The literature review presented the effects of the polyglutamate chain on the biological and nutritional properties of folates and the main methods used for folate assays, with a special emphasis on the approaches to studying intact polyglutamates. A brief introduction regarding safety aspects of folate fortification was also given.

The aim of this study was to develop a UPLC-FLR/PDA method for simultaneous determination of polyglutamyl folate vitamers. Chromatographic conditions were optimised for the resolution of polyglutamyl 5-methyltetrahydrofolates and major naturally-occurring monoglutamates. Method validation was conducted for both the UPLC method and affinity chromatography. Applicability of the validated method was evaluated on lupin flour, faba bean flour, and dry yeast, which were subjected to preparatory treatments with and without deconjugation. In addition, the effects of the sequential modification of preparatory treatments on the folate content and composition were investigated by using both the UPLC method and *Lactobacillus rhamnosus* assay.

A desirable separation of target polyglutamates and monoglutamates was successfully achieved on the BEH C\textsubscript{18} UPLC column within 11 minutes. The optimised UPLC method showed satisfactory selectivity, linearity, and sensitivity for the determination of methylated polyglutamates in the femtomole range and monoglutamates in the picogram range. Affinity chromatography showed satisfactory recoveries for polyglutamyl 5-methyltetrahydrofolates, but not for 5-formyl polyglutamates. In all three selected foods, 5-methyltetrahydrofolate was the dominant folate vitamer. Meanwhile, the analysis of undeconjugated samples showed that in the intact methylated folate pools, pentaglutamate predominated in legume flours and heptaglutamate in dry yeast. In addition, different sequences of enzyme and purification pretreatments were found to significantly affect both the total measurable folates and the folate profiles. Our standard preparatory procedures comprising simultaneous treatments with amylase and conjugase, then protease and affinity purification resulted in the greatest yield of total folates, but UPLC analysis indicated incomplete deconjugation. However, a modification in which deconjugation was conducted as the last step enhanced hydrolysis efficiency.

**Avainsanat — Nyckelord — Keywords**

UPLC, folyyl polyglutamate, folate food analysis, legume, yeast

**Säilytyspaikka — Förvaringsställe — Where deposited**

Víikki Campus Library

**Muita tietoja — Övriga uppgifter — Further information**

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PREFACE

This master thesis was conducted at Food Chemistry Division, Department of Food and Environmental Sciences, University of Helsinki. The experiment was supported by a grant kindly provided by Jenny and Antti Wihuri Foundation.

Firstly, I express my deepest thanks to my supervisor Dr. Susanna Kariluoto for her plentiful advice, guidance and encouragement during the study, and for leading me into the interesting world of folates. I would like to warmly thank MSc. Minnamari Edelmann for her patient and constant support in lab works, and Professor Vieno Piironen for carefully revising my manuscript in her precious time. My sincere appreciation also goes to Professor Marina Heinonen and everyone in Food Chemistry Division.

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Finally, I owe my everlasting gratitude and love to my parents, Qingping Yang and Xinmin Peng, for standing by me all the time. Whatever choices I made, they were always behind me, understanding me and encouraging me to pursue my dream.

Helsinki, January 2013

Yingying Yang
LIST OF ABBREVIATIONS

AACC  American Association of Cereal Chemists
AC    affinity chromatography
AOAC  Association of Official Analytical Chemists
APCI  atmospheric pressure chemical ionisation
CE    capillary electrophoresis
CHES  2-(N-cyclohexylamino)ethanesulfonic acid
CP    chicken pancreas (conjugase)
CRM   certificated reference material
CV    coefficient of variation
DAD   diode array detection
DFE   dietary folate equivalent
EL    electrochemical
ELISA enzyme-linked immunoabsorbent assays
EPBA  enzyme protein binding assays
ESI   electrospray ionisation
FBP   folate-binding protein
FDA   Food and Drug Administration
FdUMP 5-fluoro-2'-deoxyuridine-5'-monophosphate
FPGS  folylpolyglutamate synthetase
FLR   fluorescence
HEPES N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
HK    hog kidney (conjugase)
IP/RP-HPLC ion pair/reversed-phase high performance liquid chromatography
LC-MS liquid chromatography-mass spectrometry
LOD   limit of detection
LOQ   limit of quantitation
MA    microbiological assay
MALDI matrix-assisted laser desorption/ionisation
NNR   Nordic Nutrition Recommendations
NTDs  neural tube defects
pABAgluₙ p-aminobenzoypolyglutamate
PDA: photodiode array
RIA: radioimmunoassay
RP: rat plasma (conjugase)
RPBA: radiolabeled protein binding assays
R²: correlation coefficients
SAH: S-adenosylhomocysteine
SAM: S-adenosylmethionine
SAX: strong anion exchange
SCF: Scientific Committee on Foods
SPE: solid phase extraction
UPLC: ultra performance liquid chromatography
UV: ultraviolet

H₄PteGlu, THF: tetrahydrofolate
H₂PteGlu, DHF: dihydrofolate
FA/PteGlu, PGA: folic acid/pteroylmonoglutamic acid
5-CH₃-H₄PteGlu, 5-CH₃-THF: 5-methyltetrahydrofolate
5-CHO-H₄PteGlu, 5-CHO-THF: 5-formyltetrahydrofolate
5,10-CH⁺-H₄PteGlu, 5,10-CH⁺-THF: 5,10-methylenetetrahydrofolate
5,10-CH₂-H₄PteGlu, 5,10-CH₂-THF: 5,10-methylene-tetrahydrofolate
10-CHO-FA: 10-formylfolic acid
10-CHO-H₂PteGlu, 10-CHO-DHF: 10-formyl-dihydrofolate
10-CHO-H₂PteGlu, 10-CHO-THF: 10-formyl-tetrahydrofolate
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1 INTRODUCTION

Folates are a group of naturally occurring B-vitamers essential for the DNA and amino acid metabolisms, functioning as coenzymes in one-carbon transfers. They are widely distributed in foods of plant origin, especially leafy vegetables, legumes and cereals, and other good dietary sources include dairy products, liver and bread (Scott et al. 2000). The recommended daily intakes of folates in the Nordic countries are 300 μg for adults and 400 μg for pregnant women (NNR 2004). Inadequate intake of folates can cause a decrease in the serum folate concentration and an increased level of homocysteine, and ultimately lead to megaloblastic anemia. Sufficient intake of folate is particularly essential for females of childbearing ages because of its central roles in prevention of neural tube defects, which are a major cause of morbidity and mortality in newborns. In addition, increasing public attention has also been paid to preventive benefits of folates against cardiovascular diseases, some cancers, and psychiatric and mental disorders.

Folates present in most plant- and animal-derived foods are highly polyglutamylated, as is the dietary intake, with methylated and/or formylated vitamers being the major components. The glutamate chain length is biologically essential for the cellular retention of folates and the regulation of one-carbon metabolism. On the other hand, polyglutamyl folates are less bioavailable to humans compared to the supplemented form, namely folic acid, and the monoglutamate counterparts (Gregory 1989). Thus, the polyglutamylation degree is of great importance from both biological and nutritional perspectives.

In folate analysis, microbiological assay (MA) has been traditionally used as the gold standard for total folate determination, and it is the quantification method normally employed in food composition databases (Bouckaert et al. 2011). Meanwhile, liquid chromatography (LC) has also been favoured as a specific technique for determining the folate composition after enzymatic deconjugation of folate extracts, or for studying the polyglutamate distribution via conversion of all the folate vitamers into a certain species (Quinlivan et al. 2006).

During the past two decades, only a few studies were conducted for analysis of intact folyl polyglutamates by using liquid chromatography. In 1989, Selhub developed the first high performance liquid chromatography (HPLC) method combined with diode array detection for studying polyglutamate folates in tissues. The method was later applied to food folate analysis by Seyoum and Selhub (1993). In addition, some studies employed HPLC using
electrochemical (EL), fluorescence (FLR) or photodiode array (PDA) detectors, but they either were restricted to biological samples rather than foods or just focused on certain dominant forms in specific foodstuffs (Bagley and Selhub 2000; Sybesma et al. 2003; Matella et al. 2005; Naponelli et al. 2007). Recently, LC coupled with mass spectrometry (MS) was utilised to study folyl polyglutamates, exhibiting extraordinary capacity to resolve the co-eluted clusters (Garratt et al. 2005; Haandel et al. 2012). However, LC-MS has high requirements for the instrumentations, thereby restricting its extensive application.

As a novel LC technique, ultra performance liquid chromatography (UPLC) is increasingly favoured for folate analysis because of its better sensitivity, higher resolution and shorter analysis time compared to HPLC, especially for food samples with complex matrixes. However, in previous studies, UPLC combined with traditional detection modes was utilised only for the determination of monoglutamate folates in deconjugated samples, so no information on the polyglutamate chain could be obtained (De Brouwer et al. 2010; Kirsch et al. 2010; Jastrebova et al. 2011; Liu et al. 2011; Edelmann et al. 2012).

Owing to limitations of the existing methods, limited information is available for the native folate composition of various foodstuffs, and the current data is discrepant among different studies and sources. Previous studies have reported variable bioavailability of endogenous folates in different foods (Tamura and Stokstad 1973; Wei et al. 1996; Hannon-Fletcher et al. 2004). It is reasonable to believe that the polyglutamylate distribution is one of the major factors responsible for such differences. Thus, definition of folate profiles of foodstuffs can help in understanding the varied availability of dietary folates. This knowledge is the basis for formulating scientifically sound nutrition recommendations.

The main aim of this study was to develop a fast and simple UPLC method for the simultaneous determination of polyglutamyl 5-methyltetrahydrofolates and major folyl monoglutamates in food samples. The literature section of this thesis laid emphasis on nutritional and biological importance of folyl polyglutamates, and main analytical methods used in folate assays, especially approaches capable of studying intact polyglutamates. Meanwhile, safety aspects of folate fortification were also included. The experiments were mainly conducted for the optimisation and validation of the UPLC-FLR/PDA method combined with affinity chromatography for the folyl polyglutamate analysis. In addition, the validated method was used to study folate profiles of lupin flour, faba bean flour and dry yeast, and to investigate the effects of the alteration of pretreatment sequence on folate determination.
2 LITERATURE REVIEW

2.1 Introduction to folates

2.1.1 Structure and chemical properties

Folates refer to a group of heterocyclic B-vitamers exhibiting biological capacities similar to folic acid. Chemically, folates are composed of pteridine ring, \(p\)-aminobenzoate, and one or more \(\gamma\)-glutamyl residues (Figure 1). Folic acid, pteroylmonoglutamic acid (PGA), is the parent compound of folates. It was first isolated from spinach leaves by Mitchell and named after the Latin term for leaf, “folium” (Mitchell et al. 1941). Although folic acid is rarely found in nature, it is widely utilised for food fortification and pharmaceutical application because of its better stability. There is a diversity of folate derivatives differing in the oxidative state of their pteridine moiety, the one-carbon substituent at \(N^5\) and/or \(N^{10}\) positions, and/or the number of glutamyl residues. Tetrahydrofolates (\(H_4\)PteGlu\(_n\)) and their substituted derivatives are the metabolically significant forms of folates in cellular functions (Cossins 2000). They play essential roles in one-carbon metabolism by acting as single-carbon acceptors or donators.

![Figure 1. The structure of polyglutamyl tetrahydrofolates.](image-url)
Folates are ionogenic molecules, and in the pH range relevant to food and biological systems this character is greatly dependent on the glutamate carboxyl group and the N\textsuperscript{5} substitute. Generally, folates are less soluble in the mildly acidic pH condition (pH 2-4), and become more soluble above this pH range and at very low pHs (e.g., pH -0.78). Since each glutamate residue possesses a free carboxyl group, polyglutamyl folates exhibit greater anionic property in the intermediate and higher pH range, but weaker hydrophilicity at low pH values compared to their monoglutamate counterparts (Gregory 1989).

The N\textsuperscript{5} and/or N\textsuperscript{10} substitutions are of great importance to the oxidative stability of folate derivatives. As a result of steric hindrance, substituted forms are more resistant to oxidation than unsubstituted species, with substitutes at the N\textsuperscript{5} position contributing to greater stability (Scott et al. 2000). Hence, the stability of common reduced folate forms is decreased in the order of 5-CHO-H\textsubscript{4}PteGlu > 5-CH\textsubscript{3}-H\textsubscript{4}PteGlu > 10-CHO-H\textsubscript{4}PteGlu > H\textsubscript{4}PteGlu (Kariluoto 2008). On the other hand, the number of glutamate residues attached does not affect folate stability (Ye et al. 2007).

Folates are sensitive to high temperature, pH, light and oxidants. At 37°C, folate derivatives are relatively stable in the range of pH 4-8, except 5,10-CH\textsuperscript{3+}-THF and DHF, which are labile at acidic conditions. However, under typical heating conditions most folates are subject to degradation and interconversion. At 100°C, changes in pH can induce the interconversions between 5-CHO-THF and 5,10-CH\textsuperscript{2}-THF, and THF and 5,10-CH\textsuperscript{2}-THF. In addition, even without heating, acidity can cause the degradation and conversion of THF (pH < 5) and DHF (pH < 8) (De Brouwer et al. 2007). Moreover, light is potential to result in the cleavage of the C\textsuperscript{9}-N\textsuperscript{10} bond, leading to loss of vitamin activities (Arcot and Shrestha 2005). Therefore, analysis of folates must be conducted under subdued light, and glassware should be covered with aluminium foil. Furthermore, while the utilisation of reducing agents such as ascorbic acid contributes to greater retention of folates during high temperature treatments, some food additives such as sodium nitrite tend to be detrimental to folates (Ye et al. 2007).

2.1.2 Functions and nutrition

The nutritional importance of folates is mainly attributed to their functions of transferring one-carbon groups in the metabolisms of nucleic and amino acid. In remethylation of
homocysteine, 5-CH$_3$-THF acts as a co-factor and donates its methyl unit to methionine. Then, the methionine, in addition to protein synthesis, is committed to synthesis of $S$-adenosylmethionine (SAM), which is a universal methyl group donor for a variety of biological methylation reactions including that of DNA, RNA, proteins and neurotransmitters (Blom and Smulders 2011). In addition, 5,10-CH$_2$-THF and 10-CHO-THF also act as co-enzymes in the de novo synthesis of thymidylate and purine (Bailey and Gregory 1999; Mason and Choi 2000). On the other hand, polyglutamyl THF serves as the one-carbon acceptor of serine for the formations of glycine and 5,10-CH$_2$-THF (Wagner 1995).

Mammals, unlike plants and some microorganisms, lack key enzymes for folate biosynthesis, so they have to obtain folates from dietary sources (Cossins 2000). Plant-derived foods such as green leafy vegetables, legumes and some fruits (e.g., citrus and strawberries) make the greatest contribution to the dietary folate intake in European countries and the US. Other rich sources include dairy products, liver, cereals and yeast-containing foods (Scott et al. 2000; Sybesma et al. 2003). In the EU, the average folate intakes of adults were 300 μg/d in males and 250 μg/d in females, which were above the recommended daily intake of 200 μg for adults but below the level for pregnant women (400 μg) (SCF 2000).

Insufficient intake of methylated folates would result in a high serum level of homocysteine which had been recognised as an independent risk factor for cardiovascular diseases. Meanwhile, the consequent impairment of methionine metabolism leads to depletion of SAM, thereby affecting gene transcription, DNA repair and so on (Ye et al. 2007). Thus, a folate deficiency could ultimately lead to megaloblastic anemia owing to impaired DNA replication in red blood cells. Adequate folate status is particularly essential to the pregnant women and as well as the elderly. It has been widely accepted that periconceptional supplementation with folic acid is effective in decreasing the incidence of neural tube defects (NTDs). However, folic acid supplementation seems to correct a disturbed folate metabolism rather than to compensate for an insufficient folate intake (Molloy 2005). Moreover, epidemiologic data have associated the human folate deficiency with the carcinogensis of cervix, colorectum, lung, esophagus, brain, pancreas and breast, and the neurocognitive dysfunction (Alpert and Fava 1997; Mason and Choi 2000).
2.1.3 Bioavailability of folyl polyglutamates

Sauberlich et al. (1987) estimated that the relative bioavailability of food folates was ≤50% to that of folic acid, which was the cornerstone for the derivation of the dietary folate equivalents (DFEs) (Suitor and Bailey 2000). Bioavailability of dietary folates is complicated by intrinsic conditions, e.g., health, age and gastro-intestinal function, and various extrinsic factors. The polyglutamate chain of folates is an important extrinsic determinant of the bioavailability of dietary folates owing to the dominancy of polyglutamates in foods. It was estimated that in the Netherlands about two-thirds of the total folate intake from unfortified diets was derived from polyglutamates (Melse-Boonstra et al. 2002). In normal individuals, the availability of polyglutamyl folates, evaluated by urinary or blood folates, was reported to be 60-80% of that of monoglutamyl folic acid (Gregory 1989; Melse-Boonstra et al. 2004).

For normal absorption in the small intestine, folyl polyglutamates are deconjugated to their monoglutamate forms by an intestinal brush-border enzyme, namely folylpoly-γ-glutamate carboxypeptidase. This enzymatic pathway is highly pH-dependent with an optimal pH at 6.3 (Öhrvik 2009). Thus, physiological conditions or mediations that decrease the pH of the upper small intestine, such as high dose consumption of organic acids from juices, might inhibit such enzymatic processes and reduce the absorption extent of folyl polyglutamates (Wei et al. 1996; Melse-Boonstra et al. 2004). However, it has been indicated that the polyglutamylation extent of food folates was not a limiting factor in short-term bioavailability, since within the level of dietary intakes the activity of intestinal conjugases exceeded the requirement for folate deglutamylation (McKillop et al. 2002). Hannon-Fletcher et al. (2004) conducted a controlled intervention study to compare bioavailability of food folates by using two dietary sources representing different folate polyglutamylation, spinach (50%) and yeast (100%). Although the bioavailability of folates from both spinach and yeast was considerably lower than that of folic acid, no significant difference was found between two sources.

In addition, the food matrix may have an effect on folate bioavailability. Entrapment of folates in some plant foods may lead to incomplete liberation of folates from the matrixes, and thereby to some extent decrease the availability of folates. Although the bioavailability of endogenous folates varies among different foods, it is high in animal-derived foods such as liver and kidney (Gregory 1989). On the other hand, interaction of food components sometimes could improve folate bioavailability. Folate-binding proteins in milk, which are
effectively bound to folates, are able to increase folate absorption by assisting the transport of folates across the intestinal mucosa and by preventing bacterial uptake (Witthöft et al. 1999). Non-starch polysaccharides tend to improve folate status in humans by generating an optimal intestinal environment for the microbiological synthesis of folates and the following absorption (Houghton et al. 2006). It has also been reported that wheat bran increased the absorption of folate monoglutamates, but did not have significant effects on that of polyglutamates (Keagy et al. 1988).

Moreover, food processing can exert either positive or negative effects on folate bioavailability. Disruption of food matrixes, such as cutting and crushing, can assist enzymatic hydrolysis of naturally-occurring folyl polyglutamates, thereby improving folate bioavailability of the foods (Munyaka et al. 2009). On the other hand, it has also been reported that during blanching and steaming vegetables suffered a considerable loss of monoglutamates while the polyglutamate content was almost stable (Melse-Boonstra et al. 2002). When exposing to a 2.5 kGy dose of irradiation, spinach, green cabbage and sprouts lost about 10% of their folate pools, of which a greater loss was arisen from polyglutamates (Müller and Diehl 1996).

### 2.1.4 Folyl polyglutamates in biological materials

Dietary folates, which were absorbed and transported to liver and other tissues as monoglutamyl forms, must first undergo an ATP-required polyglutamylation, and then can either be retained in tissues or participate in one-carbon cycle. The glutamate tails are rapidly lengthened to five glutamate residues by folylpolyglutamate synthetase (FPGS), but further addition to more than five residues takes place slowly. Since the FPGS is apt to act on reduced folates, the majority of naturally occurring folates in biological materials exist in the forms of methyl-, formyl-, and unsubstituted THFₙ (Gregory 1989; Bagley and Sellhub 2000). On the other hand, a failure in folate polyglutamylation due to the abnormal expression of FPGS gene could retard the embryo development of plants by impairing DNA synthesis (Anukul et al. 2010).

In addition to the FPGS activity, the polyglutamylation distribution of folate pools is also influenced by a variety of factors such as metabolic states, nutritional supplement, etc. During germination, the pea cotyledon experienced a 15-fold increase in the folate concentration, and its polyglutamylation degree was increased by 35% between the 1ˢᵗ and
3rd day (Roos and Cossins 1971). Moreover, the glutamylation degree varies among different tissues (Zheng et al. 1992). For examples, while hexa- and heptaglutamates accounted for 60% of the folate pool of tomato leaves, almost 70% of folates in carrot root contained only 2 glutamate residues. Meanwhile, Zheng et al. (1992) also reported a difference in the polyglutamate distribution of various folate species in broccoli leaves where diglutamate was the primary form in the formyl and methyl folate pools and hexaglutamate in the methylene and unsubstituted folates.

Polyglutamylation, most importantly, is essential for the accumulation and compartmentalisation of cellular folates. Elongation of the glutamyl chain length decreases the affinity of folates to membrane transporters. Meanwhile, polyglutamylation can assist the retention of cellular folates by increasing protein binding affinity and by providing α-carboxyl charges (Quinlivan et al. 2006). As a result, poly-γ-glutamyl derivatives cannot cross the cell membrane, thereby being retained by tissues and involved in one-carbon metabolism (Shane 1982).

In addition, the polyglutamate chain of folates exerts roles on the regulation of one-carbon metabolism. For one thing, folates with 3-6 glutamate residues, which have a lower $K_m$ and thereby greater affinity to folate-dependent enzymes, are preferred substrates in the one-carbon cycle (Besson et al. 1993; Quinlivan et al. 2006). Changes in the chain length of polyglutamyl cofactors or inhibitors could increase or decrease catalytic efficiency of folate-dependent enzymes, thereby affecting the flux of one-carbon units through reactions of one-carbon metabolism. For another thing, alterations in the chain-length pool have been observed in response to shifts between different biosynthetic pathways in the same organ in vivo studies (Krumdieck et al. 1992). Lowe et al. (1993) also reported that triglutamate assisted the thymidylate and purine synthesis in mammalian cells as effective as longer glutamyl derivatives, but was ineffective at supporting the glycine and methionine biosynthesis. Thus, changes in the chain length might direct the intracellular flux of one-carbon fragments among competing pathways and, thereby, change the steady-state of one-carbon metabolism.
2.2 Determination of folates

2.2.1 Preparatory treatments

**Extraction**

In folate assays, efficient extraction is a prerequisite for accurate determination, involving the sample homogenisation in extraction buffers and the subsequent heating of deoxygenated homogenates. Traditional methods are based on the thermal denaturation of folate-binding and other proteins to release matrix-entrapped folates into extraction buffers, and the inactivation of enzymes to protect native folates. Due to the complexity of natural folates and their instabilities, the selection of appropriate extraction conditions including the type and pH of extractants, the temperature and the time is of great importance to the completeness of extraction and the preservation of original folate profiles (Gregory 1989; Vahteristo and Paul 2000). Antioxidants, e.g., ascorbic acid or ascorbates, together with thiols are also incorporated into the extraction buffers in order to protect the C⁹-N¹⁰ bonds and reduced folates from oxidative damages and to suppress folate interconversions (Wilson and Horne 1983; Gregory et al. 1990; Lucock et al. 1993; Patring et al. 2005b). Currently, the extraction employing the Wilson and Horne buffer (CHES/HEPES buffer containing 2% sodium ascorbate and 200 mM 2-mercaptoethanol, pH 7.85) in a 100°C water bath for 10 min (Wilson and Horne 1984) is one of the most common procedures used for foods and biological materials attributed to its superiority for complete folate extraction and increased stability of extracted folates (Gregory et al. 1990; Pfeiffer et al. 1997; Konings 1999; Rychlik et al. 2007).

**Deconjugation**

If the employed methods are unable to determine intact polyglutamyl folates, sample extracts must be subjected to enzymatic deconjugation in order to cleave the glutamate chain. Conjugases are normally obtained from hog kidney (HK), chicken pancreas (CP) and rat plasma (RP). The main deconjugation products could be either monoglutamates by HK and RP conjugases or diglutamates by CP enzyme. Hence, although all the three conjugases are suitable for microbiological assays, chicken pancreas cannot be used for liquid chromatographic analysis of monoglutamyl folates. Hydrolysis activity of the
conjugase is greatly affected by the pH condition, being the optimum at pH 4.5 for HK enzyme and pH 7.5 for CP and RP conjugases (Quinlivan et al. 2006).

In order to achieve complete extraction of folates, some researchers employed additional treatments with protease or amylase besides conjugase in order to assist liberation of the folates bound to protein or carbohydrate macromolecules in samples (Yamada 1979; Cerna and Kas 1983; Pedersen 1988). However, no attempt was made to the incorporation of all three enzymes until 1990 when Martin et al. introduced a combined extraction inclusive of conjugase, α-amylase and protease (Desouza and Eitenmiller 1990; Martin et al. 1990). Later, the trienzyme treatment gradually gained popularity due to its significant contribution to the increased measurable folates compared to conventional deconjugation, and a variety of studies have made efforts to optimise the trienzyme digestion in terms of the sequence of enzymes added, the incubation time, etc (Pfeiffer et al. 1997; Tamura et al. 1997; Hyun and Tamura 2005).

Purification

Prior to chromatographic analysis, sample purification is an indispensable step to remove matrix-derived interfering substances for desirable selectivity and sensitivity. Affinity chromatography is the most common method used for food samples containing complex components. Based on the pH-dependent affinity of folate-binding protein (FBP) for folates, pure folates can be isolated from sample extracts by retaining folates under neutral or slightly basic conditions and then eluting the FBP-bound folates with an acidic solution (Quinlivan et al. 2006). In 1988, Selhub et al. proved the adequacy of FBP columns for the isolation of pure folates from tissue extracts, and now the method has been widely combined with LC to analyse both folyl monoglutamates and polyglutamates in foods and tissue (Selhub et al. 1988; Seyoum and Selhub 1993; Pfeiffer et al. 1997; Kariluoto et al. 2001; Ndaw et al. 2001). Since the affinity of FBP varies greatly among the folate derivatives and even the isomers, the excess FBPs and the low column load for certain forms such as 5-CHO-THF are usually employed to compensate the problem (Selhub 1989; Kariluoto et al. 2001; Quinlivan et al. 2006).

Strong or weak solid phase extractions (SPEs) have also been used to purify the sample preparations meant to chromatographic analysis (Vahteristo et al. 1996; Vahteristo et al. 1997; Freisleben et al. 2003a; Jastrebova et al. 2003; Ginting and Arcot 2004). SPE gives
rise to acceptable recoveries of 70-85% for all folate forms except THF (45%) and shows equivalent retention to stereoisomeric folates (Quinlivan et al. 2006). However, since SPE cartridges would retain all the anionic compounds in samples, it is a less selective technique compared to FBP purification, resulting in chromatograms with some interfering peaks. Meanwhile, the folate recovery of SPE cartridges may be lower for food samples than standard solutions due to the saturation of sorbent capacity by nonfolate interference components (Nilsson et al. 2004).

2.2.2 Microbiological assay

Microbiological assay (MA) is the most widely used method for the total folate determination in foods and other biological samples due to its high sensitivity and relatively low expense (Quinlivan et al. 2006). Despite of the development of new technologies for folate analysis, MA is still the only approach validated by AOAC (Method 992.05 (1995), 2004.05 (2004) and 960.46 (2006)) and AACC (Method 86-47) (AOAC 2006; AACC 2000). Microbiological method obtains the folate content of samples based on turbidimetric measurement of the growth of vitamin-dependent bacteria. Three bacteria are widely used for folate analysis, *Lactobacillus rhamnosus* (ATCC No.7469), *Enterococcus hirae* (ATCC No.8043) and *Pediococcus acidilactici* (ATCC No.8081). These microorganisms have limitations in the response to certain species. While *L. rhamnosus* is unable to respond to pteroic acid, and *E. hirae* cannot grow on 5-CH₃-THF; *P. acidilactici* is only limited to 5- or 10-CHO-THF. On the other hand, none of the organisms is applicable for the folates with more than three glutamates. Remarkable decreases in the response of *L. rhamnosus* to highly glutamylated folates were observed, from 65% for PteGlu₄ to 2.4% for PteGlu₇; whereas *E. hirae* and *P. acidilactici* only grow on mono- and diglutamate derivatives (Quinlivan et al. 2006; Ye et al. 2007). Therefore, preliminary enzymatic deconjugation of folyl poly-γ-glutamates is required for microbiological assays.

In previous studies, MA results showed high variability in both the between- and within-laboratory measurements. Variation factors could be extraction methods, trienzyme digestion, sample storage conditions, microbial growth conditions and so on. Nevertheless, microbiological method is still regarded as the “gold standard”, and it is suitable for routine folate analysis (Quinlivan et al. 2006; Ye et al. 2007).
2.2.3 Ligand-binding assay

Biospecific methods using ligand binding can generally be classified into two types: immunoassays depending on the specific interaction of antibody with its antigen such as radioimmunoassay (RIA) and enzyme-linked immunoabsorbent assay (ELISA), and approaches employing labelled vitamin binding proteins including radiolabeled protein binding assays (RPBA) and enzyme protein binding assays (EPBA) (Finglas and Morgan 1994).

Although RPBA can be used in either competitive or incompetitive ways, the competitive assay is preferred in folate analysis in which the measured compounds and a known amount of vitamin binding proteins compete for a limited number of binding sites (Kariluoto 2008). The pH value should be carefully selected and controlled during reaction in order to ensure the optimum affinity (Finglas and Morgan 1994; Wigertz and Jägerstad 1995). Nowadays, RPBA employing folate-binding protein obtained from bovine milk is preferred for analysis of clinical samples. It has also been used for food samples such as berries and milk, and is particularly suitable for food samples dominated by 5-CH$_3$-THF (Wigertz and Jägerstad 1995; Strålsjö et al. 2002; Strålsjö et al. 2003). However, RPBA determination can be disturbed by a series of factors including varying affinity abilities to different folate species in foods, matrix effects of samples, pH of reaction, temperature, incubation time, and the polyglutamate chain length (Wigertz and Jägerstad 1995; Strålsjö et al. 2002).

As alternative approaches, RIA and EPBA have also been used for folate determinations in food samples. However, they have limited application in food analysis because of variable response of the individual folates to the FBP employed. Moreover, in RIA, the utilisation of radioactivity, which requires careful management, makes it unattractive in food analysis (Finglas and Morgan 1994).

2.2.4 Chromatographic methods

Compared to microbiological and ligand-binding assays, liquid chromatography is superiorly capable of studying specific folate components, normally aiming at either the one-carbon substitute or the γ-glutamyl polymer (Quinlivan et al. 2006). However, only a few studies were inclusive of both aspects.
High-performance liquid chromatography (HPLC)

For the past few years, HPLC approaches combined with ultraviolet (UV), fluorescence or electrochemical detection have been widely used for folate analysis because of their unique capability of discriminating multiple forms of folates, and even the poly-γ-glutamate chain. Due to the water-soluble nature and the differences in the ionogenic and hydrophobic properties of naturally occurring folates, reversed-phase (RP) and ion pair (IP) HPLC have an edge in the separation of folate derivatives (Vahteristo et al. 1997). In order to achieve the optimal separation, solvents should be carefully selected according to their pH, polarity and ionic property. RP-HPLC is usually conducted below pH 4, whereas IP-HPLC is performed at neutral pHs using an ion pair reagent such as tetrabutyl ammonium phosphate (Vahteristo et al. 1997; Quinlivan et al. 2006). In RP-HPLC, since various folate forms exhibit great differences in their retention times, a gradient of organic phase is normally employed. By contrast, sharp, well-resolved but relatively uniform peaks given by IP-HPLC make this mode better-matched with isocratic separation methods (Quinlivan et al. 2006).

In addition, various LC detectors can be used for folate detections, but sometimes RP- and IP-HPLC show different compatibility to some detection modes. Spectrophotometric and diode array detections are suitable for both RP- and IP-HPLC. Electrochemical detection yields a poorer response under conditions of IP-HPLC than that used for RP-HPLC. On the other hand, the acidic condition employed in RP-HPLC is ideal for combination with fluorescence detection (Quinlivan et al. 2006). In addition, an IP-HPLC separation coupled with a highly selective microbiological detection system using \textit{L. casei} assay was also proposed for the determination of folate monoglutamates in tissues, and the HPLC chromatogram was constructed according to the bacterial growth data (Belz and Nau 1998).

Ultra performance liquid chromatography (UPLC)

As a novel technique, UPLC has been recently utilised for determination of common folyl monoglutamates in various foods such as egg, vegetables, yeast, cereals and so on (Jastrebova et al. 2011; Edelmann et al. 2012). Compared to conventional HPLC, UPLC exhibits distinct advantages in the identification of folyl monoglutamates with better sensitivity and linearity, and faster analysis time while providing desirable selectivity (Jastrebova et al. 2011). Meanwhile, UPLC results showed better agreement with MA-
values than that of HPLC (Kariluoto 2008; Jastrebova et al. 2011; Edelmann et al. 2012). However, in previous researches, there was no UPLC method combined with traditional detectors developed for studying polyglutamyl folates.

**Mass spectrometry (MS)**

Compared to traditional LC detectors, MS is a highly specific and sensitive detection mode capable of providing unambiguous identification of unresolved and coeluting chromatographic peaks, which is ideally useful for studying various folate vitamers in food matrixes. In 1999, Stokes and Webb developed the first LC-MS method for analysing folyl monoglutamates in which negative electrospray ionisation (ESI) was selected for the interface rather than atmospheric pressure chemical ionisation (APCI) due to its greater signal-to-noise ratios. Since then, many efforts have been made to optimise MS parameters for optimal identification of folate derivatives. Although both positive and negative ESI are feasible for folate detection, the former is preferable because of its better sensitivity and higher MS signal intensities for derivatives with high basic $pK_a$ values (Freisleben et al. 2003b; Patring and Jastrebova 2007). Matrix-assisted laser desorption/ionisation (MALDI) MS is also applicable for semiquantitative analysis of folate (Arnold and Reilly 2000; Cha and Kim 2003). In addition, the utilisation of stable isotope labelled internal standards enables complete corrections for the folate losses during extraction, purification and mass spectrometry, contributing to enhanced method accuracy (Rychlik and Freisleben 2002).

By conducting selected reaction monitoring, LC-MS/MS method exhibits superiority in folate determination over LC-FLR/UV in terms of selectivity and sensitivity, especially for vulnerable 5-CHO-THF vitamers. In addition, though AC yields less matrix-interfering chromatograms and 10-fold enhanced sensitivity, SPE extraction also provides sufficiently purified extract required for the LC-MS/MS analysis. Thus, SPE is an adequate substitute for more sophisticated and expensive AC in LC-MS approaches (Freisleben et al. 2003a).

### 2.3 Analysis of folyl polyglutamates

The determination of folates for nutritional purposes usually requires information on both the total folate and folate composition of foods. Until now, LC is the only feasible approach to study individual folate forms in biological samples. Folate derivatives
differing in the pteridine ring structure and the number of glutamate moieties can be identified according to the retention time and instrumental responses characteristic of each derivative, and are normally quantified by using the external calibration method. Normally, the analysis of various poly-γ-glutamyl folate derivatives is achieved on either the intact polyglutamates or the converted form as poly-γ-glutamyl derivatives of a single species. LC methods that have been used for determination of folyl polyglutamates in previous studies are summarised in Table 1.

2.3.1 Analysis of intact folates

*High performance liquid chromatography*

Ion pair HPLC with diode array detection (DAD) has been used for quantitative analysis of individual folates in affinity-purified samples. Selhub (1989) investigated the elution pattern of a series of 35 folates by separating them into seven clusters on the basis of the glutamate chain length. The folates were found to elute in the sequence of increasing numbers of the glutamate residue and exhibited their characteristic UV spectra. When analysing a mixture of all 35 forms at 280 nm, 10-CHO-THF, THF and DHF were successfully separated in the groups of the mono- and diglutamyl folates, but they tended to elute in the same peak in the clusters containing longer glutamate chain; whereas 5-CH$_3$-THF and folic acid with a given number of glutamyl residues always co-eluted in the same peak. Coeluting folates are usually further identified according to their spectral features, for example 350 nm for folic acid and DHF derivatives, 258 nm for 10-CHO-THF$_n$, and 360 nm for 5,10-CH$^+$-THF$_n$ (Selhub 1989; Sybesma et al. 2003). The applicability of this method for analysising food samples was later proved by Seyoum and Selhub (1993), showing an average variability of 10% and good agreement between results obtained from the employed HPLC method and the *L. casei* assay.

In addition, electrochemical (EL) detection combined with HPLC has also been proposed for folate analysis because of its advantageous capability of determining minor amounts of folates in biological samples (Bagley and Selhub 2000; de la Garza et al. 2004; Orsomando et al. 2005; Naponelli et al. 2007). For an instance, the method developed by Bagley et al. (2000) provided limits of detection (LODs) at 0.21 pmol and 0.41 pmol for pentaglutamates of THF and 5-CH$_3$-THF, respectively. This method was able to distinguish polyglutamyl derivatives of THF, 5-CH$_3$-THF and 5,10-CH$^+$-THF except
critical couples of THF$_7$/5-CH$_3$-THF$_1$ and THF$_7$/5-CH$_3$-THF$_2$ whose quantitation were resolved according to a given equation. However, the low pH of the mobile phase was problematic for the identification of various formylated folates, all of which would be converted into 5,10-CH$^\ddagger$-THF.

By using fluorescence (FLR) detector, reduced folates are usually measured at an excitation wavelength of 290 nm and an emission wavelength of 356 nm, while 10-CHO-folic acid excites at 360 nm with a maximum emission at 460 nm (Kariluoto et al. 2001; Jastrebova et al. 2011). Matella et al. (2005) achieved adequate separation of different polyglutamates of 5-CH$_3$-THF by employing fluorescence detection. At 295 nm (excitation) all peaks showed a maximum absorption value, whereas the emission wavelengths of maximum absorption for the largest and smallest peaks were 356 nm and 325 nm, respectively. Meanwhile, simultaneous utilisation of photodiode array (PDA) detector enables identification of 10-CHO-DHF, 5-CHO-THF, 5,10-CH$^\ddagger$-THF and folic acid, and also provides spectral verification of detected peaks.

*Mass spectrometry (MS)*

While progress of the analysis of intact folyl polyglutamates is constrained by using conventional LC detectors, recent applications of LC-MS provide a breakthrough for overcoming problems arisen from the complexity of naturally occurring folates in foods. By using multiple-stage MS, the identities of detected or double peaks could be unequivocally investigated based on their structural information, thereby lowering the requirement for chromatographic separation and purification. In addition, it is also possible for the identification of various polyglutamates even if certain polyglutamyl standards are not available. By using HPLC tandem negative ESI-MS, Garratt et al. (2005) developed a method for simultaneous separation of various vitamers with up to 14 glutamate residues within 25 minutes. However, the method was limited in differentiation of 5-CHO-THF$_n$ and 10-CHO-THF$_n$ since these two clusters were identical in their mass-to-charge ratio. Meanwhile, since MS responses were found to depend on both the one-carbon substitute and the glutamate chain, determination of polyglutamates in absence of corresponding standards should be complemented by incorporating response factors (Haandel et al. 2012).

In addition, MALDI MS has also been applied for identification of polyglutamyl THF in bacteria cells (Arnold and Reilly 2000), but food samples might be susceptible to matrix-
masking problems in the low mass-to-charge range when pretreatments are omitted (Cha and Kim 2003).

*Capillary electrophoresis*

Matella et al. (2005) analysed 5-CH$_3$-THF polyglutamates in citrus products by using capillary electrophoresis (CE) with PDA detection, and compared the CE-PDA method with the HPLC-FLR approach. Despite of better precision, CE-PDA had a lower sensitivity with a LOD of 3 μM. Therefore, CE method required time-consuming steps of purification and preconcentration, which would result in a longer analysis period and a lower recovery. The authors also suggested replacing the PDA with a more sensitive detector such as fluorimetric detection.
<table>
<thead>
<tr>
<th>Method</th>
<th>Sample matrix</th>
<th>Analyte</th>
<th>Purification</th>
<th>Column</th>
<th>Mobile phase</th>
<th>Quality assurance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-HPLC-DAD</td>
<td>Tissues, various foods</td>
<td>PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, 5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, 5-CHO-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, 10-CHO-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;</td>
<td>Purified milk FBP affinity</td>
<td>C-18</td>
<td>Gradient</td>
<td>CV</td>
<td>Individual folate: 5-30% (inter-sample); ≤10% (intra-sample); Total folate: 5-19%</td>
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<td>Solution A: 5 mM tetrabutylammonium phosphate and 0.5 mM dithioerythritol in 25 mM phosphate/Tris buffer, pH 7.4 in water</td>
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<td></td>
<td>Solution B: 5 mM tetrabutylammonium phosphate and 0.5 mM dithioerythritol in 25 mM phosphate/Tris buffer, pH 7.4 in acetonitrile:ethanol:water (64:9:27)</td>
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<tr>
<td>RP-HPLC-EL</td>
<td>Tissues</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, 5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;, 5,10-CH&lt;sup&gt;+&lt;/sup&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,7&lt;/sub&gt;</td>
<td>FBP-Affiprep 10 affinity column</td>
<td>Betasil Phenyl (250 × 4.6 mm)</td>
<td>Gradient</td>
<td>CV: 0.6-16% (intra-assay), 5.2-13% (inter-assay)</td>
<td>Recovery: 89-104% LOD: 0.21-0.41 pmol</td>
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<td>Solution A: 28 mM dibasic potassium phosphate and 60 mM phosphoric acid in water</td>
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<td></td>
<td></td>
<td></td>
<td>Solution B: 28 mM dibasic potassium phosphate and 60 mM phosphoric acid in acetonitrile:water (2:8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC-EL</td>
<td>Fruits, plant tissues, bacteria</td>
<td>PteGlu, 5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,3&lt;/sub&gt;, 5-CHO-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,3,5&lt;/sub&gt;, 5,10-CH&lt;sup&gt;+&lt;/sup&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu</td>
<td>Purified milk FBP affinity</td>
<td>Prodigy ODS2 (150 × 3.2 mm, 5 μm)</td>
<td>Gradient</td>
<td></td>
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<td>Solution A: 28 mM K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt; and 0.59 H&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt; (pH 2.5)</td>
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<td>Solution B: 28 mM K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt; and 0.59 H&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt; (pH 2.5):CH&lt;sub&gt;3&lt;/sub&gt;CN (75:25)</td>
<td></td>
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<tr>
<td>HPLC-FLR/PDA</td>
<td>Bacteria</td>
<td>5,10-CH&lt;sup&gt;+&lt;/sup&gt;-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,3&lt;/sub&gt;, 5-CHO-H&lt;sub&gt;2&lt;/sub&gt;PteGlu&lt;sub&gt;1,5&lt;/sub&gt;</td>
<td>-</td>
<td>Betasil Phenyl (250 × 3 mm, 3 μm)</td>
<td>9% methanol and 1.5% formic acid, pH 3.0</td>
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</tbody>
</table>

Table 1. Continued.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample matrix</th>
<th>Analyte</th>
<th>Purification</th>
<th>Column</th>
<th>Mobile phase</th>
<th>Quality assurance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-FLR/PDA</td>
<td>Orange juice</td>
<td>5-CH$_3$-H$<em>2$PteGlu$</em>{1,7}$</td>
<td>Purified milk FBP</td>
<td>Perkin-Elmer HS3-C18 column (330 × 4.6 mm, 3 μm)</td>
<td>33 mM phosphoric acid mobile phase with 4% (v/v) acetonitrile</td>
<td>CV: 2.2%</td>
<td>Recovery: 90% LOD: 0.0155 μM</td>
</tr>
<tr>
<td>HPLC-(−)ESI-MS/MS</td>
<td>Plant, animal tissue</td>
<td>PteGlu$_{1,8}$, H$<em>2$PteGlu$</em>{1,8}$, H$<em>2$PteGlu$</em>{1,8}$, 5-CH$_3$-H$<em>2$PteGlu$</em>{1,8}$, 5-CHO-H$<em>2$PteGlu$</em>{1,8}$, 5,10-CH`-H$<em>2$PteGlu$</em>{1,8}$, 5,10-CH$_2$-H$<em>2$PteGlu$</em>{1,8}$,</td>
<td>-</td>
<td>Luna C18(2) (150 × 2.0 mm)</td>
<td>Gradient Solution A: 5 mM dimethylhexylamine in methanol:water (5:95), pH8.1 Solution B: 5 mM dimethylhexylamine in methanol</td>
<td>CV: Individual folate: 6.5-29.9% (inter-run) 3.9-18.9% (intra-run);</td>
<td>(Garratt et al. 2005; Anukul et al. 2010)</td>
</tr>
<tr>
<td>HPLC-(+)ESI-MS/MS</td>
<td>Vegetables</td>
<td>5-CH$_3$-H$<em>2$PteGlu$</em>{1,7}$</td>
<td>-</td>
<td>Sunfire C18 (150 × 4.6 mm, 5 μm)</td>
<td>Gradient Solution A: 0.1 formic acid Solution B: acetonitrile</td>
<td>CV: 1-9% (intra-assay), 5.2-1-11% (inter-assay) Recovery: 84-91% LOD: 0.06-0.66 pmol Recovery: 43.3-74.4%</td>
<td>(Wang et al. 2010)</td>
</tr>
<tr>
<td>UPLC-(−)ESI-MS/MS</td>
<td>Tissues</td>
<td>PteGlu$_{1,7}$, H$<em>2$PteGlu$</em>{1,11}$, 5-CH$_3$-H$<em>2$PteGlu$</em>{1,11}$, 5-CHO-H$<em>2$PteGlu$</em>{1,11}$,</td>
<td>-</td>
<td>BEH C18 (50 × 2.1 mm)</td>
<td>Gradient Solution A: 10 mM ammonium bicarbonate and 10 mM dimethylhexylamine in water, pH 7.5 Solution B: 5 mM dimethylhexylamine in acetonitrile</td>
<td>Recovery: 43.3-74.4%</td>
<td>(Haandel et al. 2012; Becker et al. 2012)</td>
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</table>

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<thead>
<tr>
<th>Method</th>
<th>Sample matrix</th>
<th>Analyte</th>
<th>Purification</th>
<th>Column</th>
<th>Mobile phase</th>
<th>Quality assurance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-FLR-UV</td>
<td>Various foods</td>
<td>Conversion to 5-CH$_3$H$<em>4$PteGlu$</em>{1-7}$</td>
<td>FBP affinity</td>
<td>LiChrospher 100RP 18 endcapped (250 × 5 mm, 5 μm)</td>
<td>Gradient Acetonitrile-phosphate buffer (50 mM, pH 4.6)</td>
<td>Recovery: 78-98% LOD: 0.02 pmol/injection</td>
<td>(Ndaw et al. 2001)</td>
</tr>
<tr>
<td>HPLC-Spectrometer</td>
<td>Bacteria, tissues</td>
<td>Cleavage to p-ABAGlu$_{1-7}$</td>
<td>BioGel P2 column</td>
<td>Partisil 10 SAX (250 × 4.6 mm); MicroPak AX-10 (300 × 4 mm)</td>
<td>Gradient 25 mM ammonium phosphate buffer, pH 6.5</td>
<td>Recovery: 95%</td>
<td>(Shane 1982; Shane 1986)</td>
</tr>
<tr>
<td>HPLC-FLR</td>
<td>Plant materials</td>
<td>Cleavage to p-ABAGlu$_{1-7}$</td>
<td>FBP affinity</td>
<td>Purospher Star RP-18 (150 × 4.6 mm, 5μm)</td>
<td>Gradient Solution A: 98% 0.01 M acetate buffer (pH 4.75) with acetic acid and 2% acetonitrile Solution B: 68% 0.01 M acetate buffer (pH 4.75) with acetic acid 32% and acetonitrile</td>
<td>Recovery: 101% CV: 5.91% LOD: 3.02 nM</td>
<td>(Zhang et al. 2003)</td>
</tr>
</tbody>
</table>

2.3.2 Analysis of converted folates

Owing to multiplicity of natural folates and their vulnerability to destruction, several methods have been developed to study the polyglutamate distribution of the folate pools via chemical or/and enzymatic conversion of all present folates into a single folate species.

Conversion to 5-methyltetrahydrofolates

There are several reasons for 5-CH$_3$-THF being chosen as the converted form of the folate pool. The most important superiority of 5-CH$_3$-THF is its ability to emit the strongest fluorescence yield among folate derivatives. Meanwhile, this acid-stable compound can be produced from folic acid, 5-CHO-THF and 10-CHO-THF by several feasible reaction schemes. In a study using HPLC with fluorescence detection, a low detection limit for 5-CH$_3$-H$_4$PteGlu$_{1-8}$ was determined at 0.02 pmol/injection (Ndaw et al. 2001). Hence, this approach allows the quantification of minor folate components in foods, which cannot be quantified in their original forms.

Conversion to 5,10-methylenetetrahydrofolates

5,10-CH$_2$-H$_4$PteGlu$_n$ entrapped by covalent bonds in a ternary complex with thymidylate synthetase and 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP) could be separated by polyacrylamide gel electrophoresis. As the charge of the complex is dependent on the number of glutamate residues in the 5,10-CH$_2$-THF moiety, the glutamate chain length of 5,10-CH$_2$-H$_4$PteGlu$_n$ can be indicated by its linear relationship with electrophoretic mobility of the corresponding complexes. Therefore, folate forms that can be chemically or enzymatically converted into 5,10-CH$_2$-H$_4$PteGlu can be studied by this assay, but 5,10-CH$_2$-H$_4$PteGlu$_n$ which are originally present in samples should be removed by titration with unlabeled FdUMP (Priest et al. 1981; Priest and Doig 1986).

Cleavage to p-aminobenzoylpolyglutamates

By chemical or enzymatic cleavage of naturally occurring folates at the C$^9$-N$^{10}$ bond, determination of the glutamate chain length of various folate forms could be greatly simplified to the analysis of a homologous series of unsubstituted p-
aminobenzoylpolyglutamates (pABAglu) (Shane 1982; Shane 1986; Zhang et al. 2003). The resulting pABAglu cleavage products could be purified as azo dyes of naphthylethylene diamine, which could be separated according to the glutamate chain length directly by chromatography or after regeneration of pABAglu by zinc reduction (Shane 1986; Quinlivan et al. 2006). Good baseline separation of pABAglu was observed on both strong and weak anionic exchanger, and increasing temperatures could further improve the resolution between different derivatives (Shane 1982). The fluorescent adducts of pABAglu are able to emit 50-100 fold greater fluorescence than that of other amines, thereby allowing sensitive detection of pABAglu in picomoles (Loewen 1986).

In addition, Isao and Krumdieck (1980) developed a method to allow selective cleavages of three different pools of reduced folates to pABAglu according to their responses to acid treatment. However, it has been reported that the conversion yields for individual folates to pABA greatly vary among different hydrolysis methods. Take 5-CH$_3$-THF for an example, its reaction yield could be improved from 12.2% to 97.3% by addition of hydrogen peroxide (Zhang et al. 2003).

2.4 Folate fortification and supplements

Although a variety of foods are abundant in folate vitamers, the dietary habits, the varied bioavailabilities of food folates and the losses of folates during processing, storage and preparation make it uncertain to achieve the recommended folate intake for the general populations. Therefore, food fortification and/or supplements of folates are recommended, especially for females of childbearing age. As folic acid has better stability and bioavailability compared to natural folate vitamers, it is commonly utilised in fortified foods and supplements. Folic acid becomes biologically active in the cells via a series of enzymatic reactions, starting with the reductions to DHF and then to THF followed by further conversions to 5-CH$_3$-THF and sometimes to 10-CHO-THF during transports through the gut mucosa (Scott et al. 2000).

2.4.1 Benefits

Neural tube defects are serious congenital malformations due to failure of closure of the covering of the brain or spinal cord during early development stages of the embryo, mainly
including birth defects anencephaly and spina bifida. As the neural tube forms during the 18-20\textsuperscript{th} day of pregnancy and closes during the 24-27\textsuperscript{th} day, the interventions should occur at least one month before conception until the first six weeks of pregnancy. In a number of trials and case studies, preconceptional intervention with folic acid showed strongly preventive effects against NTDs with significant reductions of 50-72\% (Molloy 2005; Pitkin 2007).

Mandatory fortification of folic acid has been conducted in more than fifty countries, recommending an additional intake of 400 $\mu$g folic acid/d for women that are planning or capable of becoming pregnant. The effectiveness of the folic acid supplement used in potential pregnant women has been investigated in some countries, showing a decline in NTDs by 8\% in New Zealand, 12\% in Australia, and 20-30\% in the US (Pitkin 2007; Dalziel et al. 2010).

2.4.2 Adverse effects

No adverse effects have been observed from excess consumption of folates from foods. On the other hand, although no toxicological information has been related to the use of synthetic folic acid, some adverse effects have been reported for folate fortification and supplementation. The FDA set a safe upper limit of 1 mg folate/d for the intakes from fortified foods and dietary supplements, and required a health claim for exceeding this level (FDA 1996).

Masking of vitamin B\textsubscript{12} deficiency symptoms

Since folates and vitamin B\textsubscript{12} are interrelated cofactors in the remethylation of homocysteine, vitamin B\textsubscript{12} deficiency can result in the entrapment of 5-CH\textsubscript{3}-THF and the resulting inavailability of 5,10-CH\textsubscript{2}-THF coenzyme for thymidine formation, which develops a secondary folate deficiency (SCF 2000) (Figure 2). Thus, insufficient supply of either folates or vitamin B\textsubscript{12} can lead to changes of the megaloblasts in the bone marrow and other replicating cells owing to disabled DNA synthesis. For people having a deficiency of vitamins B\textsubscript{12}, the administration of folic acid brings new supply of 5,10-CH\textsubscript{2}-THF, thereby repairing DNA synthesis and remitting haematological symptoms. Even a small dosage of 0.1 mg folic acid/day to patients with pernicious anaemia may restore normoblastic erythropoiesis and, thereby, suppress anaemic symptoms (Dickinson 1995).
If undiagnosed anaemia is masked by the supplement of folic acid, further development of neurological deterioration is most likely to take place. According to a large number of studies, though treatment with folic acid could correct the vitamin B\textsubscript{12}-derived anaemia; it did not prevent, but might allow and even precipitate neurological relapses, especially posterolateral spinal cord disease and peripheral neuritis. Although the theory of effects of folic acid is still not clear, it may reduce the plasma vitamin B\textsubscript{12} level or disturb vitamin B\textsubscript{12} metabolism, thereby exacerbating neurological damages (Dickinson 1995).

**Figure 2.** Overview of how folic acid fortification and supplements mask a vitamin B\textsubscript{12} deficiency; DHF: dihydrofolate, THF: tetrahydrofolate, 5-CH\textsubscript{3}-THF: 5-methyltetrahydrofolate, 5,10-CH\textsubscript{2}THF: 5,10-methylenetetrahydrofolate, 10-CHO-THF: 10-formyltetrahydrofolate, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine (Houghton et al. 2006).

**Effects on zinc absorption and deficiency**

In a human study, a marginal diet of zinc and 400 μg folic acid/d led to an increased fecal zinc level and a decreased urinary excretion of about 50% (Milne et al. 1984). Moreover, a 30% decrease in the zinc absorption was found in adults receiving folic acid supplement
Folic acid may chelate intestinal zinc to form unabsorbable complex and inhibit the transport of Zn in the lumen, thereby decreasing zinc bioavailability (Keating et al. 1987).

In addition, it has been reported that superimposition of the deficiencies in both zinc and folates may increase the incidence of abnormal morphogenesis in rat embryos (Bremert et al. 1989). In an animal study, rats with zinc deficiency were unable to convert folic acid to the metabolically active form, so the researchers concluded that teratogenesis of these subjects was possibly increased by folic acid supplementation (Quinn et al. 1990).

**Neurotoxicity**

There is no consistent evidence for possible neurotoxicity of folic acid in humans. Some animal studies have shown that folic acid exhibited neurotoxic and epileptogenic effect at very high dose levels (60-90 mg). Hunter et al. (1970) reported the development of gastrointestinal and/or nervous toxicity in healthy people after an oral administration of 15 mg folic acid/d. Meanwhile, some healthy volunteers developed gastrointestinal, neurological and psychological abnormalities in a month when receiving 5 mg of folic acid three times a day. But such results were inconsistent with other better-planned studies (Campbell 1996).

**Carcinogenicity**

Some animal and human studies have shown some evidences that a high level of folate intake from supplements and/or fortification might cause a paradoxical promotion of carcinogenicity among individuals who harboured neoplastic foci. In addition, Farber et al. (1947) reported that the administration of folic acid conjugates to patients with acute leukemia accelerated the leukemic progression. On the contrary, a folic acid antagonist, aminopterin, brought about temporary remissions in 5 out of 16 children suffering acute leukemia (Farber et al. 1948). Moreover, a promoting effect towards breast cancer has also been observed among postmenopausal women taking folic acid supplements above the level of 853 mcg/d, while a reduction of >40% in the risk of breast cancer was observed for decreasing dietary intakes of folates (Ulrich 2007; Mason 2009). In a follow-up study on the carcinogenicity of prescription drugs, folic acid showed an increasing effect on the
incidence of cancers of oropharynx, hypopharynx and so on, but the intervention of factors, such as smoking and drinking, could not be ruled out (Selby et al. 1989).

Meanwhile, it has been known that oral administrations of folic acid could saturate the conversion pathway of folic acid into bioactive forms, thereby leading to detectable levels of unmetabolised folic acid in the plasma. Increased plasma levels of folic acid have been inversely related with the decreases in the cytotoxicity of circulating natural killer cells, which acted on the destruction of growing colons of neoplastic cells (Mason 2009).

On the other hand, a great number of epidemiological and clinical studies have suggested an inverse relationship between folate status and colon cancer risk, but the dose and timing of folate supplementation seem to be critical for a safe and effective prophylaxis. It appeared that high doses of supplemental folates (>20 times of the basal daily dietary requirement) and intervention after neoplastic foci did not produce preventive effects but, in some cases, enhanced colorectal carcinogenesis (Kim 2004). Meanwhile, a folate-deficient diet was shown to significantly decrease the incidence of adenocarcinomas and tumors in small intestine, but not adenomas and large intestinal tumors (Kim 2003).

**Effects on antifolate drugs**

A number of folate antagonists have been used in antifolate therapies, including methotrexate, pyrimethamine, phenytoin, clochicine, etc. Because of their abilities to suppress the growth of malignant tumors, some antifolate drugs are used to enhance cancer-fighting effects or to protect healthy cells in treatments of various cancers, e.g. leukemia, non-Hodgkin’s lymphoma, breast cancer, bladder cancer. Other diseases employing antifolate medications include rheumatoid arthritis, bronchial asthma, psoriasis, bacterial infections and so on (SCF 2000). The antifolate drugs can directly interfere with folic acid metabolism. On the other hand, folic acid supplement may decrease patients’ serum level of phenytoin—a commonly-used antiepileptic, and may consequently increase seizure frequency. Furthermore, the methotrexate efficacy in rheumatoid arthritis treatment might be reduced by high doses of folinic acid (Campbell 1996).
3 EXPERIMENTAL RESEARCH

3.1 Aims

Folate pools of unfortified foods are predominated by highly polyglutamylated derivatives of methylated and/or formylated species. While a variety of approaches have been developed for the determination of total folates and/or vitamer distribution with a prior enzymatic deconjugation, only a few methods aimed at the analysis of intact folyl polyglutamates by using HPLC or LC-MS.

The aim of this study was to develop a UPLC method for simultaneous determination of polyglutamyl 5-methyltetrahydrofolate and major folyl monoglutamates. In addition, the applicability of the optimised UPLC method combined with an adequate purification step for analysing folate profiles of food samples was evaluated. Meanwhile, the effects of different arrangements of tri-enzyme and purification treatments on folate assays were investigated by using both UPLC and microbiological methods.

3.2 Materials and methods

3.2.1 Reagents and instrumentations

All the reagents were of analytical or HPLC grade. α-Amylase, protease, 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, >99%) and N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES, >99.5%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile was from Rathburn Chemical Ltd. (Walkerburn, Scotland). Milli-Q water was obtained from a Millipore Milli-Q System (S.A. 67120; Molsheim, France) and purified with an ICP-cartridge (CPMQ004R1 QPAK 1).

Instruments used in the studies and their sources are listed as follows:
- Spectrometer (Lambda 25; Perkin Elmer, Waltham, Massachusetts, USA)
- pH-meter (PHM220; Radiometer Analytical, Lyon, France)
- Centrifuge (Z 323; HERMLE Labortechnik, Wehingen, Germany)
- Oven (UFE 700; Memmert GmbH + Co.KG, Schwabach, Germany)
- Sterile PS-tube (4.5 mL; Greiner Bio-one, Frickenhausen, Germany)
- 96-well microplates (Costar 3596; Corning Incorporated, Corning, New York, USA)
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- Microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland)
- BD Discardit Syringe (Becton, Dickson and Company, Franklin Lakes, NJ, USA)
- GHP Acrodisc Syringe filter (Pall Corporation, MI, USA)
- Membrane filter (0.2 μm; Gelman Sciences, MI, USA)

### 3.2.2 Samples

Lupin flour was obtained by drying original blue lupin seeds (*Lupinus angustifolius* L., *Haags Blaue*; harvest 2011) at 35°C for 3 days and then grounding the dried seeds into flour. Faba bean flour was milled from the *Vicia faba* seeds (*Kontu*) harvested in 2011. Dry yeast (Sunnuntai; Ravintoraisio Oy, Raisio) was purchased from local markets. Certified reference material (CRM) was wholemeal flour BCR 121 (No.0717; Institute for reference materials and measurements, Belgium). In each sample set, a CRM sample was included, and analysis of the samples was conducted in duplicate.

### 3.2.3 Standard preparation

(6S)-Tetrahydrofolate sodium salt (H₄PteGlu), (S)-5-methyltetrahydrofolate calcium salt (5-CH₃-H₄PteGlu), (S)-5-formyltetrahydrofolate sodium salt (5-CHO-H₄PteGlu), 10-formylfolic acid (10-CHO-FA), folic acid (FA), (6R,S)-5,10-methenyltetrahydrofolate hydrochloride (5,10-CH⁺-H₄PteGlu) and 10-formylhydrofolate (10-CHO-H₂PteGlu) were obtained or synthesised from the previously reported sources (Kariluoto et al. 2004; Edelmann et al. 2012). Their stock solution were prepared by Edelmann et al. (2012) and stored at -20°C. A combined standard solution of folyl monoglutamates containing approximately 200 ng/mL of each vitamer was prepared in 10 mM acetate buffer (1% sodium ascorbate, 0.1% mercaptoethanol, pH 4.9).

Poly-γ-glutamyl folate standards were obtained from Schircks Laboratories (Jona, Switzerland) including (6R,S)-5-methyl-5,6,7,8-tetrahydropteroyl-di-, tri-, and tetra-γ-L-glutamic acid trihydrochloride salt (5-CH₃-H₄PteGlu₂₃₄) and (6R,S)-5-formyl-5,6,7,8-tetrahydropteroyl-di-, tetra- and hexa-γ-L-glutamic acid lithium salt (5-CHO-H₄PteGlu₂₄₆). The standards were dissolved, and their concentrations and purities were determined by spectrometric method (Kariluoto et al. 2004). The standard solutions were diluted in 50 mM borate buffer containing 1% ascorbic acid, and stored at -20°C. The 5-
methyltetrahydrofolate standard mixture consisted of mono-, di-, tri- and tetruglutamate (0.45 nmol/mL each).

The calibrant series were prepared by serial decimal dilutions of the standard solutions of 5-CH₃-H₄PteGlu₁₋₄ (0.45 nmol/mL each), folyl monoglutamates (200 ng/mL of each vitamer) and 5,10-CH⁺-H₄PteGlu (200 ng/mL).

### 3.2.4 Extraction and tri-enzyme treatment

Prior to extraction, 0.1-1 g of samples was mixed with 12 mL of extraction buffer (50 mM CHES/50 mM HEPES, 10 mM 2-mercaptoethanol, 2% sodium ascorbate; pH 7.85) in a 30 mL plastic tube, and flushed with nitrogen for 20 s. The homogenised samples were placed in a boiling water bath for 10 min with twice shaking during the extraction, and then cooled on ice.

The tri-enzyme treatment of samples was carried out according to the Standard Operating Procedure for microbiological assay of total folate in cereal samples (Kariluoto 2006), but a hog kidney conjugase (HK) was used instead of a chicken pancreas conjugase. HK which was available from remains of previous studies was prepared as described by Gregory et al. (1984). The activity of enzyme preparations was examined according to the method of Vahteristo et al. (1996) by measuring the amount of PteGlu₃ converted into folic acid in 45 min at 37°C.

In tri-enzyme treatment, the sample extracts which had been adjusted to pH 4.9 using HCl beforehand were treated with 1 mL of α-amylase and 1-1.5 mL of HK enzyme. The tubes were flushed with nitrogen gas, and incubated in a shaking water bath at 37°C for 3 h. After that, the pH of the extracts was adjusted to pH 7.0 with KOH, and 2 mL of protease was added. After flushing with nitrogen, the tubes were incubated in a 37°C water bath for 1 h. Then, the deconjugated extracts were heated in a boiling water bath for 5 min in order to inactivate the enzymes, and then cooled on ice. The sample extracts were centrifuged at 8 000 rpm at 4°C for 25 min. Supernatants were collected and settled to an exact volume (25 or 50 mL) with 0.5% sodium ascorbate (pH 6.1). For microbiological assays, a blank sample exclusive of the sample was included in each set of samples, and the sample extracts were diluted into a desirable concentration (0.15-0.5 ng/mL) using 0.5% sodium
ascorbate (pH 6.1). Preparations for UPLC were filtered with syringe filters (0.45 μm) before the purification step for chromatographic analysis.

### 3.2.5 Microbiological assay

Microbiological assay was carried out as the Standard Operating Procedure for microbiological assay of total folate in cereal samples (Kariluoto 2006). 5-CHO-H₄PteGlu was used as the calibrant at 1 ng/mL in 0.5% sodium ascorbate (pH 6.1). *Lactobacillus rhamnosus*-saline suspension was prepared by mixing 1 mL of thawed glycerol-cryopreserved *L. rhamnosus* (ATCC No.7469) with 2.5 mL of sterile saline in a sterile plastic tube. For preparation of the assay medium, 7.05 g of folic acid casei medium (Difco; Becton Dickinson & Co, Sparks, MD, USA) was added in 100 mL of Milli-Q water in a conical flask and heated until completely dissolved. After cooling in ice, 75 mg of ascorbic acid was added to the medium solution, and the pH was adjusted to 6.1 using acetic acid. The medium was filtered with a sterile syringe filter of 0.2 μm. The assay medium was inoculated and mixed well with 300 μL of *L. rhamnosus*-saline suspension.

Eight levels (0-80 μL) (four wells for each level) of the calibrant were pipetted into a 96-well microtiter plate, and added with 0.5% sodium ascorbate (pH 6.1) to the final volume of 100 μL. Meanwhile, the sample was diluted in two different factors, and 100 μL of each sample dilution was pipetted into four wells of the 96-well microtiter plate. Then, each well was added with 200 μL of inoculated medium. The plate was closed with the lids, and incubated in a plastic container at 35°C for 18-20 h. The optical density of the wells was measured using a microplate reader at 595 nm. The concentrations of total folates in samples were calculated based on the calibration curve derived from the calibrant serie.

### 3.2.6 Purification for UPLC analysis

Both affinity purification and solid phase extraction were tested for their adequacy in the clean-up of selected samples. Applicability in the polyglutamate analysis was evaluated in terms of the recoveries of each polyglutamate by using combined standards of 5-CH₃-H₄PteGlu₁₋₄ and 5-CHO-H₄PteGlu₁,2,4,6 with varying column loads. In the recovery test, 5-CH₃-H₄PteGlu₁₋₄ were determined by using the optimised UPLC method, but identification of 5-CHO-H₄PteGlu₁,2,4,6 was achieved on a mobile-phase gradient specifically developed
for 5-CHO-H₄PteGlu₄ by which 5-CHO-H₄PteGlu₁,₂ co-eluted but could be separated from 5-CHO-H₄PteGlu₄,₆.

**Affinity chromatography**

Affinity columns used for purification of the sample extracts were prepared by immobilising folate binding protein (Scribb's Laboratories, San Diego, CA, USA) derived from bovine milk to agarose gel (Affi-Gel 10; Bio-Rad Laboratories, Richmond, CA, USA) according to Konings’ method (Konings 1999). The prepared folate binding protein (FBP) columns were stored in phosphate buffer containing 0.2% (w/v) sodium azide at 4°C.

Utilisation of affinity chromatography was conducted according to a previous description (Kariluoto et al. 2001). The FBP columns were first equilibrated with 10 mL of 0.1 M potassium phosphate buffer (pH 7.0). Then, the filtered sample extracts were loaded onto the columns slowly. The columns were first rinsed with 5 mL of 0.025 M potassium phosphate/1 M NaCl (pH 7.0), and then with 5 mL of 0.025 M potassium phosphate (pH 7.0). After that, the columns were loaded with a void volume (1.4 mL) of elution solution (0.02 M trifluoroacetic acid/0.01 M dithiothreitol). Finally, the folates were eluted into a 5 mL flask containing 30 μL 1 M piperazine, 0.2% sodium ascorbate and 5 μL 2-mercaptoethanol by 4.95 mL of elution solution; and the solutions were filled to the mark. Purified samples were filtered with 0.2 μm filter and stored at -20°C for no more than two days before chromatographic analysis. After elution, the columns were washed with 1.5 mL of elution solution, followed by 10 mL of 0.1 M potassium phosphate buffer (pH 7.0).

**Anion exchange chromatography**

In addition, solid phase extraction was also conducted for the purification of sample extracts according to Vahteristo (1998). The strong anion exchange cartridges (Bond Elut-SAX; Agilent Technologies, Lake Forest, CA, USA) were activated with one volume (2 mL) of heptane, methanol and water, and further conditioned with 10 mL of conditioning buffer (0.01 M NaH₂PO₄-KHPO₄ buffer and 0.2% 2-mercaptoethanol, pH 7.5). The filtered sample (3-5 mL) was first diluted 2 times with water and added with 15 μL 2-mercaptoethanol. Then, the sample was applied to the column and washed with 2 × 1.5 mL conditioning buffer. The folates were eluted by using 2 mL of aqueous sodium chloride
(10%, pH 5.4) in 0.1 M sodium acetate containing 1% (w/v) ascorbic acid at a rate of <0.3 mL/min.

### 3.2.7 UPLC parameters

UPLC analysis of folates was conducted on a UPLC system (Waters, Milford, MA, USA) comprising a sample manager, a binary solvent manager, and fluorescence (FLR) and diode array (PDA) detectors. Separation of the target folyl monoglutamates and polyglutamates was tested on two Acquity UPLC columns (Waters, Ireland): BEH C$_{18}$ (1.7 μm, 2.1 × 100 mm) and HSS T3 (1.8 μm, 2.1 × 150 mm). The chromatographic conditions were proposed based on previous related studies (Jastrebova et al. 2011; Edelmann et al. 2012). Column temperature and autosampler temperature were 30-40°C and 4-6°C, respectively. The mobile phase consisting of solution A (30 mM H$_3$PO$_4$/30 mM KH$_2$PO$_4$, pH 2.2) and solution B (acetonitrile) was tested in different gradients at flow rates of 0.4-0.5 mL/min in order to achieve optimal resolution of various folate vitamers.

### 3.2.8 Validation of UPLC method

Investigation on the validity of the optimised UPLC method was carried out in terms of selectivity, specificity, linearity and sensitivity according to the methods given by Skoog et al. (2007). The elution pattern of various folate forms was studied by using a standard mixture of all target folyl mono- and polyglutamates. The retention times ($t_R$) of different vitamers were determined from the UPLC chromatograms of the standard mixture at FLR 290/356 nm and/or PDA 290 nm, and their retention factors ($k$) and numbers of theoretical plates ($N$) were calculated according to Equation 1&2. The selectivity factor ($\alpha$) and resolution ($R_s$) for adjacent peaks were calculated as follows (Skoog et al. 2007).

- Retention factor: $k = \frac{t_R - t_M}{t_M}$ \hspace{1cm} \text{(Equation 1)}
- Number of theoretical plates: $N = 16\left(\frac{t_R}{W}\right)^2$ \hspace{1cm} \text{(Equation 2)}
- Selectivity factor: $\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$ \hspace{1cm} \text{(Equation 3)}
Resolution: \[ R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B} \]  \hspace{1cm} (Equation 4)

Where \( t_M \) is the dead time for which unretained species reach the detector, and \( t_R \) is the retention time for which solute A reaches the detector. \( W \) refers to the peak width.

Moreover, linearity of the calibration curves derived from determinations of the calibrant series was evaluated involving correlation coefficients \((R^2)\) and linear ranges. Limit of detection (LOD) and limit of quantitation (LOQ) were used to assess the sensitivity of the optimised UPLC method for determining each vitamer. LOD was determined from the smallest amount of the analytes whose peak height was greater than 3 times of background noise \((S/N > 3)\). LOQ was defined as 3 times of LOD (Skoog et al. 2007).

### 3.2.9 Folate identification and quantification

In UPLC analysis, identification of \( H_4PteGlu \), \( 5-\text{CH}_3-H_4PteGlu_n \) and \( 5-\text{CHO}-H_4PteGlu \) was achieved on the FLR detector at an excitation wavelength of 290 nm and an emission wavelength of 356 nm, while 10-\text{CHO}-FA was detected at 360 nm (excitation) and 460 nm (emission). The PDA detector was used for identification of 10-\text{CHO}-H_2PteGlu, 5-\text{CHO}-H_4PteGlu and folic acid at 290 nm, and 5,10-\text{CHO}^+H_4PteGlu at 360 nm. The identity of each vitamer peak was confirmed by both comparing its retention time with that of an individual standard and inspecting its spectral characteristics in the range of 191-400 nm.

Quantitation of folate vitamers in UPLC method was based on the corresponding calibration curves obtained from external standard methods. The working standard curves were established by injecting 5-40 \( \mu L \) of the calibrant series.

Analysis of the selected foods—lupin flour, faba bean flour and dry yeast—was carried out by using the optimised UPLC method combined with affinity chromatography. In order to study both the one-carbon unit and the glutamate chain, the samples were treated both with and without HK conjugase. The undeconjugated sample enabled the study of intact poly-\( \gamma \)-glutamyl folates. Since naturally-occurring folyl polyglutamates could contain up to eight glutamate residues, quantitations of \( 5-\text{CH}_3-H_4PteGlu_{5-8} \) were based on the calibration curve of \( 5-\text{CH}_3-H_4PteGlu_4 \). On the other hand, the deconjugated sample provided information on the folate composition as monoglutamates, and its total folates was also measured by MA to examine accuracy of the UPLC method.
3.2.10 Comparison of tri-enzyme and purification treatments

In order to maximise the hydrolysis of folyl polyglutamates by HK conjugase, lupin flour was treated with different orders of tri-enzyme treatment and affinity purification (Figure 3). Among three proposed methods, A employed our standard procedure of tri-enzyme pretreatment for microbiological assay. The total folate contents were measured by microbiological assay, and UPLC analysis was also conducted for specification of the hydrolysed folate profile that resulted from different treatments.
Figure 3. Illustration on three methods (A, B and C) employing different sequences of enzyme addition and affinity purification. HK: hog kidney conjugase.
3.3 Results

3.3.1 Optimisation of chromatographic conditions

The chemistry and selectivity of two UPLC columns, namely HSS T3 and BEH C18, were evaluated for the UPLC-FLR/PDA analysis of folates by using the combined standard solution. The optimal resolutions of folate vitamers achieved on both columns are shown in Figure 4. 5-CH$_3$-H$_4$PteGlu$_n$ were sequentially eluted with increasing numbers of the glutamate residue. The PDA detection at 290 nm showed that the elution order of all target forms was H$_4$PteGlu → 5-CH$_3$-H$_4$PteGlu$_1$ → 5-CH$_3$-H$_4$PteGlu$_2$ → 5-CH$_3$-H$_4$PteGlu$_3$ → 10-CHO-H$_2$PteGlu → 5-CH$_3$-H$_4$PteGlu$_4$ → 5,10-CH$^+$-H$_4$PteGlu → 10-CHO-folic acid → 5-CHO-H$_4$PteGlu → folic acid.

Separation of 5-CH$_3$-H$_4$PteGlu$_{1-4}$ could be achieved on both columns [Figure 4(a,b)]. When using BEH C$_{18}$, sharper peaks were achieved except for 5-CH$_3$-H$_4$PteGlu$_2$ which showed a shoulder peak, but inspection of the spectra of this doubtful peak demonstrated the same compound origin. Although HSS T3 could result in good resolution of 5-CH$_3$-H$_4$PteGlu$_{1-3}$, a poor peak with shoulder was formed for the late-eluting 5-CH$_3$-H$_4$PteGlu$_4$. Meanwhile, owing to a shorter column length, BEH C$_{18}$ enabled adequate separation of 5-CH$_3$-H$_4$PteGlu$_{1-4}$ within 2 minutes compared to about 4 minutes with HSS T3. Hence, BEH C$_{18}$ was considered to be more adequate for resolution of polyglutamyl 5-CH$_3$-THF.

Regarding other folyl monoglutamates, sharp and well-resolved peaks were detected for H$_4$PteGlu and folic acid on both columns. As shown in Figure 4(c), BEH C$_{18}$ offered desirable resolution for 10-CHO-H$_2$PteGlu, 5-CH$_3$-H$_4$PteGlu$_4$, 5,10-CH$^+$-H$_4$PteGlu and folic acid. In addition, while there was no complete baseline separation between 10-CHO-FA and 5-CHO-H$_4$PteGlu, they could be alternatively determined at FLR 360/460 nm and FLR 290/356 nm, respectively. On the other hand, although 10-CHO-folic acid and 5-CHO-H$_4$PteGlu were well separated on HSS T3, the peaks were problematic for 10-CHO-H$_2$PteGlu, 5-CH$_3$-H$_4$PteGlu$_4$, 5,10-CH$^+$-H$_4$PteGlu and 10-CHO-FA. Since 10-CHO-H$_2$PteGlu could be only detected at PDA 290 nm, HSS T3 was placed at a disadvantage. Similarly, BEH C$_{18}$ allowed a faster analysis time for the optimal separation in 11 min than HSS T3 (17 min). By considering overall separation, the BEH C$_{18}$ column was thus chosen for the subsequent method validation and the analysis of the folate profiles of samples.
Figure 4. Optimal resolution of folate derivatives on BEH C18 (a, c, e, f) and HSS T3 (b, d). D: 10-formylH4PteGlu, F: 5-formylH4PteGlu, L: 10-formylfolic acid, M1-M4: 5-methylH4PteGlu1-4, N: 5,10-methenylH4PteGlu, P: folic acid, T: H4PteGlu.

During the development of the gradient system, the critical adjacent peaks which tended to co-elute together included 5-CH3-H4PteGlu and 5-CH3-H4PteGlu2, 10-CHO-DHF and 5-CH3-H4PteGlu4, and 10-CHO-FA and 5-CHO-THF. With the phosphate-acetonitrile mobile phase, a low concentration of acetonitrile assisted the resolution of easily co-eluted peaks whereas a high concentration contributed to better-shaped peaks and a shortening of elution time. As a result, the initial concentration of acetonitrile and its duration were critical in achieving adequate separation of all target analytes in the shortest possible time. Meanwhile, 5-CHO-THF, whose peak was always problematic, had to sacrifice its peak shape for the resolution of 5-CH3-H4PteGlu. Although we expected to analyse the polyglutamates of two most common natural forms, namely 5-CH3-THF and 5-CHO-THF at the same time, mono- and diglutamates of 5-CHO-THF were easily co-eluted. Our experience suggested that their separation would require the maintenance of a low concentration of acetonitrile for a sufficiently long time, which would greatly prolong the
whole analysis time and also compromise the resolution of 5-CH$_3$-H$_4$PteGlu and other derivatives. Therefore, the emphasis was laid on separation of 5-methyltetrahydrofolates. In addition, increasing the flow rate, e.g. 0.5 mL/min, accelerated the elution of analytes, but it also led to an elevated column pressure beyond the maximum limit for instruments (>15000 psi). Similarly, a higher column temperature at 40°C, which assisted the shortening of elution time, resulted in smaller peak areas compared to that at 30°C, which might imply the thermal vulnerability of certain vitamers.

The UPLC parameters for BEH C$_{18}$ were thus optimised to the following conditions: a mobile phase consisting of solution A (30 mM H$_3$PO$_4$/30 mM KH$_2$PO$_4$, pH 2.2) and solution B (acetonitrile), a flow rate of 0.4 mL/min, 30°C for column temperature and 6°C for autosampler temperature. The optimised gradient programme of the mobile phase was as follows: starting with an isocratic 3.8% of B until 4.70 min, followed by a linear increase to 6.8% (B) at 5.20 min and maintaining for 1 min, then rising to 8.0% (B) in 0.70 min and to 11.2% (B) in 2.10 min, thereafter decreasing back to 3.8% (B) during 9.00-9.50 min and staying at 3.8% for 1.50 min for a cleaning purpose.

Selectivity and specificity of BEH C$_{18}$ under above chromatographic conditions for the separation of target folates was assessed in terms of selectivity factor ($\alpha$), resolution factor ($R_s$), retention factor ($k_A$) and theoretical plate ($N$) (Table 2). All the target folates were eluted during 3.6-8.2 min, with retention factors ($k_A$) ranging from 4.67 to 13.12. Acceptable resolution factors (>1.3) reflected good separation between adjacent peaks of the target vitamers except for the critical couples of 10-CHO-H$_2$PteGlu/5-CH$_3$-H$_4$PteGlu and 10-CHO-folic acid/5-CHO-H$_4$PteGlu with resolution factors of 1.2 and 1.1, respectively.
Table 2. Selectivity and specificity of the folate separation on BEH C18 column: retention time ($t_R$), retention factor ($k_A$), number of theoretical plates ($N$), resolution factor ($R_s$), selectivity factor ($\alpha$).

<table>
<thead>
<tr>
<th>Detection</th>
<th>Vitamer</th>
<th>$t_R$ (min)</th>
<th>$k_A$</th>
<th>$N$</th>
<th>$R_s$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLR 290/356 nm</td>
<td>H$_4$PteGlu</td>
<td>3.601</td>
<td>4.67</td>
<td>1411</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td>5-CH$_3$-H$_4$PteGlu$_1$</td>
<td>5.417</td>
<td>7.11</td>
<td>1496</td>
<td>1.5</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>5-CH$_3$-H$_4$PteGlu$_2$</td>
<td>5.839</td>
<td>8.20</td>
<td>2693</td>
<td>1.4</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>5-CH$_3$-H$_4$PteGlu$_3$</td>
<td>6.273</td>
<td>8.88</td>
<td>18732</td>
<td>2.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>5-CH$_3$-H$_4$PteGlu$_4$</td>
<td>6.678</td>
<td>9.52</td>
<td>25687</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA 290 nm</td>
<td>5-CH$_3$-H$_4$PteGlu$_1$</td>
<td>6.221</td>
<td>9.74</td>
<td>76446</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10-CHO-H$_4$PteGlu</td>
<td>6.455</td>
<td>10.15</td>
<td>29630</td>
<td>1.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>5-CH$_3$-H$_4$PteGlu$_4$</td>
<td>6.627</td>
<td>10.45</td>
<td>34203</td>
<td>1.4</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>5,10-CH$^+$-H$_4$PteGlu</td>
<td>6.818</td>
<td>10.78</td>
<td>46356</td>
<td>3.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>10-CHO-folic acid</td>
<td>7.378</td>
<td>11.74</td>
<td>17995</td>
<td>1.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>5-CHO-H$_4$PteGlu</td>
<td>7.762</td>
<td>12.41</td>
<td>4426</td>
<td>1.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Folic acid</td>
<td>8.174</td>
<td>13.12</td>
<td>49697</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 Method validation

**UPLC performance**

The calibration curves for folate standards were established by plotting the peak area against the standard concentration. Linear characteristics of the standard curve for each folate are summarised in Table 3 and Table 4. The linearity for 5-CH$_3$-H$_4$PteGlu$_n$ was observed from a low amount of 2.3-13.5 fmol/injection to a high value of 13500 fmol/injection, and from 6-400 pg/injection to 4000-8000 pg/injection for other folyl monoglutamates. Good linear regression ($R^2 > 0.999$) was found for the calibrations of all the target vitamers. The resulting calibration equations of each folate were used for the quantitation of their amounts in samples. The calibration equation of 5-CH$_3$-H$_4$PteGlu$_4$ was also used for quantitation of 5-CH$_3$-H$_4$PteGlu$_5$-8.

Method sensitivity was evaluated on the basis of the LOD and LOQ for each folate derivative by analysing low concentration standards. As shown in Table 3, the proposed UPLC method was sensitive for the determination of 5-CH$_3$-THF at the fluorescence detection, with LOQs of 6.9, 4.0 and 5.4 fmole/injection for mono-, tri- and tetraglutamates, respectively, and with a relatively higher value for diglutamate (13.5 fmole/injection). Regarding other monoglutamyl derivatives, LOQs per injection at FLR or PDA detectors
were low for THF (6 pg) and 10-CHO-FA (30 pg), followed by 120 pg for folic acid, 180 pg for 5,10-CH\textsuperscript+ -THF and 240 pg for 5-CHO-THF; while a higher LOQ was found for 10-CHO-DHF (600 pg/injection).

Table 3. Linearity and sensitivity characteristics of the UPLC method for the determinations of 5-CH\textsubscript{3}-H\textsubscript{4}folate\textsubscript{1-4} standards.

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>LOD\textsuperscript{a} (fmol/injection)</th>
<th>LOQ\textsuperscript{b} (fmol/injection)</th>
<th>Linear range (fmol/injection)</th>
<th>Slope</th>
<th>Intercept</th>
<th>R\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{1}</td>
<td>2.3</td>
<td>6.9</td>
<td>4.5 – 13500</td>
<td>2.91 × 10\textsuperscript{6}</td>
<td>-5.97 × 10\textsuperscript{7}</td>
<td>0.99947</td>
</tr>
<tr>
<td>5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{2}</td>
<td>4.5</td>
<td>13.5</td>
<td>13.5 – 13500</td>
<td>3.13 × 10\textsuperscript{6}</td>
<td>-1.31 × 10\textsuperscript{5}</td>
<td>0.99936</td>
</tr>
<tr>
<td>5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{3}</td>
<td>1.3</td>
<td>4.0</td>
<td>2.3 – 13500</td>
<td>3.59 × 10\textsuperscript{6}</td>
<td>-6.00 × 10\textsuperscript{5}</td>
<td>0.99950</td>
</tr>
<tr>
<td>5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{4}</td>
<td>1.8</td>
<td>5.4</td>
<td>4.5 – 13500</td>
<td>4.07 × 10\textsuperscript{6}</td>
<td>-9.50 × 10\textsuperscript{5}</td>
<td>0.99962</td>
</tr>
</tbody>
</table>

\textsuperscript{a) LOD: limit of detection.  
\textsuperscript{b) LOQ: limit of quantitation.}

Table 4. Linearity and sensitivity characteristics of the UPLC method for the determinations of folyl monoglutamate standards.

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>LOD (pg/injection)</th>
<th>LOQ (pg/injection)</th>
<th>Linear range (pg/injection)</th>
<th>Slope</th>
<th>Intercept</th>
<th>R\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{2}PteGlu</td>
<td>2</td>
<td>6</td>
<td>6 - 4000</td>
<td>5.09 × 10\textsuperscript{6}</td>
<td>-3.46 × 10\textsuperscript{4}</td>
<td>0.99985</td>
</tr>
<tr>
<td>5-CHO-H\textsubscript{2}PteGlu</td>
<td>80</td>
<td>240</td>
<td>200 - 4000</td>
<td>4.44 × 10\textsuperscript{3}</td>
<td>-2.46 × 10\textsuperscript{4}</td>
<td>0.99975</td>
</tr>
<tr>
<td>10-CHO-H\textsubscript{2}PteGlu</td>
<td>200</td>
<td>600</td>
<td>400 - 8000</td>
<td>1.60 × 10\textsuperscript{3}</td>
<td>-1.82 × 10\textsuperscript{2}</td>
<td>0.99944</td>
</tr>
<tr>
<td>Folic acid</td>
<td>40</td>
<td>120</td>
<td>100 - 4000</td>
<td>7.72 × 10\textsuperscript{3}</td>
<td>-1.39 × 10\textsuperscript{2}</td>
<td>0.99980</td>
</tr>
<tr>
<td>10-CHO-folic acid</td>
<td>10</td>
<td>30</td>
<td>20 - 4000</td>
<td>6.60 × 10\textsuperscript{5}</td>
<td>2.45 × 10\textsuperscript{3}</td>
<td>0.99986</td>
</tr>
<tr>
<td>5,10-CH\textsuperscript{+} -H\textsubscript{4}PteGlu</td>
<td>60</td>
<td>180</td>
<td>100 - 4000</td>
<td>6.73 × 10\textsuperscript{3}</td>
<td>-1.05 × 10\textsuperscript{2}</td>
<td>0.99983</td>
</tr>
</tbody>
</table>

\textsuperscript{a) LOD: limit of detection.  
\textsuperscript{b) LOQ: limit of quantitation.}

**Affinity chromatography purification**

Based on preliminary experiments, affinity chromatography (AC) was chosen for the sample purification rather than solid phase extraction (SPE). Despite good performance in the recoveries of polyglutamyl folates (data not shown), SPE was found to be inadequate for the impurity clean-up of legume and yeast samples required for the subsequent UPLC
analysis (see Appendix 1). Affinity purification exhibited satisfactory recoveries ranging from 85% to 91% in the standard test with 5.2 μg folic acid/column.

As shown in Figure 5(a), in a given column load there was no difference in the affinity binding capacity for 5-methyltetrahydrofolates with varying glutamate chain lengths. However, a higher recovery of folates was achieved in a lower column load (1 μg/column) than in a heavier load (4 μg/column). On the other hand, there did exist competition among polyglutamyl derivatives of 5-CHO-THF, with about 20-50% decreases along with increasing numbers of the glutamate residue depending on the column load. Meanwhile, the affinity for 5-formyltetrahydrofolates was generally weaker in a heavy column load, although this trend was not consistent in the low column load levels.

![Figure 5](image)

**Figure 5.** Folate recoveries of affinity chromatography for polyglutamyl standards were investigated by using different column loads (0.5, 1 and 4 μg/column): (a) 5-methyltetrahydrofolates (5-CH₃-H₄PteGlu₁₋₄), (b) 5-formyltetrahydrofolates (5-CHO-H₄PteGlu₁,₂,₄,₆).

### 3.3.3 Determination of folate composition

The developed and validated UPLC method was used to study the folate composition in lupin flour, faba bean flour and dry yeast. Analysis of sample extracts with and without HK deconjugation provided information on the distribution of folate species and the profile of methylated polyglutamates (Table 5).
The total folates measurable in the deconjugated samples by UPLC analysis were 1058 ng/g for lupin flour, 1229 ng/g for faba bean flour, and 21041 ng/g for dry yeast. In lupin flour, 5-CH$_3$-THF was found to be the predominant derivative at 610 ng/g, followed by 5-CHO-THF (249 ng/g), and other minor vitamers included 10-CHO-FA, THF and folic acid. With regards to faba bean flour, its folate pool was composed of 5-CH$_3$-THF (429 ng/g), 5-CHO-THF (322 ng/g), 10-CHO-FA (250 ng/g), 5,10-CH$_2$-THF (180 ng/g), THF (50 ng/g) and 10-CHO-DHF in a trace amount. As a rich source of folates, dry yeast contained 19346 ng/g of 5-CH$_3$-THF, which represented approximately 92% of its total folates, and the remaining contribution resulted from 1300 ng/g of THF and 395 ng/g of 10-CHO-FA and a trace of 5,10-CH$_2$-THF.

In addition, the polyglutamylation degrees of the 5-CH$_3$-THF pool of lupin flour, faba bean flour and dry yeast were clearly revealed by chromatograms of their undeconjugated extracts. [Figure 6(a,c,f)]. Specifically, in lupin flour, about 46% of the 5-CH$_3$-THF contained five glutamate residues, followed by 5-CH$_3$-H$_4$PteGlu$_6$ (20%), 5-CH$_3$-H$_4$PteGlu$_1$ (13%) and 5-CH$_3$-H$_4$PteGlu$_2$ (10%); while other polyglutamates made less contribution of ≤ 5%. Faba bean flour showed a similar 5-CH$_3$-THF composition of which pentaglutamate accounted for 39%. Other main forms included 5-CH$_3$-H$_4$PteGlu$_1$ and 5-CH$_3$-H$_4$PteGlu$_2$, contributing to 29% and 12% of the 5-CH$_3$-THF population, respectively. On the other hand, there was no monoglutamate found in dry yeast sample, and the majority of 5-CH$_3$-THF had a glutamate chain length up to 6-8 residues. Among them, the major form—5-CH$_3$-H$_4$PteGlu$_7$—comprised 64% of the pool, and the remainder was mainly attributed to 5-CH$_3$-H$_4$PteGlu$_6$ (16%) and 5-CH$_3$-H$_4$PteGlu$_8$ (15%).
Table 5. Folate contents\(^a\) of sample extracts treated with or without HK conjugase by UPLC analysis (n = 2).

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>Lupin flour</th>
<th>Faba bean flour</th>
<th>Dry yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undeconjugated</td>
<td>Deconjugated</td>
<td>Undeconjugated</td>
</tr>
<tr>
<td>H(_4)PteGlu (ng/g)</td>
<td>8</td>
<td>58</td>
<td>17</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_1) (pmol/g)</td>
<td>158</td>
<td>1025</td>
<td>263</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_2) (pmol/g)</td>
<td>122</td>
<td>246</td>
<td>110</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_3) (pmol/g)</td>
<td>21</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_4) (pmol/g)</td>
<td>31</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_5) (pmol/g)</td>
<td>558</td>
<td>-</td>
<td>351</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_6) (pmol/g)</td>
<td>248</td>
<td>-</td>
<td>52</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_7) (pmol/g)</td>
<td>65</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>5-CH(_3)-H(_4)PteGlu(_8) (pmol/g)</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-CHO-H(_4)PteGlu (ng/g)</td>
<td>tr.</td>
<td>tr.</td>
<td>-</td>
</tr>
<tr>
<td>5,10-CH(_3)+-H(_4)PteGlu (ng/g)</td>
<td>tr.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>10-CHO-folic acid (ng/g)</td>
<td>37</td>
<td>91</td>
<td>111</td>
</tr>
<tr>
<td>5-CHO-H(_4)PteGlu (ng/g)</td>
<td>tr.</td>
<td>249</td>
<td>tr.</td>
</tr>
<tr>
<td>Folic acid (ng/g)</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Total quantifiable folates(^b) (ng/g)</td>
<td>600</td>
<td>1058</td>
<td>533</td>
</tr>
</tbody>
</table>

\(\cdot\): not detected; tr.: below limit of quantitation.

\(^a\) Mean value of the determinations.

\(^b\) 5-CH\(_3\)-H\(_4\)PteGlu\(_n\) contents were calculated into ng/g as the 5-CH\(_3\)-H\(_4\)PteGlu\(_1\) equivalent (\(M_w = 457\) g/mol).
Comparison of the total determinable folates in the deconjugated and undeconjugated samples demonstrated differences of about 33% for lupin flour, 57% for faba bean flour and 7% for dry yeast. Presence of other folyl polyglutamates besides 5-CH₃-THF might be one reason responsible for these differences. In analysis of undeconjugated samples, only THF and 10-CHO-folic acid could be found at quantifiable values in legume samples, and no other folyl monoglutamate was identified in dry yeast; which was different from that of deconjugated samples. In addition, in the undeconjugated samples, some unknown peaks were detected after the elution of a target monoglutamate and exhibited similar spectral characteristics to that of the monoglutamate [Figure 6(b,d,e,g,h)]. Thus, these peaks were very likely to be derived from the polyglutamates of the corresponding monoglutamate. In preliminary studies, we treated some food samples with and without HK to select samples with a high level of folyl polyglutamates, and percentages of the folates with more than 3 glutamate residues in the total pool were estimated to be about 37% for lupin flour, 72% for faba bean flour and 99% for dry yeast.
Figure 6. Representative chromatograms of undeconjugated extracts of lupin flour (a,b), faba bean flour (c,d,e) and dry yeast (f,g,h). L: 10-formylfolic acid, M1-M8: 5-methylH₄PteGlu₁-₈, N: 5,10-methenyl-H₄PteGlu, T:H₄PteGlu.
3.3.4 Specific investigation on the effects of tri-enzyme treatment and affinity purification

As shown in Figure 7, there were differences in the hydrolysis efficiency among different arrangements of enzyme and purification treatments. In the treatments on lupin flour, there was no significant difference in the total folate contents measured by MA between treatment A (1049 ng/g) and B (937 ng/g), while method C resulted in a much lower result (481 ng/g). Meanwhile, UPLC analysis provided detailed presentation on the hydrolysis effects of different treatments. As the major vitamer, 5-CH$_3$-H$_4$PteGlu$_n$ in lupin flour experienced a complete hydrolysis in B, followed by a relatively efficient treatment A with about 5% unhydrolysed diglutamates. In contrast, treatment C was inadequate in deconjugation where diglutamate still accounted for about 20% of the 5-CH$_3$-H$_4$PteGlu pool.

Meanwhile, the UPLC results also revealed that different treatments varied in their recovery of certain folate derivatives. While Method A and B achieved a same level of 5-CH$_3$-THF recovery, treatment C gave rise to a 40% lower value. 5-CHO-THF also showed greatest variation, being 207 ng/g and 62 ng/g in treatment A and B, respectively; and it was not detected in C. In addition, THF was determined at the highest value in B (133 ng/g) and lower contents in C (99 ng/g) and A (71 ng/g). Moreover, although 5,10-CH$^+$-THF was not detected in C and present in trace amount in B, treatment A resulted in a value of 74 ng/g. On the other hand, different treatments did not exert significant effects on the recoveries of 10-CHO-FA and folic acid.
Figure 7. Effects of arrangements of enzyme and purification treatments on the folate profiles of the lupin four. Treatment A, B and C were performed as shown in Figure 3. Detailed folate compositions were determined by the optimised UPLC method, and total folate contents were also measured by using microbiological assay (MA); 5-CH$_3$-H$_4$PteGlu$_n$ contents were calculated into ng/g as the 5-CH$_3$-H$_4$PteGlu$_1$ equivalent ($M_w = 457$ g/mol).

3.4 Discussion

3.4.1 Validity of the AC-UPLC-FLR/PDA method for folyl polyglutamate analysis

UPLC performance

Due to the emphasis on the discrimination of folyl polyglutamates, BEH C$_{18}$, which exhibited good retention and separation for the overall folates and superiority for the 5-CH$_3$-THF polyglutamates, was considered better in our study. The two tested columns, namely BEH C$_{18}$ and HSS T3, both utilise a trifunctional C$_{18}$ alkyl phase ligand-binding, thereby possessing superior low pH stability and ultra-low column bleed. BEH C$_{18}$ incorporates bridged ethylsiloxane/silica hydrid particles, and provides the widest pH operating range because of high pH stability of the 1.7 µm BEH particles (Jastrebova et al. 2011). HSS T3, which employs 100% high strength silica (HSS) particles for high-pressure compatibility, shows superiority in the polar compound retention over BEH C$_{18}$. In previous studies, both columns have been used for folate identification. Consistent with our
observation, the separation for the peaks of 10-CHO-folic acid and 5-CHO-THF was always reported to be unsatisfactory (De Brouwer et al. 2010). Javstrebova et al. (2011) and Edelmann et al. (2012) both reported that when using a binary mobile phase of 30 mM potassium phosphate and acetonitrile, HSS T3 provided better peak separation for the late-eluting couple—10-CHO-folic acid/5-CHO-THF—compared to BEH C18. However, in our studies, the gradient assisting the separation of 5-CH3-THF polyglutamates tended to compromise the shaping of subsequent eluting peaks. As a result, HSS T3 generated dull slopes for late-eluting derivatives, rendering it inadequate for our analysis.

The optimised UPLC method in our study provided the same elution pattern of folate peaks as described in previous studies (De Brouwer et al. 2010; Jastrebova et al. 2011; Edelmann et al. 2012). With the pteridine ring, polyglutamates were successively eluted with increasing numbers of the glutamate residue due to the fact that the anionic property was increased as the lengthening of the glutamate chain (Haandel et al. 2012). For the shoulder peak obtained for 5-CH3-H4PteGlu2, Kariluoto et al. (2001) and Sybesma (2003) also observed a similar phenomenon for (6R,S)-CHO-THF due to separation of the isomers. Meanwhile, the shoulder peak was not found for 5-CH3-H4PteGlu2 in real samples where the natural form existed in the S configuration, which further supported this explanation.

As previously stated, it was difficult to accomplish simultaneous separation of the polyglutamates of 5-CH3-THF and 5-CHO-THF based on current detection modes while ensuring desirable method sensitivity and shorter analysis time. For another thing, it was suspected that similar situations could also happen to the incorporation of other polyglutamyl derivatives. In previous studies, complete separation of various polyglutamyl vitmater has never been realised by using liquid chromatography coupled with traditional detectors such as FLR, DAD and EL. One solution to the co-eluted peaks was the utilisation of coincide specific expressions (Selhub 1989; Bagley and Selhub 2000). Recently, hyphenation of liquid chromatography and mass spectrometry (MS) made it possible to discriminate co-eluting clusters by using multiple-stage MS, and it also lowered the masking effects by matrixes (Garratt et al. 2005; Haandel et al. 2012).

The optimised UPLC-FLR/PDA method provided high sensitivity for the simultaneous determination of polyglutamyl 5-CH3-THF in the femtomole range and common monoglutamates in the picogram level. Due to the absence of standards, the quantitation of 5-CH3-H4PteGlu5,8 was based on the 5-CH3-H4PteGlu4 standard curve on the assumption that the fluorescence signal depended on the pteridine ring of folates rather than the
glutamate chain. Thus, the polyglutamate quantitation was calculated as the mole unit. However, when comparing CH$_3$-H$_4$PteGlu$_n$ standards, CH$_3$-H$_4$PteGlu$_4$ produced a significantly greater peak area than its mono- and diglutamate counterparts, and less significant than CH$_3$-H$_4$PteGlu$_3$. This might be attributed to a higher concentration of acetonitrile during the elution of CH$_3$-H$_4$PteGlu$_4$, which gave a sharp peak. Hence, difference in the peak areas between 5-CH$_3$-H$_4$PteGlu$_{5-8}$ and CH$_3$-H$_4$PteGlu$_4$ was assumed to be less significant at their eluting gradients. In the future, standards of other polyglutamates should be used to establish their own calibration equations.

**Effects of affinity chromatography**

According to the recovery test, FBP showed high and equivalent affinities for 5-CH$_3$-THF$_n$, but discrimination to 5-CHO-THF and its polyglutamates. It has been widely reported that there are significant differences in the binding characteristics between folate-binding protein (FBP) and various folate derivatives. According to a real time monitoring of affinity and dissociation rates of the binding interaction between FBP and pure stereoisomeric folate forms, folic acid showed the highest affinity to FBP at pH 7.4, followed by (6R)-5-CH$_3$-THF, (6S)-THF and (6S)-5-CH$_3$-THF, while the lowest was found for (6S)-5-CHO-THF (Nygren-Babol et al. 2005). In addition, the binding capacity of folates to FBP is significantly affected by pH. At pH 7.4 and 8.8 the affinity of FBP was remarkably weaker for (6R,S)-5-CH$_3$-THF than for folic acid in spite of an equal affinity at pH 9.3 (Givas and Gutcho 1975). Moreover, 5-CHO-THF was always a critical derivative in affinity purification with much lower affinity to FBP than other vitamers, so its load should be kept under 25% of the column capacity to achieve recovery of >90% (Pfeiffer et al. 1997; Kariluoto et al. 2001). Therefore, with utilisation of condition buffers at pH 7.0, our findings agreed well with previous studies.

In addition, Shane et al. (1980) and Strålsjö et al. (2002) reported that the glutamate chain length resulted in varying responses in radioprotein-binding assay (RPBA). However, these results did not actually conflict with our conclusion that the affinity of the FBP was equal for different polyglutamates of 5-CH$_3$-THF in a certain column load at neutrality, because these assays were undertaken at pH 9.3-9.5, which might be responsible for the response differences. Meanwhile, significant differences in the recoveries of 5-CHO-THF$_n$ at a
heavy column load might be due to the overload effect, so as observed such variations were lessened at low column loads.

Based on our results and previous literatures, we concluded that affinity chromatography (AC) was suitable for the purification of 5-CH$_3$-$\text{H}_4$PteGlu$_n$, but it might result in underestimation of the 5-CHO-$\text{H}_4$PteGlu$_n$ content, especially for those foods where 5-CHO-THF was the major form. Meanwhile, the binding abilities of the FBP to the polyglutamates of other vitamers were also unknown and, thereby, should be clarified in the future studies.

### 3.4.2 Pretreatments with enzymatic treatments and affinity chromatography

In our folate research, hog kidney conjugase (HK) has traditionally been used for deconjugation, and our standard tri-enzyme procedures for microbiological assays include a simultaneous treatment of amylase and HK followed by protease. HK is an endopeptidase which hydrolyses the glutamate chain by sequentially removing the terminal glutamate residues (Kozloff and Lute 1973). Hog kidney enzyme was reported to result in high microbiological-based results for foods such as garden peas and beans (Phillips and Wright 1983). Since 1990s, a variety of researches have supported that tri-enzyme treatment is essential for accurate determination of the folate content in foods. Although the increasing effect on the folate values varied among different foods, additional treatments with amylase and protease rather than conjugase alone were thought to assist more complete liberation of bound folates from food matrixes of polysaccharides and proteins (Martin et al. 1990; Peiffer et al. 1997; Tamura et al. 1997; Lim et al. 1998; Hyun and Tamura 2005). On the other hand, beneficial effects of additional amylase and/or protease treatments showed great variation depending on the type of food matrixes (Shrestha et al. 2000; Hefni et al. 2010). While the utilisation of $\alpha$-amylase increased the measurable folate values in foods high in starch and glycogen, protease made greater contributions to high-protein foods and dairy products (Yamada 1979; Cerna and Kas 1983; Pedersen 1988; Strandler et al. 2011; Mönch and Rychlik 2012). Furthermore, some studies also indicated that single-conjugase treatment was adequate for some foodstuffs such as brown rice, asparagus, soybean flour, breakfast cereals and yeast (Ndaw et al. 2001; Puwastien et al. 2005; Soongsongkiat et al. 2010). However, protease pre-treatment was thought to lower matrix
effects during sample purification and detection (Mönch and Rychlik 2012). Therefore, the necessity of amylase and/or protease seems to depend on the food types.

Among three proposed treatments, both treatment A and B exhibited their advantages with similar total folate yield. Method A which was traditional tri-enzyme treatment followed by affinity chromatography contributed to a slightly higher value of total folates and better retention of the folate pool. On the other hand, treatment B comprising α-amylase and protease treatment before purification and the final step with HK allowed complete deconjugation despite poor recovery of certain minor vitamers. As expected, its better deconjugation efficiency might give the credit to the removal of impurities before deconjugation, which contributed to maximal exposure of polyglutamyl folates to the conjugase and also protected native folates from inhibitors and destructors. It has been shown that 16% of citric acid, a known conjugase inhibitor, in white bean extracts could be removed by sample purification (Rychlik et al. 2007). However, one problem with method B for UPLC analysis was that its chromatogram contained some impurity peaks since the HK conjugase added in the last step was just removed by filtration rather than affinity purification (see Appendix 2). In addition, considering the difference in the consequent folate profile, adoption of either method A or B would be more important for quantitative methods for individual folates, but it is less relevant for total folate analysis.

In contrast, method C where sample extracts were treated with α-amylase followed by protease, then HK and finally affinity purification was unexpectedly found to be inadequate to folate determination in terms of both deconjugation efficiency and folate recovery. One of possible explanations might be that some indigenous folate conjugase inhibitors and/or enzymes were released from food matrixes after amylase and protease treatments, thereby impairing the activity of HK conjugase and destroying the folates. Rychlik et al. (2007) also reported a low conjugase activity of 45% when employing a same sequence of tri-enzyme treatment with the method C. The activity was improved to 102% by conducting a SAX purification before deconjugation, which accorded with the same difference between the method C and B found in our study. Some naturally-occurring food components have been known to inhibit the activity of folate conjugases such as organic acid, citrate, etc (Bhandari and Gregory 1990; Wei and Gregory 1998). Peas, beans and pulses were found containing a heat-activated inhibitor(s) against hog kidney conjugase, especially in the skin of the seeds (Krumdieck et al. 1973; Butterworth et al. 1975). Thus, it was possible that the boiling-water bath heating in method C that aimed to
inactivate protease turned out to activate certain inhibitor(s). However, there could be other factors leading to such significantly lower results.

In 1997, Tamura et al. obtained higher folate contents by using a consecutive treatment of amylase, protease and chicken pancreas folate conjugase than a previous study, which employed a simultaneous conjugase and amylase treatment followed by protease (Martin et al. 1990). Hence, they proposed that amylase and protease hydrolysis should be carried out before deconjugation to allow complete release of all folates from the food matrixes. Rader et al. (1998) reported increased contents of folates in cereal-grain products due to a prior treatment with protease before α-amylase and conjugase. On the other hand, the order of trienzyme extraction did not show significant effects on the contents of measurable folates in soybean flour, fish powder and breakfast cereal (Puwastien et al. 2005). However, all of these studies did not take into account sample purification and utilised different samples from ours. Meanwhile, since only one food sample was tested, it could not confirm whether the inefficiency of method B was typical to the tested sample or not.

In addition, it was uncertain whether the eluent employed in affinity chromatography affected microbiological responses. Nevertheless, it was clear that the sequential alteration of enzymatic and purification treatments would affect folate recovery and hydrolysis efficiency, though the effects might also depend on the sample matrixes. Hence, a series of further studies is necessary to investigate all possible factors involved and to optimise the orders of enzyme addition and purification in food folate assays or even for specific food types.

### 3.4.3 Applicability of the method to selected food samples

The validated UPLC method combined with affinity chromatography was successfully applied to the identification of 5-CH$_3$-THF with 1-8 glutamate residues and other major folyl monoglutamates in food samples. The differences of UPLC values relative to MA results ranged from 81 to 102% depending on factors such as the folate composition of samples and the injection volume. Since some folyl derivatives were present in foods at very low levels, they might be detected by the method but fell below their LOQs. Meanwhile, some analytes including 10-CHO-DHF and 5-CHO-THF had quite high limits for confident quantitation in our method, which limited the determination of small amounts of these components. In addition, if 5-CHO-THF was an abundant vitamer in samples,
affinity discrimination of FBPs for the species could be a possible factor responsible for relatively lower UPLC results. In previous studies, the UPLC method gave rise to 2-28% lower results than MA (Edelmann et al. 2012), and more significant differences (45-80%) were reported for the HPLC approaches (Konings 1999; Konings 2001; Ginting and Arcot 2004).

However, owing to the lack of available polyglutamate standards of other derivatives, our method only aimed at identification of methylated polyglutamates. As stated in the section 3.3.3, some peaks, which were possibly derived from other polyglutamyl derivatives, were detected in undeconjugated samples. Incapability of quantifying these peaks as well as traces of some naturally occurring monoglutamates might lead to different degrees of underestimation of the total folate content, depending on the methylated polyglutamylation of samples. Therefore, with this method, we recommended treating samples also with conjugase to obtain information on the folate composition as monoglutamates in addition to undeconjugated samples for the 5-CH$_3$-THF$_n$ profile. Alternatively, this method can be used to study the polyglutamylate distribution of the total folate pool by converting all the folate components into 5-methyltetrahydrofolates.

### 3.4.4 Folate composition of selected food samples

**Lupin flour**

Our study obtained a total folate content of 1058 ng/g in the flour from lupin seeds grown in 2011, a result which is similar to the value (1130 ng/g) reported by Mäkelä (2012) who used samples from the same harvest. However, folate contents of the blue lupin seeds from the 2010 harvest were found to be much higher; and the contents differed significantly between growing locations, ranging from 3420 ng/g (Mikkeli) to 3990 ng/g (Helsinki), but were not affected by the cultivars (Lizarazo et al. 2011; Rekola 2011). According to the USDA nutrient data, raw lupin seeds contain 3550 ng folic acid/g. Hence, the folate contents of lupin could be greatly influenced by extrinsic factors such as growing conditions and the harvest year.

Meanwhile, Rekola (2011) identified the folates in the lupin seed of *Boruta* harvested in Helsinki, which constituted 5-CH$_3$-THF (60%), 5-CHO-THF (29%) and minor components including THF, 5,10-CH$_3^+$-THF, 10-CHO-FA and folic acid, a profile which was in an
agreement with our findings. However, they found same folyl components with a different
distribution in the same cultivar grown in Mikkeli. Thus, the folate profile of a certain
cultivar seemed to be less affected by the harvest year in spite of great variations in the
total folate content.

In our study, the 5-CH$_3$-THF pool of lupin flour was highly polyglutamylated at 87%, with
pentaglutamate as the predominated form. In plants and animals, folyl pentaglutamates
occupied a large proportion of the folate pools (Pfeiffer and Gregory 1996; Scott et al.
2000; Matella et al. 2005; Becker et al. 2012). The major folates in pea cotyledons, leaves
and chloroplasts were found to be tetra- and pentaglutamyl derivatives (Imeson et al. 1990;
Besson et al. 1993). In lima beans, the majority of folates were pentaglutamates of 5-CHO-
THF (47%), 5-CH$_3$-THF (35%), 10-CHO-THF (9%) and THF (9%) (Seyoum and Selhub
1993). In plants, the glutamate chain length of cellular folates is affected by physiological
states and nutritional status (Crosti et al. 1993; Cossins 2000), and it regulates the one-
carbon metabolism by affecting the intracellular flux of one-carbon units among competing
pathways (Krumdieck et al. 1992). During germination and developmental stages, the
folate pool was dominated by highly glutamylated 5-CH$_1$-THF derivatives, which implied
their importance in the largest anabolic flux for methionine synthesis and turnover of S-
adenosylmethionine (SAM) (Cossins 2000). Meanwhile, the polyglutamylation degree

**Faba bean flour**

Faba bean is a folate-rich dietary source, and increasing the consumption of faba beans has
been associated with an enhanced folate intake (Bermejo et al. 2009). Our UPLC
measurement was about 13% lower than the average content of 1500 ng folates/g
determined by Konings et al. (2001), which might be due to the loss during affinity
purification and a high LOQ for 10-CHO-DHF. Meanwhile, the folate contents of faba
beans showed significant differences between cultivars and sites, ranging from 1500 to
3000 ng/g (Rekola 2011). In addition, the dominancy of pentaglutamyl 5-CH$_3$-THF was
also found in faba bean flour as expected.

When comparing our results with literature data, the folate composition of faba bean were
found to vary among different sources. Rekola (2011) determined the folate composition of
the *Kontu* variety, which was mainly comprised of 5-CH$_3$-THF (47%), 5,10-CH$^+$-THF
(28%), 5-CHO-THF (17%) and THF (4%), and of Tattoo variety where there was 34% 5-CH$_3$-THF, 25% 5-CHO-THF, 23% 10-CHO-FA and 14% 5,10-CH$_2$-THF. In a study conducted by Hefni et al. (2010), faba beans contained 960 ng folic acid/g with a folate profile of 160 ng THF/g, 730 ng 5-CH$_3$-THF/g and 100 ng 10-CHO-FA/g. Such discrepancies in the folate profile of faba beans might be attributed to both intrinsic and extrinsic factors. Firstly, the folate distribution in plants is associated with the physiological conditions and/or the folate metabolism in different compartments (Cossins 2000). While 5-CH$_3$-THF is involved in the conversion of homocysteine to methionine; 5-CHO-THF might implicate the biosynthesis of purine and formylmethionyl-tRNA, and 5,10-CH$_2$-THF for the formations of thymidylate and pantothenate (Hanson and Roje 2001). Moreover, during sample preparation stages, the heat treatment and the pHs of extraction buffer and purification eluent could induce non-enzymatic interconversion of various folates (De Brouwer et al. 2007).

**Dry yeast**

In 1931, yeast or a yeast extract was found their curative effects on tropical macrocytic anaemia in the Indian pregnant patients, which was later found to result from the benefits of the folate components in yeasts (Wills et al. 1931). Our sample is belonged to the common strain used for commercial baker’s yeasts—Saccharomyces cerevisiae—which is regarded as an abundant source of folates. An agreement was found between our results and previous data, which were reported in a range of 10-40 $\mu$g folates/g (Witthöft et al. 1999; Patring et al. 2005a; Patring et al. 2009). Hjortmo et al. (2005) studied the concentrations of total folates in 44 yeast strains, which ranged from 40 to 145 $\mu$g/g. According to our data, the folate derivatives in dry yeasts were primarily present in the forms of methylated polyglutamates of which heptaglutamate contributed to the greatest proportion, which was in accordance with previous data. The distribution of 5-CH$_3$-H$_4$PteGlu derivatives in yeasts varied greatly from 33 to 92% among different strains in the research of Hjortmo (2005), and was above 65% in all strains tested by Roje et al. (2002). In 1976, Bassett et al. have already estimated that the folate distribution during the growth cycle of yeast extracts was 12-16% for hexa-, 67-71% for hepta-, and 10-13% for octaglutamates. According to the HPLC analysis conducted by Seyoum and Selhub (1998), yeast folates were comprised of unsubstituted (20%) and methylated (77%) polyglutamates,
with 17% 5-CH$_3$-H$_4$PteGlu$_6$, 70% 5-CH$_3$-H$_4$PteGlu$_7$ and 13% 5-CH$_3$-H$_4$PteGlu$_8$ in the latter cluster.

Owing to both their high folate contents and ability to synthesis folates, baker’s yeasts have been widely reported for their great potential for the folate enhancement in bread (Keagy et al. 1975; Kariluoto et al. 2004; Hjortmo et al. 2005). During fermentation, the folate contents of rye sourdough experienced a considerable increase compared to that of flour, with enrichment of 54% and 128% depending on the flour cultivars (Kariluoto et al. 2004). Thus, by selecting most effective strains, yeast could be a vehicle for the folate enhancement in yeast-containing foods such as bread, kefir, cheese, beer, etc. In addition, the utilisation of yeast tended to modify the folate profile of used flour, primarily leading to a high proportion of 5-CH$_3$-THF in breads (Patring et al. 2009).
4 CONCLUSIONS

To our knowledge, this was the first UPLC-FLR/PDA method enabling simultaneous determination of intact polyglutamyl 5-methyltetrahydrofolates and common monoglutamyl folates including THF, 5-CHO-THF, 10-CHO-DHF, 10-CHO-FA, 5,10-CH\(^{\text{+}}\)-THF and folic acid. The chosen UPLC column BEH C\(_{18}\) provided adequate selectivity, specificity and linearity for the analysis of target folate forms without sacrificing peak shapes. The optimised UPLC method enabled fast and sensitive determination of folate polyglutamates in the femtomole level (LOQs ≤ 13.5 fmol/injection) and monoglutamates in the picogram range (LOQs: 6-600 pg/injection). Meanwhile, as an indispensable preparatory step for LC analysis, affinity chromatography (AC) was feasible for the purification of 5-methyl polyglutamates with desirable recoveries, but it resulted in varying losses of the polyglutamates of 5-CHO-THF, depending on the column load and the polyglutamylation degree. In addition, AC was shown to efficiently result in chromatograms devoid of matrix-derived interfering peaks for legume and yeast samples.

Alteration of the sequence of tri-enzyme and purification treatments could significantly affect the total measurable folates and consequent folate composition of the lupin flour. Our standard procedure—a simultaneous amylase and conjugase treatment followed by protease treatment and finally AC—was ideal for microbiological method, yielding the greatest amount of total folates. On the other hand, a modified method where deconjugation was conducted after successive treatments with amylase, protease and AC showed the best deconjugation efficiency and a slightly lower MA-derived content. Meanwhile, different procedures exhibited varying discrimination to vitamers, resulting in varied recoveries for certain folates. However, other possible factors such as the type of sample and the MA response to AC eluent should be further investigated. Nevertheless, it is certain that appropriate pretreatments should be carefully selected and optimised according to the purpose of the study and the determination method.

With the commitment to food analysis, the validated AC-UPLC-FLR/PDA method was successfully applied for the identification of native 5-CH\(_{3}\)-H\(_{4}\)PteGlu\(_{1-8}\) in the lupin flour, faba bean flour and dry yeast, which underwent standard pretreaments omitting deconjugation. The UPLC results of the total folates showed good agreement with MA determinations. As the major species, the methylated folate pools were predominated by pentaglutamate in legume flours and heptaglutamate in dry yeast. In addition, based on
comparison of the results of deconjugated and undeconjugated samples, it was reasonable to conclude that other polyglutamylated species were also present in the samples, especially in legumes. This finding, therefore, raised the necessity to develop a universal method for all possible polyglutamylated derivatives when commercial standards were available. Also, the recoveries of other polyglutamates on AC should be carefully investigated in further studies.
REFERENCES


Kariluoto MS. 2006. Standard operating procedure #3 Microbiological assay of total folate in cereal samples.


Appendix 1. Example chromatograms of sample extracts purified by solid phase extraction: (a) faba bean flour, (b) dry yeast.
Appendix 2. Chromatogram of the lupin flour extract undergone method B at FLR 290/356 nm (M: 5-methylH₄PteGlu, T: H₄PteGlu).