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HPLC determination of folates in raw and processed beetroots

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Abstract

A sensitive HPLC method with fluorescence detection and gradient elution has been developed for the determination of folates in vegetables. The method involved extraction of folates from food matrix by heat treatment, deconjugation of folate polyglutamates to monoglutamates by incubation with hog kidney conjugase and purification of food extracts by solid-phase extraction with strong-anion exchange cartridges. The chromatographic separation of folates was achieved on Zorbax SB C_8 column, which was found to be superior over conventional C_{18} column in terms of selectivity and sensitivity. Validation of the method included linearity tests, the addition of standard folates for the determination of recovery, repeatability and stability tests. The method developed was applied to analysis of raw and processed beetroots; 5-methyltetrahydrofolate was found to be the main folate form in beetroots. Cultivar differences and growing conditions were found to have a pronounced effect on the folate content in beetroots. Processing resulted in considerable losses of folates, whereas losses during storage appeared to be moderate.

Keywords: Folate; Food processing; Food analysis; HPLC; Beetroot

1. Introduction

Folates are a group of naturally occurring B vitamins of great nutritional importance related to pteroyl monoglutamic acid (folic acid). They exhibit biological activity like folic acid and usually exist in the polyglutamate form typically with five to seven molecules of glutamic acid (Ball, 1998; Eitenmiller & Landen, 1999; Gregory, 1989; Lucock, 2000; Scott, Rébeille, & Fletcher, 2000). Research in recent years concerning health-protecting roles of folates versus neural tube defects, coronary heart diseases, certain forms of cancer and impaired cognitive functions have resulted in increased recommendations of folate intake in many Western countries (Selhub & Rosenberg, 1996). Good dietary sources of folates are vegetables, fruits and berries with folate content of 50–200 μg/100 g (Witthoft, Forssén, Johannesson, & Jägerstad, 1999). However, reliable data on folate content in foodstuffs of plant origin are still lacking, especially data regarding effects due to cultivar differences, growing area, storage conditions as well as processing. The currently available data on dietary folates in food databases are often contradictory because of difficulties associated with quantification and characterisation of folates due to their instability and wide variety of forms.

Owing to high sensitivity, microbiological assay still remains the method of choice for determination of food folates despite the fact that it is extremely laborious and provides only a sum of folate forms (Eitenmiller et al., 1999; Tamura, 1998). HPLC methods allow determination of individual folate forms and are less time-consuming. A number of HPLC methods for folates in various food matrices have been reported recently (Bagley & Selhub, 2000; Eitenmiller et al., 1999; Konings, 1999; Lucock, Green, Priestnall, Daskalakis, Levene, & Hartley, 1995; Ndaw, Bergaentzlé, Aoudé-Werner, Lahély, & Hasselmann, 2001; Osseyi, Wehling, & Albrecht, 2001; Vahteristo & Finglas, 2000). However, these methods can not offer high sensitivity like

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microbiological assay (Vahteristo & Finglas, 2000). Furthermore, different endogenous constituents present in food extracts may interfere with folates, which makes quantification of some folate forms difficult (Finglas, Wigertz, Vahteristo, Witthöft, Southon, & de Froidmont-Görtz, 1999; Ruggeri, Vahteristo, Aguzzi, Finglas, & Carnovale, 1999; Vahteristo, Lehikoinen, Ollilainen, & Varo, 1997). Therefore, more specific and reliable HPLC methods are needed.

This paper describes the development of a sensitive, selective and robust HPLC method for the quantification of folates in food matrices of plant origin. The validation of the method is performed by the use of certified reference material CRM 485 (lyophilised mixed vegetables). The validated method is applied to analysis of beetroots, which are a good source of folates, containing up to 150 μg folate/100 g (Witthöft et al., 1999). In Scandinavia the major part of beetroots is consumed in pickled form, but the data on the losses of folates due to processing and storage are lacking, as well as the data concerning folate content in raw beetroots in relation to cultivar differences, growing conditions and post-harvest storage. The present study provides new data on folate content in raw and processed beetroots, which can be useful for food databases.

2. Materials and methods

2.1. Reagents

Acetonitrile and methanol were of HPLC grade. Other reagents were of p.a. grade. All chemicals were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, USA).

Hog kidney acetone powder (Sigma-Aldrich, USA) was used for preparation of hog kidney conjugase (γ-carboxypeptidase).

2.2. Standards

Folic acid, pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and the reduced forms of monoglutamic folates: (6R,S)-5,6,7,8-tetrahydrofolic acid sodium salt (H_4folate), (6R,S)-5-HCO-5,6,7,8-tetrahydrofolic acid, sodium salt (5-HCO-H_4folate) and (6R,S)-5-CH_3-5,6,7,8-tetrahydrofolic acid, sodium salt (5-CH_3-H_4folate) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland) and stored at −80 °C until use. The purity of the reduced forms of folates and folic acid was checked according to the procedure of van den Berg, Finglas, and Bates (1994) using the molar extinction coefficients reported by Blakley (1969).

The standard stock solutions of folates of 100 μg/ml (concentration corrected for purity) were prepared under subdued light in 0.1 M phosphate buffer pH 6.0 containing 1% ascorbic acid and 0.1% 2-mercaptoethanol. Aliquots of the standard stock solutions were placed in separate tubes, flushed with nitrogen and stored below −80 °C at most 3 months. The calibration solutions were prepared immediately before use by dilution of the stock solutions with elution buffer (0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol).

2.3. Samples

Certified reference material CRM 485 (a lyophilised mixed vegetable sample) was obtained from The Institute for Reference Materials and Measurements (Geel, Belgium) and stored as vacuum-packed sub-samples (2 g) at −80 °C until analysis.

All samples of raw and pickled beetroots were obtained from food company Procordia Food located in the southern Sweden (Eslöv). Three different cultivars of beetroots (Boltardy, Ricky and Kim) were grown in sandy humus-rich soil at two different locations (farmer 1 and farmer 2) in southern Sweden. Commercial and natural fertilisation was supplied during cultivation. The beetroots were harvested in September 2000. Following storage either for 1 week or for 3 months at ambient temperature and darkness, the raw beetroots were transported to the Department of Food Science, Swedish University of Agricultural Sciences in Uppsala for folate analysis. They were vacuum-packed in plastic bags immediately after delivery and stored frozen at −20 °C until sample preparation (no more than 1 week). A representative sample of raw beetroots was usually prepared by pooling 5–7 beetroots. The three cultivars of beetroots originating from "farmer 1" were processed into pickled sliced beetroots, either from harvested beetroots stored for 1 week or for 3 months. Following washing, peeling and slicing, the beetroots were filled into glass jars together with a water solution in the proportion two parts beetroots to one part water solution. The water solution contained acetic acid, sugar, preservative (potassium sorbate) and spices. The filled jars were locked by aluminium caps and pasteurised for 40 min at 90 °C. Representative jars (1 week, 3 months and 15 months after processing, respectively) were sent to the Department of Food Science, Swedish University of Agricultural Sciences in Uppsala for folate analysis. The jars were stored at 8 °C until sample preparation (no more than 2 days).

The prepared sample extracts were immediately frozen at −20 °C. The storage time for prepared sample extracts at −20 °C until HPLC-analysis was kept to a minimum, being at most 1 week.

2.4. Enzyme preparation

Suspension of hog kidney folate conjugase was prepared according to Phillips and Wright (1983) by

homogenisation of 10 g hog kidney acetone powder in 100 ml L-cysteine hydrochloride solution (10 mg/ml, pH 4.6 adjusted with NaOH) and incubation for 4 h at 37 °C followed by centrifugation at 27 000 *g* for 30 min. The enzyme was isolated from resulting supernatant according to Gregory, Sartain, and Day (1984) by precipitation with ammonium sulphate (75% saturation) followed by centrifugation and subsequent re-dissolving of pellet in 100 ml of 50 mM potassium acetate buffer (pH 4.5) containing 10 mM 2-mercaptoethanol. To remove endogenous folates the obtained enzyme suspension was dialysed in two steps using 2 l of the acetate buffer in each step (2 h and then overnight). Dialysis was performed at 4 °C with stirring. The dialysed folate conjugase preparation was stored in small aliquots at −20 °C until use (no more than 3 months).

Activity of each enzyme preparation was checked using $PteGlu_3$ as substrate in 0.1 M acetate buffer (pH 4.9) containing 1% sodium ascorbate at 37 °C as described by Vahteristo, Ollilainen, Koivistoinen, and Varo (1996). Concentrations of $PteGlu_3$ and produced folic acid were measured by means of UV-detection using HPLC-method for reduced folate forms described later.

2.5. Sample preparation

All beetroot samples were analysed as duplicates. The certified reference samples CRM 485 were analysed as triplicates. Sample pre-treatment was performed under subdued light. Previously minced and weighted beetroot samples (6 g) were homogenised with an Ultra-Turrax T25 homogeniser (IKA, Germany) in 20 ml of extraction buffer (0.1 M phosphate buffer pH 6.0 containing 2% sodium ascorbate and 0.1% 2-mercaptoethanol) until a homogeneous suspension formed. For samples CRM 485, 0.35 g was weighed and homogenised in 20 ml of extraction buffer. The tubes containing homogenates were flushed with nitrogen, tightly capped and heated in a boiling water bath for 10 min. The tubes were shaken twice during heat extraction. The extracts were rapidly cooled on ice, the pH of the extracts was adjusted to 4.9 with acetic acid and 3 ml of hog kidney conjugase was added. The tubes were flushed with nitrogen, capped and incubated in a water bath at 37 °C under shaking for 3 h. The incubated extracts were heated for 5 min in a boiling water bath to inactivate the enzymes, cooled in ice and centrifuged at 27 000 *g* for 15 min at 4 °C. The supernatants were transferred to 50 ml volumetric flasks and the residues were re-dissolved in 20 ml 0.1 M phosphate buffer pH 6.0 containing 0.1% 2-mercaptoethanol, flushed with nitrogen and re-centrifuged at 27 000 *g* for 15 min at 4 °C. The new portions of supernatants were added to the same volumetric flasks as before, filled to 50 ml with 0.1 M phosphate buffer pH 6.0 containing 0.1% 2-mercaptoethanol and mixed thoroughly. Aliquots of the obtained sample extracts were placed in separate tubes, flushed with nitrogen and frozen below −20 °C. Blank samples containing only hog kidney conjugase and phosphate buffer were prepared and treated in the same way as the beetroot samples to check if the enzyme preparation contained any endogenous folates.

2.6. Sample clean-up

Purification of sample extracts was carried out prior to HPLC analysis by solid-phase extraction (SPE) on strong anion exchange (SAX) Isolute cartridges (500 mg, 3 ml, International Sorbent Technology, UK). A Visiprep SPE Vacuum Manifold (Supelco, USA) was used for elution under reduced pressure. The cartridges were preconditioned with methanol (2×2.5 ml) and water (2×2.5 ml) at a flow-rate of 1–2 drops/s. Aliquots (2.5 ml) of the sample extracts were applied to the cartridges and passed slowly with flow-rate not exceeding 1 drop/s. The cartridges were washed with water (2×2.5 ml) to remove matrix interfering components (flow-rate 1–2 drops/s). The elution of retained folates was performed slowly (flow-rate not exceeding 1 drop/s) with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol. The first portion (0.7 ml) of eluate was discarded and the second portion (3.8 ml) was collected and weighed.

2.7. HPLC analysis

All experiments were performed using an Agilent 1100 HPLC system, equipped with a gradient quaternary pump, a thermostatted autosampler, a thermostatted column compartment, a diode array (DAD) detector and a fluorescence detector. An Agilent Chemstation software was used for control of the HPLC system and data processing.

Two analytical columns were tested for separation of folates: a LiChrospher 100 RP-18, 125×4.0 mm, 5 μm (Merck, Darmstadt, Germany) with a matching guard column (4×4.0 mm, 5 μm) and a Zorbax SB C_8, 150×4.6 mm, 5 μm (Agilent Technologies, USA) with a matching guard column (12.5×4.6 mm, 5 μm).

The chromatographic conditions for gradient elution were as follows: flow-rate, 0.4 ml/min; volume injected, 20 μl; column temperature, 23 °C; autosampler temperature, 8 °C; fluorescence detection, 290 nm excitation and 360 nm emission; UV detection, 290 nm. 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 total run time was 33 min. The isocratic elution was performed with mobile phase containing 8% acetonitrile in 30 mM potassium phosphate buffer at pH 2.3,

total run time was 26 min, other chromatographic conditions were as mentioned above.

Retention times were used for peak identification; comparison of ratio of sample peaks from fluorescence and DAD detectors to ratio of standard peaks as well as fluorescence and diode array spectra were used for verifying peaks.

Capacity factors (k') were calculated as $k' = (t_r\text{-}t_0)/t_0$, where t_0 is retention time of unretained solvent and t_1 is retention time of analyte. Number of theoretical plates (N) was calculated from peak width at half height ($w_{0.5}$) using the formula $N = 5.54(t_r/w_{0.5})^2$. Peak asymmetry (A_s) was estimated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak. Resolution factor (R_s) for two adjacent peaks with retention times $t_2 > t_1$ was calculated as $R_s = 1.18(t_2 - t_1)/(w_2 + w_1)$, where w_1, w_2 are peak widths at half-height.

2.8. Quantification

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

The limit of detection (LOD) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of 10.

2.9. Recovery tests

Recovery tests were performed by adding the standard solutions to the food sample before homogenisation. The spiked samples were then processed through the entire procedure. The recovery (R) was calculated according to Rodriguez, Campana, Barrero, Linares, and Ceba (1995) as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is concentration in spiked sample, C_{sample} is concentration in sample before spiking and C_{added} is added concentration.

2.10. Dry matter

Dry matter of raw and processed beetroots was determined by the central laboratory of Procordia Food according to NMKL method for determination of moisture and ash (NMKL, 1991).

3. Results and discussion

3.1. Sample preparation and purification

Folates were extracted from food matrix using 0.1 M phosphate buffer at pH 6.0 with added antioxidants (sodium ascorbate and 2-mercaptoethanol) and enzyme treated with hog kidney conjugase at pH 4.9 as described by Vahteristo et al. (1996, 1997). To choose optimal incubation time, a deconjugation test was performed using $PteGlu_3$. A 10-fold amount of $PteGlu_3$ compared to amount of native folates in food samples was incubated for 2 and 3 h with 3 ml of hog kidney conjugase and the concentrations of $PteGlu_3$ and produced folic acid were measured by HPLC. The deconjugation was found to be complete after 2 h incubation. However, a longer incubation time (3 h) was chosen to assure the complete deconjugation of folates in all food samples, regardless of possible weak inhibition effects from some matrices as shown by Engelhardt and Gregory (1990) and Vahteristo et al. (1996). The stability of folates during heat extraction and enzyme treatment was tested at various concentrations of sodium ascorbate (0.5–2%) and the same concentration (0.1%) of 2-mercaptoethanol. The use of 2% of sodium ascorbate was found to provide a better stability of H_4folate during heat treatment in comparison with 1% of sodium ascorbate, whereas the stability of other folate forms was good even at lower concentrations of sodium ascorbate (Fig. 1). However, the high ascorbate level might complicate the UV-detection of folates due to shifting of baseline at the chromatogram. Because of this, a phosphate buffer not containing sodium ascorbate was used for re-dissolving and dilution of sample extracts after heat treatment and incubation to keep the final ascorbate level at 1%.

A slightly modified SPE-procedure with SAX sorbent developed by Vahteristo et al. (1996) was employed for purification of food extracts. The capacity of the Isolute SAX cartridges (500 mg) was tested by applying different volumes (1.5–5.0 ml) of beetroot extract onto the SPE cartridges. It was possible to apply up to 3.5 ml of beetroot extract without losses of folates during application step. Dilution of sample extract with water prior to application step to lower salt concentration did not improve the recovery of folates when applying larger (>3.5 ml) sample volumes. The application volume 2.5 ml was chosen for all samples with the aim to avoid the possible overloading of SPE cartridges due to variations in matrix composition. The use of water instead of 0.01 M phosphate buffer pH 7 during the conditioning and washing steps was found to be preferable because of losses of folates when employing phosphate buffer during these steps. The volume of eluate needed for quantitative recovery of folates was checked for a standard solution and a beetroot extract taken through SPE-procedure (Table 1). All three folate forms (H_4folate, 5-CH_3–H_4folate and 5-HCO–H_4folate) eluted effectively with 3.8 ml elution buffer (fraction 2 and 3), the first elution fraction (0.7 ml) did not contain any folate form and could be discarded.

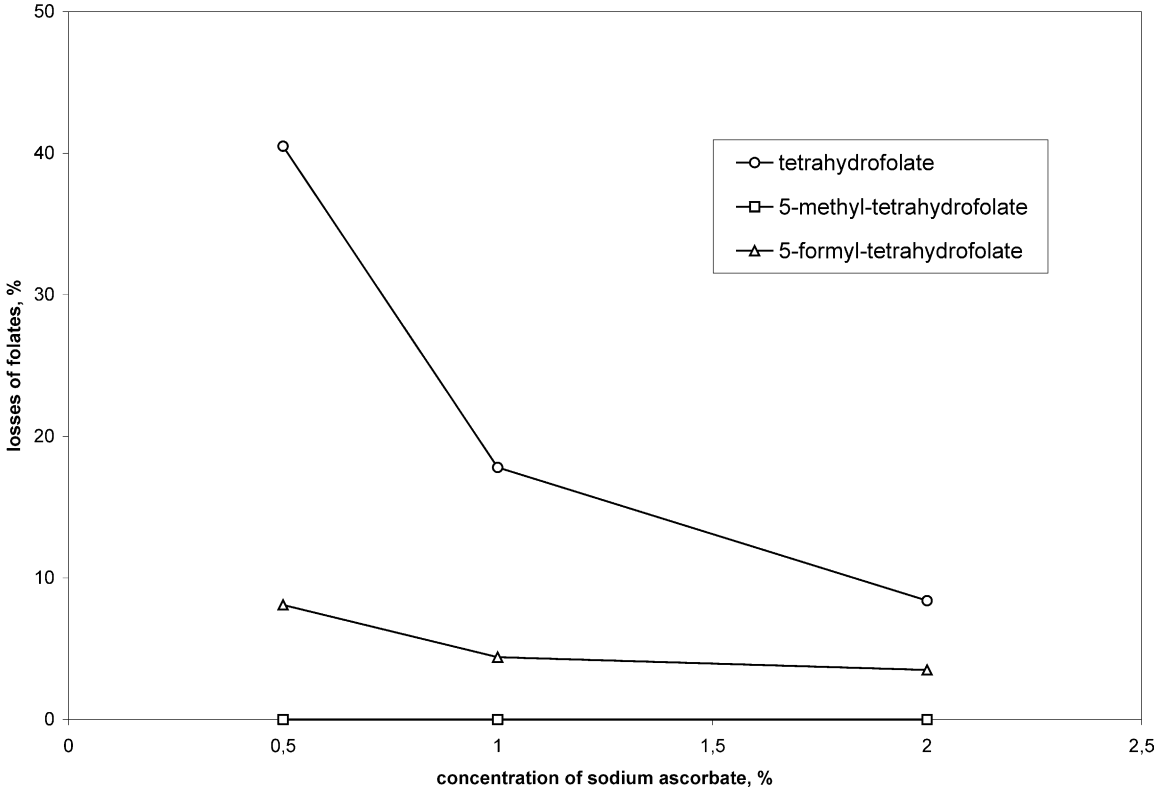

Fig. 1. Losses of folates during heating and enzyme treatment depending on concentration of sodium ascorbate in extraction buffer.

Table 1
Recovery and distribution of folates between different elution fractions of standard solution and beetroot extract taken through SPE-procedure (applied volume 2.5 ml)

Sample	Elution fraction	Recovery (%)[a]		
		H_4folate	5-CH_3–H_4folate	5-HCO–H_4folate
Standard solution[b]	Fraction 1 (0.7 ml)	0	0.1	0
	Fraction 2 (3.2 ml)	97.7	102.1	95.4
	Fraction 3 (0.6 ml)	1.1	1.4	0
	Sum	98.8	103.6	95.4
Beetroot extract	Fraction 1 (0.7 ml)	ND[c]	0	ND
	Fraction 2 (3.2 ml)	ND	98.5	ND
	Fraction 3 (0.6 ml)	ND	1.5	ND
	Sum		100	

[a] For standard solution recovery is calculated as percentage of initial standard amount applied on SPE-column (values are means of duplicate determinations); for beetroot extract recovery is calculated relative to sum of all fractions (values are means of triplicate determinations).

[b] Standard solution (100 ng/ml of H_4folate, 5-CH_3–H_4folate and 5-HCO–H_4folate) in 0.1 M phosphate buffer pH 6 containing 1% sodium ascorbate and 0.1% 2-mercaptoethanol.

[c] ND, not detected.

3.2. Optimisation of chromatographic conditions

Most HPLC analyses of food folates were carried out using conventional C_{18} columns (Konings, 1999; Ndaw et al., 2001; Osseyi et al., 2001; Pfeiffer, Rogers, & Gregory, 1997; Seyoum & Selhub, 1993; Stokes & Webb, 1999; Vahteristo et al., 1996; Wigertz & Jägerstad, 1995). However, water-soluble polar compounds such as folates can exhibit better separation and improved peak symmetry on a C_8 column due to better coverage of surface silanols. Therefore a Zorbax SB C_8 column was tested and compared with conventional Lichrospher RP-18 column. The use of Zorbax Stable Bond (SB) stationary phases can also be preferable for folate analysis because these phases are based on ultra-pure (99.995%) silica particles and have long lifetimes in aqueous mobile phases with low pH due to steric protection of the siloxane bonds (Vervoort, Debets, Claessens, Cramers, & de Jong, 2000). A mobile phase containing an aqueous buffer with low pH (2–3.5) is necessary for folate analysis to achieve the maximum fluorescence intensity (Gounelle, Ladjimi, & Prognon, 1989).

The separation of H_4folate, 5-CH_3–H_4folate and 5-HCO–H_4folate was tested for both isocratic and

gradient elution. For isocratic elution, a mobile phase containing 8% acetonitrile in 30 mM phosphate buffer (pH 2.3) was chosen, a slightly modified method of Wigertz et al. (1995). For gradient elution, a linear 20-min acetonitrile gradient from 6 to 25% in 30 mM phosphate buffer (pH 2.3) was used, a slightly modified method of Vahteristo et al. (1996). As seen from Table 2, the peak efficiency for all folate forms was much higher using the gradient elution. The Zorbax SB C_8 column was found to exhibit superior efficiency, resolution and peak symmetry in comparison with Lichrospher RP-18 column (Table 2). Therefore, the gradient elution with the Zorbax SB C_8 column was chosen for further experiments. Fig. 2 shows representative chromatograms from a mixed standard solution, an extract of certified reference material CRM 485 and an extract of pickled beetroots.

The fluorescence detection was chosen for quantification of reduced folate forms because of much higher fluorescence response for H_4folate and 5-CH_3–H_4folate compared to UV-detection and less interfering peaks. During tests of enzyme activity the UV-detection was used for quantification of $PteGlu_3$ and folic acid (retention times 20.6 and 21.7 min, respectively). The folate peaks on chromatogram were verified by using fluorescence emission spectra, collected in the range 315–640 nm, and diode array spectra, collected in the range 190–400 nm. The use of fluorescence emission spectra for peak verification was found to be the most suitable due to high fluorescence response of folates. The spectra for interfering peaks on chromatograms (these peaks are marked by "x" on Fig. 2) were also checked and were much different from folate spectra, suggesting that these peaks might be some endogenous compounds from the food matrix like organic acids, which can co-eluate with folates during purification step.

3.3. Evaluation of the HPLC method

The detector response was linear over the concentration ranges tested for all three folate forms and calibration curves had a correlation coefficient higher than 0.9999. Linearity and sensitivity data are presented in Table 3. The repeatability of a measurement (RSD for 10 successive injections of the same standard solution at concentration of 100 ng/ml) was better than 0.9% for all folate forms. The limits of quantification were 0.3 and 0.5 ng/ml for 5-CH_3–H_4folate and H_4folate, respectively, whereas for 5-HCO–H_4folate the limit of quantification was 4 ng/ml due to lower fluorescence of 5-HCO–H_4folate and a relatively broader peak in comparison with other folate forms (see Fig. 2).

The repeatability (intra- and inter-assay) of the entire analytical procedure was evaluated by analysing certified reference material CRM 485 in triplicates over 3 days. The intra- and inter-assay RSD were no more than 8% for 5-CH_3–H_4folate and H_4folate (Table 4). The 5-HCO–H_4folate was not found in the sample, therefore no repeatability data are presented for this folate form. As seen from Table 4, the mean value obtained for 5-CH_3–H_4folate in this study agreed quite well with indicative value for 5-CH_3-H_4folate determined by HPLC in intercomparison study of the European Commission's Standard, Measurement and Testing Programme (Finglas et al., 1999). Total folate content obtained in the present study was 22% lower than certified microbiological value, which might be due to different analytical methodologies and presence of other folate forms not quantified in the present study. Accuracy of the HPLC method was determined by recovery tests. The certified sample CRM 485 was spiked with a standard solution of H_4folate and 5-CH_3–H_4folate (folate forms detected in the sample) at three different concentrations. The method showed good recoveries that were close to 100% (Table 5), this indicates that the method is accurate. The recoveries of 5-CH_3–H_4folate were found to be 95–97%, whereas the recoveries of H_4folate were somewhat lower (85–90%) due to higher lability of H_4folate.

The stability of samples taken through the assay procedure was tested by re-injection of duplicate samples of CRM 485 stored in autosampler for 48 h at 8 °C. The difference between initial (0 h) and replicate (48 h)

Table 2
Column performance data for folates[a]

Column	Mobile phase	H_4folate			5-CH_3–H_4folate				5-HCO–H_4folate			
		k'	A_s	N	k'	A_s	R	N	k'	A_s	R	N
Zorbax	I[b]	1.20	1.33	9740	1.58	1.17	3.97	10 170	6.42	0.95	11.4	1550
LiChrospher	I	2.37	1.74	3780	3.13	1.17	3.41	5250	8.11	0.80	6.92	900
Zorbax	II[c]	2.58	1.16	31 490	2.90	1.00	4.48	63 060	4.05	0.94	11.2	19 520
LiChrospher	II	4.48	1.58	29 500	4.90	1.21	3.41	39 310	6.05	0.79	6.59	14 610

[a] Abbreviations: k'—capacity factor, A_s—peak asymmetry, N—number of theoretical plates (efficiency), R—resolution factor; see Section 2, HPLC analysis for calculations.

[b] Mobile phase I: 8% acetonitrile in 30 mM potassium phosphate buffer (pH 2.3), isocratic elution.

[c] Mobile phase II: a binary gradient mixture of acetonitrile and 30 mM potassium phosphate buffer (pH 2.3), linear gradient from 6 to 25% acetonitrile; see Section 2, HPLC analysis for more details.

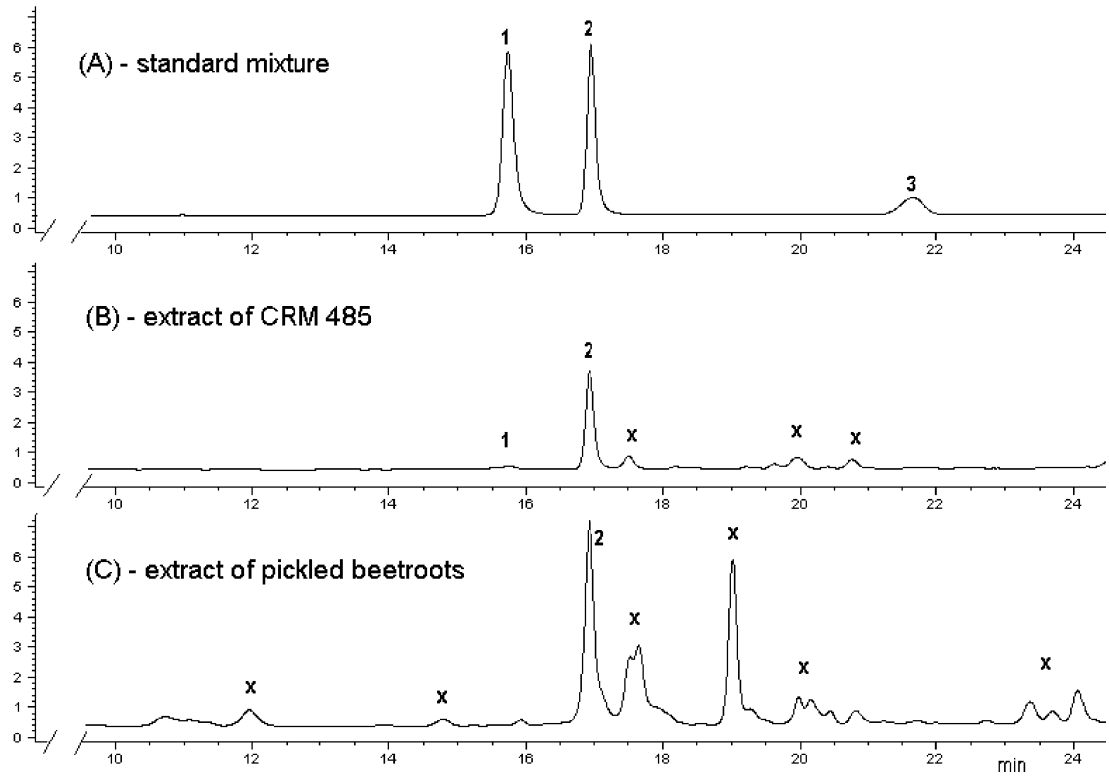

Fig. 2. Chromatograms of reduced folate monoglutamates detected by fluorescence (λ_{ex} = 290 nm, λ_{em} = 360 nm). Samples: (A) standard mixture containing H_4folate 50 ng/ml, 5-CH_3–H_4folate 30 ng/ml and 5-HCO–H_4folate 200 ng/ml in elution buffer; (B) extract of certified reference material CRM 485; (C) extract of pickled beetroots. Peaks: 1—H_4folate; 2—5-CH_3–H_4folate; 3—5-HCO–H_4folate; ×— interfering compounds from food matrix. Column: Zorbax SB C_8 (150 × 4.6 mm, 5 μm). Mobile phase: acetonitrile-phosphate buffer (pH 2.3) with acetonitnle gradient from 6 to 25% (see Section 2 for details). Flow 0.4 ml/min, injection volume 20 μl.

Table 3
Linearity and sensitivity of the method[a]

Folate form	Linearity range (ng/ml)	Correlation coefficient[b]	RSD (%)	LOD (ng/ml)	LOQ (ng/ml)
H_4folate	0.3–100	0.99997	0.3	0.1	0.3
5-CH_3–H_4folate	0.2–100	0.99999	0.2	0.07	0.2
5-HCO–H_4folate	4–300	0.99991	0.9	1.5	4.0

[a] Abbreviations: LOD—limit of detection, LOQ—limit of quantification, RSD—relative standard deviation, see Section 2, Quantification for calculations.
[b] Value represents average for eight calibration curves.

Table 4
Precision of the method for folate determination in certified reference material CRM 485

Folate form	Mean content (μg/100 g)	RSD (%) (intra-assay)	RSD (%) (inter-assay)	Certified/ indicative value (μg/100 g)
H_4folate	7.84±0.64	5.6	8.1	Not reported
5-CH_3–H_4folate	247±2	0.9	1.0	214±42[a]
Total folate as folic acid	246±3	1.0	1.1	315±28[b]

[a] Indicative HPLC-value for 5-CH_3–H_4folate resulted from intercomparison study (Finglas et al., 1999).
[b] Certified microbiological value for total folate (Finglas et al., 1999).

Table 5
Recovery of method for folate determination in certified reference material CRM485

Folate form	Amount in sample (μg/100 g)	Added (μg/100 g)	Found (μg/100 g)	Recovery (%) (n = 3)
H_4folate	7.84	11.0	17.2	86±6
		22.0	26.6	85±2
		31.5	36.0	90±2
				87±4 (mean)
5-CH_3-H_4folate	247	111	351	94±3
		221	461	97±2
		317	548	95±2
				95±2 (mean)

values was less than 5%. The prepared samples were stable for at least 48 h in autosampler.

3.4. Folates in raw and pickled beetroots

The folate content of raw and pickled beetroots obtained with the reported method is presented in Table 6. 5-CH_3–H_4folate was found to be the dominant folate form in all beetroot samples, which is in agreement with previously published data for pickled beetroots (Vahteristo et al., 1997). Recovery tests performed

Table 6
Content of 5-CH_3–H_4folate in μg per 100 g fresh weight or dry matter[a] in raw and pickled beetroots

Cultivar	No.[b]	Raw beetroots		Pickled beetroots (drained)			
		Storage time after harvest		Storage time after processing			
		1 week	3 months	1 week[c]	3 months[c]	15 months[c]	1 week[d] (3 months)
Boltardy	1	94.6 (681)	88.1	46.3 (289)	43.4	40.8	38.9 (234)
Boltardy	2	93.3 (521)					
Ricky	1	90.4 (735)	74.2	39.6 (257)	34.0	27.9	36.7 (240)
Ricky	2	89.8 (565)					
Kim	1	76.0 (503)	72.1	39.9 (240)	32.1	30.8	32.5 (182)
Kim	2	71.9 (431)					

[a] Folate content in μg per 100 g dry matter is given in brackets.
[b] 1 and 2 are different locations (farmer 1 and farmer 2).
[c] Raw beetroots were stored for 1 week before processing.
[d] Raw beetroots were stored for 3 months before processing.

by spiking at two different concentrations of about 50 and 100% of the amounts present in the sample showed good recovery for 5-CH_3–H_4folate (88 ± 4%, $n = 6$). The precision of the quantification of 5-CH_3–H_4folate in beetroot samples was satisfactory (the difference between two separate values for a duplicate was less than 10% for all samples). Other folate forms, e.g. H_4folate and 5-HCO–H_4folate, were below limits of determination (0.4 and 5 μg/100 g fresh weight, respectively). The dry matter of raw beetroots varied between 12.3 and 17.9% (w/w). Similar ranges (15.3–17.9%) of dry matter were obtained for the processed beetroots.

The results for raw beetroots showed variations in folate concentrations with both cultivar and growing conditions. As seen from Table 6, cultivars Boltardy and Ricky contained 89–95 μg of 5-CH_3–H_4folate/100 g fresh weight (521–735 μg 5-CH_3–H_4folate/100 g on dry matter basis), while cultivar Kim contained only 72–76 μg of 5-CH_3–H_4folate/100 g fresh weight (431–503 μg 5-CH_3–H_4folate/100 g on dry matter basis). In this manner the cultivar differences in folate concentrations ranged up to 30 and 46%, calculated on fresh weight and dry matter basis, respectively. On dry matter basis, the location of growing caused 15–25% variation in folates, while on fresh weight basis, the variation was below 10%. After 3 months of storage of raw beetroots, the concentrations of 5-CH_3–H_4folate had decreased by 5–18% on fresh weight basis, probably due to oxidative degradation of the 5-CH_3–H_4folate. The folate concentrations found in raw beetroots analysed in the present study agree quite well with data of Swedish, Danish, Norwegian, Finnish and German national food tables (Moller, 1996; Rastas, Seppanen, Knuts, Hakala, & Karttila, 1997; Rimestad et al., 2001; SLV, 1996; Souci, Fachmann, & Kraut, 2000), which report folate concentrations between 68 and 93 μg folate/100 g. In contrast, British food tables (Holland, Welch, Unwin, Buss, Paul, & Southgate, 1991) report 150 μg folate/100 g. Most of food table data for folates are based on microbiological assays and present only total folate concentration.

Pickled sliced beetroots analysed after 1 week of storage contained around 40–46 μg 5-CH_3–H_4folate/100 g wet weight (or 240–289 μg 5-CH_3–H_4folate/100 g dry matter) after draining. This is in accordance with one previous study (Vahteristo et al., 1997), which reported 37 μg 5-CH_3–H_4folate/100 g pickled beetroots. Overall, the preservation by pickling resulted in an average retention of 43–53% of 5-CH_3–H_4folate calculated on wet weight. Similar retention patterns were found when beetroots were processed after 3 months of storage after harvest. Folate losses during processing can be explained by leakage and chemical oxidative degradation due to heating at acidic pH. It is well known that 5-CH_3–H_4folate is sensitive for oxidation induced by heating and light and an acidic pH is less favourable for the stability of 5-CH_3–H_4folate (Gregory, 1996; Hawkes & Villota, 1989). Storage of pickled beetroots for 3 and 15 months at ambient temperature induced a further loss of 5-CH_3–H_4folate of 6–20% and 22–28%, respectively. The concentrations of 5-CH_3–H_4folate in pickled beetroots still varied with cultivar, but these differences appeared to be dependent on storage period. They were minor (up to 17%) in pickled beetroots stored for 1 week and increased to 46% in pickled beetroots stored for 15 months. According to various national food tables (Holland et al., 1991; Moller, 1996; Rastas et al., 1997; Rimestad et al., 2001), pickled beetroots contain between 2 and 53 μg folate/100 g based on microbiological assays. These variations in folate concentrations of pickled beetroots (drained) might be due to cultivars, a variety of pickling procedures and different microbiological assays used.

The stability of 5-CH_3–H_4folate was found to be dependent on cultivar in both raw and processed beetroots, which can be explained by different concentrations of endogenous antioxidants in various cultivars. The stability appeared to be lowest in cultivar Ricky

both during processing and storage of raw and pickled beetroots.

4. Conclusions

The results of validation procedure show that the proposed method is selective, precise, accurate and sensitive. The advantage in using Zorbax SB C_8 analytical column is the improved chromatographic efficiency and selectivity as well as longer column life compared to conventional columns. The method was successfully applied for determination of folates in beetroots. We expect this method to be suitable for the routine determination of folates in vegetables and other food matrices of plant origin.

This is the first study to our knowledge, where the determination of individual folate forms has been performed in raw and processed beetroots in relation to cultivar and growing conditions. 5-CH_3–H_4folate was shown to be the dominant folate form in beetroots. Both cultivar differences and growing conditions were found to have a pronounced effect on the folate content in beetroots. Processing resulted in considerable losses of folates, whereas losses during storage appeared to be moderate.

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