Determination of Folate for Food Composition Data

Development and Improvement of Analytical Methods

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Cover: Brimstone butterfly. Folates belong to a group of compounds called pteridines. The first pterin was isolated in 1925 from the wing of brimstone butterfly and was hence named after the Greek word for wing, pteron

(Photo modified from Wikipedia: "Gonepteryx rhamni" from the collection of Cologne Zoo, Germany. 2007-09-17 by Sarefo)

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Abstract

Folate is B-vitamin that is required for maintaining normal blood status. Recommendations on daily intake are based on information on blood folate status in relation to folate intake and bioavailability. Folate intake from a diet is evaluated by folate content in the food consumed. In Sweden folate intake is evaluated on a regular basis in food consumption surveys by the National Food Agency (NFA). Folate content in foods is determined at NFA for the Swedish food composition database with a microbiological assay (MA), an international standardized method. In 2008 sampling of eggs was made for reassessment of folate content and for this study for investigation on effects on folate content from production form and processing.

In folate determination the extraction procedure is a critical step that needs to be validated for the folate forms present in the food analysed. Due to the labile nature of some folate forms, the vitamin might be destroyed during the extraction process with underestimation of folate content as a consequence. With MA total folate content is determined and another approach has to be used in order to matrix validate the extraction in respect of stability and glutamate forms of the present folates.

In eggs the chemically stable folate derivatives 5-methyl-tetrahydrofolate (5-CH_3-H_4folate) and 10-formyl folic acid (10-HCO-folic acid) in their monoglutamate forms were found using high performance liquid chromatography and mass spectrometry detection (LC/MS). The extraction procedure of MA was improved according to these findings, validated and used for analysis of the sampled eggs. There were no significant differences related to production form, the breed of hen or the feed used, organic or conventional. The retention factor for folate in boiled eggs was determined to 91 %.

A method with a new technique, ultra performance liquid chromatography (UPLC) was developed. It was more sensitive and faster when compared with an HPLC method. Further comparison with the MA will show if this can be an alternative method for folate determination of foods.

Keywords: Folate, folic acid, microbiological assay, HPLC, UPLC, LC/MS, food

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Svensk sammanfattning

Folat är ett B-vitamin som behövs för att upprätthålla normal blodstatus (undvika anemi). Rekommendation om dagligt intag baseras på information om blodstatus i relation till intag och biotillgänglighet av folat. Intag av folat från dieten beräknas utifrån vilka livsmedel som konsumerats och hur mycket samt deras folatinnehåll. Livsmedelsverket utvärderar folatintaget i Sverige genom regelbundna matvane-undersökningar. Man analyserar också matens folatinnehåll för den svenska livsmedelsdatabasen med hjälp av en mikrobiologisk metod. 2008 provtogs ägg för uppdatering av bland annat folatinnehållet. I samband med det provtogs även ägg för undersökning av effekter av produktionsform och beredning på folatinnehållet.

Extraktionen är ett kritiskt steg i metoder för bestämning av folat och behöver anpassas efter de folatformer som finns i det livsmedel som ska analyseras. Vissa folatformer är instabila och kan förstöras under extraktionen vilket i så fall leder till en underskattning av folatinnehållet. Eftersom den mikrobiologiska metoden bara ger totalinnehåll av folat måste en annan teknik användas för att bestämma vilka former som finns i livsmedlet. I ägg hittades de kemiskt stabila formerna 5-metyltetrahydrofolat (5-CH_3-H_4folat) och 10-formylfolsyra (10-HCO folsyra) som monoglutamater. Extraktionen för ägg med den mikrobiologiska metoden förbättrades med hänsyn till dessa former, validerades och användes för analys. Inga signifikanta skillnader för ägg kunde relateras till hönsras eller foder, ekologiskt eller konventionellt. Retentionsfaktorn för folat i ägg vid kokning bestämdes till 91 %.

En metod med ny vätskekromatografisk teknik (UPLC) utvecklades. Jämfört med en HPLC metod var den snabbare och kunde analysera lägre halter. Ytterligare jämförelse med den mikrobiologiska metoden behövs för att avgöra om UPLC metoden kan ersätta den mikrobiologiska.

Our greatest glory is not in never failing,
but in rising up every time we fail.
Ralph Waldo Emerson

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Strandler, H.S., Jastrebova, J., and Mattisson, I. (2011). Folate content in Swedish eggs – influence of breed, feed and processing. *European Food Research and Technology.* 233, (6): 923-930

II Jastrebova, J., Strandler, H.S., Patring, J., Wiklund, T. (2011). Comparison of UPLC and HPLC for analysis of dietary folates. *Chromatographia.* 73 (3):219-225.

The contribution of Hanna Sara Strandler to the papers included in this thesis was as follows:

I Planned the study with main supervisor, performed the analytical work, evaluated the results and had main responsibility for writing and revising the paper.

II Planned and performed parts of the analytical work and evaluated the results, participated in writing and revising the paper.

Abbreviations

H_4folate	tetrahydrofolate
5-CH_3-H_4folate	5-methyl-tetrahydrofolate
5-HCO-H_4folate	5-formyl-tetrahydrofolate
10-HCO-H_4folate	10-formyl-tetrahydrofolate
10-HCO-folic acid	10-formyl-folic acid

TR	true retention
ACN	acetonitrile
LOD	limit of detection
LOQ	limit of quantification
MA	microbiological assay
HPLC	high performance liquid chromatography
UPLC	ultra performance liquid chromatography
SPE	solid phase extraction
SAX	strong anion exchange
UV	ultraviolet
FL	fluorescence
RSD	relative standard deviation
MS	mass spectrometry
LC/MS	HPLC method with MS-detection
AOAC	Association of Analytical Communities
NFA	National Food Agency (Livsmedelsverket in Swedish)
NMKL	Nordic Committee on Food Analysis
USDA NDL	U.S. Department of Agriculture's Nutrient Data Laboratory
CEN	Comité Européen de Normalisation, European Comimittee for Standardisation

1 Introduction

The term folate refers to folic acid and its naturally occurring derivatives (vitamers) that have biological significance as vitamin for humans. They are required for one-carbon metabolism, amino acid biosynthesis as well as cellular replication and growth (Ball, 2004). The vitamin is especially important where there is a rapid cell growth such as blood production or during pregnancy, but also for cancer development.

Supplementation with the synthetic form of the vitamin, folic acid, has shown preventive effect on neural tube defects in pregnancies (MRC, 1991). Mandatory fortification has therefore been implemented in several countries in recent years (Flour fortification initiative). However, an excess of the synthetic form for the population as a whole is not necessarily beneficial (Smith *et al.*, 2008).A main concern is that folic acid might mask vitamin B_{12} deficiency (Ball, 2004). Concerns have also been raised regarding a possible increase of cancer risk. Animal studies have shown that supplementation with folic acid has a dual role depending on the folate status in the blood, enhancing the risk of colorectal cancer if folate is sufficient for normal blood status (Kim, 2003) but reducing the risk if status is low (Kim, 2004). Observational studies and randomised trials have not conclusively confirmed or disproved this concern, partly due to lack of differentiation between dietary folate and folic acid by fortification and supplement (EFSA, 2009).

Since no adverse effects are known for the native food folates in physiological amounts, provision for the daily need of folate with a diet rich in natural folate is to prefer. Intake of folate is estimated from data on folate content for the food consumed and the amount of folate that can be utilized is determined by folate bioavailability. Adequacy of intake is evaluated by folate status in the blood. Based on this information, recommendations on daily intake are set. (NCM, 2005).

Hence, in order to evaluate folate intake and set recommendations, reliable data on folate content in food is needed. In order to produce reliable data, adequate methods are required for determination. Choice of method for determination and quantification is based on what is "fit for purpose". Microbiological assays measures total folate content, while it is possible to identify the different vitamers using HPLC-methods (Ball, 2005). Competitive binding radio-assays also measure total content but are less suitable for food analysis due to their varying affinity for the different vitamers (Stralsjo *et al.*, 2002). Due to the complex nature of the vitamin, the variety of compounds and their different stability, determination of folate content has proven to be a challenge. Especially the extraction procedure is a critical step for folate determination (Gregory, 1996).

Folate content in food is regularly determined at the National Food Agency (NFA) for the Swedish food composition database with a microbiological assay (MA), an international standardized method. In this study the extraction procedure for determination of the folate forms in eggs with MA was improved and validated. Since MA only determines total folate content another approach had to be used in order to matrix validate the extraction in respect of stability and glutamate forms of the present folates. The chemically stable folate derivatives 5-methyl-tetrahydrofolate (5-CH_3-H_4folate) and 10-formyl folic acid (10 HCO-folic acid) in their monoglutamate forms were found using high performance liquid chromatography and mass spectrometry detection (LC/MS). The extraction procedure of MA was improved according to these findings and validated. Also a method with a new technique, ultra performance liquid chromatography (UPLC), was developed and compared with a validated HPLC-method The UPLC was tested as an alternative method for folate determination of foods. In connection to a project for the Swedish food composition database (Gard *et al.*, 2010), sampling of eggs was made for investigation on the effects on folate content from production form (breed of hen used and type of feed) and from processing (boiling). Egg samples were analysed with the validated microbiological assay.

2 Background

2.1 Discovery and significance of folate

"The excellence of her work on tropical megaloblastic anaemia has long been recognized by nutritionists and haematologists. Every medical student has heard of its cure by her discovery of the Wills factor in yeast extract, which paved the way for the subsequent work on folic acid. It was one of the simple but great observations which are landmarks in the history and treatment of the nutritional anaemias. . . ."
Obituary in the British Medical Journal May 1964, p 1445
(Obituary Notices, 1964)

Figure 1. Lucy Wills (Family picture, Wikipedia, the picture is somewhat trimmed)

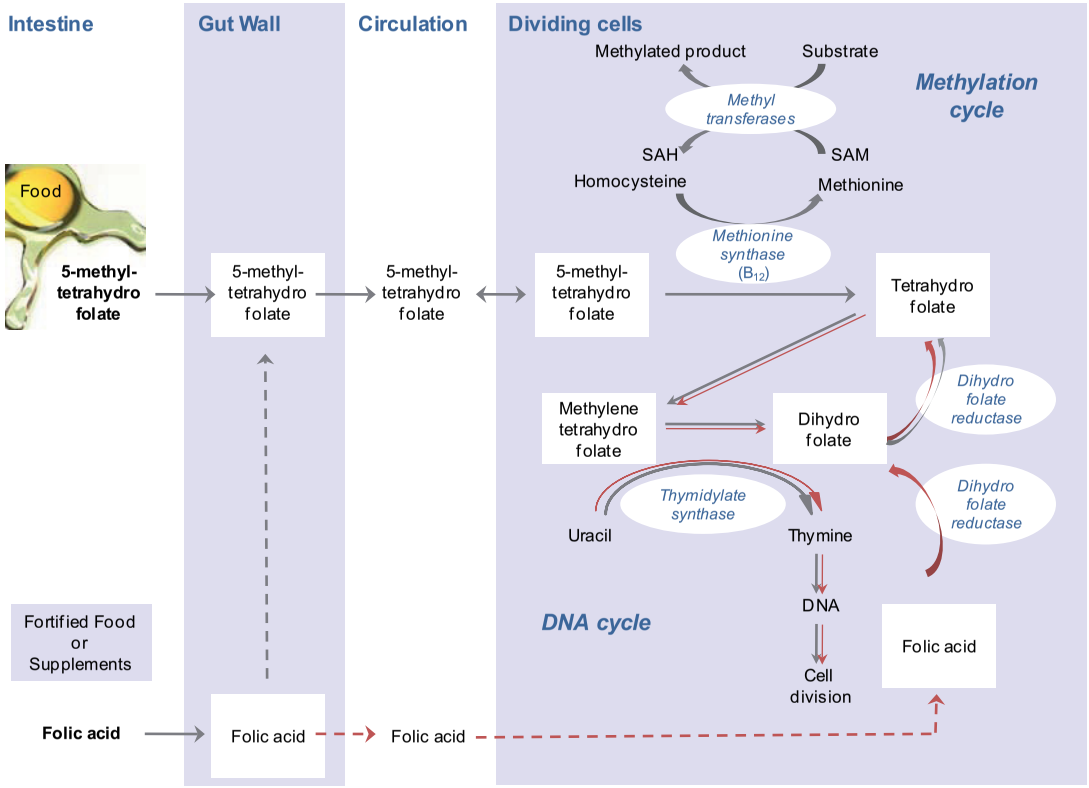

Figure 2. Human folate and folic acid metabolism. Modified from Scott (EFSA, 2009). In higher doses folic acid can pass into circulation and by-pass the body's regulation of folate metabolism. It will enter into the DNA cycle. .

Many diseases that are caused by vitamin deficiency were first thought to be caused by bacteria or a toxic substance. Lucy Wills' research for a treatment of pregnancy anaemia among Indian women was no exception, but her work brought her to another conclusion.

> The question must, however, remain unsettled until further work can decide the common factor in the two extracts which is active. At present it is only possible to state that in marmite, and probably in other yeast extracts, there appears to be a curative agent for this dread disease which equals liver extract in potency, and has the advantage in India of being comparatively cheap and of vegetable origin." (Wills, 1931, p.1064).

According to Blakley's review (Blakley, 1969) of the historical development, three lines of studies followed where a factor from liver, yeast and spinach was proven to have an effect on megaloblastic anaemia in monkeys, growth in chicks and lactic acid bacteria. In 1940 Mitchell's group could isolate an acidic substance which was necessary for the growth of *Lactobacillus case (L.casei).* They named the new substance folic acid from the Latin word for leaf, *folium*, probably because they had to prepare four tons of spinach in order to isolate it. In another five years Angier's group managed to synthesise a compound identical with the *L. casei* factor from liver (Angier *et al.*, 1945) and later they determined the structure (Angier *et al.*, 1946). Synthetic folic acid was then used for treatment of anaemia and on her request, folic acid was brought to Lucy Wills in Birmingham, where she successfully treated her patients (Roe, 1978).

Anaemia as a symptom of folate deficiency is due to the vitamin's biochemical function. Folates have a unique role in the body through their ability to transport one-carbon units at different oxidation levels. This is important for body chemistry, such as the biosynthesis of DNA and for methylation reactions (Fig.2) (Scott *et al.*, 2000). Methylated reduced folates are cofactors in the synthesis of three of the four nucleotides for DNA and are involved in amino acid metabolism. Methylation is often a way to activate or deactivate signal substances or gene expression (Wagner, 1995). In the rapidly growing blood cells there is an increased need of DNA and consequently of folate. Folate deficiency causes abnormal nuclear maturation in the blood cells which in turn leads to megaloblastic anaemia (Ball, 2004).

2.2 Nutritional requirement of folate

The requirement of folate varies individually. However, recommendations for the population have to be set to meet the needs of most individuals. The Nordic Nutrition Recommendation (NNR) 2004 (NCM, 2005) identifies four intake levels for folate (Fig.3):

LI – The lower intake level, below this intake deficiency symptom can appear in some individuals.

AR – The average requirement for normal levels of folate in blood, serum and red blood cells.

RI – Recommended intake is based on AR and a safety margin.

UL – The upper limit is an estimate of the highest level of intake that carries no appreciable risk of adverse health effect.

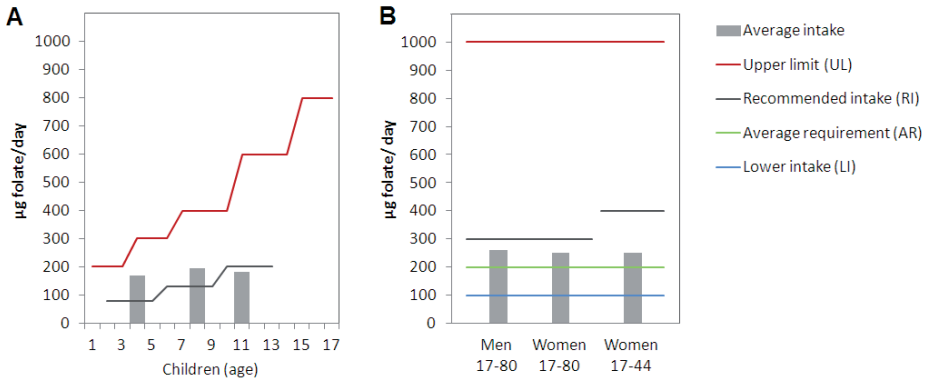

Figure 3. **A.** Folate intake for children - recommendations in NNR (NCM, 2005) and average folate intake, Riksmaten barn 2003 (Enghardt Barbieri *et al.*, 2003). **B.** Folate intake for adults - recommendations in NNR (NCM, 2005) and average folate intake, Riksmaten 97-98 (Becker & Pearson, 2002).

The recommended intake is

"to be interpreted as guidelines for the nutritional composition of a diet which provides a basis for good health" (NCM, 2005, p.10)

"valid for groups of healthy individuals" and for "the average intake over a longer period of time, *e.g.* over at least a week" (NCM, 2005, p.11).

Hence they are not intended to be used on an individual basis and, due to their inherent uncertainty they can only be used to determine the probability of an adequate intake. When evaluating the intake in a population the AR should be used with LI and RI as reference values. The UL is used to estimate the risk of negative effects. For assessment of individual adequate intake, the nutritional status needs to be measured biochemically (NCM, 2005, p. 48).

2.3 Food composition data – production and use

> "A knowledge of the chemical composition of foods is the first essential in the dietary treatment of disease or in any quantitative study of human nutrition. Both of these have become increasingly important in recent years and there has in consequence been a demand for better and more up-to date information about the chemistry of food."
>
> The chemical composition of foods. (McCance and Widdowson, 1940, p.5).

In order to evaluate a diet, food composition data of good quality are needed. (Gregory, 1998). Data quality in food composition tables can be assessed by evaluation of the representativeness of the samples and analytical quality. The U.S. Department of Agriculture's (USDA) Nutrient Data Laboratory (NDL) has developed an evaluation scheme of food composition data (Greenfield & Southgate, 2003; Holden *et al.*, 2002) that can also be used in the planning an analytical project. The following should be considered and documented:

Sampling plan – to assure representativeness
: Representativeness determines what conclusions that can be drawn in general from the results. How representative is the sample, geographically, time wise, brands?

Sample handling - treatment that does not destroy the analyte of interest.
: If necessary, was the sample homogenised? Was only the edible portion analysed? Was the sample stored correctly?

Number of samples - to assure a representative value.
: How many samples were taken? Were they analysed individually or as composite samples?

Analytical method – to assure validity of the analytical method used.
: Does the method used meet the criteria for determination of the specific analyte?

Analytical quality control – to assure the validity of the method as used by the laboratory, e.g. the use of reference materials and recovery tests.

The importance of food description and identification of the analyte (EuroFIR, 2009), the use of relevant matrices and concentrations when choosing reference materials, and participation in proficiency testing programmes is also emphasised (NMKL, 2011).

Aspects to keep in mind when comparing data for plant foods, is to question whether the observed differences are due to artefacts (changes in analytical method, calculation, definition of nutrient) and/or real differences (variety, degree of ripeness, cultivation, climate, storage or dry weight) (Mattisson *et al.*, 2008).

Analysis is often performed on raw foods. The ratio of folate content in the food after preparation compared with the folate content in the raw food is called retention factor. In the Swedish food composition database the retention factor for folate is presently set to 70 % as an average of factors compiled by Bergström (Bergström, 1997). However, the ranges for retention factors in this compilation are large e.g. vegetables 35 – 70 %, fruit 20 – 60 %, eggs 50 - 95 %, and the lack of factors for many foods indicates that there is a need for determination of such.

The amount of folate utilized in the body is affected by the bioavailability. Bioavailability is therefore considered when setting reference values. On an average the bioavailability of natural folates is estimated to be 50 % (NCM, 2005), but the range is large (30 % - 98 %) as reviewed by Öhrvik and Witthöft (Öhrvik & Witthoft, 2011). A review by Caudill (Caudill, 2010) concludes with an average for bioavailability of 65 % with the range of 40-80 %. The bioavailability of folic acid is considered to be 100 % but appears to be lower (85 %) if consumed in combination with food (Pfeiffer *et al.*, 1997a).

Food composition databases are provided on a national basis, in Sweden by the National Food Agency. Governmental national surveys of food consumption are performed on regular basis in Sweden and used with the food composition database for evaluation of folate intake in the Swedish population (Fig.3) (Enghardt Barbieri *et al.*, 2003; Becker & Pearson, 2002; Becker, 1994). Intake data together with information of folate blood status in the population is a prerequisite for a solid baseline if considering folic acid fortification (NCM, 2005, p.55).

The Swedish food composition database comprises 52 nutrients for 2050 foods and dishes today. Folate content in most foods is below 50 µg/100 g (Fig.4). Actual intake is determined by portion size. Each year folate in foodstuffs is determined in different projects for reassessment of previous data (Öhrvik *et al.*, 2010) and for inclusion of new data (Öhrvik *et al.*, 2011). In 2008 eggs were sampled for reassessment of nutrient data (Gard *et al.*, 2010).

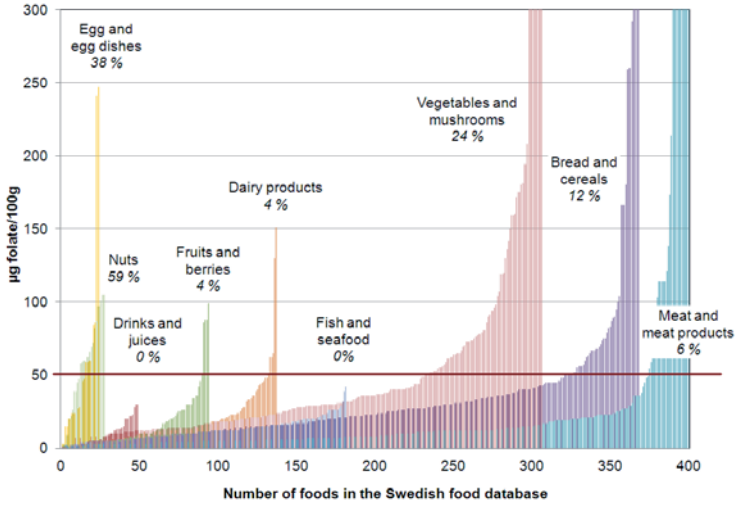

Figure 4. Folate content in different food groups in the Swedish food composition database (Livsmedelsverket, 2012) and percentage within each group for folate amount > 50 µg/100

Eggs contributed to 3 % of the folate intake in Sweden (Fig.5) according to the last food consumption survey, Riksmaten 97-98 (Becker & Pearson, 2002). Consumption of eggs was on average 15 g/day both for men and women, not including mixed dishes (pancakes, pies) and cakes, cookies etcetera (Becker & Pearson, 2002). However, consumption was not normally distributed in the population. Based on food consumption statistics per capita from the Swedish Board of Agriculture, the total consumption of egg year 2000 was about 25 g egg/day and year 2009 almost the same, 24 g egg/person (Swedish Board of Agriculture, 2011).

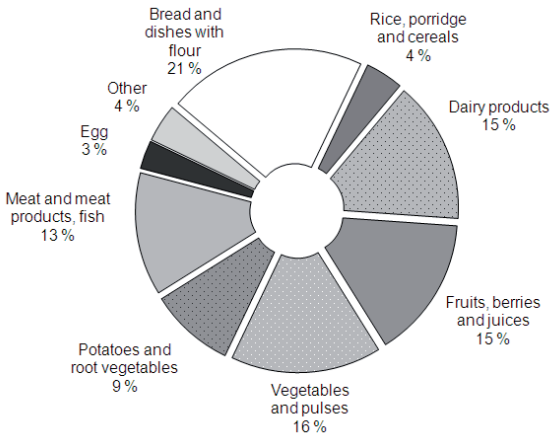

Figure 5. Contribution from different food groups to the folate intake of the Swedish population, Riksmaten 97-98 (Becker & Pearson, 2002).

2.4 Folate chemistry

Knowledge of the analyte is the starting point for analysis.

2.4.1 Structure and nomenclature

Folate is a generic term for folic acid and derivates of the vitamin (Blakley, 1987). The basic structure of folate (Fig.6, Folic acid and reduced folate) comprises a pteridine ring joined to *p*-aminobenzoic acid with a methylene group. To the *p*-aminobenzoic acid an L-glutamic acid residue is linked. Additional glutamates can be coupled in the γ-position, commonly to form a chain of totally five to seven residues. The cell uses the attachment of glutamates to retain the folate.

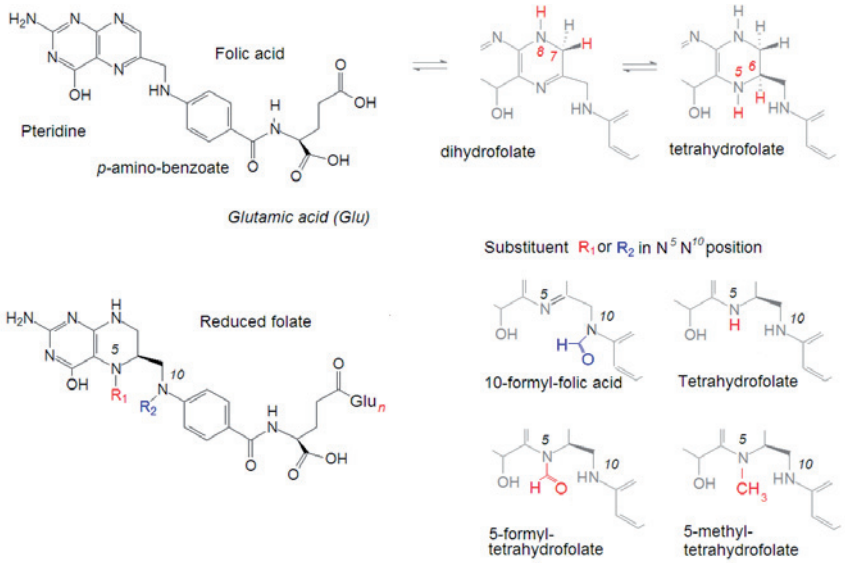

Figure 6. Structures of folic acid and the folate forms determined in this study.

Since natural folates function as one-carbon carrier in the body's chemical reactions it occurs in several structures. The one-carbon groups are attached in the N-5 and/or N-10 position (Fig.6, Substituent R_1 or R_2 in N^5 N^{10} position). Common for the enzymatically active forms is that the pteridine ring is reduced to tetrahydrofolate (H_4-folate). Each folate form is named after its substituent and the reduction state of the pteridine ring (Fig.6). Reduction of the pteridine ring brings a steric centre at the sixth carbon (6S, 6R) (Fig.6 Reduced folate). There is also a steric centre at the α-carbon (αS, αR) in the glutamate residue, where the αS- or L-glutamate is the biologically active (Gregory, 1996).

2.4.2 Stability of folates

The stability of the different folates varies and depends of the chemical environment such as oxidants, pH and catalysts, even the type of buffer ions influence the vitamers (Gregory, 1996). Vitamin activity is lost if the C^9-N^{10} bond is cleaved, but changes in the pteridine ring might also have an impact. The vitamers with a substituent at N^5 or N^{10} position as a steric hindrance are less prone for cleavage (Gregory, 1996).

According to the study of O'Broin (O'Broin *et al.*, 1975) the most labile form is H_4folate followed by, 10-HCO-H_4folate and 5-CH_3-H_4folate, while 5-HCO-H_4folate and folic acid has good stability. However, folate was quantified with a microbiological assay that gives response to all biologically active forms. The authors thought it likely that 10-HCO-H_4folate was converted to the more stable form 5-HCO-H_4folate and/or was oxidised to 10-HCO- folic acid, which also have vitamin activity (Gregory *et al.*, 1984).

The use of reducing agents such as ascorbic acid and/or thiols improves the stability of 5-CH_3-H_4folate greatly (Gregory, 1996). A combination of ascorbic acid and a thiol is necessary in order to prevent interconversions of folate forms caused by formaldehyde formed from ascorbic acid when heated (Wilson & Horne, 1984; Wilson & Horne, 1983). This combination is also necessary during sample-preparation and storage in order to protect the most labile form H_4folate (Patring *et al.*, 2005b). The thiol 2, 3-dimercapto-propanol was more efficient than 2-mercapto-ethanol for this purpose. However, these thiols are more or less toxic and therefore not user friendly. De Brower et al. found the non-toxic 2-thiobarbituric acid to be as efficient as 2, 3-dimercapto-propanol (Brouwer *et al.*, 2010).

2.5 Analytical methods for folate determination

Analytical techniques used for determination of folate content are biological, such as microbiological and competitive-binding assays or chemical, such as HPLC-methods (Arcot & Shrestha; Ball, 2005; Gregory, 1996)

The competitive binding assay, using a folate-binding protein and a radiolabeled folate to compete with the folate to be determined, gives total folate content. The Biacore method, certified by AOAC for folic acid determinations, also uses a folate binding protein and measures the amount of vitamin with a plasma surface resonance technique (Boström Caselunghe & Lindeberg, 2000). However, the folate binding protein does not have the same affinity to the various folate forms (Stralsjo *et al.*, 2002). Therefore these methods are appropriate only if it is possible to calibrate against the same form of folate as the one present in the sample, such as folic acid in fortified foods. They are less useful for food analysis with more complex matrices and several combinations of folate forms.

For food analysis the microbiological assay, utilizing the nutritional requirement of a microorganism, is suitable for determination of total folate content. However, if the focus of interest is on the individual folate forms a HPLC method, which separates and detects the different forms based on their chemical properties, has to be used. There are standard methods assessed in collaborative studies established for microbiological assays, for fortified infant formula (AOAC, 2000), cereals (AOAC, 2004) and for foodstuffs (CEN, 2003). There are no standard methods established yet for chromatographic methods. Common for both the microbiological and HPLC method is that they are labour-intensive and require skilled users (Kariluoto, 2008). Microbiological assays are commonly used for food composition data in Europe, sometimes complemented with an HPLC method for control of folic acid in fortification (Bouckaert *et al.*, 2011). Inter-laboratory comparisons for folate determination show large differences for foods with naturally occurring folates relative standard deviation (RSD) >45% (Koontz *et al.*, 2005) and RSD 24-35 % (Puwastien *et al.*, 2005). Usually one type of analytical technique is used for folate determination, but combinations have also been used such as separation on HPLC and quantification with a microbiological assay (Hoey *et al.*, 2009; Kelly *et al.*, 1996).

Folate determination comprises three main parts; extraction, deconjugation and detection/quantification (Fig.7). Crucial during the whole procedure for the result is the use of antioxidants. Also, caution has to be taken to reduce the UV-light.

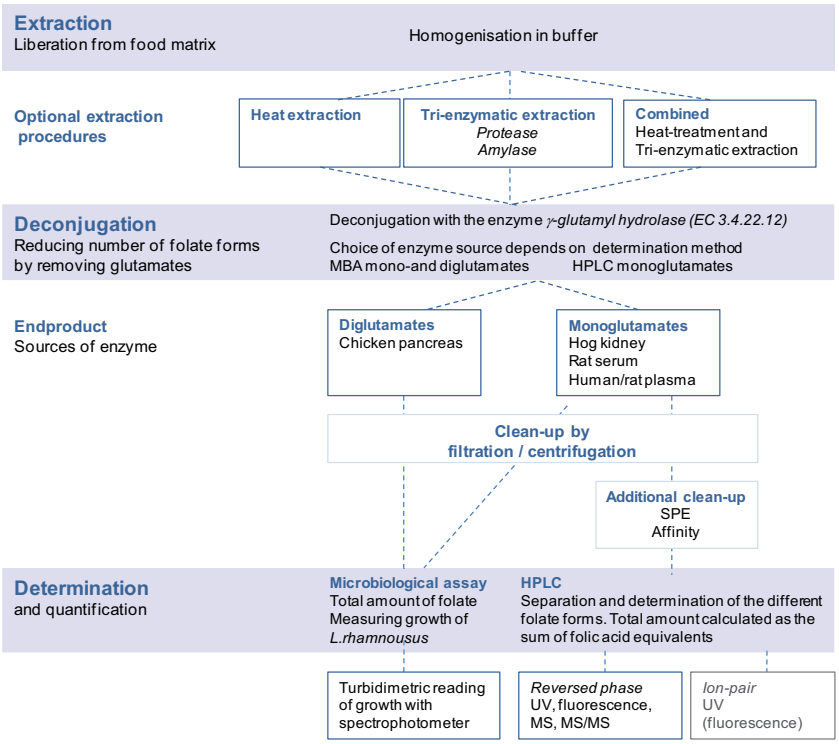

Figure 7. Schematic overview of analytical methods for folate determination modified from reviews of (Ball, 2005; Gregory, 1996). SPE = solid phase extraction.

2.5.1 Extraction and deconjugation

Early, the need of a deconjugation enzyme, reducing the number of glutamate residues on folates to di-glutamates, was recognised in order to achieve equal bacterial growth response to all forms in the microbiological assay (Blakley, 1969). During the 1950's the need of an antioxidant was recognized to be necessary for protection of folates and over time ascorbic acid was included in extraction buffers (Blakley, 1969). Therefore folate data before the 60's are not as reliable as more recent data (Gregory, 1996). During the 1990's the use of protease and amylase during extraction was recognised to increase the result of folate content in some foods and tri-enzymatic extractions were introduced (Hyun & Tamura, 2005; Gregory *et al.*, 1990; Martin *et al.*, 1990). As

mentioned earlier the need of an additional antioxidant was identified to improve the results (2.4.2). The use of an additional antioxidant is not applied in the microbiological standard assays (AOAC, 2004; CEN, 2003; AOAC, 2000). Considering the long incubation times at elevated temperatures in the microbiological assays this might lead to an underestimation of folate content in foods with H_4-folate, the most labile form.

Three major procedures are used for liberation of folate from the food matrix (Fig.7). One option is to denature the folate binding protein with heat treatment, e.g. 100-121 °C, 15 min (CEN, 2003). Another option is enzymatic extraction using protease and amylase for liberation of protein-and starch bound folate. In their review Hyun and Tamura suggest a procedure with treatment of the food sample with a protease preparation from *Streptomyceus griseus* (Type XIV) followed by treatment with an amylase preparation from, *Aspergillus oryzae.* Hyun and Tamura mean that heat-treatment is unnecessary if protease treatment is applied (Hyun & Tamura, 2005). The enzymatic procedure is often called trienzymatic since the extraction is followed by the enzymatic deconjugation. A third option is to combine heat- and the trienzymatic treatment. This procedure is used in the standard method for cereals (AOAC, 2004). In the European standard it is optional to use either the heat-extraction or the combined heat- and tri-enzymatic procedure (CEN, 2003).

In order to limit the number of forms for determination, mono- or diglutamates are achieved by enzymatic deconjugation of the glutamate residues. A purified enzyme of γ-glutamyl hydrolase (EC 3.4.22.12) for deconjugation is not available at a reasonable cost. Instead enzyme preparations are prepared from different crude sources, depending on the method used for determination (Fig.7). The temperature during incubation with enzymes is usually 37 °C and the buffer pH depends on the optima of the enzymes (Hyun & Tamura, 2005). Folate content may be affected by the long incubation times at this temperature and the enzyme treatment needs to be optimised (Pfeiffer *et al.*, 1997b; Pedersen, 1988). It is necessary to determine and/or eliminate endogenous folate in the enzyme preparation or otherwise the results will be affected. Elimination can be made by charcoal treatment (Brouwer *et al.*, 2008; Tamura, 1998; Pedersen, 1988)] or dialysis (Patring *et al.*, 2005a). Endogenous folate in the enzyme preparations should be determined, with enzyme correction of the result if necessary.

2.5.2 Microbiological assays

Folates and folic acid are growth factors for the bacteria *L. casei, subspecies rahmnosus* American type culture collection (ATCC) 7469. *L.casei* responds equally to all different forms at pH 6.2 (Phillips & Wright, 1983). The bacteria utilize the mono- and diglutamate forms of folate, the growth response decline with additional glutamate residues (Tamura *et al.*, 1972). *L. casei* are lactic acid bacteria and thereby not harmful to work with. Also, they are not prone for mutation (Ball, 2005). The culture of *L.casei* can either be kept as a stock culture on broth (Ball, 2005) which means more attendance and preparation, or cryoprotected (Wilson & Horne, 1982) which simplifies the assay and gives better repeatability. The assay of Molloy (Molloy & Scott, 1997) also uses a penicillin-resistant strain which makes it possible to work in non-sterile conditions. A common procedure described by Ball (Ball, 2005) is that aliquots of sample extract or standard are mixed with a medium that is complete with nutrients for the growth of *L.casei* except for the vitamin; the vitamin added hence becomes growth determining. Either tubes (5 mL) or microtiterplates (300 μL) (Molloy & Scott, 1997; Newman & Tsai, 1986; Wilson & Horne, 1982) can be used. After addition of the microorganism and 22-24 h incubation at 37 °C the growth rate is measured by reading the turbidity spectrophotometrically (Ball, 2005).

2.5.3 Chromatographic methods

Both ion-pair and reversed phase HPLC can be used for folate separation (Fig.7), but reversed phase is more frequently applied due to its more versatile choices of detection (Ball, 2005; Gregory, 1996). Applications commonly use C18 silica columns as stationary phase, mobile phase with an acidic buffer such as phosphate buffer (UV-/fluorescence detection) or formic or acetic acid buffer (MS, MS/MS detection) and a gradient with acetonitrile and/or methanol (Ball, 2005).

UPLC, a new technique has also emerged for faster separation. By using packing materials with smaller particle size, < 2 μm, it is possible to increase the column efficiency. The sensitivity will increase since higher efficiency is related to narrower and higher peaks. (Swartz, 2005). This leads to higher requirements on the detection system, e.g. cell volume of the detector that has to be smaller to maintain the increased resolution and data sampling that has to be fast enough to handle the more narrow peaks. However, the use of smaller particles will increase back-pressure and the equipment used has to be able manage this. Columns used in UPLC need to have stationary phases with enhanced mechanical stability (Swartz, 2005).

Determination and quantification

When using HPLC, the choice of calibrants becomes more crucial since only the forms that are used as calibrants can be identified in the sample. Due to interconversions and stability problems, there are limitations to which folate forms can be found in food extracts and this has been reviewed by Patring (Patring, 2007). Determination can be made by using UV-, fluorescence or MS detection. The reduced folates have native fluorescence of different intensity and wavelength maximum (Gounelle *et al.*, 1989). Folic acid does not fluorescence and is better determined with UV detection (Eitenmiller & Landen, 1999). This means that a combination of UV- and fluorescence detection at different excitation/emission wavelengths is necessary for quantification of all folate forms. Interfering substances can make it difficult to determine and quantify especially 5-HCO-H_4folate which is low in fluorescent (Kariluoto, 2008).

Higher specificity is achieved by using MS and MS/MS detectors. Analytes are ionized, giving a specific mass to charge pattern and folates can be determined in both positive and negative mode (Patring & Jastrebova, 2007). Quantification is usually performed with external calibration in HPLC methods using when UV and fluorescence detection. In methods with MS, MS/MS detection the use of internal standards , deuterium (Freisleben *et al.*, 2003) or radiolabeled folates (Chandra-Hioe *et al.*, 2011; Brouwer *et al.*, 2008), can compensate for losses during sample preparation and for ion-suppression during chemical analysis but should be evaluated (Wieling, 2002)

Sample purification

Chromatographic methods are more sensitive to interfering substances and have higher demands on sample clean-up than the microbiological assays. Solid phase extraction (SPE) can be used both to remove interfering substances and for concentration of the analyte. Sample extracts are applied to a column packed with a material that will retain the analyte of interest. Interfering substances are rinsed with a washing solution and the analyte is then eluted using a solution with chemical properties that releases the analyte from the stationary phase. SPE-columns are commercially available and the clean-up procedure can be automated (Ball, 2005). Affinity chromatography, clean-up using folate binding protein applied on columns can be used to increase sensitivity. However, the same issue as for folate binding assays of different affinity to the various folate forms (Stralsjo *et al.*, 2002) is a concern also for this technique.

3 Objectives

Accurate data on folate content in foods is a prerequisite for reliable evaluation on folate intake from the diet and when setting recommendations. Accurate data require adequate methods for folate determination. For food composition data folate is generally determined by a microbiological assay. The main objectives for this study have been:

To improve the extraction procedure of a routinely used microbiological method for folate determination in respect of the folate forms present in eggs.

To evaluate UPLC as an alternative analytical technique

To investigate the effects on folate content in eggs due to differences in production such as breed of hen or feed used.

To determine the retention factor for folate in boiled eggs.

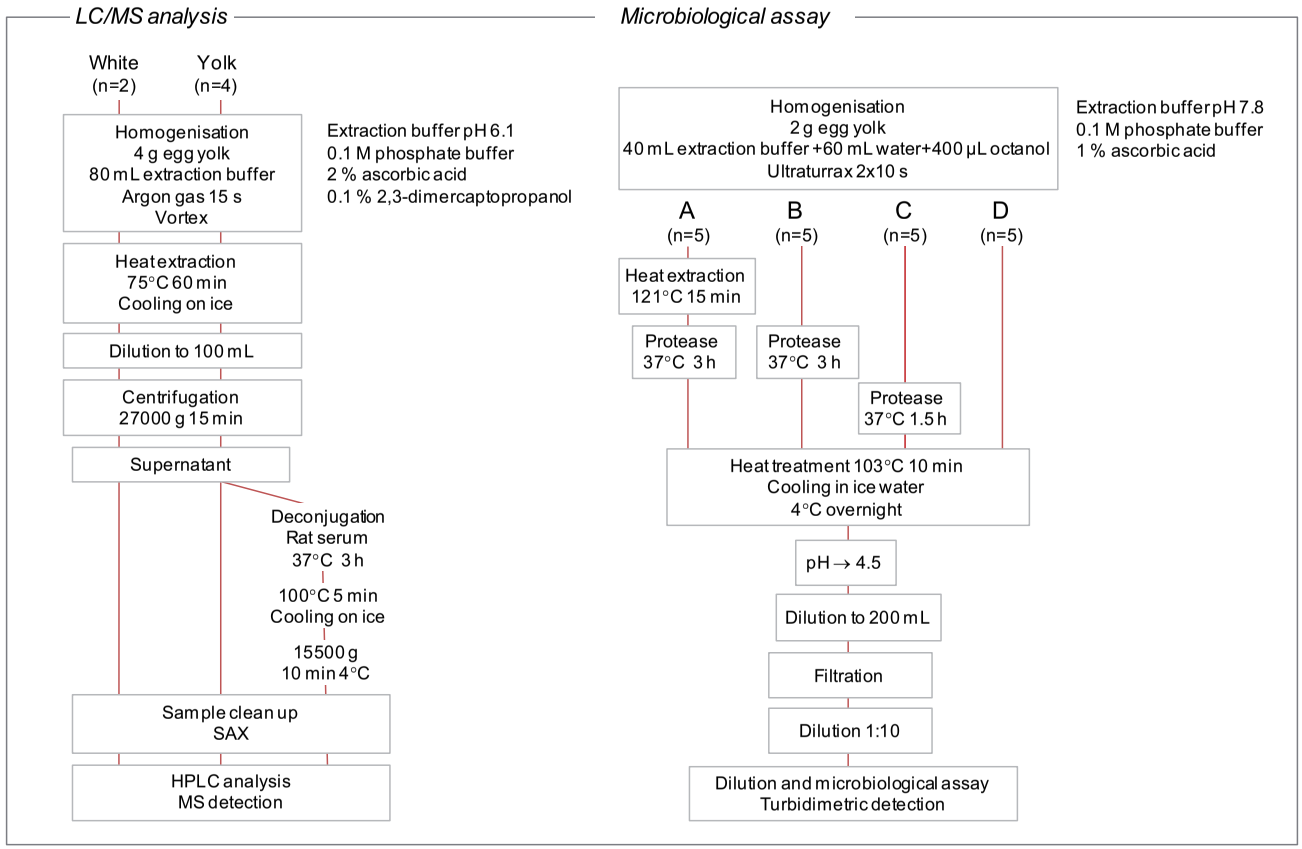

Figure 8. Methods used in Paper I. LC/MS for detection of folate forms. Extraction procedures compared in microbiological assay. A=standard procedure B= proposed procedure.

4 Materials and methods

4.1 Calibrants for folate determination

Calibration was performed with external standards in all assays. In the microbiological assay (Paper I) folic acid was used, in the HPLC method (Paper I and II) and UPLC method (Paper II) standard solutions of folic acid, (6S)-H_4folate, (6S)-5-formyl-H_4folate, (6S)-5-methyl-H_4folate, 10-formyl folic acid were used. More detailed descriptions of the calibrants and preparations used are found in the Paper I and Paper II.

4.2 Study designs

4.2.1 Folate content in Swedish eggs – influence of breed, feed and processing (Paper I).

Outlines for the methods used are shown in Figure 8, detailed descriptions of the procedures, materials and instrumentation are found in Paper I.

The folate forms in egg yolk were first determined by LC/MS. Also, extracts treated with conjugase were compared with untreated extracts to determine if there were polyglutamate folate forms present. Thereafter the extraction procedures for folate in eggs by the microbiological assay were assessed in accordance to the findings from LC/MS. The standard extraction procedure A (Fig.8) was compared with proposed alternative procedures, two enzymatic procedures (B and C) and one with heat-treatment only (D) (Fig.8). Procedure B was used, after validation, for quantification of folate in the egg samples (4.3.1) (Fig.8 and Fig.10). An in-house control-sample was analysed in each analysis. Dry matter was determined for all samples.

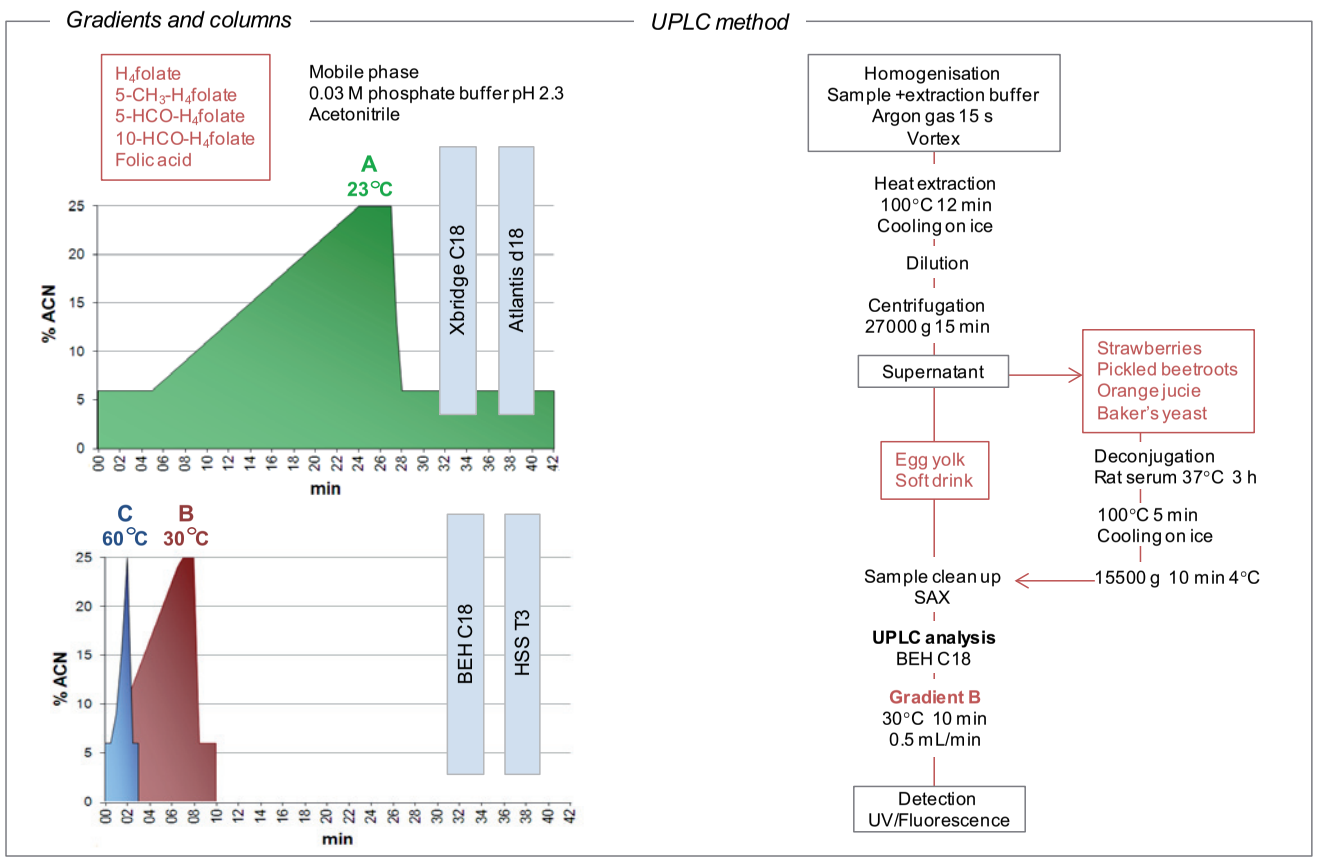

Figure 9. Methods and samples used in Paper II. Xbridge C18 and Atlantis 3.5 µm,150×4.6 mm, Acquity HSST3 and BEH C18 1.7 um,100×2.1 mm

For estimation of the retention factor for folate in boiled eggs (4.3.1) (Fig.10), calculation was performed according to the recommendation in "Nutrient losses and gains in the preparation of foods" for true retention (TR). (Bergström, 1997)

$$\%\ \mathrm{TR} = \frac{\text{folate content per g cooked food} \times \text{g food after cooking}}{\text{nutrient content per g raw food} \times \text{g food before cooking}} \times 100$$

4.2.2 Comparison of UPLC and HPLC for analysis of dietary folates (Paper II).

Outlines for the methods used are shown in Figure 9, more detailed descriptions of the procedures, materials and instrumentation are found in Paper II.

An UPLC method was developed for comparison with the validated HPLC method of Jastrebova (Jastrebova *et al.*, 2003) regarding sensitivity, linearity and selectivity. In each system two columns were tested (Fig.9). Standard solutions (4.1.1) in nine concentrations were determined with the HPLC-method, gradient A at 23 °C (Fig.9). The same solutions were then analysed with two different gradients with the UPLC-method, gradient B at 30 °C and gradient C at 60 °C (Fig.7).

The UPLC method, BEH C18 column and gradient B (Fig.9), was assessed for food analysis. Different food matrices (4.3.2) (Fig.8) were prepared according to Patring and Jastrebova (Patring & Jastrebova, 2007) and purified according to Jastrebova (Jastrebova *et al.*, 2003).

4.3 Samples

4.3.1 Folate content in Swedish eggs – influence of breed, feed and processing (Paper I).

Sampling of eggs for determination of nutrients for the Swedish food composition database was performed between late February and early March 2008 (Gard *et al.*, 2010). Eggs from three different breeds of hen; Lohmann Selected Leghorn (LSL), Bovans and Hy-Line, were sampled. Since organic production has been promoted in Sweden in recent years, eggs from hen fed with an organic feed (O) was sampled for comparison with eggs from hens fed with one of two conventional feed, "Lantmännen" (C1) or "Svenska foder" (C2). The number of eggs required for a representative samples was calculated according to the recommendations in "Food composition data. Production, management and use". (Greenfield & Southgate, 2003, p.214-215).

Eggs were collected at 43 farms from hens aged 37-53 weeks. From these 43 farms 18 were chosen, using one of nine combinations of breed and feed, in a nested design for evaluation by two-way ANOVA (Fig.10). A composite sample of four egg yolks was made from each farm. An in-house control sample was prepared by homogenising one egg yolk from each farm.

Sample 4 (Fig.10), eggs from Bovans hens fed with a conventional feed, was used for determination of folate forms in egg yolks and egg white with LC/MS (Paper I) and in egg yolks with UPLC (Paper II).

From one farm with LSL-hens fed with a conventional feed from Lantmännen, 22 eggs were sampled for determination of folate retention when boiling. Of the 22 eggs, 11 were boiled at 90 °C for 10 min.

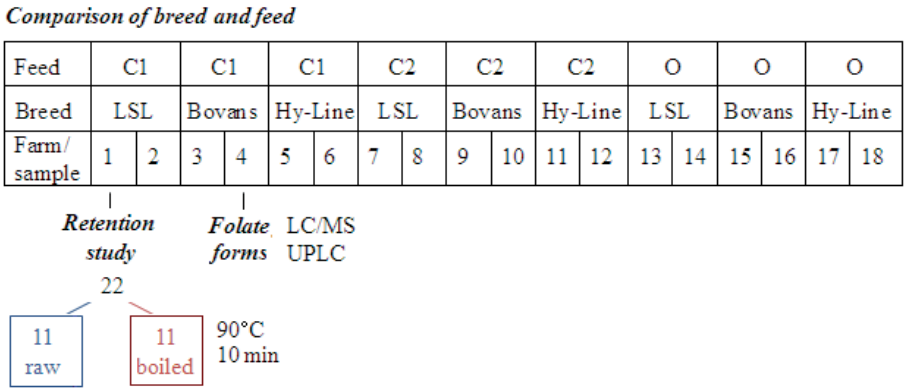

Figure 10. Sampling layout. C= Conventional feed from Lantmännen. C2=Conventional feed from Svenska Foder, O=Organic feed from Lantmännen. Retention study (4.2.1), folate forms (4.2.1 and 4.2.2)

4.3.2 Comparison of UPLC and HPLC for analysis of dietary folates (Paper II).

For evaluation of the UPLC method for determination of folates in foods, samples representing different food groups and with different forms of folate were chosen. Orange juice, pickled beetroots, baker's yeast and a fortified soft drink were obtained from retail outlets. The egg yolk sample used was from the sampling of Swedish eggs (Fig.10), Bovans hen fed with the conventional feed from Lantmännen.

5 Results and discussion

5.1 Folate content in Swedish eggs – influence of breed, feed and processing (Paper I).

Improvement and validation of the microbiological assay

Folate forms found in egg yolk by using LC/MS (Fig.7) were 5-CH_3-H_4folate (292 µg/100 g) and 10-HCO-folic acid (48 µg/100 g) whereas no folates were found in egg white. The same results were achieved for extracts treated with conjugase and untreated extracts, indicating that no polyglutamate forms were present in egg yolk.

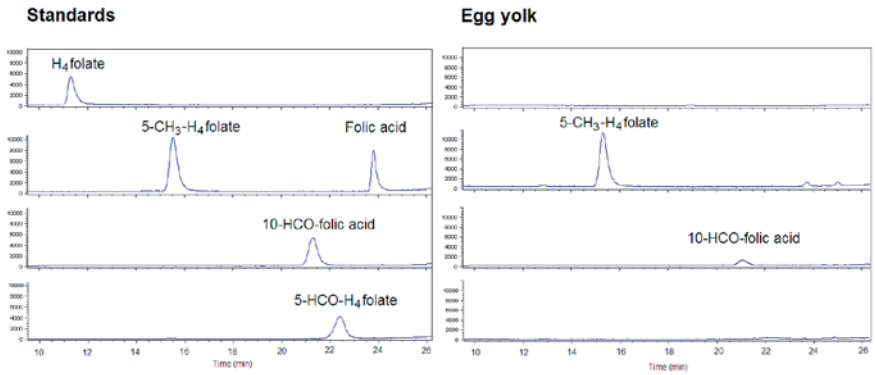

Figure 11. Folate forms analysed with LC/MS in Paper I. 5-CH_3-H_4folate (292 µg/100 g) and 10-HCO-folic acid (48 µg/100 g)

Since there were no polyglutamates the deconjugation treatment with chicken pancreas could be excluded. This made the procedure less laborious and was beneficial for folate stability since the samples could be kept at 4°C over-night instead of being treated with conjugase for 16 h at 37°C.

Due to the improvements in the extraction procedure and the fact that the folate forms found are stable (2.4.2), an additional antioxidant was considered unnecessary. Comparison of extraction procedures (Fig.8) resulted in replacement of the standard procedure with a matrix validated method (Paper I) which was used for folate determination in eggs.

Effects of production form on folate content in eggs.

The folate content showed a large variation (Fig.12) and no significant difference in folate content could be related to either the feed or breed of hen. The overall average was 252 ± 49 µg/100 g ranging from 195–382 µg/100 g

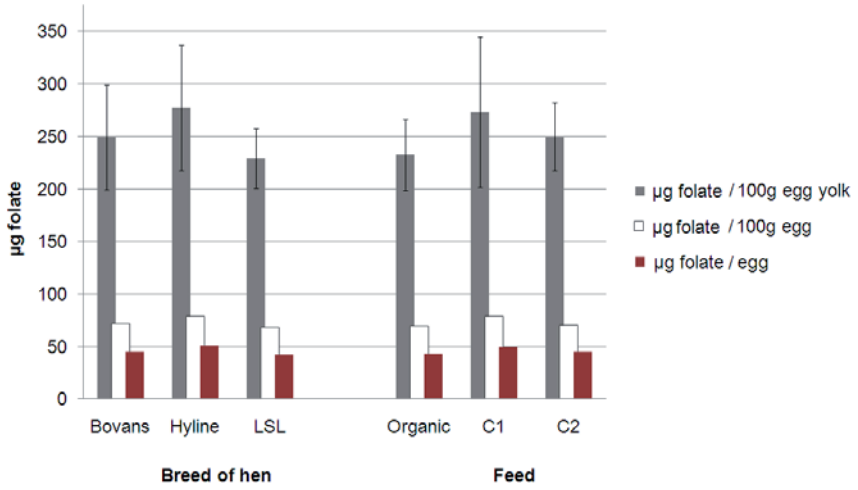

Figure 12. Influence of breed of hen and feed on folate content in eggs, outcome of two-way ANOVA analysis. The values are on fresh weight basis.

Retention study

Folate content before and after boiling, 233 ± 25 µg/100 g for raw compared to 213 ± 29 µg/100 g for boiled egg yolk, indicated a folate loss of 9 %. The retention factor of 91 % (4.2.1) is higher than the factor of 70 % (2.3) used in the Swedish food composition database. This emphasizes the need for determination of separate retention factors for different foods since this would give more accurate estimates of folate intake.

The individual variation between eggs was 11 % and 14 % respectively for raw and boiled egg which is smaller than the overall variation of 25 % from the study of different breed and feed. Repeating the experiment with eggs from all the breeds and feeds will add to what general conclusions that can be made regarding folate retention in eggs.

Folate intake

One raw egg of 60 g supplies 45-50 µg of folate according to our study. With retention of 91 % this means an intake of 41-46 µg folate which corresponds to 14-15 % of RI or 20-23 % of AR. Previous corresponding data for folate content in egg was 28 µg (Livsmedelsverket, 2001). In consequence the contribution from egg to the folate intake in Sweden (Fig.5) will show an increase although the consumption is somewhat lower. This highlights the need of a reassessment of folate content in foods. Especially data for foods that are good folate sources, due to content of naturally occurring folates and/or consumption level, should be evaluated to ensure accurate estimates of folate intake (Gregory, 1998).

5.2 Comparison of UPLC and HPLC for analysis of dietary folates (Paper II).

Available HPLC methods were not considered sensitive enough to replace the microbiological assay and comparisons show lower results for HPLC methods on average 30 % (Kariluoto, 2008) and 40% (Phillips *et al.*, 2010). However, HPLC methods could be considered for eggs and foods with higher folate content (>50 µg/100 g) (Fig.4). UPLC might even be sensitive enough to replace the microbiological assay, with the advantage of also differentiating between naturally occurring folates and added folic acid. The difference is large in sensitivity and calibration range between the microbiological assay and chromatographic methods, in this case UPLC (Fig.13).

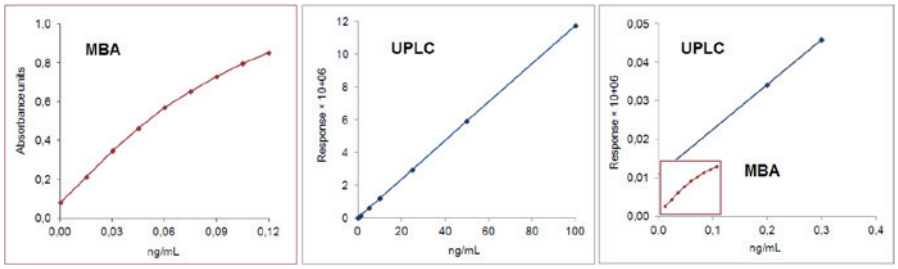

Figure 13. Comparison of calibration curves MA=microbiological assay (non-linear), UPLC method (linear) and comparison of calibration range for MA and UPLC.

Comparison of limit of the detection (LOD) for calibration solutions with the UPLC method, gradient B (Fig.9) and the HPLC method, gradient A (Fig.9) showed promising improvement in sensitivity due to a lower signal-to-noise ratio (Fig.14).

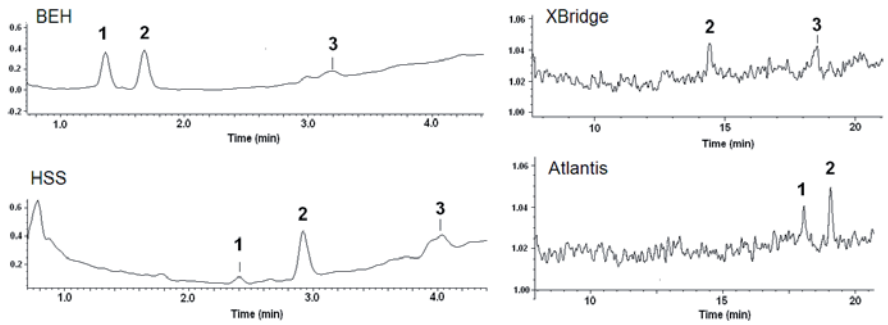

Figure 14. Separation of folates on Acquity BEH C18 and Acquity HSS T3 using gradient program B at 30 °C and XBridge C18 and Atlantis d18 using gradient program A at 23 °C. Peaks: 1=H_4folate (0.1 ng/mL); 2= 5-CH_3-H_4folate (0.1 ng/mL); 3= 10-HCO-folic acid (1 ng/mL).

LOD in UPLC was lower for standard solutions for all folate forms, for BEH C_{18} (0.02-2.5 ng/mL) compared with XBridge C_{18} (0.4-8 ng/mL). Runtime was reduced with a factor 4 from 42 min to 10 min with maintained resolution. However, the attempt to further decrease runtime by increase of temperature, gradient C (Fig.9) resulted in several interfering peaks, possibly due to degradation products of either the thermally unstable forms of folate or the antioxidants used. The BEH C_{18} column and gradient B was the best option at low concentrations when comparing the different columns and gradients (Paper II). In analysis of food samples 4.3.2 (Fig.9) more narrow peaks (Fig.11, Fig.15) gave good separation of all folate forms from interfering matrix compounds (Paper II).

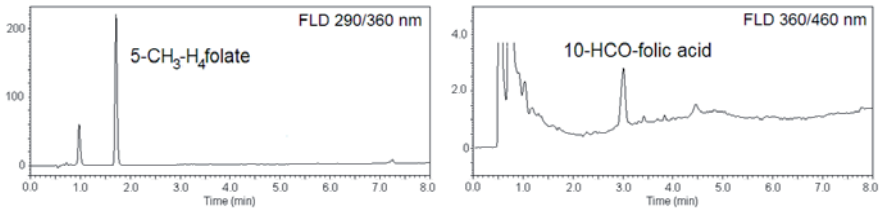

Figure 15. UPLC analysis of egg yolk, 5-CH_3-H_4folate (226 µg/100 g) and 10-HCO-folic acid (46 µg/100 g) was found.

In a comparison of HPLC and microbiological methods Kariluoto concludes that not only sensitivity is an issue between the two techniques, but also analysis time and sample-through put. Although there was 18 h incubation in the microbiological assay, the overall analysis time was longer for HPLC, mainly due to the additional sample purification step and data processing. As a consequence sample through-put was higher for MA.

Masking of 5-HCO-H_4folate by matrix interferences was also identified as a problem for correct quantification (Kariluoto, 2008; Jastrebova *et al.*, 2003). UPLC with MS or MS/MS detection might overcome these issues. Automation of sample purification could be helpful in sample preparation as well as for data processing. Information of folate forms achieved by using chromatographic techniques could be included in the food composition databases. As shown in this study, matrix validation also of the microbiological assay can be performed more efficiently with this knowledge.

6 Conclusions and future research

The findings of this study can be summarised as follows:

Analytical aspects

The extraction procedure of the microbiological assay routinely used for determination of folates could be simplified and shortened and was validated for folate determination in eggs.

An UPLC method suitable for determination of folate in foods was developed. The UPLC method showed better sensitivity and maintained resolution with shorter run-time compared with the validated HPLC method and was suitable for determination of folate in food samples.

Aspects of folate content in eggs

The folate forms in egg were determined by LC/MS to be 5-CH_3-H_4folate and 10-HCO-folic acid as monoglutamates.

There was no significant difference in folate content in eggs due to the breed of hen or due to the feed used, conventional or organic.

The retention factor for folate in boiled eggs was determined to 91 % .

The experiences from this study have highlighted the following needs:

Aspects for the future

Determination of folate forms in other food matrices should be performed for assessment of the extraction procedure of the microbiological assay routinely used for folate determination.

The effect of an additional antioxidant during the extraction of foods with H_4-folate needs to be evaluated for the microbiological assay.

The UPLC method for folate determination needs to be validated for food analysis and compared with the microbiological assay to determine if the microbiological assay can be replaced.

The retention factors for individual foods need to be determined for more accurate estimates on folate content.

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