African Journal of Biotechnology Vol. 11(8), pp. 2087-2098, 26 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2491
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

A rapid high-performance liquid chromatography (HPLC) method for the extraction and quantification of folates in dairy products and cultures of *Propionibacterium freudenreichii*

Jessy Van Wyk[1]* and Trevor J. Britz[2]

[1]Department of Food Technology, Cape Peninsula University of Technology, Bellville 7535, South Africa.
[2]Department of Food Science, University of Stellenbosch, Stellenbosch 7600, South Africa.

Accepted 31 October, 2011

Nutritional folate deficiencies in Southern African communities necessitated mandatory fortification. Current microbiological assays (MA) used to measure food folates, essential for quality control and regulatory purposes, are time-consuming. This study describes an alternative extraction and detection method for folates in dairy products and *Propionibacterium freudenreichii* cultures. Folates were extracted by heating with a phosphate buffer (pH 6.0). Polyglutamates were deconjugated with chicken pancreas and hog kidney deconjugases. Samples were purified using strong anion exchange solid phase extraction. Reversed-phase high-performance liquid chromatography (HPLC) using an acetonitrile-phosphate buffer (pH 2.2) gradient effectively separated four vitamers. Fluorescence (tetrahydrofolate (THF), 5-CH_3-THF and 5-CHO-THF), and UV detection (folic acid) were used, calibration curves were linear (R^2>0.0997), and detection and quantification limits were 0.0006 to 0.015 and 0.002 to 0.05 μg/ml, respectively. Accuracy was 80 to 108% and intra- and inter-day precision [%relative standard deviation (%RSD)] were lower than 4%. The method, validated against the standard MA, is a selective, sensitive, reliable and rapid alternative.

Key words: *Propionibacterium freudenreichii*, microbiological folate assay, high-performance liquid chromatography (HPLC), folate.

INTRODUCTION

"Folate" is the generic name for a large number of chemical derivatives of pteroylglutamic acid (PGA), also known as folic acid. Folic acid, the oxidised and most stable vitamer (or folate form), do not occur naturally, but are widely used in vitamin supplements and fortified food products (Shane, 2010; Pfeiffer et al., 2010). Biological samples contain reduced forms of PGA (Ball, 2006; Ye et al., 2008), comprising mainly tetrahydrofolic acid (THF), 5-methyl-THF (5-CH_3-THF), 5-formyl-THF (5-CHO-THF) and 10-formyl-THF (10-CHO-THF). Folinic acid (5-CHO-THF) is the most stable reduced vitamer, while stabilised salts of 5-CH_3-THF have been developed recently (Shane, 2010) confirming a growing interest in researching various aspects of this vitamin (Pfeiffer et al., 2010) while, 90 to 95% of the folate in cows' milk is 5-methyl-THF (Vahteristo et al., 1997a; Ye et al., 2008), 5-formyl-THF predominates in buttermilk and yoghurt (Vahteristo et al., 1997a). In milk, 5-CH_3-THF is strongly and specifically bound to folate binding protein (Shane, 2010), but heating for 5 min at 100°C denatures the binding proteins (Lim et al., 1998). Most folates occur as polyglutamates, containing two to eight glutamate

*Corresponding author. E-mail: vanwykj@cput.ac.za. Tel: +27 21 959 6176 or +27 0822006876. Fax: +27 21 959 6095.

Abbreviations: **AOAC**, Association of Official Analytical Chemists; **HPLC**, high-performance liquid chromatography; **MA**, microbiological assay; **NTDs**, neural tube defects; **PAB**, propionibacteria; **PGA**, pteroylglutamic acid; **THF**, tetrahydrofolate; **RSD**, relative standard deviation.

residues (Ndaw et al., 2001). Unable to traverse the cell membrane (Shane, 1982, 2010), folylpolyglutamates are likely to be retained intracellularly when synthesised by micro-organisms such as *Propionibacterium freudenreichii*, an excellent candidate for folate bio-fortification of dairy products (Van Wyk et al., 2011). However, digestive processes (*in vivo*) (Iyer and Tomar, 2009), or heating for 6 min at 100°C (*in vitro*) was found to release intracellular folates (Shane, 1982, 2010).

Folate is of great nutritional importance due to its role in one-carbon metabolism which is critical for DNA synthesis and repair, for all cell replication, including normal foetal development, for protein synthesis and for remethylation of homocysteine to methionine (Iyer and Tomar, 2009; Stover, 2010). Its therapeutic use against the deficiency disease megaloblastic anaemia has been practiced for over 50 years (Shane, 2010). However, one of the most significant public health discoveries of the century is that periconceptual intake of folate significantly reduces the risk of neural tube defects (NTDs) such as spina bifida and anencephaly. Global mandatory folate fortification ensued, commencing in the USA, with other countries, including Canada, Mexico and Hungary, following suit (Oakley, 2009). In Southern Africa, not only were NTDs linked to maternal folate deficiency (Ubbink et al., 1999), but serious folate deficiencies were also demonstrated among the poor (Labadarios et al., 2000). As a result, mandatory folate fortification of a number of foodstuffs was promulgated (Anonymous, 2003). Furthermore, adequate folate intake possibly protect against cardiovascular disease by decreasing serum homocysteine levels (Iyer and Tomar, 2009; Stover, 2010). Mounting evidence from epidemiological studies indicates that elevated folate intakes may reduce the risk of colorectal cancer (Iyer and Tomar, 2009; Kim, 2007). Due to the increasing significance of folates in health and disease, there is a need for the development of rapid methods to give reliable results when measuring food folates (Arcot and Shrestha, 2005).

As the only food folate method given official status by the Association of Official Analytical Chemists (AOAC) (Iyer and Tomar, 2009), the microbiological assay (MA) is still the most widely used analytical method (Ye et al., 2008). Based on the specific growth requirement of *Lactobacillus rhamnosus* (ATCC 7469) for folate, this method is sensitive, low cost and requires simple instrumentation. However, it measures only total folates, is labour intensive, even with semi-automated procedures, less precise than other methods, and results are only available after two to five days (Pfeiffer et al., 2010).

Liquid chromatography-mass spectrometry (LC-MS and LC-MS/MS) methods (de Brouwer et al., 2008; Patring et al., 2009) are considered the most specific, sensitive and selective. However, due to the cost and complexity of operation of the equipment, these are not within the reach of the average analytical or research laboratory (Iyer and Tomar, 2009; Pfeiffer et al., 2010). Hence, high-performance liquid chromatography (HPLC) folate assays are currently the most viable alternatives to the MA (Ball, 2006). Several HPLC methods exist for the determination of folates in foodstuffs (Hefni et al., 2010; Lebiedzińska et al., 2008; Ndaw et al., 2001; Vahteristo et al., 1997a, b; Yazynina et al., 2008). The differences in ionic properties and hydrophobicity of the folate vitamers facilitate their separation by reversed-phase or ion exchange liquid chromatography (Gregory, 1984). Electrochemical, fluorescence, and UV detection methods are the most common (Gregory et al., 1984; Lebiedzińska et al., 2008; Vahteristo et al., 1997a, b).

Extraction procedures are designed to liberate protein-bound folates and deconjugate folylpolyglutamates. As a result, the procedure includes a heat treatment to denature proteins (Lim et al., 1998), followed by enzymatic deconjugation. Since chicken pancreas (CP) conjugase yields diglutamyl folates, it is essential to use either hog kidney (HK), human or rat plasma conjugase to produce monoglutamates for HPLC assays (Strålsjö et al., 2002; Ye et al., 2008). The trienzyme method has been used by several researchers (Lim et al., 1998; Pfeiffer et al., 2010), but the reactions with the protease and α-amylase are time-consuming, which often have no effect on folate levels (Ndaw et al., 2001; Strålsjö et al., 2002) and sometimes result in folate losses (Yazynina et al., 2008). Combining CP conjugase, a low cost product (Pfeiffer et al., 2010) which possesses significant amylolytic (Pedersen, 1988) and proteolytic activity (Strålsjö et al., 2002), and HK conjugase, Vahteristo et al. (1996) attained complete folate extraction and conjugation without long incubation times.

All folates are sensitive to oxidative degradation, which is enhanced by oxygen, heat and particularly light. Hence, the extraction is always conducted in the presence of an antioxidant and under subdued light (Vahteristo et al., 1996, 1997a; Pfeiffer et al., 2010).

The aim of this study was to develop an extraction protocol as well as an HPLC method that is sensitive, selective, reliable and rapid for quantifying folates in milk and fermented milk products, as well as of synthetic media cultured with *P. freudenreichii* (PAB) strains.

MATERIALS AND METHODS

Chemicals, reagents and other materials

Folic acid, Neat (Supelco, Bellefonte, USA); THF (Sigma, St. Louis, USA); 5-CH_3-THF disodium salt (Fluka, Buchs, Switzerland); and 5-CHO-THF calcium salt (Sigma, St. Louis, USA) were used as external standards during the HPLC analyses. Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) was obtained from Schircks Laboratories (Jona, Switzerland). HK acetone powder (Porcine type II) (Sigma, St. Louis, USA) and CP (Difco, Detroit, USA) conjugases, both γ-glutamyl hydrolases (EC 3.4.19.9), were used to deconjugate folylpolyglutamates. The certified reference material of choice CRM421 (spray-dried milk powder), was discontinued by the

supplier, the Institute for Reference Materials and Measurements (Geel, Belgium). A substitute was obtained by combining a food grade folic acid fortificant [DSM Nutritional Products, Isando, South Africa (SA)] with fat free milk powder (Elite, Clover, Roodepoort, SA) to yield a folate concentration of 100 mg per 100 g. The resultant reference material was subsampled (2 to 5 g) and stored at –80°C. Methanol (BDS, Johannesburg, SA) and Acetonitrile (BDS, Johannesburg, SA) were of HPLC grade. All other chemicals were analar grade. Ultrapure water (18.2 MΩ), purified with a Milli-Q system (Millipore, Bellville, SA), was used for all solutions and dilutions.

During the HPLC assays, six different buffers were used for folate extraction, purification and analysis. The function and composition of these buffers were as follows: (1) dissolution of stock standard solutions (0.1 M phosphate buffer, pH 7.0); (2) preparation of working standard (calibrant mixture) (0.01 M acetate buffer with 1.0% (m/v) Na-ascorbate, pH 4.9); (3) extraction buffer (0.15 M phosphate buffer with 52 mM ascorbic acid/ascorbate mixture and 0.1% (v/v) 2-mercaptoethanol (MCE), pH 6.0); (4) solid phase extraction (SPE) conditioning buffer (0.01 M phosphate buffer with 0.1% (v/v) MCE, pH 7.0); (5) SPE elution buffer (0.1 M acetate buffer with 10% (m/v) NaCl and 1% (m/v) ascorbic acid, pH 4.5); and (6) mobile phase (30 mM potassium phosphate buffer, pH 2.2).

The medium (B_{12} medium), used to enhance folate synthesis by the seven *P. freudenreichii* strains, was prepared as described by Van Wyk and Britz (2010). The inoculum volume was 200 µl per 100 ml broth, with a viable cell count 1 X 10^7 cfu/ml (variation of 5% at final count). The test organism used in the microbiological assay, *L. rhamnosus* (ATCC 7469), was obtained from the South African Bureau of Standards (SABS, Johannesburg, SA).

For the microbiological folate assay, media were prepared according to the instructions of the supplier. These included deMan, Rogosa and Sharpe (MRS) agar and MRS broth (Biolab, Johannesburg, SA), Bacto® Lactobacilli broth AOAC (Difco, Detroit, USA) and the folate-deficient medium, vitamin folic acid assay broth base (Merck, Darmstadt, Germany).

Pasteurised fat free milk, 0.5% fat (m/v) and “amasi”, 5% fat (m/v), a fermented milk product generally consumed in Southern Africa, were purchased from a local supermarket and analysed the same day. Kefir grains, obtained from the Department of Food Science, University of Stellenbosch, were used to prepare kefir, both with and without co-inoculation with strain J15, as described previously (Van Wyk and Britz, 2010; Van Wyk et al., 2011). Nitrogen 5.0 gas (Air Liquide, Cape Town, SA), containing <2 mg/kg oxygen, was used to displace the air in the headspace of folate standards and samples.

Preparation of standard solutions

The purity of all standards was checked in phosphate buffer (pH 7.0), as described by van den Berg et al. (1994), using appropriate molar extinction coefficients (Strålsjö et al., 2002). Stock solutions of the four vitamers were prepared by dissolving 10.0 mg (purity corrected) per 100 ml 0.1 M phosphate buffer (pH 7.0) and rapidly adding 1.0% (m/v) Na-ascorbate (Aldrich, St. Louis, USA) immediately after dissolution. The 0.1 M phosphate buffer (pH 7.0) used to dissolve THF contained 0.1% (v/v) 2-mercaptoethanol (MCE) (Amresco, Solon, USA) to prevent oxidation of this extremely labile compound. All stock solutions were flushed with nitrogen to prevent oxidative decomposition and stored at –20°C for a maximum of two weeks.

A working standard mixture (or calibrant mixture) containing THF, folic acid, 5-CHO-THF and 5-CH_3-THF was prepared by combining aliquots of the stock solutions, followed by dilution to 100 ml with 0.01 M acetate containing 1.0% (w/v) sodium ascorbate (Aldrich, St. Louis, USA) at pH 4.9. The calibrant mixtures were immediately flushed with nitrogen and stored at –20°C for a maximum of one week.

Folate extraction from dairy products and cultures of propionibacteria (PAB)

The extraction protocol included a heat treatment, a prerequisite for liberation of protein-bound folates (Vahteristo et al., 1996, 1997a). 10 ml extraction buffer was added to 2 ml or 2 g sample, followed by 15 s homogenisation using a Polytron® homogeniser (Kinematica, Lucerne, Switzerland), under a constant flow of nitrogen. 2-Octanol (Merck, Darmstadt, Germany) was used to prevent excessive foaming. The homogenate was transferred to a 50 ml centrifuge tube, flushed with nitrogen and heated in a microwave oven at 75% power (610 kW) for 1 min. The tube was then placed in a boiling water bath at 100°C for 10 min with gentle agitation. After rapid cooling on ice, centrifugation at 15 000 × *g* for 20 min at 2°C, and decanting the supernatant into clean centrifuge tubes, 60 mg CP conjugase was added per sample and dispersed using a Vortex-Genie2™ mixer (Scientific Industries, Inc., New York). The tubes were flushed with nitrogen, capped and incubated at 37°C for 2 h. The pH of the extract was then adjusted to 4.9 with glacial acetic acid, followed by the addition of 20 mg HK acetone powder (HK conjugase), dispersion and incubation as for CP conjugase. Extracts were then transferred to a boiling water bath (100°C) for 5 min to inactivate the enzymes, followed by rapid cooling on ice and centrifugation at 20 000 × *g* for 20 min at 2°C. 5 ml of the supernatant was dispensed into a 25 ml volumetric flask, followed by 15 µl MCE and Milli-Q water to the mark, resulting in a 5-fold dilution.

Deconjugation efficiency in each sample type was tested by adding 110.4 nmol of $PteGlu_3$ to the sample extract prior to the addition of the enzymes. Control samples, in which 110.4 nmol of $PteGlu_3$ was added after inactivation of the deconjugase enzymes, were also assayed.

In order to establish whether the enzyme preparations contained any folate, an enzyme blank was assayed, with 60 mg CP and 20 mg HK conjugases dispersed in 12 ml extraction buffer (pH 6), followed by enzyme inactivation, centrifugation and dilution as described previously.

Purification of folate extracts

SPE columns were used to purify the folate extracts with a SPE-12 vacuum manifold (Lida, Rochester, New York). Quaternary amine-based strong anion exchange columns (Strata SAX/3 ml/500 mg) (Phenomenex, Torrance, California) were used. n-Hexane, followed by HPLC grade methanol and Milli-Q water activated the columns. Conditioning was achieved by applying 10 ml conditioning buffer, at a flow rate of 1 ml/min. This was followed by 10 ml sample extract, aspirated at 0.7 to 0.8 ml/min. Impurities were removed by washing with 3 ml conditioning buffer at 0.3 ml/min. This was followed by elution, using 2 ml elution buffer at 0.2 to 0.3 ml/min, thus achieving a 5-fold concentration, hence cancelling out the 5-fold sample dilution that occurred during sample extraction. The eluate was collected in clean sample vials, followed by syringe filtration (0.22 µm Cameo, Osmonics, Minnetonka, USA) into amber glass vials prior to the HPLC analyses.

Due to the labile nature of reduced folates (Ball, 2006), measures were taken throughout to minimise oxidative degeneration induced by light and oxygen during preparation and handling of all standards and samples. These included expedient handling under subdued light (gold fluorescent), the use of opaque containers or amber glassware, inclusion of antioxidants into solutions and flushing with nitrogen gas.

Folate determination by high performance liquid chromatography

An Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), with a Rheodyne 7125 injection valve (with a 20 µl sample loop), a quaternary pump, a thermostatted column compartment and a scanning fluorescence detector with excitation and emission wavelengths set at 290 and 356 nm was used. Folic acid and $PteGlu_3$ were detected with the variable wavelength UV detector at a wavelength of 290 nm. The Chemstation software (Agilent Technologies, Waldbron, Germany) was used for integration, calculation and recording peak areas. It was also used for peak purity analyses, as well as for calibration, based on least square regression analysis.

Optimum chromatographic conditions were established when the analytical columns were a 3 µm Luna-C_{18} column (Phenomenex, Torrance, California), 150 × 4.6 mm internal diameter (i.d.), followed by a 3.5 µm Zorbax SB-C_{18} column (Agilent Technologies, Waldbron, Germany), 150 × 3.0 mm i.d. The first analytical column was protected by disposable C_{18} guard cartridges. The column temperature was maintained at 30 ± 1°C during all assays.

The vitamers were separated by means of gradient elution with acetonitrile and a 30 mM potassium phosphate buffer (pH 2.2) at a flow rate of 0.45 ml/min. The gradient was started at 5% acetonitrile and then linearly increased to 13% over 18 min. The gradient was then further increased to 25% acetonitrile over the next 7 min. To ensure complete column regeneration between injections, this was followed by a 10 min cycle with 5% acetonitrile: 95% Milli-Q water, followed by recycling to initial conditions before the following injection.

As mentioned, using the calibration function of the Chemstation software to construe the calibration plot, quantification of the folate levels of samples was performed by the external standard method; hence, a calibrant mixture, diluted to a known concentration during the day, was analysed in duplicate, and the software programmed from the resultant peak areas was used to calculate the concentration of the folate vitamers in the samples. The amounts were expressed as µg of the pure folate in its free acid form per 100 ml or 100 mg sample matrix.

The retention time of the folate peaks recorded for the external standard solutions and spiked samples was used to identify the peaks for the sample. However, impurities co-eluting with the analytes may diminish the accuracy of the identification process. Peak purity analyses ensure greater accuracy when calculating the concentrations of analytes in the samples by obviating incorrect inclusion of fluorescing impurities or impure peaks (Ye et al., 2001). Since fluorescence spectra of all standards and samples were recorded during all assays, peak purity analyses were routinely conducted using Chemstation. All purity tests pertained only to the reduced folates, THF and 5-CH_3-THF, 5-CHO-THF, since only these vitamers exhibited sufficient native fluorescence (Ball, 2006; Gounelle et al., 1989). As a result, spectra could not be recorded for PGA and purity tests were, therefore, not possible.

Since folate antibody (Biogenesis, Bournemouth, England) effectively bind folates in the extract, this was used to obtain further confirmation of the identity of folate peaks via complete absence or a diminished peak area.

Validation of performance parameters of the HPLC method

Linearity, limits of detection (LOD) and quantification (LOQ)

The inherent sensitivity of the HPLC method was determined by analysing a series of folate calibrant mixtures, increasing linearly in concentration from 0.001 to 20 ng per 20 µl injection. The full range of concentrations were: 0.001; 0.002; 0.005; 0.01; 0.05; 0.1; 0.5; 1.0; 5.0; 10.0; and 20.0 ng per 20 µl injection. The range was selected based on folate levels reported by Vahteristo et al. (1997a, b) (converted to ng 20 µl injection): 0.002 (LOD); 0.98 (milk); 3.0 (Edam cheese), 18.8 (Brussels sprouts).

Seven samples per concentration were analysed (n = 7) and the average peak area vs. folate concentration was plotted. A linear regression line, calculated by the method of least squares, was indicated. The correlation and regression coefficients were also calculated (Figure 1).

Accuracy

Each sample matrix type was spiked with a known quantity of calibrant mixture before sample extraction. Three different levels of addition was employed, namely 25, 50 and 100% of the expected native folate content. B_{12} medium was included as a blank sample matrix. Folates were determined in five replicate samples at each level of addition, the percentage recovery reported and the relative standard deviation (RSD) calculated.

Seven strains of *P. freudenreichii* (J9–J12, J15–J17), cultivated in B_{12} medium at 32°C, were sampled at 208 h and extracted. In addition, samples of pasteurised fat free milk, kefir and "amasi" were extracted. The HPLC results of all of the foregoing samples were verified by performing the microbiological assay on a portion of the same extract, the latter being the only food folate method with official AOAC status (Iyer and Tomar, 2009). Three replicate samples were assayed in all cases. Quality control samples of milk powder fortified with folate (100 mg per 100 g) were also analysed in duplicate in our laboratories as well as by an accredited laboratory (Microchem Specialised Lab Services, Cape Town, SA).

Precision

Repeatability precision is precision measured within a laboratory by analysing one sample multiple times. This includes simultaneous and consecutive replicates of the sample (Chen and Eitenmiller, 2007). Six replicates of the B_{12} medium spiked with the calibrant mixture were each analysed in two sessions on one day (simultaneous replicates). The intermediate precision was determined by analysing six replicates of the spiked B_{12} medium on consecutive days. In both cases, the mean ± standard deviation (SD) and the RSD for repeatability (RSD_r) were calculated. The Horwitz criterion of acceptance was used to evaluate repeatability precision. This criterion was determined as the $HORRAT_r$ value and compared to the acceptable range for single laboratory precision, namely 0.3 to 1.3 (Chen and Eitenmiller, 2007).

Folate determination by microbiological assay

The method as described by Angyal (1996), with modifications, was employed. Repeated transfers of the test organism, *L. rhamnosus* (ATCC 7469), to MRS broth (Biolab, Johannesburg, SA) ensured optimum culture activity, with definite turbidity visible 2 to 4 h after inoculation. One day prior to the assay, the culture was transferred to the test tubes containing 10 ml aliquots of the liquid culture medium (Bacto® Lactobacilli broth AOAC) (Difco, Detroit, USA) and incubated for 16 to 21 h at 37°C. On the day of the assay, the cultures were centrifuged (10 000 × *g*, 10 min, 4°C), the cells were washed and resuspended in sterile physiological saline solution. After dilution with sterile saline to 60%T (read against sterile saline set at 100 %T), the cell suspension, equivalent to a dried cell weight of 0.5 to 0.75 mg per tube, was used as inoculum.

A 100 µg/ml stock solution of 5-CH_3-THF Disodium salt was prepared as described under "standard solutions". On the day of assay, an intermediate solution was prepared by diluting the stock solution with 0.1 M phosphate buffer (pH 7.0), containing 1.0%

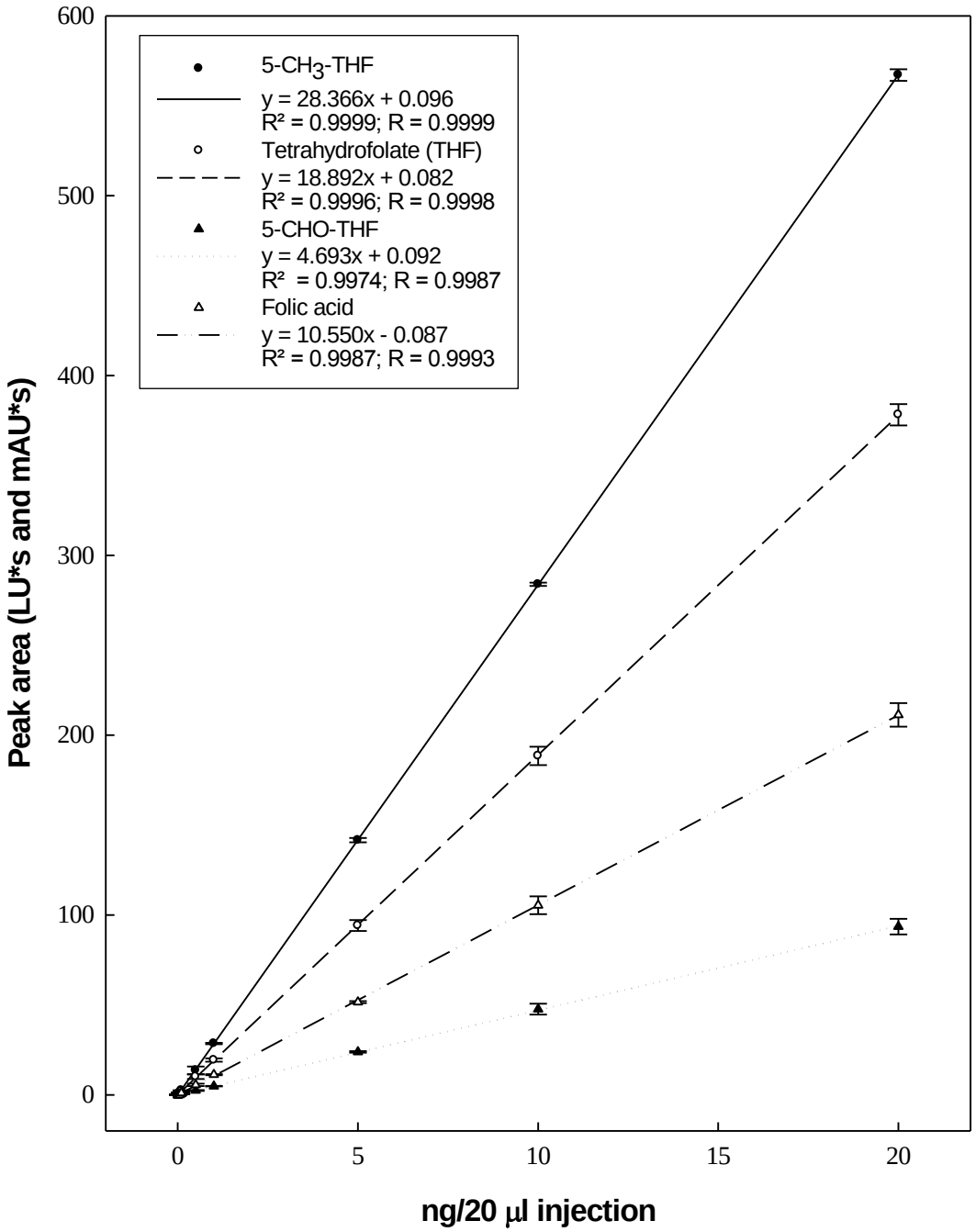

Figure 1. Linearity of folate analyses (n = 7). Error bars indicate the standard deviation.

(m/v) Na-ascorbate, to 1.0 µg/ml. The intermediate solution was then further diluted to produce the primary and two secondary standard solutions. The diluent was a phosphate buffer at pH 6.8, containing 1% ascorbic acid. The folate concentration in the primary standard was 0.2 ng/ml, while the two secondary standards contained 0.12 and 0.3 ng/ml folate, respectively.

Since replicate samples were HPLC assayed, the extraction and deconjugation procedure was as described in the "sample extraction" section.

After preparation and sterilisation of the standard and assay tubes, a Distriman repeater pipette equipped with a gamma-sterilised tip (Gilson, Villiers-le-Bel, France), was used to deposit one drop of inoculum directly onto the surface of the tube contents. The tubes were then incubated at 37°C for up to 72 h. When maximum growth was obtained, turbidity was measured at 620 nm and the results calculated by quadratic regression analysis.

Data analyses

All statistical analyses of data were performed using SPSS 17.0 for Windows®. The relative standard deviation (RSD) was calculated

Table 1. Limits of detection (LOD) and quantification (LOQ) of folate vitamers.

Vitamer	LOD		LOQ
	ng/20 µl injection	µg/ml sample	(µg/ml sample)
Tetrahydrofolate (THF)	0.002	0.0006	0.002
5-CH_3-THF	0.002	0.0006	0.002
5-CHO-THF	0.05	0.015	0.05
Folic acid (PGA)	0.05	0.015	0.05

for the recovery data as a measure of intra and inter-assay variability.

RESULTS AND DISCUSSION

Performance parameters of the HPLC method

Linearity, limits of detection (LOD) and quantification (LOQ)

The linearity of the method was confirmed by analysis of 10-point calibration standards over a range of five orders of magnitude, with correlation coefficients (R^2) > 0.997 and regression coefficients (R) > 0.998 (Figure 1). The minimum levels of the individual folate vitamers that could be detected (LOD) and measured (LOQ) were very low (Table 1). The LODs for THF, 5-CH_3-THF, 5-CHO-THF agreed closely with those reported previously (Vahteristo et al., 1997b; Yazynina et al., 2008), while PGA was detectable at 0.05 ng per 20 µl (Table 1) compared to 1.4 (Vahteristo et al., 1997b) and 0.02 ng per 20 µl (Yazynina et al., 2008). The HPLC method was, therefore, linear over a wide range of concentrations and sufficiently sensitive to be used as an analytical tool.

The potential presence of 10-formyl-THF was not monitored in this study for several reasons. Firstly, it was not found in milk and fermented milk products (Vahteristo et al., 1997a). Secondly, it was reported to be unstable under anaerobic conditions, such as the nitrogen flushing applied to standards and samples in this study, and it is converted to 5-formyl-THF upon heating or prolonged standing (Gounelle et al., 1989). Lastly, it cannot be measured under the acidic liquid chromatographic (LC) conditions that were used in this study (pH 2.2) (Vahteristo et al., 1997b).

Accuracy

The recovery values reported in Table 2 ranged from 80 to 108% overall, with RSD ranging from 0.79 to 9.75%. These values were within the acceptable range of recovery limits provided by the AOAC (Anonymous, 2002), confirming the quantitative validity or accuracy of the extraction process and other preparative as well as the determinative phases of this assay method.

The results of the microbiological and the HPLC analyses of the same samples showed good agreement (P > 0.05; Table 3). The HPLC method is, therefore, a valid alternative to the microbiological assay.

The closeness of the analytical results for the reference sample confirmed the accuracy of the method. An average folate content of 100.61 ± 0.65 mg per 100 g sample was obtained (Table 4), while the concentration of added folate was 100 ± 1.40 mg per 100 g sample. The good agreement between the results of our laboratory and that of the accredited facility (103.47 ± 0.64 mg per 100 g) (Table 4), further verifies the accuracy of the method.

Precision

The HORRAT value is typically used as the parameter of the Horwitz criteria of acceptance for method performance studies (Chen and Eitenmiller, 2007). Repeatability precision of simultaneous replicates (intra-assay) and of consecutive replicates (inter-assay) is presented in Table 5. The $HORRAT_r$ values of intra-assay and inter-assay precisions for all the folate vitamers were within the acceptable range of 0.3 to 1.3, with the %RSD all below 4% (Table 5). These results, therefore, indicate that the method was sufficiently precise.

Peak identification and peak purity

The identification of folate vitamers was performed by using their retention times (t_R) and the quantification was achieved by measuring the peak areas relative to standards. The mean and SD of the t_R of the vitamers, resulting from 20 injections of the calibrant mixture (n = 20) over a period of one month, were calculated. The results (in min) were: THF (16.41 ± 0.41); 5-CH_3-THF (17.84 ± 0.31); 5-CHO-THF (22.81 ± 0.87); and PGA (24.74 ± 0.55). The SD values indicated that the t_R of the vitamers were reproducible, confirming their suitability for peak identification and the suitability of the method for routine analyses. Sample chromatograms for a calibrant mix, kefir, milk and a PAB broth culture extract (strain J15) are presented in Figures 2A to D. The excellent

Table 2. Recovery results for HPLC determination of folates in PAB cultures and dairy products.

Sample with added calibrant mixture (n = 5)	Spiking range (µg/100 ml sample)	Percentage (%) recovery[1]							
		THF[2]		5-CH_3-THF		5-CHO-THF		Folic acid	
		Range	Mean ± SD[3] (%RSD)[4]	Range	Mean ± SD (%RSD)	Range	Mean ± SD (%RSD)	Range	Mean ± SD (%RSD)
B_{12} medium	4.0–16.0	83–97	88.2 ± 3.6 (4.08)	80–99	89.9 ± 5.1 (5.67)	86–100	91.9 ± 6.0 (6.53)	92–100	93.1 ± 2.1 (2.26)
Strain J15 in B_{12} medium	4.0–16.0	82–98	87.5 ± 5.2 (5.94)	82–102	90.2 ± 6.3 (6.98)	84–102	93.2 ± 5.2 (5.58)	89–97	91.8 ± 3.3 (3.59)
Amasi	3.0–15.0	94–106	100.1 ± 5.0 (4.99)	93–108	99.2 ± 6.9 (6.96)	88–107	96.4 ± 9.4 (9.75)	96–105	99.9 ± 4.1 (4.10)
Kefir	3.0–15.0	89–99	94.7 ± 4.2 (4.44)	84–93	90.0 ± 3.4 (3.78)	82–108	95.4 ± 8.7 (9.11)	80–94	89.1 ± 5.3 (6.30)
Pasteurised fat free milk	1.2–5.0	88–100	94.9 ± 5.7 (6.01)	100–103	101.7 ± 0.8 (0.79)	102–105	103.5 ± 1.2 (1.16)	102–108	104.0 ± 2.1 (2.02)

[1]% Recovery (percentage recovery of added standard) = $(C_s - C_p/C_a) \times 100$. C_s, Folate concentration in spiked sample; C_p, folate concentration in the native sample; C_a, amount of folate added. [2]Tetrahydrofolate, [3]standard deviation, [4]relative standard deviation reported in brackets. RSD (%) = (SD/Mean) × 100.

Table 3. Comparison of folate results from HPLC and microbiological assays[1].

Sample (n=3)	Microbiological assay	HPLC results	P-value[3]
J9[2]	19.56 ± 0.99	19.36 ± 0.68	0.79
J10	11.02 ± 0.68	11.33 ± 0.17	0.49
J11	8.83 ± 0.20	8.96 ± 0.45	0.67
J12	7.61 ± 0.43	7.65 ± 0.07	0.88
J15	15.25 ± 0.43	15.67 ± 0.17	0.19
J16	21.13 ± 0.29	21.46 ± 0.07	0.13
J17	19.77 ± 0.65	19.78 ± 0.02	0.98
Fat free pasteurised milk	4.98 ± 0.24	5.07 ± 0.18	0.63
Kefir	15.88 ± 0.79	15.97 ± 0.22	0.86
"Amasi"	13.07 ± 0.42	13.19 ± 0.17	0.67

[1]Results are reported as mean ± standard deviation (SD) in µg per 100 ml sample. [2]*P. freudenreichii* strains J9–J17 were cultivated in B_{12} medium and sampled at 208 h. [3]The student's t-test (independent samples) was performed to establish whether the results of the two methods differed significantly. $P \leq 0.05$ indicates significance.

peak resolution and similar retention times exhibited by samples and standards confirm the selectivity of the method (Anonymous, 2002).

Peak identification can only be made unequivocally by means of LC-MS (Brouwer et al., 2008). However, by using a folate antibody (Biogenesis, Bournemouth, England) to effectively bind folates in the extract, the folate peaks were absent, while present in the sample not treated with antibody. This further confirmed that the peaks were correctly identified as folates.

The purity tests routinely performed using the "Check purity" option in the Chemstation software ensured that only peaks with a purity factor within the threshold limit were used in calculations of folate concentrations. Purity factors obtained throughout were > 990, where 1 000 signifies a perfect peak.

Table 4. Folate levels in dairy products and PAB cultures[1].

Sample	THF[2]	5-CH_3-THF	5-CHO-THF	Folic acid (PGA)	Total
"Amasi" (n = 8)	2.02 ± 0.06	3.96 ± 0.14	7.33 ± 0.29	0	13.32 ± 0.26
Kefir (n = 8)	2.63 ± 0.29	4.71 ± 0.18	8.89 ± 0.32	0	16.23 ± 0.42
Kefir co-inoculated with J15[3] (n = 6)	15.10 ± 0.12	4.74 ± 0.13	14.32 ± 1.18	0	34.16 ± 0.15
Pasteurised fat free milk (n = 7)	1.00 ± 0.06	4.18 ± 0.14	0	0	5.18 ± 0.15
J15[4] (aerobic) (n = 6)	1.99 ± 0.32	9.37 ± 1.13	2.82 ± 0.27	0	14.19 ± 1.40
J16[4] (aerobic) (n = 6)	1.87 ± 0.27	8.43 ± 0.96	3.79 ± 0.43	0	14.09 ± 1.35
J15[5] (anaerobic) (n = 26)	5.64 ± 0.27	17.37 ± 0.77	3.48 ± 0.28	0	26.57 ± 0.94
Ref1 Lab1[6] (fortified milk powder) (n = 2)	ND[7]	ND	ND	100.61 ± 0.65[8]	ND
Ref1 Lab2[9] (fortified milk powder) (n = 2)	ND	ND	ND	103.47 ± 0.64[8]	ND

[1,8]Results are reported as mean ± standard deviation (SD) in µg per 100 ml or in µg per 100 g sample[1] and in mg per 100 g sample[8]. [2]Tetrahydrofolate, [3]sampled at 72 h,[4,5]*P. freudenreichii* (strains J15 and J16) cultured in B_{12} medium, sampled at 88[4] and 94 h[5]. [6,9]Reference sample analysed in our laboratory[6] and by an accredited facility[9], [7]ND = not determined.

Table 5. Method precision based on repetitive analyses of folate standards.

Intra-assay[1]	Session 1				Session 2			
	Mean[2]	SD[3]	RSD_r (%)[4]	$HORRAT_r$[5]	Mean	SD	RSD_r (%)	$HORRAT_r$
THF (n = 6)	64.71	2.49	2.86	0.34	65.46	2.29	3.50	0.31
5-CH_3-THF (n = 6)	271.60	7.25	2.67	0.31	276.90	8.09	2.92	0.34
5-CHO-THF (n = 6)	172.80	5.49	3.18	0.32	170.40	5.66	3.32	0.34
Folic acid (PGA) (n = 6)	115.90	3.69	3.29	0.32	126.60	4.33	3.42	0.33

Inter-assay[1]	Day 1				Day 2			
	Mean	SD	RSD (%)	$HORRAT_r$	Mean	SD	RSD (%)	$HORRAT_r$
THF (n = 6)	64.65	2.37	3.67	0.32	64.97	2.44	3.76	0.33
5-CH_3-THF (n = 6)	279.20	7.96	2.85	0.33	279.30	7.65	2.74	0.31
5-CHO-THF (n = 6)	172.50	6.47	3.75	0.38	170.60	6.38	3.74	0.38
Folic acid (PGA) (n = 6)	121.40	4.48	3.69	0.35	111.70	3.95	3.54	0.34

[1]Repeatability precision of simultaneous replicates (intra-assay) and consecutive replicates (inter-assay), [2]folate concentration in µg per 100 ml measured with the HPLC method [3]standard deviation [4]relative standard deviation observed. $RSD_{r\ observed}$ (%) = (SD/Mean) × 100. [5]$HORRAT_r = RSD_{r\ observed} / RSD_{r\ predicted}$. $RSD_{r\ predicted} = 2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction in g/g) (Chen and Eitenmiller, 2007).

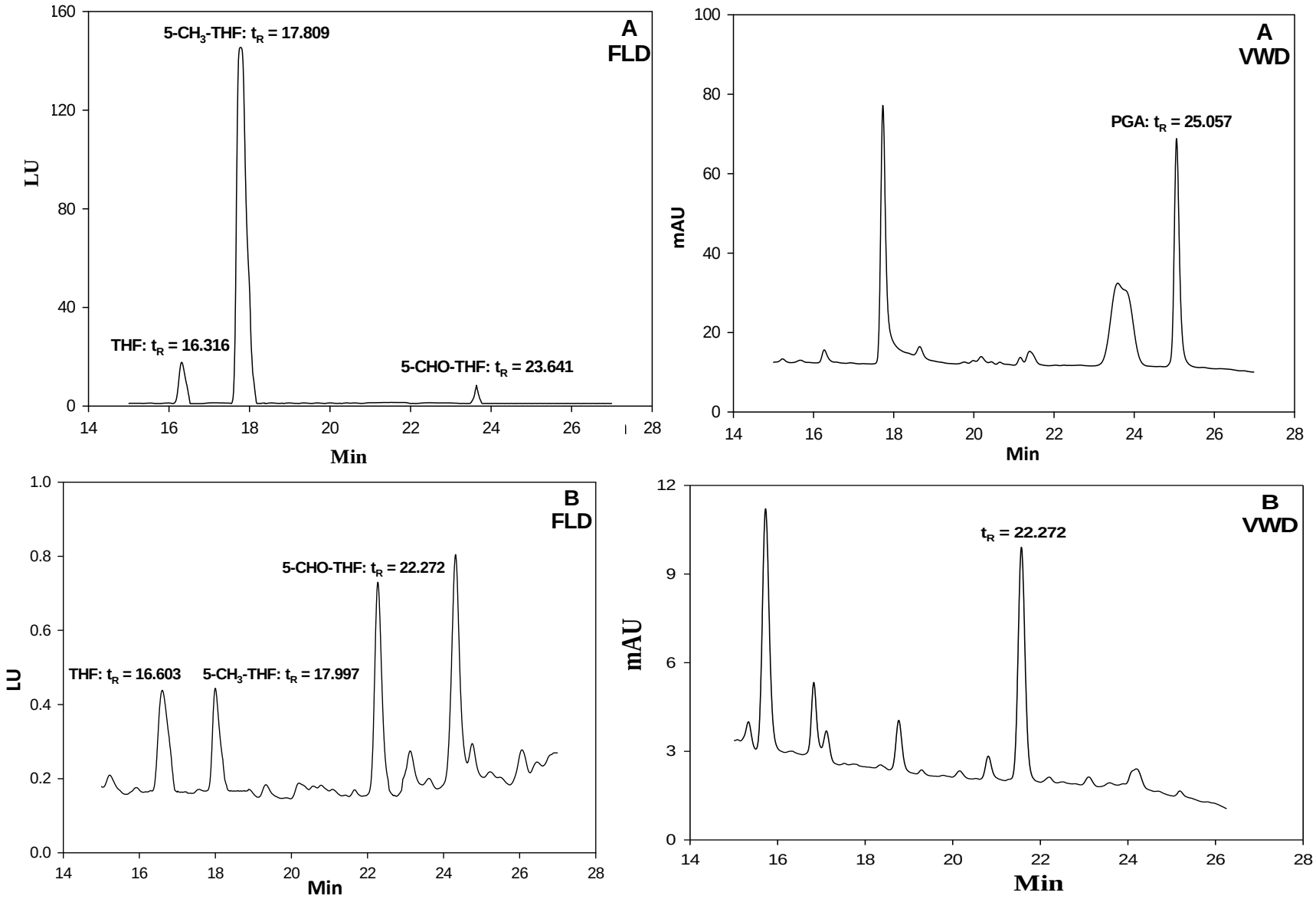

Figure 2. HPLC chromatograms of folate standards (calibrant mix) (A), a kefir sample extract (B), a milk sample extract (C) and a broth sample extract (strain J15) (D). FLD = Fluorescence detection used for tetrahydrofolic acid (THF), 5-methyl-THF (5-CH_3-THF) and 5-formyl-THF (5-CHO-THF). VWD = UV detection used for folic acid (PGA). t_R = retention time.

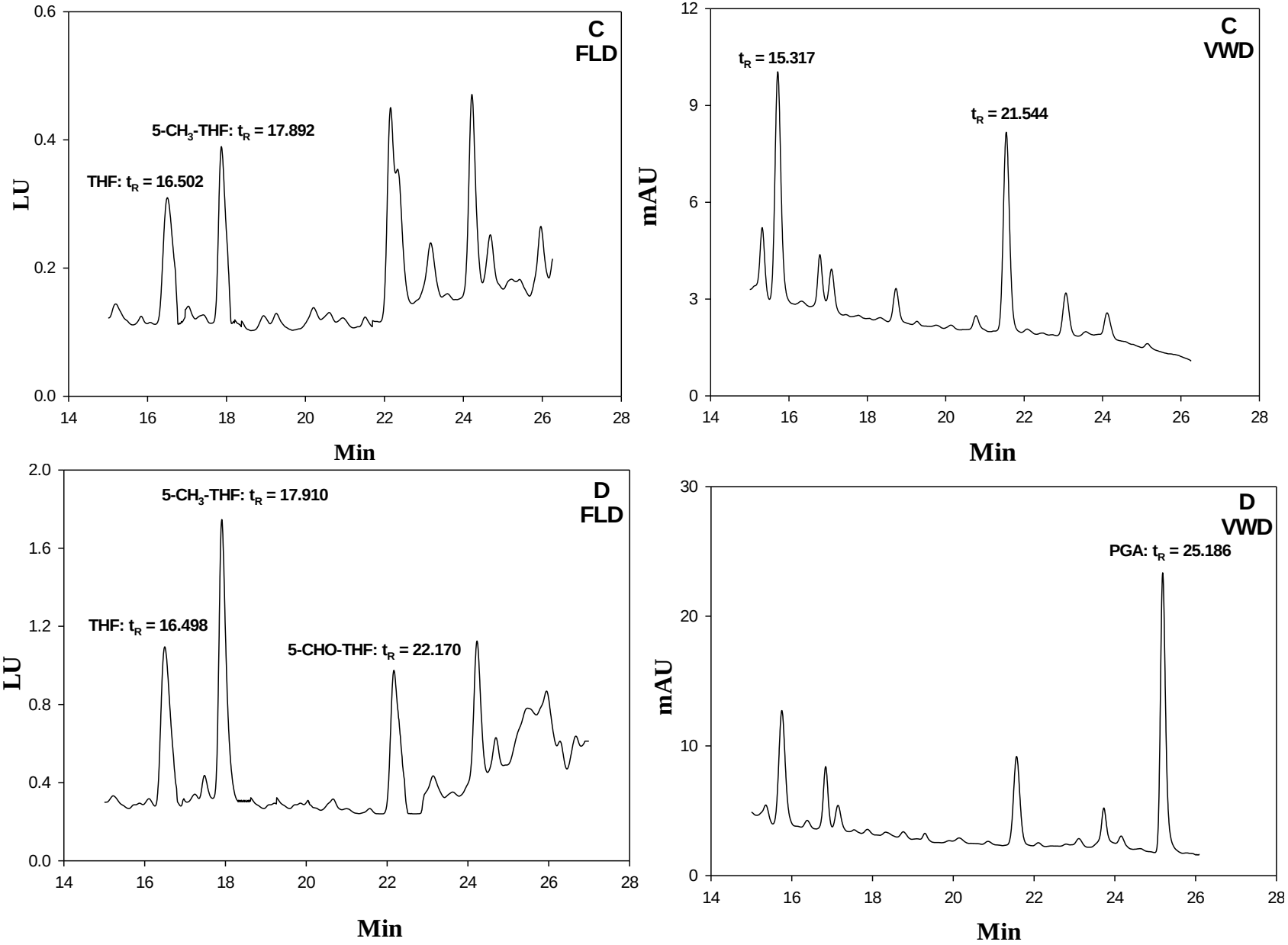

Figure 2. Contd.

Folate levels in deconjugase enzymes and deconjugation efficiency

The total folate content of the enzyme blank (n =3) was 60.0 ng (27.3 ng THF and 32.7 ng 5-CH_3-THF). When calculating folate concentrations, these values were subtracted from the folate levels measured in the samples.

The doses of the CP and HK conjugases were based on those employed by Pedersen (1988) and Strålsjö et al. (2002), respectively. The CP was added first since it contains amylolytic and proteolytic activity, which liberates folates from oligosaccharides, glycoproteins and proteins (Lim et al., 1998; Strålsjö et al., 2002). It is important to ascertain whether the deconjugation protocol afforded complete deconjugation in all sample extracts. Complete deconjugation would be signified by the complete conversion of $PteGlu_3$ to folic acid (PGA) by the deconjugase enzymes. In other words, the presence of a substantial PGA peak where the $PteGlu_3$ was added prior to heat denaturation of the deconjugases, in contrast to the absence of PGA (or a notably smaller peak) where the $PteGlu_3$ was added after denaturation of the deconjugases. In this study, complete deconjugation was observed for all sample types since a substantial concentration of PGA was present in the sample to which $PteGlu_3$ was added with deconjugases intact, while this peak was replaced by a $PteGlu_3$ peak when $PteGlu_3$ was added after heat denaturation of the deconjugases. It can, therefore, be concluded that the deconjugases effected complete deconjugation of folylpolyglutamates in the samples.

Folate concentrations in dairy products and PAB cultures

The ratios of the folate vitamers measured in this study (Table 5) do not completely agree with the results reported by Gregory et al. (1984) and Vahteristo et al. (1997a). While those authors measured 90 to 95% 5-CH_3-THF in cows' milk, this vitamer was also found to predominate in this study, but in a ratio of 81% 5-CH_3-THF to 19% THF. However, in agreement with Vahteristo et al. (1997a), the predominant vitamer in fermented dairy products was found to be 5-CHO-THF. The ratios measured in this study were as follows: 15% THF, 30% 5-CH_3-THF and 55% 5-CHO-THF ("Amasi"); 16% THF, 29% 5-CH_3-THF and 55% 5-CHO-THF (kefir). Furthermore, the total folate content measured (µg per 100 ml) in milk, 5.18, in kefir, 16.23, and in "amasi", 13.3 (Table 5), agreed well with those reported previously 4.9 (milk), 9.0 (yoghurt) (Kauwell et al., 2010), 11.2 (yoghurt) (Johnston et al., 2001) and 15.0 (buttermilk) (Vahteristo et al., 1997a).

Conclusions

The extraction protocol developed in this study was shown to effect satisfactory recovery of folates from the sample matrices and the deconjugation procedure afforded complete deconjugation. The analytical method performance parameters indicated that the method was linear over the normal working concentration range, was selective, sensitive, adequately precise and accurate (reliable). The accuracy was confirmed by the results obtained when analysing the reference material (fortified fat free milk powder), both in terms of agreement with the amount of folate added as well as with the results obtained by an accredited laboratory. The method also produced satisfactory peak purity. The good agreement in terms of quantified folate content obtained with the MA, verified the HPLC method. The method, therefore, is a rapid alternative to the time-consuming microbiological assay, suitable to be adopted for routine quality control of folate-enriched products.

ACKNOWLEDGEMENTS

Research funding provided by the National Research Foundation and by Cape Peninsula University of Technology (CPUT) is gratefully acknowledged. The authors also thank Mr. Ndumiso Mshicileli (Agrifood Technology Station, CPUT) for assistance with vitamin analyses and Prof. Victoria Jideani (Department of Food Technology, CPUT) for proofreading the manuscript.

REFERENCES

Anonymous (2002). AOAC requirements for single laboratory validation of chemical methods. Draft 2002-11-07. Available at URL http://www.aoac.org/Ag_Materials/additives/aoac_slv.pdf.

Anonymous (2003). Regulations relating to the fortification of certain foodstuffs, GNR 2003/2003. Foodstuffs, Cosmetics and Disinfectants Act and Regulations. Act no. 54 of 1972. Department of Health. Johannesburg, South Africa: Lex Patria Publishers.

Arcot J, Shrestha A (2005). Folate: methods of analysis. Trends Food Sci. Tech. 16: 253-266.

Ball GFM (2006). Vitamins in Foods, Analysis, Bioavailability and Stability. Boca Raton, Florida, USA: CRC Press. pp. 231-273, 585-733.

Chen L, Eitenmiller RR (2007). Single laboratory method performance evaluation for the analysis of total food folate by trienzyme extraction and microplate assay. J. Food Sci. 72: C243-C247.

De Brouwer V, Storozhenko S, van de Steene JC, Wille SMR, Stove CP, van der Straeten D, Lambert WE (2008). Optimisation and validation of a liquid chromatography-tandem mass spectrometry method for folates in rice. J. Chromatogr. A 1215: 125-132.

Gounelle JC, Ladjumi H, Prognon P (1989). A rapid and specific extraction procedure for folates determination in rat liver and analysis by high-performance liquid chromatography with fluorometric detection. Anal. Biochem. 176: 406-411.

Gregory JF (1984). Determination of folacin in foods and other biological materials. J. Assoc. Off. Anal. Chem. 67: 1015-1019.

Gregory JF, Sartain DB, Day BPF (1984). Fluorometric determination of folacin in biological materials using high performance liquid chromatography. J. Nutr. 114: 341-353.

Hefni M, Öhrvik V, Tabekha M, Witthöft C (2010). Folate content in foods commonly consumed in Egypt. Food Chem. 121: 540-545.

Iyer R, Tomar SK (2009). Folate: a functional food constituent. J. Food Sci. 74: R114-R122.

Johnston KE, DiRienzo DB, Tamura T (2001). Folate content of dairy products measured by microbiological assay with trienzyme treatment. J. Food Sci. 67: 817-820.

Kauwell GPA, Diaz ML, Yang Q, Bailey LB (2010). Recommended intakes, consumption and status. In: Bailey LB (Ed.), Folate in Health and Disease, (2nd ed.). CRC Press, Boca Raton, Forida, USA. pp. 467-490.

Kim YI (2007). Folate and colorectal cancer: an evidence-based critical review. Mol. Nutr. Food Res. 51: 267-292.

Labadarios D, Maunder E, Swart R, Bagriansky J, Steyn NP, MacIntyre U, Gericke G, Huskisson J, Dannhauser A, Voster HH, Nesamvuni AE (2000). General discussion and recommendations. In: Labadarios D (Ed.), The National Food Consumption Survey (NFCS): Children aged 1–9 years, South Africa, 1999. Directorate: Nutrition, Department of Health, Stellenbosch, Republic of South Africa. pp. 855-946.

Lebiedzińska A, Dąbrowska M, Szefer P, Marszałł M (2008). High-performance liquid chromatography method for the determination of folic acid in fortified food products. Toxicol. Mech. Method. 18: 463-467.

Lim HS, Mackey AD, Tamura T, Wong SC, Picciano MF (1998). Measurable human milk folate is increased by treatment with α-amylase and protease in addition to folate conjugase. Food Chem. 63: 401-407.

Ndaw S, Bergaentzlé M, Aoudé-Werner D, Lahély S, Hasselmann C (2001). Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates. J. Chromatogr. A 928: 77-90.

Oakley GP (2009). The scientific basis for eliminating folic acid-preventable spina bifida: a modern miracle from epidemiology. Ann. Epidemiol. 19: 226-230.

Patring J, Wandel M, Jägerstad, M Frølich W (2009). Folate content of Norwegian and Swedish flours and bread analysed by use of liquid chromatography-mass spectrometry. J. Food Compost. Anal. 22: 649-656.

Pedersen JC (1988). Comparison of y-glutamyl hydrolase (conjugase; EC 3.4.22.12) and amylase treatment procedures in the microbiological assay for food folates. Br. J. Nutr. 59: 261-271.

Pfeiffer CM, Fazili Z, Zhang M (2010). Folate analytical methodology. In: Bailey LB (Ed.), Folate in Health and Disease, (2nd ed.). CRC Press, Boca Raton, Forida, USA. pp. 517-574.

Shane B (1982). High performance liquid chromatography of folates: identification of poly-γ-glutamate chain lengths of labelled and unlabelled folates. Am. J. Clin. Nutr. 35: 599-608.

Shane B (2010). Folate chemistry and metabolism. In: Bailey LB (Ed.), Folate in Health and Disease, (2nd ed.). CRC Press, Boca Raton, Forida, USA. pp. 1-24.

Stover PJ (2010). Folate biochemical pathways and their regulation. In: Bailey LB (Ed.), Folate in Health and Disease, (2nd ed.). CRC Press, Boca Raton, Forida, USA. pp. 49-74.

Strålsjö L, Arkbåge K, Witthöft C, Jägerstad M (2002). Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk. Food Chem. 79: 525-534.

Ubbink JB, Christianson A, Bester MJ, Van Allen MI, Venter PA, Delport R, Blom HJ, Van der Merwe A, Potgieter H, Vermaak WJH (1999). Folate status, homocysteine metabolism, and methylene tetrahydrofolate reductase genotype in rural South African blacks with a history of pregnancy complicated by neural tube defects. Chem. Pathol. 48: 269-274

Vahteristo LT, Ollilainen V, Koivistoinen PE, Varo P (1996). Improvements in the analysis of reduced folate monoglutamates and folic acid in food by High-Performance Liquid Chromatography. J. Agric. Food Chem. 44: 477-482.

Vahteristo LT, Ollilainen V, Varo P (1997a). Liquid chromatographic determination of folate monoglutamates in fish, meat, egg and dairy products consumed in Finland. J. Assoc. Off. Anal. Chem. 80: 373-378.

Vahteristo LT, Lehikoinen K, Ollilainen V, Varo P (1997b). Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland. Food Chem. 59: 589-597.

Van den Berg H, Finglas PM, Bates C (1994). FLAIR intercomparisons on serum and red cell folate. Int. J. Vit. Nutr. Res. 64: 288-293.

Van Wyk J, Britz TJ (2010). A rapid HPLC method for the extraction and quantification of vitamin B_{12} in dairy products and cultures of *Propionibacterium freudenreichi*. Dairy Sci. Technol. 90: 509-520.

Van Wyk J, Witthuhn RC, Britz TJ (2011). Optimisation of vitamin B_{12} and folate production by *Propionibacterium freudenreichii* strains in kefir. Int. Dairy J. 21: 69-74.

Yazynina E, Johansson M, Jägerstad M, Jastrebova J (2008). Low folate content in gluten-free cereal products and their main ingredients. Food Chem. 111: 236-242.

Ye L, Eitenmiller RR, Landen WO (2008). Vitamin Analysis for the Health and Food Sciences, (2nd ed.). Boca Raton, Florida, USA: CRC Press. pp. 443-505.