In western countries, heat treated donor milk is predominantly used in the health care of premature born infants. Unfortunately, heat treated breast milk supplies the premature infant with maximum 25% of the RDA (*Paper IV*). Low folate intake is a risk factor for impaired brain development for infants (Georgieff 2007). Folate enrichment is one option to improve a premature born infant's supply with folate. In Sweden, it is common to fortify donor milk (Omarsdottir *et al.* 2008).

In conclusion, folate concentrations in untreated breast milk of healthy, well nourished mothers provided sufficient amounts of folate for infants up to eleven months of age. Folate losses caused by the examined heat treatment procedures were acceptable. Heat treated breast milk for premature born infants should be fortified to ensure sufficient folate supply and to support regular growth and development.

5. Concluding remarks and future perspectives

Determination of folate in food and human samples is challenging because of the high number of different naturally occurring vitamers, their low stability under extraction conditions and their presence in trace amounts. Instrumental shortcomings, *e.g.* insufficient LC separation or low detector sensitivity, are further obstacles. Folate determination by SIDA including LC-MS/MS detection has been shown to outperform conventional HPLC methods (Freisleben *et al.* 2003a). Several SIDAs for the quantification of folate in food and human samples have been described lately (Nelson *et al.* 2003, Pfeiffer *et al.* 2004, Rychlik *et al.* 2007, Mönch *et al.* 2010). Because MS/MS is capable of differentiating unlabelled, [$^2H_4$]-labelled and [$^{13}C_5$]-labelled folate unequivocally, improved SIDAs could be developed that allowed for the simultaneous quantification of unlabelled and [$^{13}C_5$]-labelled intact folate and folate catabolites in plasma, ileostomal effluent and urine by the respective [$^2H_4$]-labelled ISs (*Paper I*).

For the simultaneous quantification of unlabelled and [$^{13}C_5$]-labelled folate catabolites in urine by SIDA, [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG had to be synthesized. Previous research showed the [$^2H_4$]-label to be stable and the mass increment of four between IS and analyte to be sufficient for unequivocal spectral differentiation of the isotopologues (Freisleben *et al.* 2002). Different synthetic pathways have been described to obtain [$^2H_4$]-labelled intact folate (Maunder *et al.* 1999, Freisleben *et al.* 2002). [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG were synthezised successfully starting from p-ABA (*Paper II*). NMR spectroscopy and MS analysis of the reaction products proofed their suitability as ISs and confirmed former results (Maunder *et al.* 1999, Freisleben *et al.* 2002, Mönch *et al.* 2010).

The need for unequivocal differentiation of unlabelled endogenous folate and isotopically labelled test folate has been emphasized in studies on folate bioavailability (Rogers *et al.* 1997, Finglas *et al.* 2002). The new SIDAs offer the possibility (i) to quantify unlabelled endogenous folate and (ii) to follow a [$^{13}C_5$]-labelled tracer simultaneously. Working with [$^{13}C_5$]-labelled test folate, endogenous folate will not hamper studies (i) on folate absorption and metabolism or (ii) folate retention and interconversion during food processing in the future.

Folate intake and bioavailability determine the nutritional status (Gregory III *et al.* 2005). Appropriate dietary recommendations require substantiated knowledge on folate bioavailability. In a stable-isotope AUC/ileostomy model, the bioavailability of [$^{13}C_5$]-5-CH_3-H_4folate and [$^{13}C_5$]-PteGlu fortificants from wholemeal bread was high (90%) and not affected by a breakfast meal matrix (*Paper III*). Fortification of bread may be a means to increase the low folate intake of European populations (de Bree *et al.* 1997, Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz 2008). Today, food is mostly fortified with synthetic PteGlu because of its high stability and low costs during synthesis. But, there is concern that high concentrations of systemic PteGlu are associated with negative health effects (Wright *et al.* 2007). 5-CH_3-H_4folate, a naturally occurring vitamer, may be a suitable alternative for PteGlu. Future research needs to focus on the possibilities to use 5-CH_3-H_4folate as food fortificant commercially.

For hygienic and health protective reasons, donor milk is heat treated in milk banks all over the world. Diverse effects of heat treatment on concentrations of vitamins, minerals and other nutritive factors have been reported (Goldblum *et al.* 1984, Tully *et al.* 2001, Israel-Ballard *et al.* 2008). Low temperature pasteurization, Holder pasteurization and RHT treatment decreased breast milk folate content similarly (about 20%; *Paper IV*). Folate content in heat treated donor milk supplied at maximum 25% of a preterm infant's RDA (*Paper IV*), which may be critical to normal brain development (Georgieff 2007). Thus, the recommended aliment for preterm infants in neonatal care is enriched breast milk (Agostoni *et al.* 2010). To ensure sufficient folate supply of preterm infants future recommendations need to consider folate losses during heat treatment of donated breast milk.

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A. Synthesis of folate standards

A.1. Unlabelled Ap-ABG

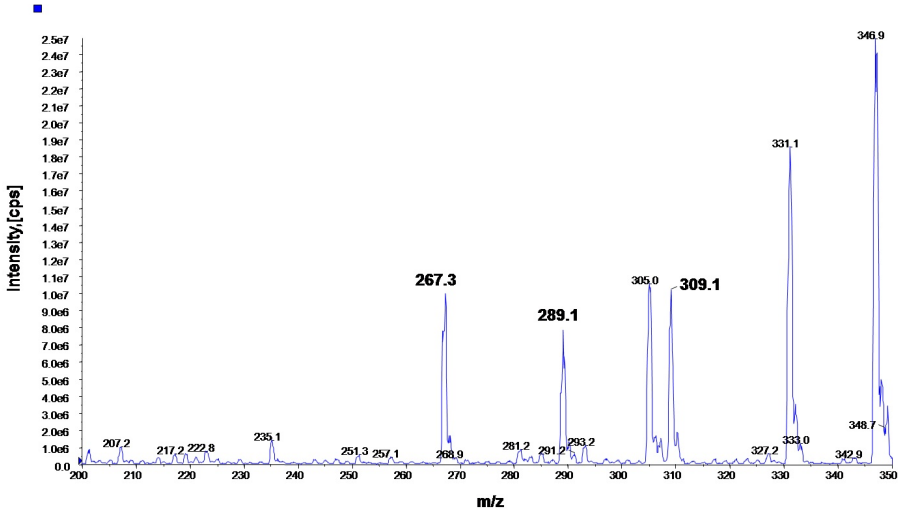

Figure A.1.: Mass spectrum (Q1-Scan) of Ap-ABG (ESI^+: m/z 309; m/z 331: Sodium adduct) prior to preparative HPLC.

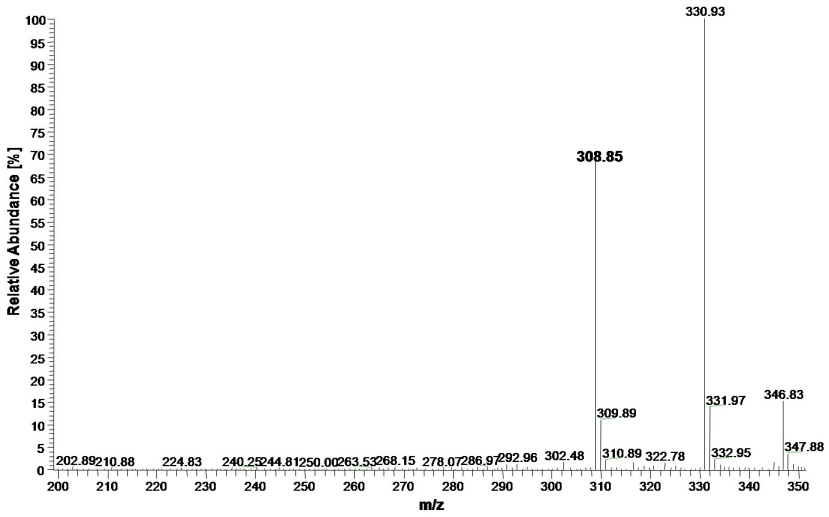

Figure A.2.: Mass spectrum (Q1-Scan) of Ap-ABG (ESI^+: m/z 309; m/z 331: Sodium adduct) after preparative HPLC.

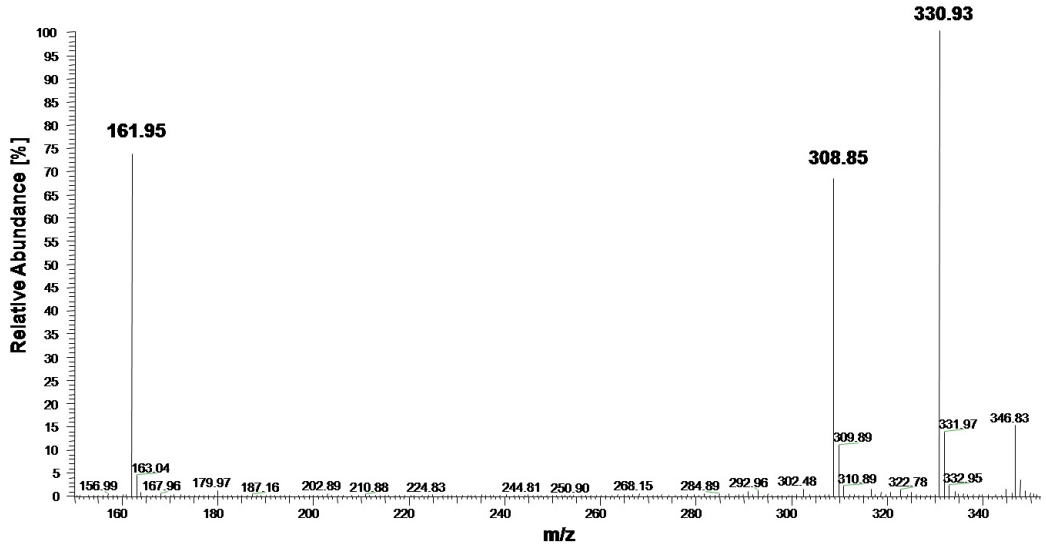

Figure A.3.: Mass spectrum (Product ion-Scan) of Ap-ABG (molecular ion: ESI^+: m/z 309; m/z 331: Sodium adduct; fragment ion (loss of m/z 147, *i.e.* glutamyl moiety): ESI^+ m/z: 162).

A.2. $[^2H_4]$-p-ABG and $[^2H_4]$-Ap-ABG

A.2.1. Deuteration of p-ABA

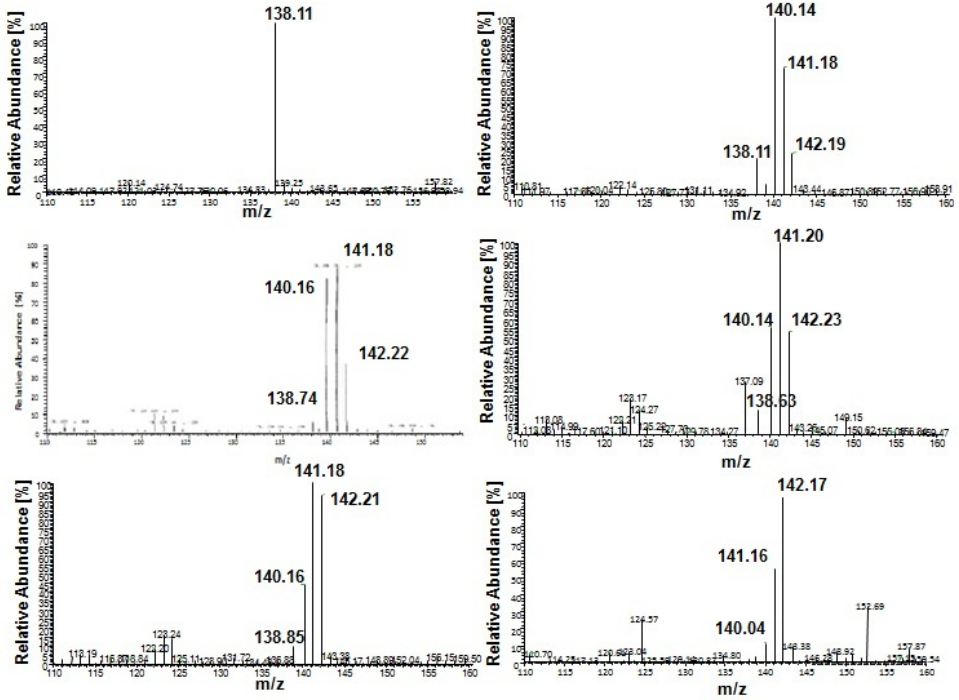

Figure A.4.: Deuteration progress: p-Aminobenzoic acid (top left; ESI$^+$: m/z 138), after cycle 1 (top right), after cycle 2 (middle left; scan only), after cycle 3 (middle right), after cycle 4 (bottom left) after cycle 5 (bottom right).

A.2.2. Protection of $[^2H_4]$-p-ABA

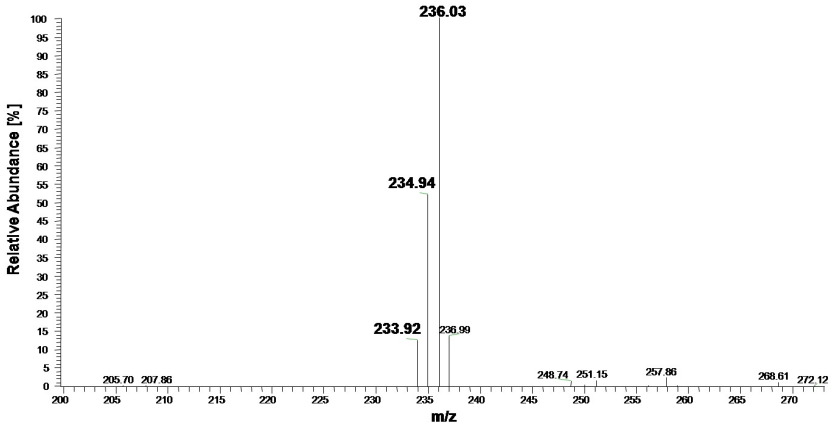

Figure A.5.: Mass spectrum (Q1-Scan) of $[^2H_4]$-N-TFA-p-aminobenzoic acid (ESI^-: m/z 236) after cleanup by preparative HPLC (TFA: Trifluoracetyl).

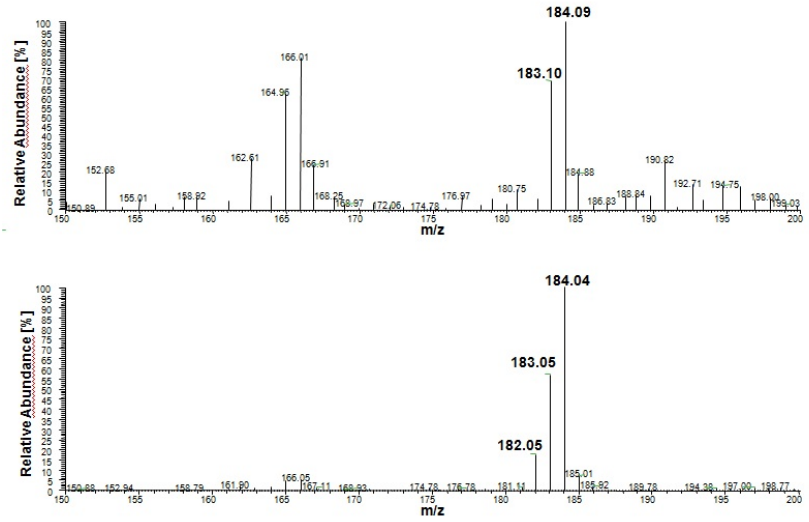

Figure A.6.: Mass spectrum (Q1-Scan) of $[^2H_4]$-p-acetamidobenzoic acid (ESI^+: m/z 184) before (top) and after (bottom) preparative HPLC cleanup.

A.2.3. Coupling of [$^2H_4$]-p-ABA and L-glutamic acid

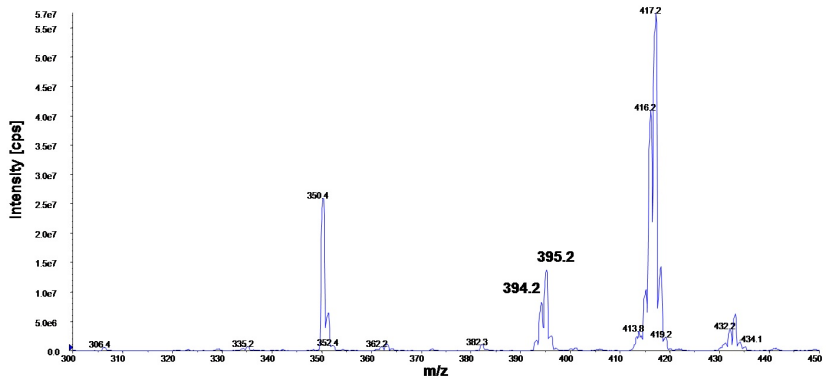

Figure A.7.: Mass spectrum (Q1-Scan) of [$^2H_4$]-N-TFA-p-aminobenzoyl glutamate-di-methylester (ESI$^+$: *m/z* 395; *m/z* 417: Sodium adduct; TFA: Trifluoracetyl).

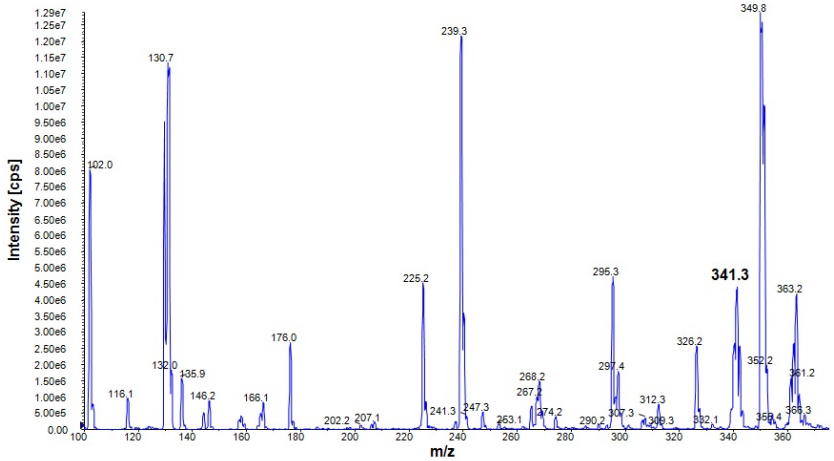

Figure A.8.: Mass spectrum (Q1-Scan) of [$^2H_4$]-p-acetamidobenzoyl glutamate-di-methylester (ESI$^+$: *m/z* 341; *m/z* 363: Sodium adduct).

A.2.4. Cleavage of protection groups and mass spectral analysis of the final synthesis products

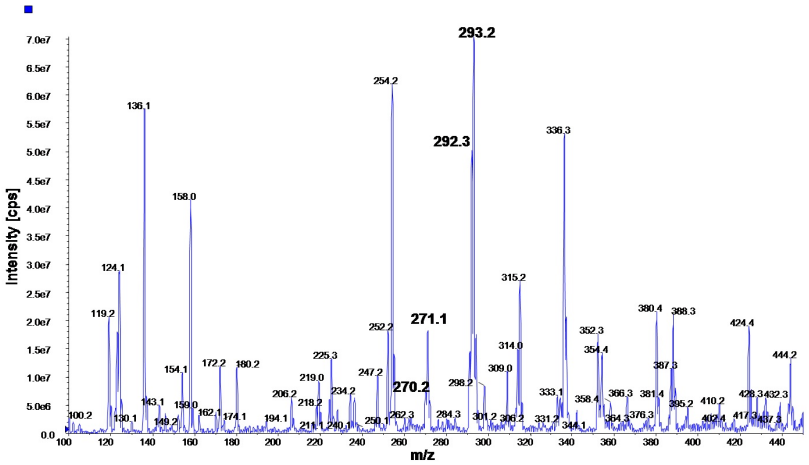

Figure A.9.: Mass spectrum (Q1-Scan) of $[^2H_4]$-p-ABG prior to preparative HPLC (ESI$^+$: m/z 271; m/z 293: Sodium adduct).

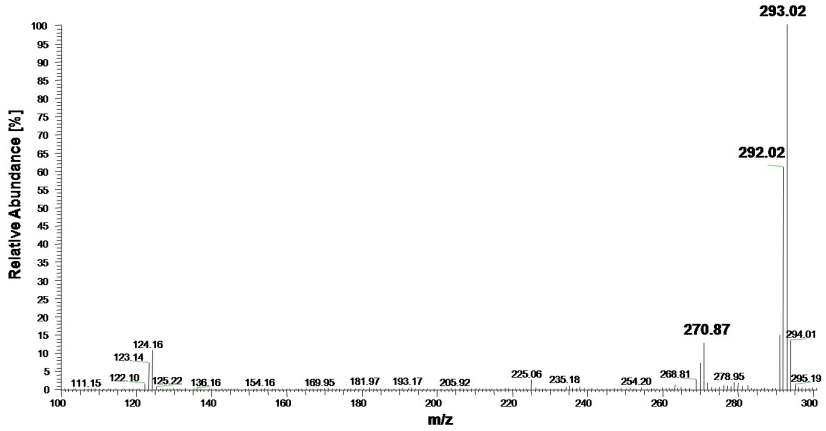

Figure A.10.: Mass spectrum (Q1-Scan) of $[^2H_4]$-p-ABG after preparative HPLC (ESI$^+$: m/z 271; m/z 293: Sodium adduct).

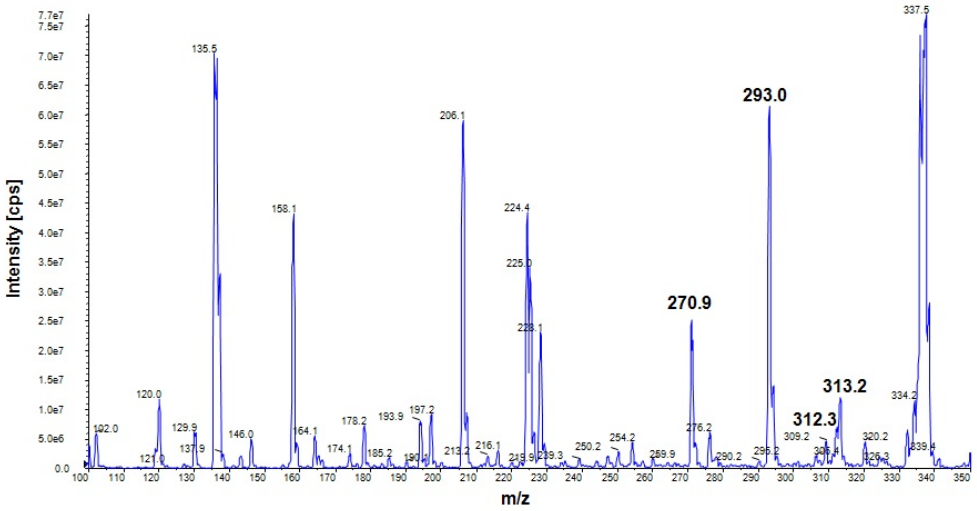

Figure A.11.: Mass spectrum (Q1-Scan) of [$^2H_4$]-Ap-ABG prior to preparative HPLC (ESI$^+$: m/z 313).

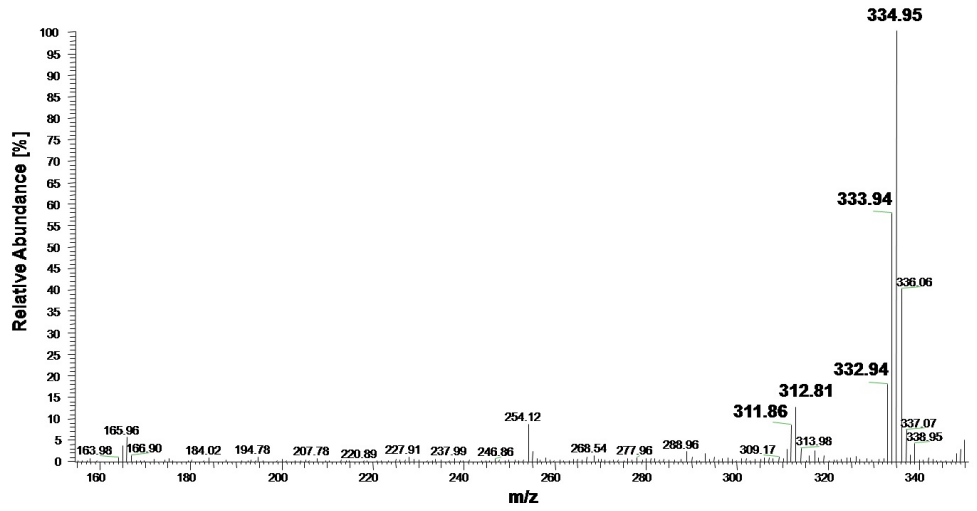

Figure A.12.: Mass spectrum (Q1-Scan) of [$^2H_4$]-Ap-ABG after preparative HPLC (ESI$^+$: m/z 313; m/z 335: Sodium adduct).

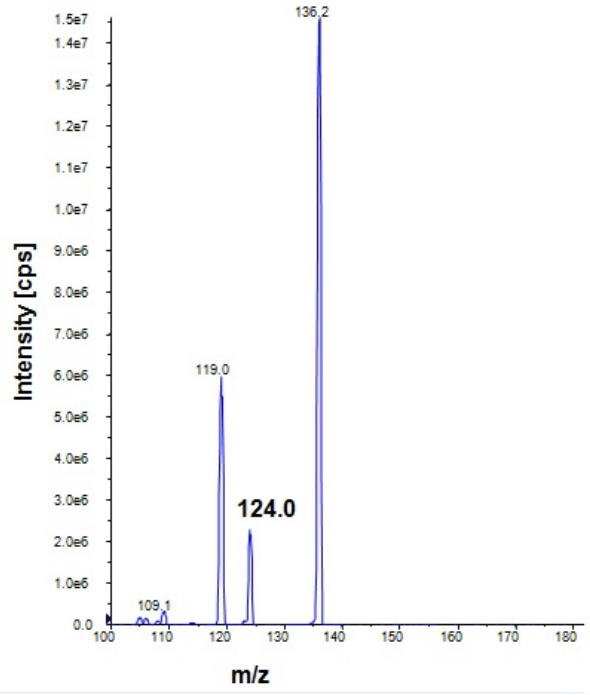

Figure A.13.: Mass spectrum (Product ion-Scan) of [$^2H_4$]-p-ABG (fragment ion (loss of *m/z* 147, *i.e.* glutamyl moiety): ESI$^+$: *m/z* 124).

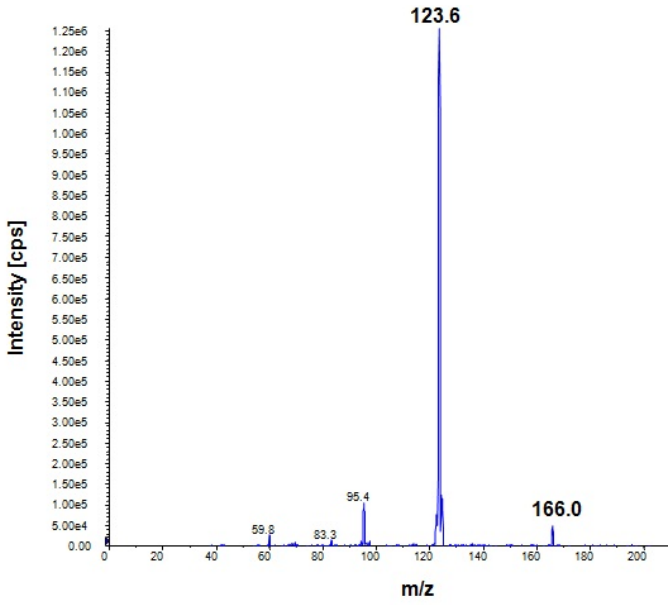

Figure A.14.: Mass spectrum (Product ion-Scan) of [$^2H_4$]-Ap-ABG (fragment ion I (loss of *m/z* 147, *i.e.* glutamyl moiety): ESI$^+$: *m/z* 166; fragment ion II (loss of *m/z* 189, *i.e.* glutamyl and acetyl moieties): ESI$^+$: *m/z* 124).

B. Development of SIDAs for analysis of human samples

B.1. Precipitation of plasma protein

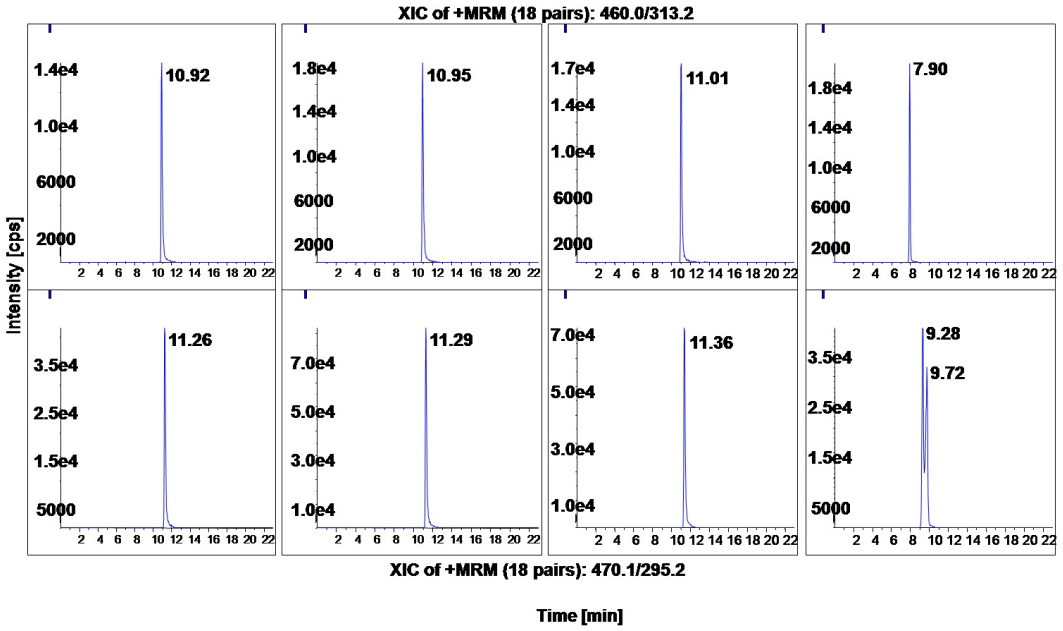

Figure B.1.: Chromatograms of a pooled plasma sample after protein precipitation with 100% acetonitrile (concentration in the extract: 60%), 100% methanol (concentration in the extract: 60%), modified extraction buffer II and modified elution buffer (Table 2 in *Paper I*; from the left) of 5-methyltetrahydrofolate (top) and 10-formylfolic acid (bottom); note that the y-axis differs between panels.

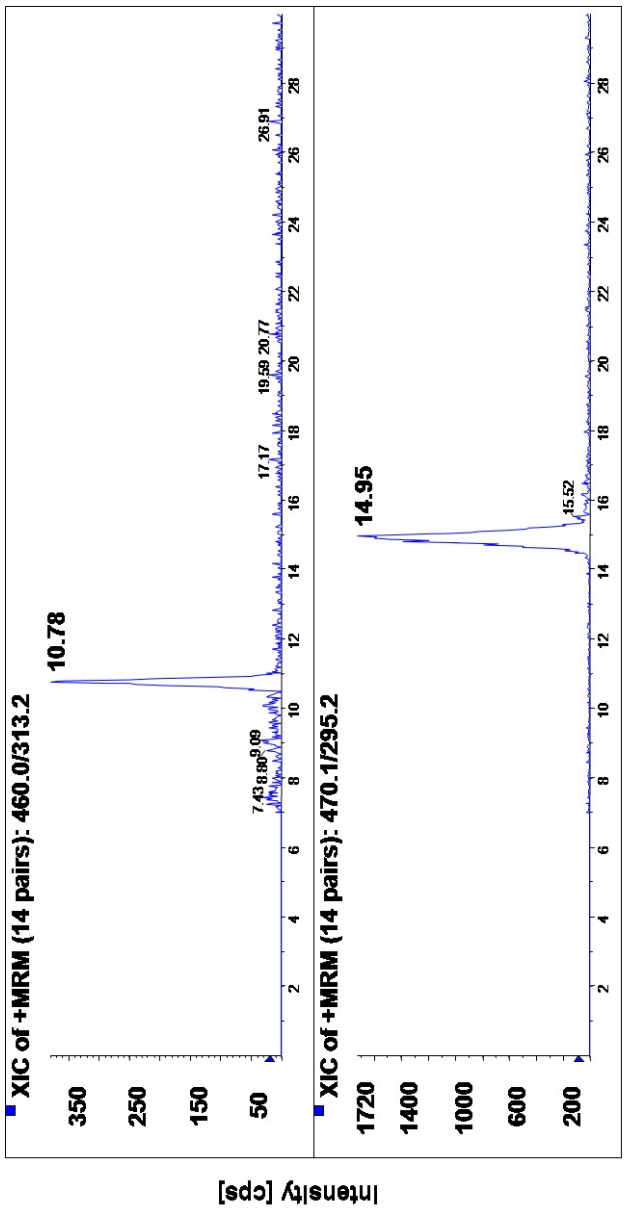

Figure B.2.: Chromatograms of a pooled plasma sample after cleanup by solid phase extraction on a novel HybridSPETM-Precipitation-Cartridge of 5-methyltetrahydrofolate (top) and 10-formylfolic acid (bottom); 400 μL plasma, 10-formylfolic acid as internal standard and 500 μL extraction buffer were stirred for 37 minutes at room temperature, the extract was applied to the cartridge and the resulting eluate was analyzed by LC-MS/MS; note that the y-axis differs between the panels.

B.2. Preparation of ileostomal effluent samples

Table B.1.: Peak areas in chromatograms of three ileostomal effluent samples (number 1–3) extracted including mono- or di-enzyme treatment. [counts]

Folate vitamer	**1**		**2**		**3**	
	Di[1]	**Mono[2]**	**Di[1]**	**Mono[2]**	**Di[1]**	**Mono[2]**
H_4folate	5.0 e^4	4.1 e^4	1.1 e^5	7.3 e^4	4.7 e^4	9.7 e^3
5-CH_3-H_4folate	7.0 e^4	6.3 e^4	**7.0 e^5**	**7.3 e^5**	**9.2 e^4**	**1.8 e^5**
[$^2H_4$]-5-CH_3-H_4folate	8.0 e^4	7.1 e^4	9.8 e^4	9.7 e^4	1.2 e^5	2.4 e^4
[$^{13}C_5$]-5-CH_3-H_4folate	4.2 e^3	4.2 e^3	n.d.	n.d.	2.5 e^3	2.5 e^2
10-HCO-PteGlu	3.6 e^4	2.6 e^4	**1.8 e^5**	**3.0 e^5**	1.0 e^6	2.5 e^4
[$^2H_4$]-10-HCO-PteGlu	3.1 e^4	2.4 e^4	**5.1 e^4**	**9.2 e^4**	6.2 e^4	1.4 e^3
5-HCO-H_4folate	**1.1 e^4**	**1.2 e^4**	3.8 e^4	2.9 e^4	8.7 e^5	1.2 e^4
[$^2H_4$]-5-HCO-H_4folate	2.4 e^4	2.3 e^4	4.6 e^4	3.9 e^4	5.8 e^4	2.6 e^4
PteGlu	6.5 e^3	6.2 e^3	9.9 e^3	7.7 e^3	**4.4 e^4**	**5.2 e^4**
[$^2H_4$]-PteGlu	4.9 e^3	4.2 e^3	6.5 e^3	6.0 e^3	8.1 e^3	6.1 e^3
[$^{13}C_5$]-PteGlu	1.6 e^5	1.4 e^5	8.5 e^4	6.7 e^4	n.d.	n.d.

[1] Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C).

[2] Mono-enzyme treatment: 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

n.d.: Not detected.

[$^2H_4$]-H_4folate was not deteced.

Peak areas were corrected for peak areas of endogenous folate content in enzyme preparations.

The bold face typed numbers indicate where decreased peak areas were detected after di-enzyme treatment.

Table B.2.: Peak area ratios (A(Std)/A(Ana)) in chromatograms of three ileostomal effluent samples (number 1–3) extracted including mono- or di-enzyme treatment.

Folate vitamer	**1**		**2**		**3**	
	Di[1]	**Mono[2]**	**Di[1]**	**Mono[2]**	**Di[1]**	**Mono[2]**
5-CH_3-H_4folate	0.9	0.9	7.1	7.5	**0.8**	**7.5**
[$^{13}C_5$]-5-CH_3-H_4folate	0.1	0.1	0.01	0.01	0.02	0.01
10-HCO-PteGlu	1.2	1.1	3.5	3.3	16	18
5-HCO-H_4folate	0.5	0.5	0.8	0.7	**15**	**0.5**
PteGlu	1.3	1.5	1.5	1.3	**5.4**	**8.5**
[$^{13}C_5$]-PteGlu	33	33	13	11	n.c.	n.c.

[1] Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C).

[2] Mono-enzyme treatment: 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

n.c.: Not calculated.

No ratio was calculated for H_4folate: [$^2H_4$]-H_4folate was not deteced.

The bold face typed numbers indicate where peak areas changed remarkably after di-enzyme treatment.

Figure B.3.: Chromatograms of an ileostomal effluent sample after mono-enzyme (left) or di-enzyme (right) treatment (from top to bottom: 5-methyltetrahydrofolate, $[^2H_4]$-5-methyltetrahydrofolate, $[^{13}C_5]$-5-methyltetrahydrofolate). Note that the y-axis differs between panels.
Mono-enzyme treatment: 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C), Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

Figure B.4.: Chromatograms of an ileostomal effluent sample after mono-enzyme (left) or di-enzyme (right) treatment (from top to bottom: 5-formyltetrahydrofolate, $[^2H_4]$-5-formyltetrahydrofolate, 10-formylfolic acid, $[^2H_4]$-10-formylfolic acid).
Note that the y-axis differs between panels.
Mono-enzyme treatment: 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C), Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

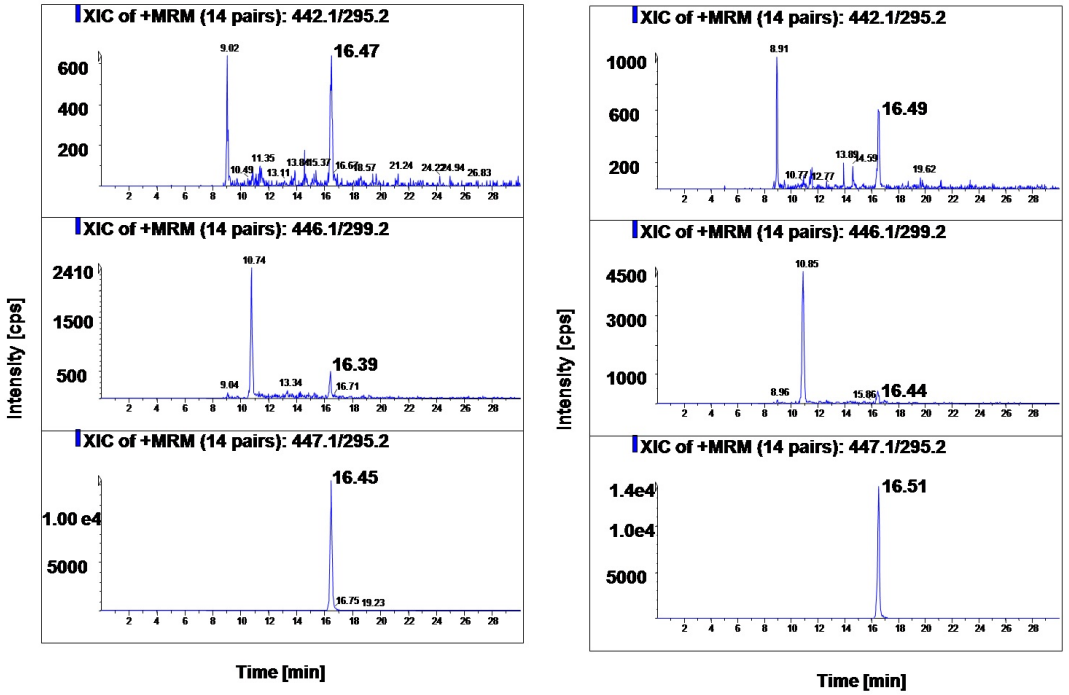

Figure B.5.: Chromatograms of an ileostomal effluent sample after mono-enzyme (left) or di-enzyme (right) treatment (from top to bottom: folic acid, $[^2H_4]$-folic acid, $[^{13}C_5]$-folic acid).
Note that the y-axis differs between panels.
Mono-enzyme treatment: 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C), Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

Table B.3.: Peak areas in chromatograms of two ileostomal effluent samples (number 1–2) after elution from the SPE cartridge with 1.5 mL or 1 mL+1 mL elution buffer. [counts]

Folate vitamer	**1**			**2**		
	1.5	**1+1 I[1]**	**1+1 II[2]**	**1.5**	**1+1 I[1]**	**1+1 II[2]**
H_4folate	2.8 e^5	2.6 e^5	1.9 e^5	1.4 e^5	1.8 e^5	7.1 e^4
5-CH_3-H_4folate	8.5 e^5	6.3 e^5	6.5 e^5	1.3 e^5	1.5 e^5	9.7 e^4
[$^2H_4$]-5-CH_3-H_4folate	6.5 e^4	4.9 e^4	4.8 e^4	5.7 e^4	6.3 e^4	4.1 e^4
[$^{13}C_5$]-5-CH_3-H_4folate	5.2 e^3	4.1 e^3	4.2 e^3	1.5 e^5	1.6 e^5	1.8 e^5
10-HCO-PteGlu	6.2 e^4	1.0 e^5	5.1 e^4	9.3 e^4	1.4 e^5	3.1 e^4
[$^2H_4$]-10-HCO-PteGlu	3.1 e^4	4.7 e^4	2.3 e^4	3.4 e^4	5.1 e^4	1.2 e^4
5-HCO-H_4folate	6.6 e^3	1.4 e^4	6.9 e^3	2.7 e^4	4.1 e^4	9.3 e^3
[$^2H_4$]-5-HCO-H_4folate	1.5 e^4	2.9 e^4	1.3 e^4	2.5 e^4	3.8 e^4	8.0 e^3
[$^{13}C_5$]-5-HCO-H_4folate	8.8 e^3	1.7 e^4	8.4 e^3	n.d.	n.d.	n.d.
PteGlu	1.3 e^4	4.8 e^3	1.2 e^4	7.7 e^3	5.7 e^3	6.9 e^3
[$^2H_4$]-PteGlu	6.7 e^3	2.6 e^3	5.6 e^3	5.7 e^3	4.8 e^3	5.7 e^3

[1] Peak areas in the mL eluted first (I; collected separately from the mL eluted second).

[2] Peak areas in the mL eluted second (II; collected separately from the mL eluted first).

n.d.: Not detected.

[$^2H_4$]-H_4folate was not deteced.

Peak areas were corrected for peak areas of endogenous folate content in enzyme preparations.

Table B.4.: Peak area ratios (A(Std)/A(Ana)) in chromatograms of two ileostomal effluent samples (number 1–2) after elution from the SPE cartridge with 1.5 mL or 1 mL+1 mL elution buffer.

Folate vitamer	**1**			**2**		
	1.5	**1+1 I[1]**	**1+1 II[2]**	**1.5**	**1+1 I[1]**	**1+1 II[2]**
5-CH_3-H_4folate	13.1	12.9	13.5	2.3	2.4	2.4
[$^{13}C_5$]-5-CH_3-H_4folate	0.08	0.08	0.09	2.6	2.5	4.4
10-HCO-PteGlu	2.0	2.1	2.2	2.7	2.7	2.6
5-HCO-H_4folate	0.4	0.5	0.5	1.1	1.1	1.2
[$^{13}C_5$]-5-HCO-H_4folate	0.6	0.6	0.6	n.c.	n.c.	n.c.
PteGlu	1.9	1.8	2.1	1.4	1.2	1.2

[1] Peak areas in the mL eluted first (I; collected separately from the mL eluted second).

[2] Peak areas in the mL eluted second (II; collected separately from the mL eluted first).

n.c.: Not calculated.

No ratio was calculated for H_4folate: [$^2H_4$]-H_4folate was not deteced.

Table B.5.: Peak heights in chromatograms of three ileostomal effluent samples (number 1–3) extracted including mono- or di-enzyme treatment. [cps]

Folate vitamer	**1**		**2**		**3**	
	Di[1]	**Mono[2]**	**Di[1]**	**Mono[2]**	**Di[1]**	**Mono[2]**
H_4folate	4.5 e^3	2.4 e^3	9.7 e^3	6.2 e^3	4.3 e^3	1.1 e^3
5-CH_3-H_4folate	**4.9 e^3**	**5.8 e^3**	**5.0 e^4**	**5.8 e^4**	7.7 e^4	1.7 e^4
[$^2H_4$]-5-CH_3-H_4folate	6.1 e^3	5.3 e^3	9.4 e^3	8.8 e^3	1.1 e^4	2.2 e^3
[$^{13}C_5$]-5-CH_3-H_4folate	3.5 e^2	5.8 e^2	1.0 e^2	8.0 e^1	3.0 e^2	9.0 e^1
10-HCO-PteGlu	2.5 e^3	1.8 e^3	**1.3 e^4**	**1.9 e^4**	5.4 e^4	1.9 e^3
[$^2H_4$]-10-HCO-PteGlu	1.8 e^3	1.7 e^3	**3.9 e^3**	**5.8 e^3**	3.7 e^3	1.1 e^2
5-HCO-H_4folate	7.5 e^2	5.3 e^2	2.1 e^3	1.7 e^3	3.9 e^4	7.8 e^2
[$^2H_4$]-5-HCO-H_4folate	1.1 e^3	9.4 e^2	2.3 e^3	2.1 e^3	2.3 e^3	n.d.
PteGlu	**6.0 e^2**	**6.4 e^2**	8.1 e^2	5.2 e^2	**2.8 e^3**	**6.1 e^3**
[$^2H_4$]-PteGlu	**4.5 e^2**	**5.2 e^2**	4.4 e^2	3.0 e^2	**4.5 e^2**	**6.3 e^2**
[$^{13}C_5$]-PteGlu	1.4 e^4	1.3 e^4	6.2 e^3	4.6 e^3	n.d.	n.d.

[1] Di-enzyme treatment: 2 mg protease/0.5 g lyophilized sample (4 hours 37 °C), 150 μL rat serum (RS) and 2 mL chicken pancreas (CP) solution/0.5 g lyophilized sample (overnight 37 °C).

[2] Mono-enzyme treatment: 150 μL RS and 2 mL CP solution/0.5 g lyophilized sample (overnight 37 °C).

n.d.: Not detected.

[$^2H_4$]-H_4folate was not deteced.

Peak heights were corrected for peak heights of endogenous folate content in enzyme preparations. The bold face typed numbers indicate where decreased peak heights were detected after di-enzyme treatment.

Table B.6.: Peak heights in chromatograms of two ileostomal effluent samples (number 1–2) after elution from the SPE cartridge with 1.5 mL or 1 mL+1 mL elution buffer. [cps]

Folate vitamer	**1**			**2**		
	1.5	**1+1 I[1]**	**1+1 II[2]**	**1.5**	**1+1 I[1]**	**1+1 II[2]**
H_4folate	2.1 e^4	2.4 e^4	1.8 e^4	1.4 e^4	1.7 e^4	6.7 e^3
5-CH_3-H_4folate	5.5 e^4	5.2 e^4	5.3 e^4	1.1 e^4	1.3 e^4	7.8 e^3
[$^2H_4$]-5-CH_3-H_4folate	4.4 e^3	4.0 e^3	3.7 e^3	4.8 e^3	5.5 e^3	3.4 e^3
[$^{13}C_5$]-5-CH_3-H_4folate	4.2 e^2	4.1 e^2	4.4 e^2	1.3 e^4	1.5 e^4	8.8 e^3
10-HCO-PteGlu	3.7 e^3	6.9 e^3	4.0 e^3	6.6 e^3	9.5 e^3	2.4 e^3
[$^2H_4$]-10-HCO-PteGlu	1.8 e^3	3.3 e^3	1.8 e^3	2.6 e^3	3.7 e^3	1.0 e^3
5-HCO-H_4folate	3.5 e^2	8.8 e^2	4.9 e^2	1.7 e^3	2.1 e^3	5.7 e^2
[$^2H_4$]-5-HCO-H_4folate	5.7 e^2	1.4 e^3	7.8 e^2	1.3 e^3	2.0 e^3	4.4 e^2
[$^{13}C_5$]-5-HCO-H_4folate	3.8 e^2	9.5 e^2	5.2 e^2	n.d.	n.d.	n.d.
PteGlu	9.6 e^2	4.9 e^2	1.2 e^3	7.7 e^2	6.3 e^2	6.9 e^2
[$^2H_4$]-PteGlu	5.1 e^2	2.4 e^2	5.2 e^2	5.5 e^2	4.3 e^2	4.6 e^2

[1] Peak areas in the mL eluted first (I; collected separately from the mL eluted second).

[2] Peak areas in the mL eluted second (II; collected separately from the mL eluted first).

n.d.: Not detected.

[$^2H_4$]-H_4folate was not deteced.

Peak heights were corrected for peak heights of endogenous folate content in enzyme preparations.

B.3. Determination of folate catabolites in urine

Table B.7.: Peak heights in chromatograms of a urine sample extracted according to Mönch *et al.* 2010 and *Paper II*. [cps]

Folate vitamer	**Mönch *et al.* 2010**	***Paper II***
H_4folate	7.0 e^2	1.0 e^3
5-CH_3-H_4folate	n.d.	1.9 e^3
10-HCO-PteGlu	n.d.	2.7 e^2
p-ABG	n.d.	4.0 e^2
Ap-ABG	3.9 e^2	1.5 e^4

n.d.: Not detected.

Baseline in the 460.0/313.2-trace (5-CH_3-H_4folate) was around 5.0 e^2 for analysis according to Mönch *et al.* 2010.

5-HCO-H_4folate and PteGlu were investigated but not deteced with neither one of the two methods.

Table B.8.: Concentrations of folate catabolites in three different urine samples (1 – 3) determined by a simplified "Standard addition" procedure (A) and stable isotope dilution assay (B). [μg/100 g]

Sample	**p-ABG**		**[^{13}C$_5$]-p-ABG**		**Ap-ABG**		**[^{13}C$_5$]-Ap-ABG**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
1	2.3	0.6	0.6	0.1	20.6	4.2	9.0	2.5
2	1.0	0.4	0.2	0.1	7.0	1.6	5.3	0.2
3	2.7	0.4	0.7	0.1	9.3	2.0	3.1	0.9

C. Bioavailability of folate from wholemeal bread

C.1. Plasma response

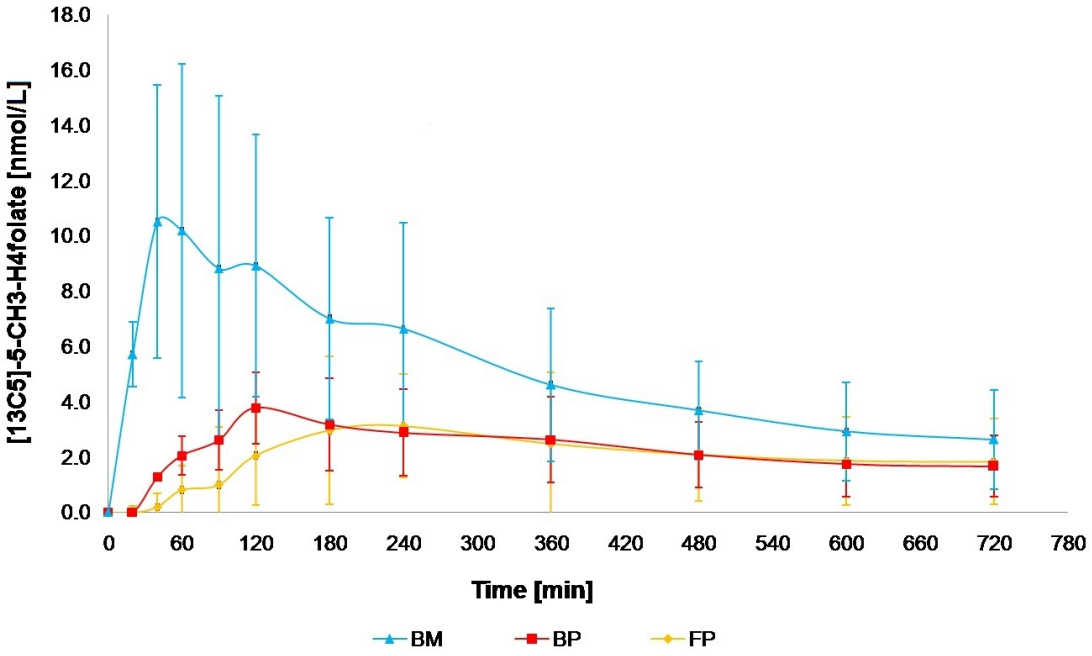

Figure C.1.: Plasma response curves of $[^{13}C_5]$-5-CH_3-H_4folate after a single testdose of folate fortified bread (median, range depicted by bars; n = 8; analyzed in dublicate).
Dose: 450 nmol $[^{13}C_5]$-labelled folate, BM: Wholemeal bread fortified with $[^{13}C_5]$-5-CH_3-H_4folate ingested with pear sauce and a glass of water, BP: Wholemeal bread fortified with $[^{13}C_5]$-PteGlu ingested with pear sauce and a glass of water, FP: Wholemeal bread fortified with $[^{13}C_5]$-PteGlu ingested with a breakfast meal consisting of liver pâté, orange juice, cornflakes and fermented milk.

C.2. Folate in ileostomal effluent

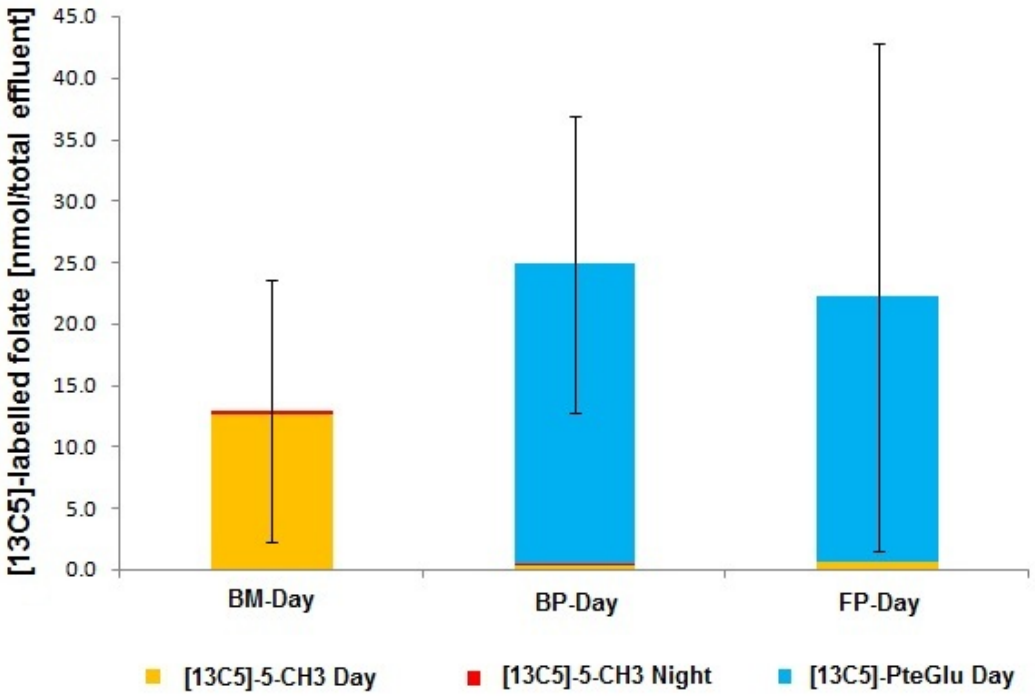

Figure C.2.: Content of [$^{13}C_5$]-labelled 5-CH_3-H_4folate and PteGlu folate in ileostomal effluent collected during 24 hours post dose (median, range depicted by bars; n = 8; analyzed in dublicate).
Dose: 450 nmol [$^{13}C_5$]-labelled folate, BM: Wholemeal bread fortified with [$^{13}C_5$]-5-CH_3-H_4folate ingested with pear sauce and a glass of water, BP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with pear sauce and a glass of water, FP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with a breakfast meal consisting of liver pâté, orange juice, cornflakes and fermented milk, Day: Ileostomal effluent collected 0 – 12 hours post dose, Night: Ileostomal effluent collected 12 – 24 hours post dose.
[$^{13}C_5$]-PteGlu was not detected in the samples collected 12 – 24 hours post dose, *i.e.* there was no [$^{13}C_5$]-PteGlu Night.

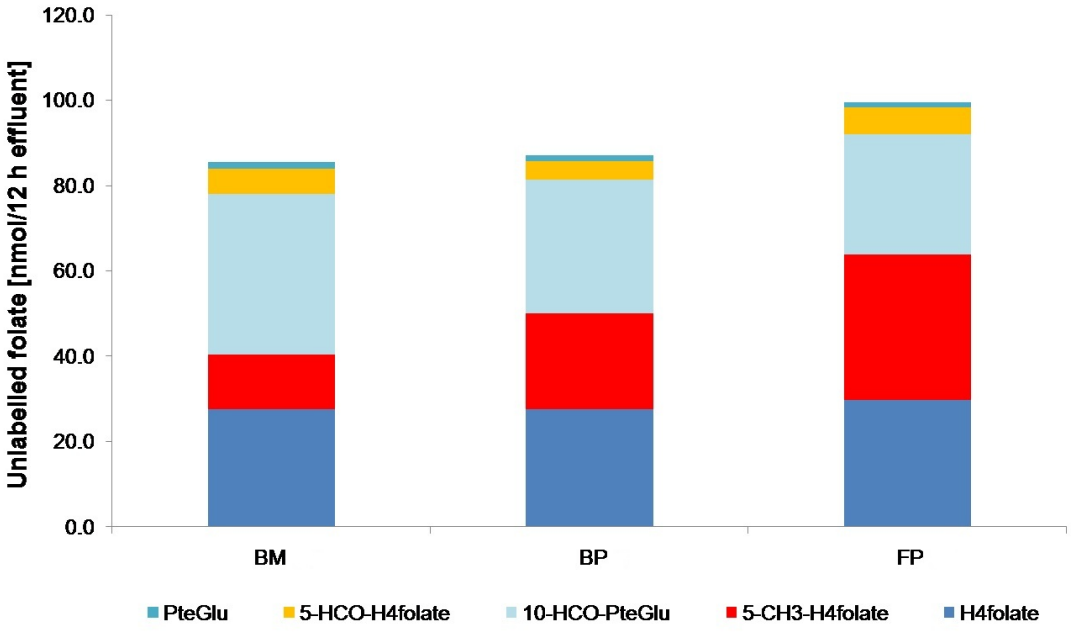

Figure C.3.: Individual unlabelled intact folate in ileostomal effluent collected 0 – 12 hours post dose (median; n = 8; analyzed in dublicate).
Dose: 450 nmol [$^{13}C_5$]-labelled folate, BM: Wholemeal bread fortified with [$^{13}C_5$]-5-CH_3-H_4folate ingested with pear sauce and a glass of water, BP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with pear sauce and a glass of water, FP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with a breakfast meal consisting of liver pâté, orange juice, cornflakes and fermented milk.
0 – 12 hours post dose: controlled diet, *i.e.* intake of unlabelled folate: 340 nmol (FP), 115 nmol (BP, BM).
Folate contents were corrected for endogenous folate content in enzyme preparations.

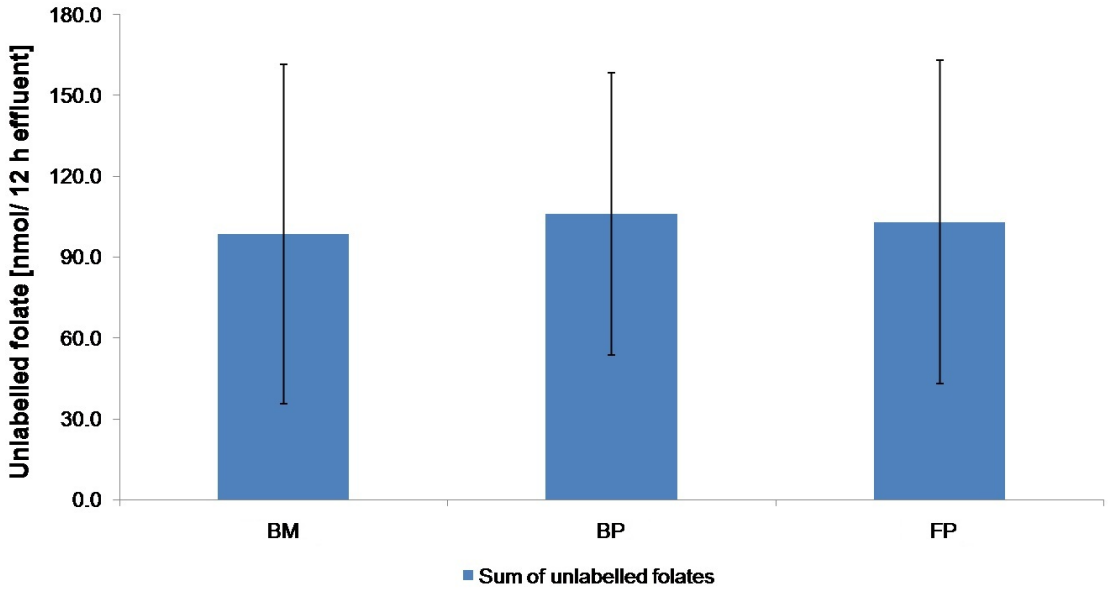

Figure C.4.: Sum of unlabelled intact folate in ileostomal effluent collected 0 – 12 hours post dose (median, range depicted by bars; n = 8; analyzed in duplicate).
Dose: 450 nmol [$^{13}C_5$]-labelled folate, BM: Wholemeal bread fortified with [$^{13}C_5$]-5-CH_3-H_4folate ingested with pear sauce and a glass of water, BP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with pear sauce and a glass of water, FP: Wholemeal bread fortified with [$^{13}C_5$]-PteGlu ingested with a breakfast meal consisting of liver pâté, orange juice, cornflakes and fermented milk.
0 – 12 hours post dose: controlled diet, *i.e.* intake of unlabelled folate: 340 nmol (FP), 115 nmol (BP, BM).
Sum of unlabelled folates: Sum of 5-methyltetrahydrofolate, folic acid, 10-formylfolic acid, 5-formyltetrahydrofolate and tetrahydrofolate.
Folate contents were corrected for endogenous folate content in enzyme preparations.

D. Folate stability during heat treatment of breast milk

D.1. Preparation of breast milk samples

Table D.1.: Effect of protease treatment (different amounts of protease [mg] and incubation durations [h]) on breast milk folate concentration (mean ± Stdev; n = 3 each treatment). [nmol/L]

Folate vitamer	**Di-enzyme treatment[1]**			**Tri-enzyme treatment[2]**		
	2.5 mg 3h	**5.0 mg 2h**	**5.0 mg 3h**	**2.5 mg 3h**	**5.0 mg 2h**	**5.0 mg 3h**
H_4folate	17.3±1.2^{3a}	21.3±0.7^{3b}	20.1±1.6$^{3b,\#}$	15.8±2.4^{4a}	10.4±0.7^{4a}	12.6±2.5$^{4a,\#}$
5-CH_3-H_4folate	42.1±2.5^{3a}	63.1±2.8^{3b}	80.9±1.4$^{3c,\#}$	46.6±1.0^{4a}	64.7±2.2^{4b}	85.2±1.6$^{4c,\#}$
10-HCO-PteGlu	6.2±0.1^{3a}	8.2±0.1^{3a}	8.5±1.2$^{3a,\#}$	8.5±0.5^{4a}	8.5±1.0^{4a}	8.4±0.8$^{4a,\#}$
Sum of folates	65.6±3.8^{3a}	92.6±3.3^{3b}	109.4±3.7$^{3c,\#}$	71.0±3.5^{4a}	83.6±2.0^{4b}	106.1±4.9$^{4c,\#}$

[1] Protease treatment: 37 °C, conjugase treatment: 50 μL rat serum (RS; 3 hours 37 °C).

[2] 60μL α-amylase during heat extraction, protease treatment: 37 °C, conjugase treatment: 50 μL RS (3 hours 37 °C).

\# No statistically significant difference between di-enzyme und tri-enzyme treatment for incubation with 5.0 mg protease for 3h.

Sum of folates: Sum of H_4folate, 5-CH_3-H_4folate and 10-HCO-PteGlu contents.

Folate contents were corrected for endogenous folate content in enzyme preparations.

Values with the same number but different letters superscript in the same row were significantly different ($p < 0.05$; Examination of the difference by a "2-sample t-test", Minitab® 15.1.0.0, Minitab Ltd., Coventry, UK).

Table D.2.: Breast milk folate concentration after incubation of 7 g breast milk with different amounts of protease (heat extraction, protease 3 hours 37 °C, 50 μL rat serum 3 hours 37 °C; mean ± Stdev; n = 3). [nmol/L]

Folate vitamer	**5.0 mg protease**	**10.0 mg protease**	**p[1]**
H_4folate	33.3±0.5	33.1±1.1	0.832
5-CH_3-H_4folate	78.4±0.6	81.1±7.0	0.579
10-HCO-PteGlu	Traces (< 2 nmol/L)		
Sum of folates	111.7±1.1	114.2±5.9	0.549

[1] Significance for $p < 0.05$ (Examination of the difference between application of 5 mg and 10 mg protease by a "2-sample t-test", Minitab® 15.1.0.0, Minitab Ltd., Coventry, UK).

Sum of folates: Sum of H_4folate and 5-CH_3-H_4folate contents.

Folate contents were corrected for endogenous folate content in enzyme preparations.

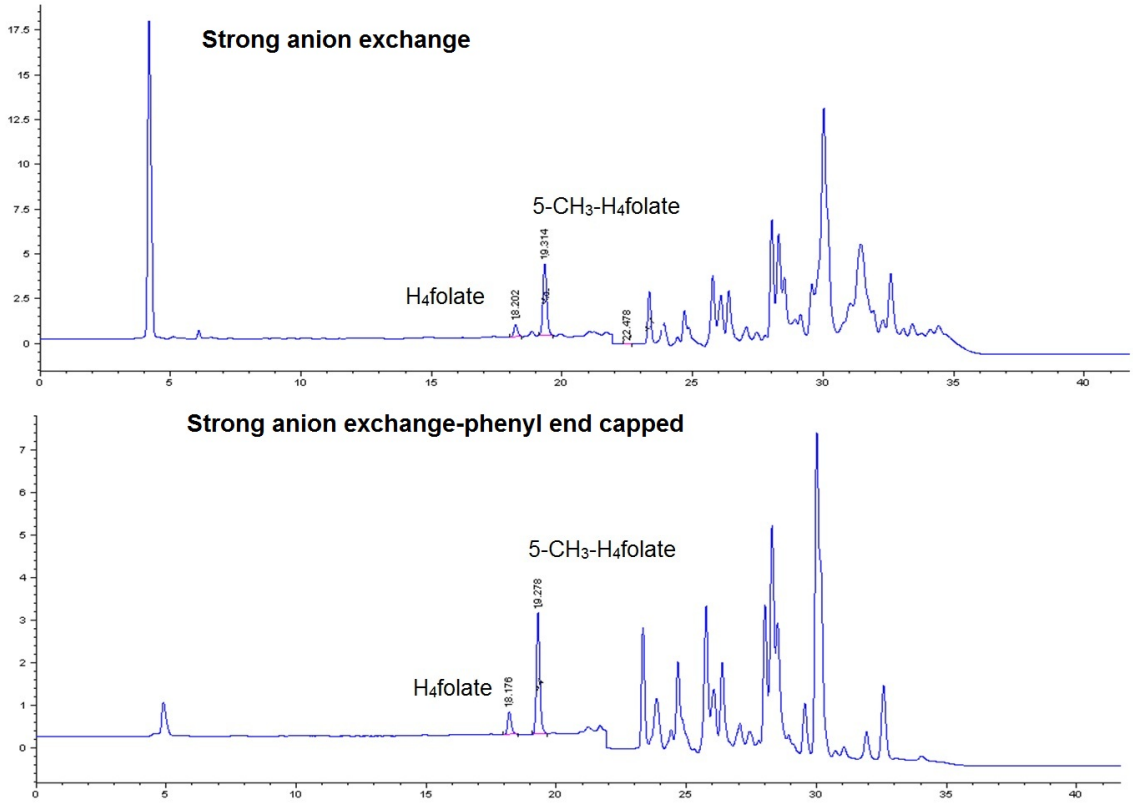

Figure D.1.: Chromatograms (Fluorescence detector) of a pooled breast milk sample after solid phase extraction on different materials.
(H_4folate: 18.2 min; 5-CH_3-H_4folate: 19.3 min).
Note that the y-axis differs between panels.

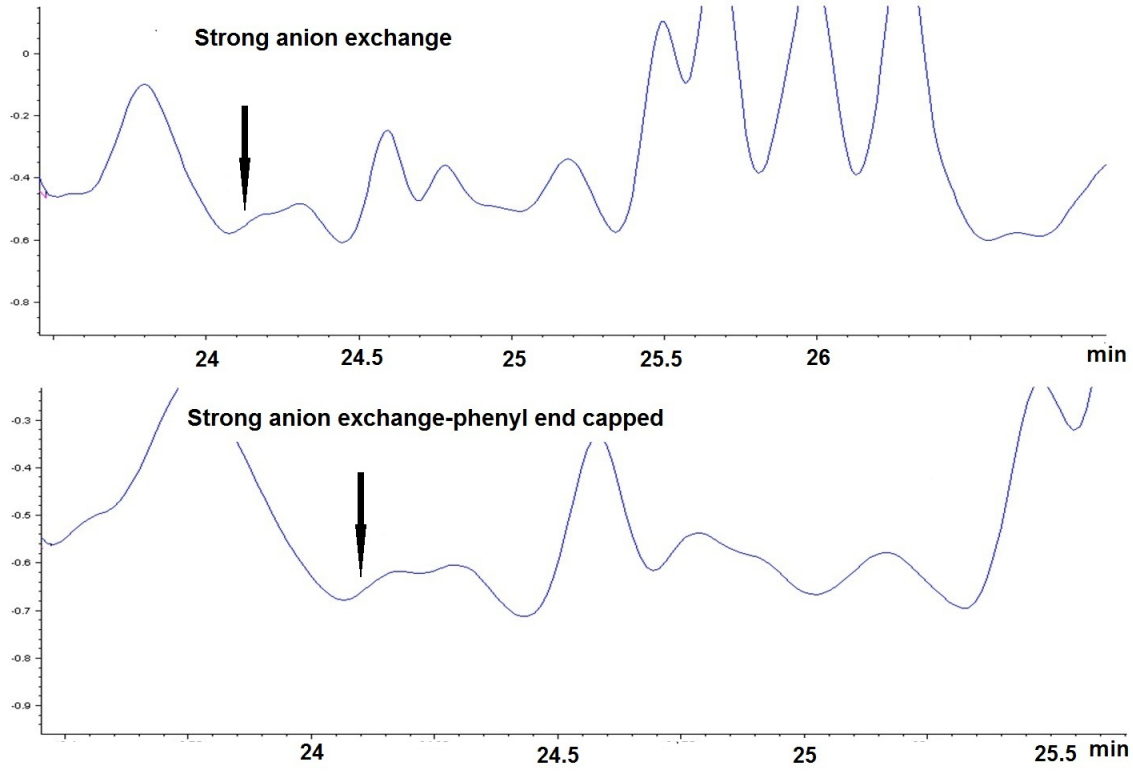

Figure D.2.: Chromatograms (UV detector) of a pooled breast milk sample after solid phase extraction on different materials
(Folic acid: 24.1 min; here: not detected, peak location indicated by arrow; figure just to show improved baseline).

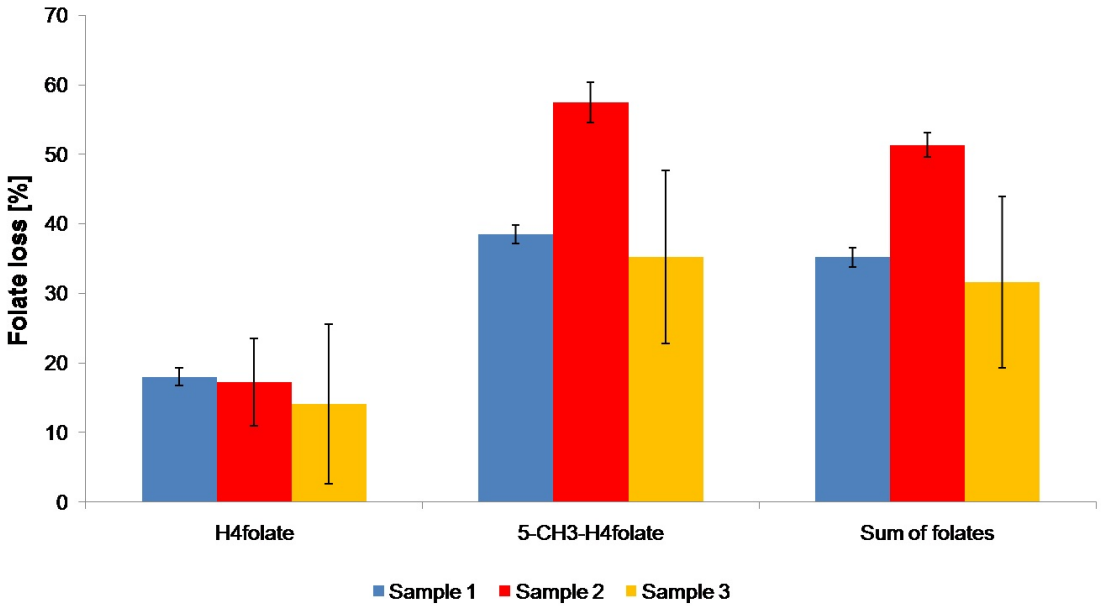

Figure D.3.: Folate losses in three different samples (1 – 3) of breast milk after a combined cleanup by solid phase extraction on strong anion exchange-phenyl end capped materials (expressed as percentage of the folate content determined after cleanup on strong anion exchange material only; mean ± Stdev; n = 3 each sample).
Sum of folates: Sum of H_4folate and 5-CH_3-H_4folate contents.

D.2. Adjustment of the heat treatment procedures

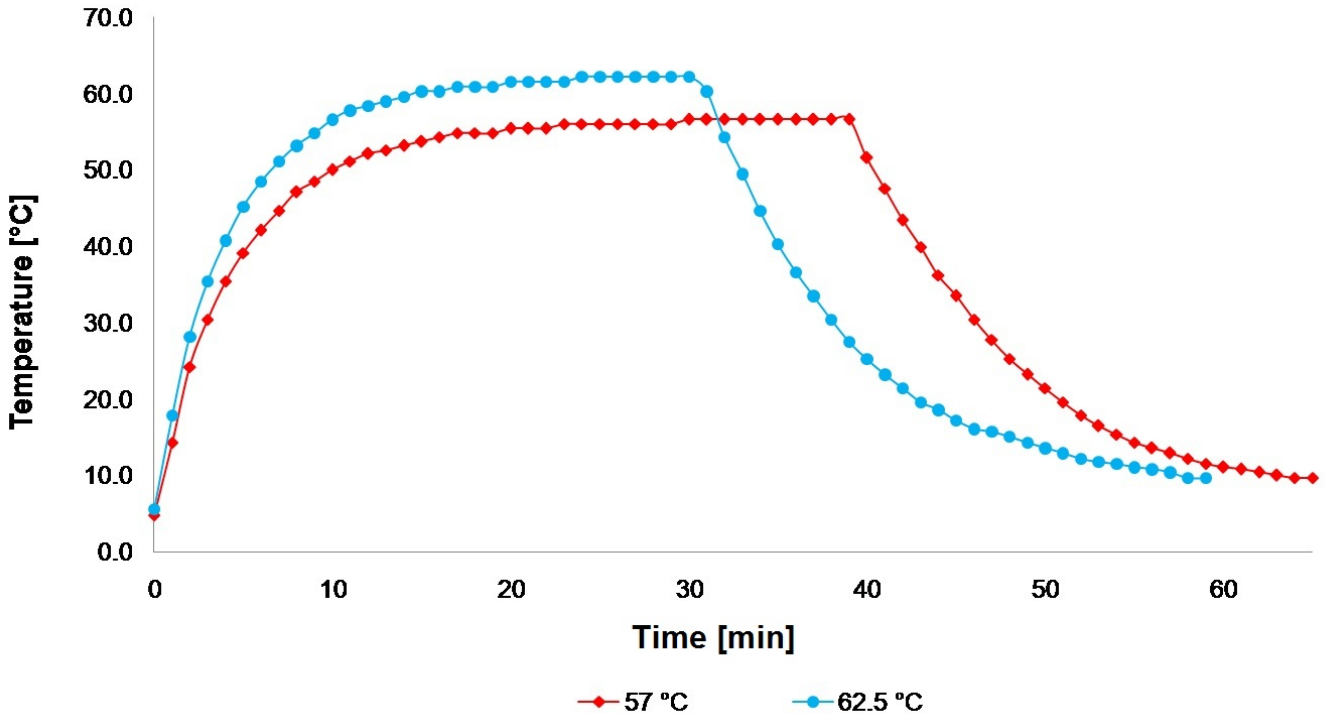

Figure D.4.: Temperature courses after adjustment of the pasteurization time to 22 minutes and 12 minutes at 57 °C and 62.5 °C, respectively.

D.3. Heat treatment of breast milk

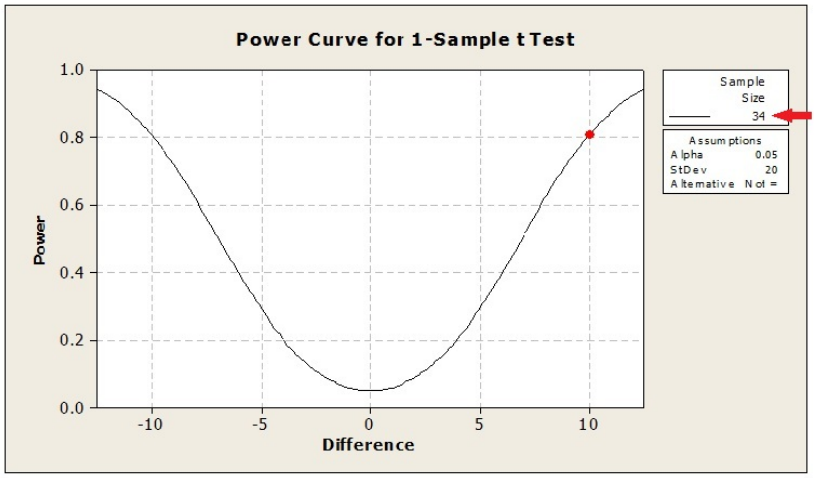

Figure D.5.: Calculation of the minimum sample size (s. red arrow) for sufficient statistical power (0.8; Minitab® 15.1.0.0, Minitab Ltd., Coventry, UK).

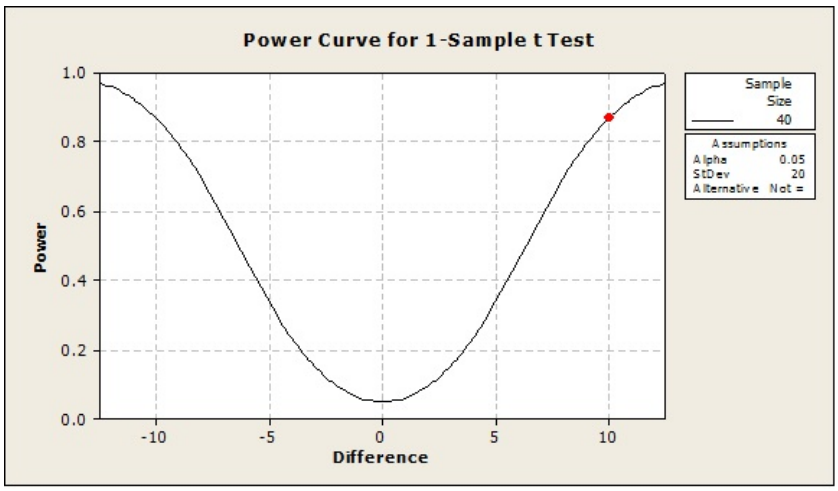

Figure D.6.: Calculation of the statistical power for sample size n = 40 (Minitab® 15.1.0.0, Minitab Ltd., Coventry, UK).

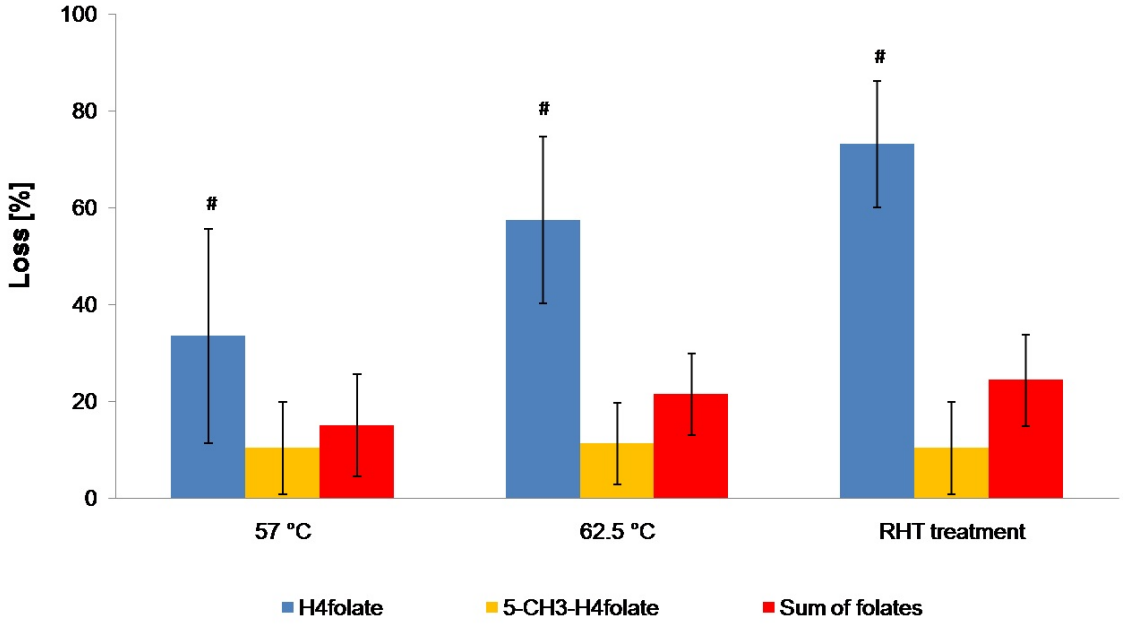

Figure D.7.: Folate losses after three heat treatments of 25 mL samples of breast milk (mean ± Stdev; n = 38, at 62.5 °C: n = 37). [%]
57 °C: Pasteurization at 57 °C for 22 minutes, 62.5 °C: Pasteurization at 62.5 °C for 12 minutes, RHT treatment: Heating in the boiling water bath for 4 minutes and 20 seconds (initial temperature: 17 °C, final temperature: 73 °C).
#: Significant difference in comparison to raw breast milk ($p < 0.05$).
Sum of folates: Sum of H_4folate and 5-CH_3-H_4folate contents.
Folate contents were corrected for endogenous folate content in enzyme preparations.

E. Publications

E.1. Paper I - Summary

New SIDAs were developed for the simultaneous determination of unlabelled and [$^{13}C_5$]-labelled intact folate vitamers, *i.e.* 5-methyltetrahydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, tetrahydrofolate and folic acid, in human plasma and ileostomal effluent.

Plasma protein had to be removed during folate extraction to prevent precipitation on the HPLC-column and clogging, which makes the column useless. The need to replace a HPLC column frequently means a high financial burden. Barbara Büttner created and tested several precipitation reagents on a pooled plasma sample. While the plasma protein removal had to be complete, degradation of the instabile folate molecule had to be prevented. Based on the obtained results, she included protein precipitation by 100% methanol into the SIDA for folate determination in plasma.

Ileostomal effluent samples are of great value in human studies on folate bioavailability as the folate content in the ileostomal effluent is less affected by microbial folate synthesis in the colon than in stools samples. Folate quantification in ileostomal effluent was based on preceding methods for food developed by this research group. Barbara Büttner tested (i) the effect and necessity of protease treatment during folate extraction from ileostomal effluent and (ii) different elution procedures during sample cleanup by SPE. Based on the obtained results, she included protease treatment (2 mg/0.5 g lyophilized sample, 4 hours, 37 °C) into the SIDA and decreased the elution volume from the SPE cartridge to 1.5 mL. Thereby, she could improve the sensitivity of the assay.

Subsequently, Barbara Büttner validated the new assays. For this purpose she determined the intra-assay CV, the inter-assay CV and the LOD. Folate-free surrogate matrices were spiked with known amounts of unlabelled and [$^{13}C_5$]-labelled folate, the samples were analyzed by the new SIDAs and the validation parameters were determined by statistical methods.

Additionally, Barbara Büttner shared the responsibility for writing and revising this publication with her main supervisor.

E.2. Paper II - Summary

New SIDAs were developed for the simultaneous determination of unlabelled and [$^{13}C_5$]-labelled folate catabolites in human urine, *i.e.* p-ABG and Ap-ABG. The respective [$^{2}H_4$]-labelled isotopologues were used as ISs.

For the development of the intended dual-label SIDAs, all unlabelled, [$^{2}H_4$]-labelled and [$^{13}C_5$]-labelled folate catabolites had to be available. So, Ap-ABG, [$^{2}H_4$]-p-ABG and [$^{2}H_4$]-Ap-ABG had to be synthesized prior to assay development. Ap-ABG was synthesized by acetylation of p-ABG. [$^{2}H_4$]-p-ABG and [$^{2}H_4$]-Ap-ABG were synthesized according to a four-step pathway starting from p-ABA. First, p-ABA was deuterated with deuterium oxide over a palladium catalyst. Subsequently, the amino moiety was protected and the protected molecule was coupled to L-Glu-di-methylester. During the final step, the protection groups were removed. Experiments by NMR spectroscopy and MS confirmed the idendity of the synthesis products. Barbara Büttner conducted all synthesis steps and performed and evaluated the confirming experiments.

The quantification of folate catabolites in human urine was based on preceding methods developed by this research group. Barbara Büttner introduced the application of a higher sample volume to the SPE cartridge during cleanup and the simultaneous quantification of the unlabelled and [$^{13}C_5$]-labelled folate catabolites p-ABG and Ap-ABG. Thereby, she could improve the sensitivity of the assay. Subsequently, Barbara Büttner validated the new assays as far as possible. For this purpose she determined the intra-assay CV, the inter-assay CV and assessed the LOD in a folate-free surrogate matrix. This matrix was spiked with known amounts of unlabelled and [$^{13}C_5$]-labelled folate catabolites, the samples were analyzed by the new SIDAs and the LOD was assessed based on the "Signal-to-Noise-Ratio".

The new SIDAs were applied successfully to the determination of the concentration of unlabelled and [$^{13}C_5$]-labelled folate catabolites in a urine sample derived from a human bioavailability trial.

Additionally, Barbara Büttner had the main responsibility for writing this publication and shared the responsibility for revising the publication with her main supervisor.

E.3. Paper III - Summary

Investigations of folate bioavailability revealed (i) differences in absorption kinetics between reduced folate and folic acid and (ii) shortcomings in studies using a dose of unlabelled folate, *e.g.* no differentation between endogenous and dose folate.
In a novel human stable-isotope AUC/ileostomy model, the influence of (i) ingested vitamer and (ii) food matrix on folate bioavailability from wholemeal bread was investigated. With her co-author and her secondary supervisor, Barbara Büttner participated in two study days in Umeå, Sweden, where plasma and ileostomal effluent samples were collected from healthy ileostomists after a single dose of either [$^{13}C_5$]-5-CH_3-H_4folate- or [$^{13}C_5$]-PteGlu-fortified wholemeal bread.
The ileostomal effluent samples were pooled as one day and one night sample at SLU, Uppsala, Sweden, prior to shipping to TUM, Freising, Germany and folate analysis by SIDA. Barbara Büttner participated in the preparation of the clinical samples for analysis, *i.e.* she thawed, pooled, homogenized and re-froze the ileostomal effluent samples. She performed the analysis of food and human samples by SIDA, *i.e.* extraction, cleanup and LC-MS/MS analysis, and introduced the methods to three laboratory technicians: A. Berrang and M. Dötsch analyzed the majority of the plasma samples, while C. Zeck analyzed ileostomal effluent and urine samples. Barbara Büttner was responsible for the evaluation of the primary results.
Based on the primary results, folate plasma kinetics and folate excretion with ileostomal effluent were calculated. The maximum plasma concentration of [$^{13}C_5$]-labelled folate after ingestion of a test dose (450 nmol), the time when this concentration was reached and the AUC differed significantly between reduced and oxidized folate. But, when ingested together with the [$^{13}C_5$]-PteGlu-fortified bread, a complete breakfast meal did not seem to affect bioavailability of [$^{13}C_5$]-PteGlu from wholemeal bread. Excretion of [$^{13}C_5$]-labelled folate via ileostomal effluent was low and ranged between 2% and 9% of the dose. This suggested a high rate of absorption of the test dose. The majority of [$^{13}C_5$]-labelled folate was detected in the first twelve hours post dose. Based on the folate content in plasma and ileostomal effluent, folate bioavailability from wholemeal bread was estimated as about 90%.
Additionally, Barbara Büttner contributed several sections to this publication.

E.4. Paper IV - Summary

Breast milk is the recommended aliment for newborn infants. Milk banks provide donated breast milk when the mother's own milk is not available. Due to the rapid growth during the first months in life, infants have particularly high folate requirements. These have to be met by the folate content in breast milk. Donated breast milk is heat treated in milk banks which causes folate losses.

The effect of three commonly applied heat treatment procedures, *i.e.* pasteurization at 57 °C for 30 minutes, Holder pasteurization (62.5 °C for 30 minutes) and a RHT treatment (73 °C for several seconds), on the folate content and the pattern of the folate vitamers in breast milk was investigated. The examined heat treatment procedures had to be adjusted to the smaller sample size in this trial (25 mL). Barbara Büttner planned, performed and evaluated all experiments required for the adjustment of the heat treatment procedures.

Breast milk samples were collected from four milk banks in Sweden and divided into four sub-samples each. Three sub-samples were heat treated according to the adjusted procedures at 57 °C, at 62.5 °C and at 73 °C, the fourth sub-sample was stored untreated until analysis by HPLC-UV/FD. Two folate vitamers were detected in Swedish milk banking samples: 5-CH_3-H_4folate and H_4folate. 5-CH_3-H_4folate was the predominant vitamer. Heat treatment caused folate degradation. Especially H_4folate was affected, while 5-CH_3-H_4folate content remained stable. The sum of folates in breast milk was decreased by 15 – 24 %, but there were no significant differences between the three investigated heat treatment procedures. Barbara Büttner shared the responsibility for the sample collection with her co-author. She had the main responsibility for the heat treatment of the trial samples and the determination of the folate content and pattern in the samples by HPLC-UV/FD. She shared the responsibility for the statistical evaluation of the results with her co-author.

Additionally, Barbara Büttner had the main responsibility for writing and revising this publication.

Acknowledgements

Viele Menschen haben es mir durch ihre Unterstützung ermöglicht meine Arbeit so wie sie nun vorliegt zu erstellen. An dieser Stelle möchte ich mich herzlich dafür bedanken. Ich weiß, dass es ohne diese Hilfe nicht gegangen wäre. Mein Dank gilt ganz besonders:

Meinem Doktorvater, Prof. Dr. Michael Rychlik, für die Möglichkeit, dieses Thema im Rahmen seiner Kooperation mit der SLU, Uppsala, Schweden, zu bearbeiten. Durch die Mitarbeit in zwei verschiedenen Arbeitsgruppen erhielt ich Einblick in verschiedenste Forschungsansätze und -schwerpunkte sowie ein weites Spektrum von Methoden. Ich hatte die Chance viele international anerkannte Forscher kennenzulernen und weitreichende Kontakte zuknüpfen. Bei der Organisation meiner Wechsel zwischen Deutschland und Schweden ebenso wie bei der Arbeit an den verschiedenen Projekten in Deutschland gewährte er mir sehr viel Freiraum und unterstützte mich nach Kräften dort, wo es nötig war.

Meiner Doktormutter, Prof. Dr. Cornelia Witthöft, für ihre Bereitschaft mich als Doktorandin zu betreuen und die freundliche Aufnahme in ihre Arbeitsgruppe an der SLU, Uppsala, Schweden. Dort lernte ich die Folatforschung von Grund auf kennen. Ich erhielt weitreichenden Einblick in Studienplanung und -durchführung sowie die statistische Auswertung von Studienergebnissen. Mit ihren kritischen Fragen half sie mir Hintergrundwissen zu erschließen und brachte mich, wenn meine Arbeit ins Stocken geraten war, immer wieder weiter.

Inger Öhlund (PhD) och sina kollegor vid Barn- och Ungdomskliniken, Norrlands Universitetssjukhus, Umeå, Sverige, för allt stöd med studien om folatstabilitet i bröstmjölk. Med sin stora erfarenhet i vård av prematura barn bidrog de mycket till gestaltningen av studiestrukturen och artikeln om våra spännande resultat.

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