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Analytical Methods

Low folate content in gluten-free cereal products and their main ingredients

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ABSTRACT

Folate content in some gluten-free cereal products and their main ingredients was determined using a validated method based on reversed-phase high performance liquid chromatography (HPLC) with fluorescence and diode array detection. The main folate forms found in gluten-free products were 5-methyl-tetrahydrofolate and tetrahydrofolate. Starches and low protein flours commonly used as main components in gluten-free products appeared to be poor folate sources with folate content $\leqslant 6$ μg/100 g fresh weight. Folate content in gluten-free breads was higher (15.1–35.9 μg folate/100 g fresh weight) due to use of bakery yeast which is a rich folate source. Overall, folate content in gluten-free products was lower than in their gluten-containing counterparts. Therefore, fortification of gluten-free products with folic acid or enrichment of these products with nutrient-dense fractions of cereals naturally free from gluten (such as buckwheat, quinoa, amaranth or millet) can be of interest.

1. Introduction

Coeliac disease is a life-long intolerance to gluten proteins. A decade ago, coeliac disease was considered an uncommon disorder in the world, with prevalence rates of 1 in 1000 or lower (Feighery, 1999). However, recent population studies have reported a much higher prevalence and it is now estimated that coeliac disease may affect one in 100 of the population, including both adults and children (Mendoza & McGough, 2005). In addition, there are geographic differences, and in Finland, for instance, the prevalence of coeliac disease in adult Finns achieved 1.99% in 2000–2001 (Lohi et al, 2007). People suffering from coeliac disease react with inflammation of the small intestine, leading to malabsorption of several important nutrients including iron, folate, calcium and fat soluble vitamins (Feighery, 1999; Murray, 1999). Clinical and epidemiological studies have showed coeliac disease to be a risk factor for cancer (Silano et al., 2007), osteoporosis (Sategna-Guidetti et al., 2000), thyroid disease (Sategna-Guidetti et al., 1998), female infertility (Stazi & Mantovani, 2000), neurological and psychiatric disorders (Ludvigsson, Osby, Ekbom, & Montgomery, 2007; Tengah, Wills, & Holmes, 2002). Therefore a strict gluten-free diet – without cereals containing gluten proteins (wheat, barley, rye, triticale, dinkel and kamut) is essential (Gallagher, Gormley, & Arendt, 2004). Such a diet improves the health-related quality of life in terms of less symptoms of the disease and normalised microvilli, which is of utmost importance for optimal gastrointestinal functions (Johnston, Rodgers, & Watson, 2004).

The definition of gluten-free food varies in different countries. In the US a gluten-free diet includes mostly rice and maize that are naturally gluten-free. In Scandinavia and the UK, a gluten-free diet may include wheat starch that has been rendered gluten-free (Peraaho et al., 2003; Thompson, 2001). However, the concentrations of folates in gluten-free products are much lower than those in their gluten-containing counterparts (Thompson, 2000), which can lead to reduced folate intake in coeliac patients. Recently, it was demonstrated that the coeliac patients consuming gluten-free products have a daily folate intake of 186 μg for women and 172 μg for men (Hallert et al., 2002), which is much lower than recommended daily intake of 400 μg for women in fertile age and 300 μg for adults (Becker et al., 2004). These figures of daily folate intake among coeliac patients are in the lower range of the folate intake of the populations in European countries yet not having introduced mandatory folic acid fortification, i.e. 168–320 μg/day for women and 197–326 μg/day for men (De Bree, van Dusseldorp, Brouwer, van het Hof, & Steegers-Theunissen, 1997). The increase of folate content of gluten-free products therefore seems to be necessary. However, up to now no countries have performed mandatory folic acid fortification of gluten-free products, whereas mandatory fortification of white flour has been introduced in several countries, such as US (1998), Canada (1998) and Chile (2000). Furthermore, there are limited data in food data bases regarding gluten-free products which makes the decision-making difficult.

The objectives of the present investigation were to determine folate content in some gluten-free products typically consumed in Sweden. These data can be useful for food databases and for authorities that take decision on folic acid fortification.

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2. Materials and methods

2.1. Materials

2.1.1. Reagents and folate standards

All reagents were of *p.a.* grade, except methanol and acetonitrile which were of HPLC grade. The water was purified using Milli-Q system. All chemicals were purchased from VWR International (Darmstadt, Germany).

Folic acid, (6S)-5-formyl-5,6,7,8-tetrahydrofolate, sodium salt (5-HCO–H_4folate), (6S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH_3–H_4folate) and (6S)-5,6,7,8-tetrahydrofolate, sodium salt (H_4folate) were all donated by Merck Eprova AG, Schaffhausen, Switzerland. Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-formyl-folic acid, sodium salt (10-HCO-folic acid) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). The purity of the folates and folic acid was checked in phosphate buffer, pH 7.0 according to van den Berg, Finglas, and Bates (1994) and purities calculated using molar extinction coefficient reported by Baggott and Johanning (1999) for 10-HCO–folic acid and molar extinction coefficients reported by Eitenmiller and Landen (1999) for other folate derivatives. All stock solutions, of 200 μg/ml (purity corrected) were prepared under subdued light in 0.1 M phosphate buffer pH 6.1 containing 1% ascorbic acid (AA) and 0.1% 2-mercaptoethanol (MCE). Aliquots of stock solutions were placed in separate tubes, flushed with nitrogen and stored in −80 °C for maximum 90 days.

2.1.2. Enzyme preparations

Thermostable α-amylase solution (E-BLAAM, 3000 U/ml) was obtained from Megazyme International, Ireland and used for sample pre-treatment without additional preparation. Protease (Cat. No. P5147) was purchased from Sigma Chemical Co. (St. Louis, USA). Protease solution (5 mg/ml) was prepared in 0.05 M phosphate buffer pH 6.1 containing 0.1% MCE and dialysed at 4 °C during stirring in three steps using 800 ml of the 0.05 M phosphate buffer pH 6.1 containing 0.1% MCE in each step. The dialysed protease solution was stored in small portions at −20 °C for maximum of one month.

Lyophilised chicken pancreas (CP) from Difco (Detroit, USA) and rat serum (RS) from Scanbur (Sollentuna, Sweden) were used as folate conjugase (γ-glutamyl hydrolase) sources. CP solution (5 mg/ml) was prepared according to Strålsjö, Arkbåge, Witthöft, and Jägerstad (2002). CP (50 mg) was ground in a mortar with 10 drops glycerol and 10 ml water was added; the obtained suspension was centrifuged. The supernatant was used freshly prepared. RS was dialysed to remove endogenous folates according to Patring, Jastrebova, Hjortmo, Andlid, and Jägerstad (2005) and was stored in small portions (0.5 ml) at −20 °C for a maximum of one month. Activity of each enzyme preparation was checked before use as described by Patring et al. (2005).

2.2. Samples

Two different flours, one kind of lunch rolls, two kinds of crisp bread and three starch products were purchased at the local market in Uppsala, Sweden. All products were immediately vacuum-packed in plastic bags and frozen at −20 °C. The lunch rolls were sliced before freezing. Prior to analysis the samples of crisp breads were ground in a food mixer and then milled in a type ZM 1 ultra-centrifugal mill with a 0.5-ring sieve (Retsch, Haan, Germany). The slices of frozen lunch rolls were first freeze-dried during 45 h in a Labconco freeze-drier (AB Ninolab, Upplands-Väsby, Sweden) and then ground in a food mixer and milled. All milled samples were immediately flushed with nitrogen and stored at −20 °C. The storage time for the milled samples at −20 °C until analysis was kept to a minimum, being at most 2 weeks. Information on the ingredients in the products, according to the labelling, is given in Table 1.

2.3. Sample preparation

To prevent folate oxidation, all samples were protected by nitrogen, subdued light and cooled on ice throughout sample preparation. All samples were analysed at least as duplicates and some in triplicates.

2.3.1. Monoenzyme treatment

The sample (1 g) was added to 80 ml fresh extraction buffer (0.1 M phosphate buffer, pH 6.1, 2% (w/v) sodium ascorbate and 0.1% (v/v) MCE) and extracted for 1 h at 75 °C after being flushed with nitrogen. The extract was rapidly cooled on ice, diluted to exact 100 ml with fresh extraction buffer and centrifuged at 27,000*g* (Sorvall RC 5B, Du Pont Instruments) for 15 min at 4 °C. The obtained supernatant was used for two separate deconjugation procedures.

To deconjugate folate polyglutamates to monoglutamates, 144 μl of dialysed RS was added to 5.75 ml supernatant and the sample was flushed with nitrogen, capped and incubated in shaking water bath (37 °C) for 3 h. To deconjugate folate polyglutamates to diglutamates, 750 μl of CP solution (5 mg/ml) was added to 30 ml supernatant prior to incubation in shaking water bath (37 °C) for 3 h. The enzymes were then inactivated by treatment in a boiling water bath for 5 min. The sample was cooled on ice and centrifuged. The optimisation of deconjugation steps was performed by checking deconjugation efficiency when adding

Table 1
Ingredients in the gluten-free breads, flours and starches according to the producer labelling

Gluten-free product	Ingredients	Protein (%)	Carbohydrates (%)	Fat (%)
Maize starch (Maizena)	Maize starch	<1	87	0
Potato starch (Lyckeby stärkelsen)	Potato starch	<0.5	80	<0.5
Rice flour (Risenta)	Rice flour	7.0	80	<1
Flour mix (Finax)	Wheat starch, sugar, guar gum, iron, thiamine, riboflavin, niacin, B_6	0.3	86	<0.1
Coarse flour mix (Semper)	Rice flour, maize starch, modified potato starch, sugar-beet fibre, maize fibre, thickener, salt, sugar, baking powder, iron, thiamine, riboflavin, niacin, B_6	2.5	75	<0.5
Coarse crisp bread (Semper)	Rice flour, maize starch, modified potato starch, sugar, sugar-beet fibre, maize fibre, yeast, vegetable margarine, thickener, salt, baking powder, iron, thiamine, riboflavin, niacin, B_6	3.5	80	3
Crisp bread (Wasa) (lactose free)	Rice flour, maize flour, maize starch, potato starch, vegetable fat, water, yeast, buckwheat flour, sugar-beet fibre, sugar, syrup, thickener, salt, raising agent, emulsifier	6.0	71	6.5
Lunch rolls (Schär)	Water, maize starch, rice flour, potato starch, dextrose, psyllium, soy protein isolate, yeast, vegetable oil, thickener, salt, tartaric and citric acid, ethyl alcohol	3.3	49.4	2.4

different amounts of enzyme preparations to extracts of coarse crisp bread spiked with $PteGlu_3$ as described previously (Patring et al., 2005).

The obtained supernatants were directly purified by SPE or stored at 0 °C prior to SPE (no more than 12 h). The correction for dilution of yeast extracts due to addition of rat serum was made. Blank enzyme samples containing only CP or RS and extraction buffer were prepared and treated in the same way as the real samples to check whether the enzyme preparations contained any endogenous folates.

2.3.2. Dienzyme treatment

The same sample preparation procedure as described for the monoenzyme treatment was performed except the extraction step that was conducted at 100 °C (10 min) on addition of α-amylase solution (40 μl) to the extraction buffer (80 ml).

2.3.3. Trienzyme treatment

As for dienzyme treatment, 40 μl of α-amylase solution was added during the extraction step which was performed either at 100 °C (10 min) or at 75 °C (1 h). Thereafter 0.8 ml of protease solution was added to sample extract that was incubated in shaking water bath (37 °C) for 1 h. The obtained extract was boiled in a water bath for 5 min to inactivate protease, rapidly cooled on ice, diluted to exact 100 ml with fresh extraction buffer and centrifuged at 27,000*g* for 15 min at 4 °C. The obtained supernatant was used for deconjugation procedure with dialysed RS as described above for monoenzyme treatment.

2.4. Purification of extracts

Purification of the sample extracts was carried prior to HPLC analysis with solid-phase extraction (SPE) on Isolute phenyl-endcapped (PH EC) bonded silica (500 mg, International Sorbent Technology, Mid-Glamorgan, UK) as described by Nilsson, Johansson, Yazynina, Strålsjo, and Jastrebova, (2004). A Visiprep SPE Vacuum Manifold (Supelco, USA) was used for elution under reduced pressure. The PH EC cartridges were conditioned by rinsing with methanol (2 × 2.5 ml) followed by 0.03 M H_3PO_4 containing 1% (w/v) ascorbic acid (AA), 2 × 2.5 ml. The cartridge was loaded with 5 ml extract and washed with 5 ml 0.03 M H_3PO_4 containing 1% (w/v) AA. Folates were eluted with 3.5 ml 0.1 M sodium acetate containing 11% (v/v) acetonitrile, 1% (w/v) AA and 0.1% (v/v) MCE.

In addition to purification on the PH EC cartridges the most complex matrices such as rice flour, coarse crisp bread and lactose-free crisp bread were subjected to a combined SPE procedure in order to better eliminate the interferences and to facilitate the identification of folate peaks (Nilsson et al., 2004). Aliquots of extracts (5 ml) were applied on PH EC cartridges and treated as described above. Thereafter eluates were applied onto SAX cartridges preconditioned with methanol (2 × 2.5 ml) and water (2 × 2.5 ml). The cartridges were washed with water (2 × 2.5 ml) to remove matrix interfering components. The folates were eluted with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) MCE. The first portion (0.7 ml) of eluate was discarded and the second portion (3.8 ml) was collected. The purified samples were analysed directly on HPLC and were not stored more than 24 h at 8 °C in the thermostated autosampler before analysis.

2.5. HPLC analysis

An HPLC method described previously by Patring et al. (2005) was used to determine individual folate forms. An Agilent 1100 HPLC system was used, equipped with a gradient quaternary pump, a thermostated autosampler, a thermostated column compartment, a diode array (DAD) detector and a fluorescence (FLD) detector. Agilent Chemstation software was used to control the HPLC system and data processing.

Folates were separated on an Aquasil C_{18} column (150 × 4.6 mm, 3 μm, Thermo Electron-Corporation, US) with a C_{18} (1 mm) guard column (Optimize Technologies). The column temperature was 23 °C, the autosampler temperature 8 °C and the injection volume was 20 μl. The mobile phase was a binary gradient mixture of 30 mM potassium phosphate buffer at pH 2.3 and acetonitrile. The gradient started at 6% (v/v) acetonitrile maintained isocratically for the first 5 min, thereafter the acetonitrile content was raised linearly to 25% within 20 min. The flow rate was 0.4 ml/min and total run time was 42 min. The excitation and emission wavelength in the FLD detector was set to 290 nm and 360 nm respectively; DAD wavelengths were set to 269, 290 and 340 nm. The FLD detector was used for detection and quantification of H_4folate, 5-CH_3–H_4folate and 5-HCO–H_4folate, whereas DAD detector was used for detection and quantification of 10-HCO–H_4folate and folic acid. Retention time was used for peak identification; comparison of ratio of sample peak heights and areas from FLD and DAD detectors (at different wavelengths) to ratio of standard peak heights and areas as well as fluorescence spectra were used to verify individual folates if necessary.

The detector response was linear up to concentration 100 ng/ml for 5-CH_3–H_4folate and H_4folate and 600 ng/ml for 5-HCO–H_4folate, 10-HCO–folic acid and folic acid. The calibration curves had a correlation coefficient higher than 0.9998 for all folate forms. The limits of detection were 0.05, 0.1, 2.0, 4.0, and 1.0 ng/ml for 5-CH_3–H_4folate, H_4folate, 5-HCO–H_4folate, 10-HCO–folic acid, and folic acid, respectively.

2.6. Quantification

Quantification of folate monoglutamates was based on external standard method in which the peak area was plotted against concentration and least-squares regression analysis was used to fit lines to the data. A multilevel calibration curve was used ($n = 7$) and the amount of each folate form was calculated in its free acid form.

Quantification of folate polyglutamates was performed indirectly, namely, by comparison of results from two monoenzyme treatments with the use of RS or CP. When using RS for deconjugation step, total content of mono- and polyglutamates could be determined as RS deconjugated all folate polyglutamates to monoglutamates. When using CP, polyglutamates were deconjugated to diglutamates, whereas native monoglutamates remained intact. Therefore the concentration of folate polyglutamates could be calculated by subtraction of the concentration of folate monoglutamates found in sample extract deconjugated by using CP (corresponding to native folate monoglutamates) from the concentration of folate monoglutamates found in sample extract deconjugated by using RS (corresponding to sum of native folate mono- and polyglutamates).

Because enzyme preparations contained small amounts of endogenous 5-CH_3–H_4folate, the concentration of 5-CH_3–H_4folate in real food samples was corrected by subtraction of the concentration of 5-CH_3–H_4folate in blank enzyme sample (for preparation see Section 2.3.1 monoenzyme treatment) from the concentration of 5-CH_3–H_4folate in extracts of real food samples.

2.7. Check of accuracy and precision of the final method

The gluten-free coarse crisp bread from Semper was used as in-house control material to check accuracy and precision of the final method based on monoenzyme treatment. To measure folate losses during the entire analytical procedure known amounts of H_4folate

and 5-CH_3–H_4folate were added to the samples before extraction. The recovery (R) was calculated as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is the concentration in the spiked sample, C_{sample}, the concentration in the sample prior to spiking and C_{added}, the concentration of the added standard. The repeatability (intra- and inter-assay) of the entire analytical procedure was evaluated by analysing in-house control material in triplicates during 3 different days.

3. Results and discussion

3.1. Optimisation of sample pre-treatment for cereal matrices

Commonly used sample pre-treatment in folate analysis involves extraction and deconjugation steps. Extraction by heat treatment during 10–12 min is usually sufficient to achieve good recovery. However, for starch-rich cereals the first step (heat treatment) can fail due to formation of gel or very fine suspension which is impossible to filtrate or purify. Moreover, it is suggested that folates are bound to cereal proteins and other components of complex cereal matrix. Therefore, trienzyme treatment has been recommended by several researchers for complex matrices such as cereals (De Souza & Eitenmiller, 1990; Pfeiffer, Rogers, & Gregory, 1997). This treatment involves the use of α-amylase and protease during sample pre-treatment in addition to conjugase. Protease breaks down the proteins by splitting the peptide bonds, whereas α-amylase breaks down the large starch molecules to short-chain carbohydrates and in this way prevents the aggregation and gelatinisation of starches. It is also suggested that folate bindings to the matrix are disrupted by breaking down the proteins and starches. However, the necessity of trienzyme treatment is still questionable because of contradictory results. Some research groups have reported increase in total folate content due to trienzyme treatment (Pedersen, 1988; Pfeiffer et al., 1997; Rader, Weaver, & Angyal, 1998), whereas other groups have found lower folate content when using trienzyme treatment (Shrestha, Arcot, & Paterson, 2000).

In the present study, routinely used extraction method by heat treatment at 100 °C (Patring et al., 2005) was slightly modified to prevent formation of gel when extracting folates from cereal matrices. Instead of boiling a more soft treatment at 75 °C (1 h) was used. It showed to be effective in prevention of gel formation. To achieve complete extraction of folates, the optimal sample to buffer ratio was evaluated. The same volume of extraction buffer (80 ml) was added to different amounts of crisp bread (0.6, 1.0, 2.0 and 3.0 g) and 1.0 g was chosen since there were no differences in recovery of folates (data not shown) between these amounts of samples.

Deconjugation efficiency was checked by adding different amount of rat serum (100, 144, 250 and 400 μl) to 5.75 ml extract of crisp bread. Complete deconjugation was already observed when 144 μl of rat serum was added (data not shown); this amount of rat serum was therefore chosen for deconjugation step.

The monoenzyme treatment described above was compared with di- and trienzyme treatments to check if it provided efficient release of folates from cereal matrix. Four gluten-free samples (coarse crisp bread, lactose-free crisp bread, lunch rolls and rice flour) were prepared with different enzyme treatments. As shown in Table 2, di- and trienzyme treatment did not increase the folate yield in any of the investigated samples which indicated that heating at 75 °C was sufficient to release folates from cereal matrices. By contrast, trienzyme treatment resulted in considerable losses of folates: the decrease in H_4folate and 5-CH_3–H_4folate content ranged up to 100% and 40%, respectively (Table 2). This can be explained by longer duration of trienzyme treatment including more heating steps compared to monoenzyme treatment. The use of higher temperature during trienzyme treatment (100 °C instead for 75 °C) further increased the losses of folates (Table 2). For lunch rolls the tri-enzyme treatment was found not to be suitable due to formation of a suspension consisting of tiny particles which could not be separated by centrifugation or filtration (clogging the filter) nor purified by SPE (clogging the SPE cartridge). On the basis of the obtained data, monoenzyme treatment was chosen as the most suitable for the investigated cereal matrices.

Table 2
Comparison of different enzyme treatments[A] regarding folate recovery[B]

Gluten-free product	Recovery of H_4folate (%)				Recovery of 5-CH_3–H_4folate (%)			
	Mono[A]	Di[A]	TriA[A]	TriB[A]	Mono[A]	Di[A]	TriA[A]	TriB[A]
Coarse crisp bread	100[a]	93[b]	80[c]	ND	100[a]	95[b]	98[a]	ND
Crisp bread (lactose free)	100[a]	88[b]	88[b]	90[b]	100[a]	99[a]	97[a]	98[a]
Lunch rolls	100[a]	76[b]	ND	ND	100[a]	93[a]	ND	ND
Rice flour	100[a]	86[a]	0[b]	0[b]	98[a]	100[a]	42[b]	58[c]

ND – no data.
[A] Mono – monoenzyme treatment with extraction at 75 °C (1 h); Di – dienzyme treatment with extraction at 100 °C (10 min); TriA – trienzyme treatment with extraction at 100 °C (10 min); TriB – trienzyme treatment with extraction at 75 °C (1 h).
[B] Recoveries of folates for each gluten-free product are calculated relative to maximal recovery that is taken as 100%; different letters in superscript (horizontal) show statistically significant ($p < 0.05$) differences in recoveries.

The stability of folates in cereal extracts was checked during storage. The freezing/thawing of extracts prior to SPE procedure resulted in considerable losses of folates possibly due to oxidative degradation induced by freeze-thaw cycle and endogenous oxidants from food matrix. This is in agreement with our previous results on folate susceptibility to freeze-thaw cycles (Patring, Johansson, Yazynina, & Jastrebova, 2005). For H_4folate these losses ranged up to 80% and for 5-CH_3–H_4folate up to 30%. By contrast, the storage of extracts at 0 °C prior to SPE procedure was found to provide good stability of folates. No noticeable decrease in folate content was found when extracts were stored at 0 °C up to 15 h. Therefore, the freezing/thawing step prior to SPE was eliminated to prevent losses of folates. The extracts were purified directly by SPE or stored at 0 °C prior to SPE (no more than 12 h). The stability of folates in purified extracts in autosampler at 8 °C was also checked. The extracts were stable for at least 24 h in autosampler.

3.2. Evaluation of the final method based on monoenzyme treatment

Checking for precision of the entire analytical procedure gave relative standard deviation between 1 and 11% for gluten-free crisp bread (Table 3). Accuracy of the method was determined by recovery tests. Crisp bread samples were spiked (addition of standard solution to the sample before extraction) at two different concentrations (Table 4). The mean recovery for 5-CH_3–H_4folate was 78% at both spiking levels, whereas the recoveries for H_4folate were considerable lower, 48–56%, which may be explained by higher susceptibility of H_4folate to oxidative degradation.

3.3. Determination of folates in gluten free products

The main folate forms found in gluten-free products were 5-CH_3–H_4folate and H_4folate. 5-CH_3–H_4folate was the most abundant

Table 3
Intra- and inter-day precision (expressed as RSD) of the final monoenzyme method for folate determination in gluten free coarse crisp bread

Folate form	Intra-assay, RSD (%)			Inter-assay, RSD (%)
	Day 1 ($n = 3$)	Day 2 ($n = 3$)	Day 3 ($n = 5$)	($n = 11$)
H_4folate	1.0	3.0	2.0	11.3
5-CH_3–H_4folate	1.9	6.9	2.2	4.0

Table 4
Recovery of the final monoenzyme method for folate determination in gluten free coarse crisp bread

Folate form	Amount in sample (μg/100 g)	Added (μg/100 g)	Found (μg/100 g)	Recovery (%), mean ± SD (n = 3)
H_4folate	4.11	4.92	6.47	48 ± 3
		7.82	8.22	53 ± 6
5-CH_3–H_4folate	27.1	24.1	45.9	78 ± 1
		34.4	53.9	78 ± 3

form, its contribution to the total folate content ranged from 78% to 100%, whereas H_4folate occurred in a lesser proportion (0–22%). The highest concentrations of 5-CH_3–H_4folate and H_4folate were found in samples of crisp breads and rolls, whereas starches and low-protein flour mixes contained only trace amounts of folates (Table 5). The determination of other folate forms, such as 5-HCO–H_4folate and 10-HCO-folate, in extracts of crisp breads and rolls was complicated by appearance of interfering peaks on FLD chromatograms when using one SPE step on PH EC cartridges (see Fig. 1). The fluorescence spectra of these interfering peaks differed radically from those of 5-HCO–H_4folate and 10-HCO–folate peaks. The ratio of peak heights and areas from FLD and DAD detectors at 269, 290 and 340 nm was also different from that of folate peaks, which confirmed that these interfering peaks were not related to folates. To further confirm the absence of these peaks the combined SPE (purification on PH EC cartridges followed by purification on SAX cartridges) was performed. However, the necessity of combined SPE makes the developed method more time consuming. The use of mass spectrometric detection seems to be necessary to quantify these folate forms with sufficient selectivity and sensitivity.

The obtained results showed that removal of protein-rich fractions from flours may result in dramatic depletion of folates. As seen from Table 5, only trace amounts of folates could be detected in flour mix based on wheat starch, whereas common wheat flour with protein content of 8.5% contains 21 μg folate/100 g (Livsmedelsverket, 2002). Similar to wheat starch, the maize starch contained only trace amounts of folates compared to 10 μg folate/100 g maize flour with protein content of 7.8% (Livsmedelsverket, 2002). Unlike starches, the rice flour with 7% of proteins contained considerably higher amounts of folates, 6.3 μg/100 g (Table 5), and appeared to be superior to starches from the nutritional standpoint. Generally, the use of nutrient-poor starches as the main ingredients of gluten-free products may markedly impair the nutritional density of such products and result in reduced intake of folates and other vitamins in coeliac patients. The use of nutrient-dense ingredients

Table 5
Folate content[a] in gluten-free products, flours and starches (μg/100 g fresh weight), determined by monoenzyme method

Gluten-free product	H_4folate, mean ± SD (n = 3)	5-CH_3–H_4folate, mean ± SD (n = 3)	Total folate (expressed as folic acid)	Polyglutamates (%)	Total folate (literature data[b])
Maize starch	ND (< 0.7)	Trace amounts (⩽0.4)	–	–	0
Potato starch	ND (< 0.7)	Trace amounts (⩽0.4)	–	–	0
Flour mix	ND (< 0.7)	Trace amounts (⩽0.5)	–	–	No value
Rice flour	1.4 ± 0.1	5.0 ± 0.1	6.3 ± 0.2	11	4
Coarse flour mix	ND (< 0.7)	1.0 ± 0.1	1.0 ± 0.1	0	No value
Coarse crisp bread	4.1 ± 0.1	27.1 ± 0.5	30.1 ± 0.6	92	48
Crisp bread (lactose free)	5.1 ± 0.2	31.9 ± 0.4	35.9 ± 0.6	90	50
Lunch rolls	3.4 ± 0.3	12.1 ± 0.3	15.1 ± 0.6	75	26[c]

ND – not detected.
[a] Results are not corrected for recoveries.
[b] Livsmedelsverket (2002).
[c] Value for similar product.

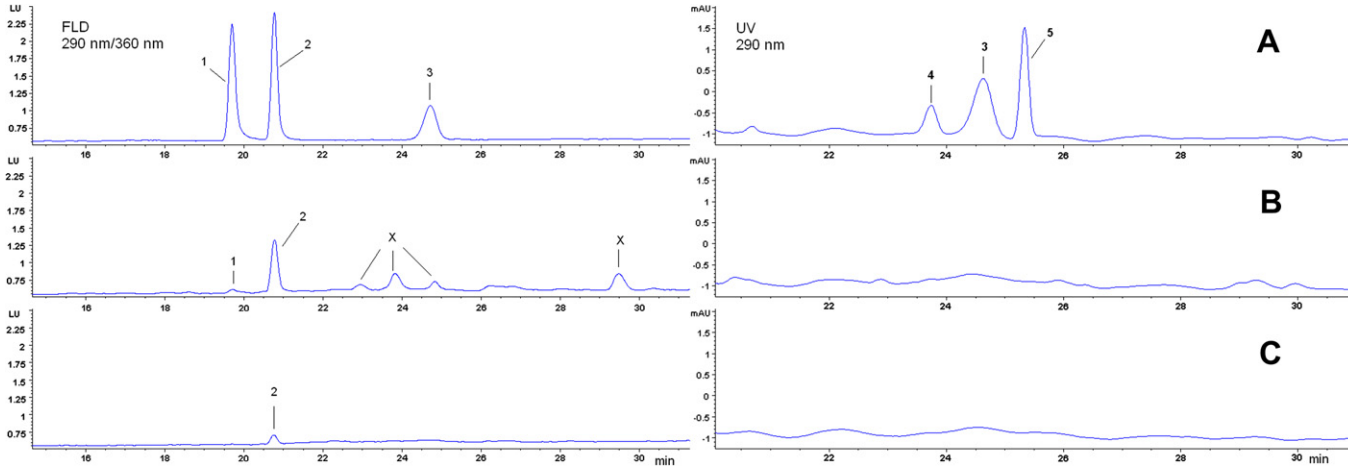

Fig. 1. Representative FLD- and UV–chromatograms of folate derivatives. Samples: (A) – standard mixture containing H_4folate 15 ng/ml, 5-CH_3–H_4folate 10 ng/ml, 5-HCO–H_4folate 170 ng/ml, 10-HCO–folic acid 170 ng/ml and folic acid 165 ng/ml in elution buffer; (B) – purified extract of coarse crisp bread; (C) – purified blank sample containing rat serum. Peaks: 1 – H_4folate; 2 – 5-CH_3–H_4folate; 3 – 5-HCO–H_4folate; 4 – 10-HCO–folic acid, 5 – folic acid and x – interfering compounds. Column: Aquasil C_{18} (150 × 4.6 mm, 3.5 μm). Mobile phase: acetonitrile–phosphate buffer (pH 2.3) with acetonitrile gradient from 6% to 25% (see Section 2 for details). Flow 0.4 ml/min, injection volume 20 μl.

seems therefore to be important to improve the nutritional quality of gluten-free products. Yeast is one example of such ingredients. It is a rich folate source and may contribute considerably to folate content in bakery products (Keagy, Stokstad, & Fellers, 1975). As evident from Table 5, the gluten-free yeast breads had folate content 15-36 μg/100 g that was comparable with folate content of similar gluten-containing breads – 26–75 μg/100 g (Livsmedelsverket, 2002). Other components that can improve nutritional quality of gluten-free products are buckwheat, quinoa, amaranth and millet (Gallagher et al., 2004) (Alamprese, Casiraghi, & Pagani, 2007).

The contribution of folate mono- and polyglutamates to the total folate content of different gluten-free products was determined by comparing results of two mono-enzyme treatments: deconjugation to monoglutamates by using RS with resulting total content of mono- and polyglutamates, and deconjugation to diglutamates by using CP which yielded concentration of native monoglutamates (see Section 2.6 Quantification for more details). The determination of native monoglutamates in non-deconjugated extracts was not possible due to coelution of mono- and polyglutamates as we have shown previously (Patring et al., 2005). Therefore the use of deconjugation step with CP was necessary to obtain pure peaks of native monoglutamates without interference from polyglutamatic forms. As Table 5 illustrates, monoglutamates were the dominant form of folates in rice flour and coarse flour mix, whereas yeast breads contained mostly polyglutamates (75–92%), probably from yeast, which is known to contain only folate polyglutamates (Bassett, Weir, & Scott, 1976).

4. Conclusion

An HPLC method to determine folates in cereal matrices was optimised and validated. It was demonstrated that heat treatment at 75 °C followed by deconjugation with rat serum or chicken pancreas was sufficient to release folates from cereal matrices. An additional treatment with α-amylase and protease provided no increase in folate yield; instead, it resulted in enhanced losses of folates, possibly due to increased oxidative degradation because of longer treatment at elevated temperatures. The freezing/thawing of extracts prior to SPE procedure was found to impair the stability of folates, especially H_4folate, and should be replaced by storage of extracts at 0 °C prior to SPE procedure if necessary.

The main folate forms found in gluten-free products were 5-CH_3–H_4folate and H_4folate. Starches and low protein flours commonly used as main components in gluten-free products appeared to be poor folate sources; they contained no more than 6 μg folate/100 g fresh weight. This demonstrates that folates are probably bound to cereal proteins; therefore the removal of protein-rich fractions from cereal matrix may cause depletion of folates. Compared to low-protein flours and starches, folate content in gluten-free breads was higher (15.1–35.9 μg folate/100 g fresh weight) due to use of bakery yeast which is a rich folate source. Overall, folate content in gluten-free products was lower than in their gluten-containing counterparts. Therefore, fortification of gluten-free products with folic acid or 5-CH_3–H_4folate or enrichment of these products with nutrient-dense fractions of cereals naturally free from gluten (such as buckwheat, quinoa, amaranth or millet) can be of interest.

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