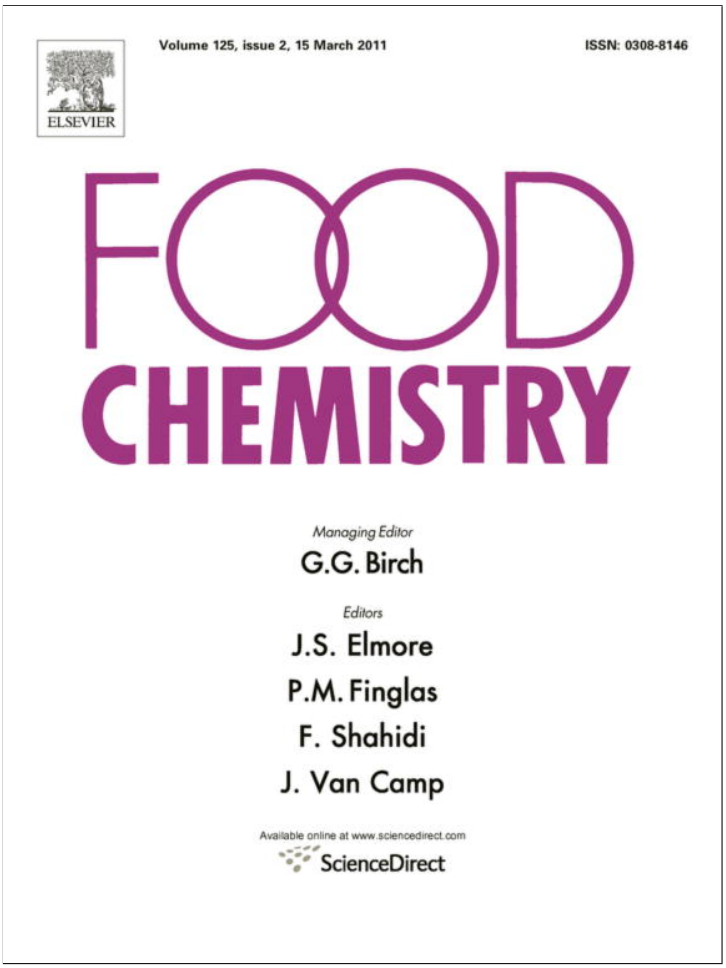

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Analytical Methods

Naturally-occurring folates in foods: Method development and analysis using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

An accurate method for detecting and quantifying both synthetic (folic acid) and naturally-occurring folates in foods is described. A system capable of analysing the five most commonly occurring folates (pteroylglutamic acid, 5-methyltetrahydrofolate, tetrahydrofolate, 10-formylfolate and 5-formyltetrahydrofolate) in 20 min using liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed. Quantification of folates was performed using ^{13}C labelled internal standards. This paper outlines the development of a comparatively fast LC–MS/MS method, method validation using commercially available folate standards and establishment of the method's suitability for quantification using selected reaction monitoring (SRM) mass spectrometry. The application of the system was verified by analysing several certified reference materials and comparing results with certified values as determined by microbiological assay. LC–MS/MS promises to be an ideal tool for the quantitative analysis of folates in food.

1. Introduction

Folates, a group of interconvertible water soluble nutrients have assumed great significance in recent years in the area of human nutrition and health. This is particularly true in view of its documented linkage with the reduction of neural tube defects (Czeizel & Dudas, 1992) and reduced risk factors for both heart disease (Christopherson, 1996) and certain types of cancer (Lucock, 2000).

Folates and its derivatives exist as polyglutamates in nature (Forssen, Jagerstad, Wigertz, & Witthoft, 2000).

Using HPLC methods, 5-methyltetrahydrofolate was found to be the major form in fruits and vegetables (Vahteristo, Lehikoinen, Ollilainen, & Varo, 1997), 5-formyltetrahydrofolate, 10-formylfolate and 5-methyltetrahydrofolate were found in cereals (Pfeiffer, Rogers, & Gregory, 1997), and 5-methyltetrahydrofolate and tetrahydrofolate were dominant in animal products (Vahteristo et al., 1997). The most widely used microbiological assay can only measure total folate in foods using *Lactobacillus casei* where the growth response of the organism to the mixture of folates present is measured turbidimetrically (Tamura, 1998).

High pressure liquid chromatography separation techniques with ultraviolet and/or fluorescent detection have been documented to detect the different forms of folate. In many instances these methods lack specificity. Consistently HPLC data for total folate was much lower (30–50%) than the microbiological assay data reported in literature (Ginting & Arcot, 2004; Kariluoto, Vahteristo, & Piironen, 2001). Improved methodology offering superior specificity and sensitivity for analysing the different forms of folates in foods is critical as these compounds differ in their bioavailability (Tamura & Stokstad, 1973; Finglas et al., 2006).

Gas chromatography and liquid chromatography mass spectrometric techniques (including tandem MS) are currently available for quantification of folate forms in both biological samples and food (Garbis, Melse-Boonstra, West, & van Breemen, 2001; Hart et al., 2002; Pawlosky, Flanagan, & Pfeiffer, 2001; Rychlik, Netzel, Pfannebecker, Frank, & Bitsch, 2003; Santhosh-Kumar & Kolhouse, 1997; Toth & Gregory, 1988). The main disadvantage of the GC–MS method is the fact that different folate forms within the sample cannot be distinguished since they all require derivatisation to para-amino benzoyl glutamate (Lin, Dueker, & Clifford, 2003). Thus the folate molecule is poorly suited for GC–MS analysis in its intact form due to thermal instability and low volatility (Vahteristo & Finglas, 2000).

The problems associated with GC–MS have been overcome with the advent of LC–MS. Recently, there have been a number of

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; SRM, selected reaction monitoring; HPLC, high pressure liquid chromatography; GC–MS, gas chromatography–mass spectrometry; ESI, electrospray ionisation; APCI, atmospheric pressure chemical ionisation; m/z, mass to charge ratio; R_f, response factor; SPE-SAX, solid phase extraction-strong anion exchange; CID, collision induced dissociation; CRM, certified reference materials.

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published validated LC–MS and LC–MS/MS methods for the analysis of folate in plasma (Garbis et al., 2001; Hart et al., 2002; Nelson, Pfeiffer, Margolis, & Nelson, 2003; Pfeiffer, Fazili, McCoy, Zhang, & Gunter, 2004), fortified foods (Pawlosky et al., 2001) and urine (Hart et al., 2002). Of all the methods published in literature, Stable Isotope Dilution Assays (SIDA) with LC–MS detection are considered most accurate because of the use of internal standards to correct for losses of the vitamers during sample clean-up. In addition it also compensates for the variation in ionisation efficiency due to matrix interferences. Until recently, there have been two labelled folates used in LC–MS studies employing internal standards. First, $^{13}C_5$ labelled 5-methyltetrahydrofolate and pteroylglutamic acid by Pawlosky, Flanagan, and Doherty (2003) and second quadruple deuterated vitamers by Freisleben, Schieberle, and Rychlik (2003a) and in this study, $^{13}C_5$ labelled tetrahydrofolate, 5-formyltetrahydrofolate (commercially available) and 10-formylfolate (commercially not available and therefore synthesised in-house).

This paper outlines the development and optimisation of a fast LC–MS/MS method to profile and quantify the folate vitamers (pteroylglutamic acid, 5-methyltetrahydrofolate, tetrahydrofolate, 10-formylfolate and 5-formyltetrahydrofolate) commonly found in foods with good on-column sensitivity comparable to Freisleben, Schieberle, and Rychlik (2003b). Linearity of instrument response to folate concentration is demonstrated using folate standards; the suitability of carbon-labelled standards were established and the application of the method has been tested using commercially-available standard reference materials and other food samples representing complex food matrices.

2. Materials and methods

2.1. Unlabelled folate standards

Pteroyl glutamic acid (PteGlu), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5-formyltetrahydrofolate (5-CHO-H_4folate), 10-formylfolate (10-CHO-PteGlu) and tetrahydrofolate (H_4folate) were obtained from Schircks Laboratories, Jona, Switzerland. The purity of the unlabelled standards was verified using UV spectrophotometry based on Zhang et al. (2003). Stock solutions (1 mg/mL) of the standards were prepared by dissolving the powdered folate standards in 0.05 M HEPES–CHES buffer, pH 7.85 containing 2% (w/w) sodium ascorbate and 0.01 M 2-mercaptoethanol.

Folate derivatives are easily degraded by light and are easily oxidised under atmospheric conditions. Therefore, all folate stock solutions were prepared under subdued light. When not in use, solid powders and stock solutions were stored at −80 °C in the dark. The folate standards were not used for more than 3 months. Aliquots of stock solutions were prepared and used throughout analysis to minimise the freeze–thaw cycles.

2.2. Isotopically labelled folate standards

The LC–MS/MS method described in this paper involves the use of commercially-available carbon (C13) labelled isotopomers of the folate standards as internal standards. The commercially-available carbon labelled folate standards: Pteroyl glutamic acid Pte[$^{13}C_5$]Glu, free acid form, 5-methyltetrahydrofolate (6S)-5-CH_3-H_4Pte[$^{13}C_5$]-Glu, ca-salt, 5-formyltetrahydrofolate (6S)-5-CHO-H_4Pte[$^{13}C_5$]Glu, ca-salt, tetrahydrofolate (6S)-H_4Pte[$^{13}C_5$]Glu, free acid form were purchased from Eprova, Switzerland. The only commercially unavailable labelled vitamer 10-formylfolate (10-CHO Pte[$^{13}C_5$]Glu) was synthesised in-house based on the reaction in which isotopically-labelled folic acid reacts with formic acid under heat at 50–60 °C to yield labelled 10-formyl folic acid (Kay, Osborn, Hatefi, & Huennekens, 1960). Purity was checked by LC–MS/MS analysis described in this publication. One hundred microliters of unlabelled 10-formylfolate (1000 ng/mL working standard solution) was injected with equal amounts of the in-house synthesised carbon labelled 10-formyl folic acid (n = 5) and peak areas were compared. Labelled standards (10 μg/mL) were prepared in 0.05 M HEPES–CHES buffer, pH 7.85 containing 2% (w/w) sodium ascorbate and 0.01 M 2-mercaptoethanol and were stored under nitrogen at −80 °C.

2.3. Sample extraction

The foods chosen were all mixed diet matrices that consisted of cooked meals prepared with cereals, vegetables, and legumes. Milk and a fruit matrix were also analysed. The mixed diet consisting predominantly of a cereal and vegetable matrix with selected spices was semolina, a thick soup made of split pigeon pea to represent the pulse based matrix, cooked carrots sautéed in oil, salt and spices to represent the vegetable matrix, milk and banana (Morris variety) to represent milk and fruit based matrices, respectively. The original samples were split into two. One half was then assayed using a microbiological assay as part of a parallel study. The method of analysis and results have been published recently (Vishnumohan, Arcot, Sini, Uthira, & Ramachandran, 2009) and the other half was assayed using the new LC–MS/MS method described in this paper. The sum of individual vitamers obtained using the LC–MS/MS method was compared against the total folate values generated using the microbiological assay.

The prepared foods were homogenised individually in a domestic kitchen blender and composite samples were prepared and freeze-dried. Sample extraction was carried out in subdued light, with all glassware wrapped with aluminium foil. To 0.5–1 g of freeze-dried sample was added 10 mL of extraction buffer (0.05 M HEPES–CHES buffer, pH 7.85 containing 2% (by mass) sodium ascorbate and 0.01 M 2-mercaptoethanol) and mixed thoroughly by shaking until the sample was completely dispersed in the buffer. The sample homogenate was placed in a water bath at 100 °C for 10 min, then immediately cooled.

2.4. Moisture determination

Duplicate samples were separately analysed for moisture content immediately following the homogenisation using a vacuum oven at 70 °C overnight (AOAC, 2007).

2.5. Tri-enzyme treatment and deconjugation

The pH of the extract was adjusted to 4.5 using 1 M HCl. To a 10 mL sample, 1.6 mL of protease preparation (diluted 2 mL of 50 mg/mL protease solution (megazyme, subtilisin A from B. licheniformis) in 50 mL water) (2 mg/mL) was added and incubated at 37 °C for 16 h. The reaction mixture was heated for 5 min at 100 °C in a water bath to stop enzymatic activity. The mixture was then cooled, the pH adjusted to 6.1 and further treated with 1.6 mL of α-amylase (A-3176, Sigma Chemical Co., St. Louis, MO 63178) (20 mg/mL of water) for 4 h at 37 °C. The pH of the enzyme hydrolysed extract was adjusted to 7.2 using 1 M NaOH. Ten milliliters of hydrolysed extract was treated with 1.0 mL of human plasma (Red Cross Society, Prince of Wales Hospital, Sydney, Australia) and incubated for 3 h at 37 °C. The deconjugated extract was heated in a boiling water bath for 5 min, cooled and centrifuged at 10,000 rpm for 10 min. The supernatant was retained and stored at −20 °C for a maximum period of 1 week.

2.6. Sample purification

Purification was carried out using solid phase extraction with a strong anion exchange cartridge (3 mL/500 mg of quaternary amine) as described by Freisleben et al. (2003b) with slight modifications. All the solutions and extracts were applied to the cartridges using a Supelco 24-port vacuum manifold. The cartridges were conditioned sequentially with 3 mL of hexane, methanol and water and then equilibrated with 10 mL of conditioning solution (0.01 M potassium phosphate buffer containing 0.1% (w/v) ascorbic acid and 0.01% (v/v) mercaptoethanol). Three milliliters of the enzyme treated sample extracts were spiked with 10 μL of the working internal standard solution mix (containing 100 ng of each folate vitamer) and loaded on to the SPE cartridge at a rate of less than 1 mL/min. The cartridge was then washed twice with 1.5 mL of conditioning solution. Finally, folate compounds were eluted with 1.0 mL of aqueous elution buffer: (sodium chloride 5% (w/v) containing 1% (w/v) sodium ascorbate and 0.1 mol/L sodium acetate). The eluate was reduced to dryness using a vacuum centrifuge and resuspended in 100 μL of 0.1% formic acid for analysis with LC–MS/MS. The purified extracts were analysed immediately without any freeze–thaw storage.

2.7. Preparation of standards for linearity curve

Standard mixes containing all the five forms of folate (0.2, 0.4, 0.6, 0.8, 1.0, 2, 4, 6, 8 and 10 μg/mL) were purified as described earlier. The analysis was performed in replicates ($n = 6$) as outlined before. The linearity curve for each folate standard was obtained by plotting the peak area against the ten concentrations of folate standards injected (20–1000 ng/injection). The linearity curve was assessed to find the suitable linear region to quantify the folate in the food samples. Detection and quantitation limits were estimated based on the calibration curve.

2.8. LC–MS instrumentation

The LC–MS system used in this study consisted of a ThermoFinnigan Surveyor LC and autosampler coupled directly to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer via an electrospray interface. LC was performed on a C18 reversed phase column (Zorbax Eclipse, 5 μm, 2.1 mm by 150 mm) as previously described by Freisleben et al. (2003b). Ten microliters of the sample solutions were analysed using gradient elution with aqueous formic acid (0.1%, solvent A) and acetonitrile (solvent B), at a flow of 0.2 mL/min. The solvents were chosen based on Freisleben et al. (2003b) but the gradient was optimised to get a better separation. An initial 4-min hold of 7% acetonitrile was ramped to 30% acetonitrile over 12-min before being brought back to the initial mixture for another 4-min to allow for column re-equilibration. During the first 3 min of the gradient programme, the column eluent was diverted to waste to prevent salt and hydrophilic buffer components contaminating the electrospray source.

The mass spectrometer was operated in the positive ion mode with a spray voltage of 5 kV. The heated capillary was maintained at 250 °C and the capillary voltage was set to 24.3 V. The maximum ion injection time was set to 200 min. Prior to sample analysis the ion optics were optimised for sensitivity using the Xcalibur software 'autotune' function on a continually infused 10 μg/mL standard mix solution containing all the five target analytes. The automatically performed optimisation was confirmed manually. A collision energy of 30 (ThermoFinnigan arbitrary units) was considered satisfactory to induce optimal fragmentation of the ions in the SRM mode. The mass spectrometer was programmed to 10 scan events to scan the relevant ions sequentially.

2.9. LC–MS/MS analysis of folates

Data was acquired using SRM due to the lower possibility of interference.

Recording of the chromatograms and data analysis were performed using ThermoFinnigan XCalibur software. The mass transitions chosen for labelled and unlabelled standards are as follows: (a) pteroylglutamic acid (m/z 442–295), (b) tetrahydrofolate (m/z 446–317), (c) 5-methyltetrahydrofolate (m/z 460–313), (d) 10-formylfolate (m/z 470–452), (e) 5-formyl tetrahydrofolate (m/z 474–327), (f) Pte[$^{13}C_5$]Glu (m/z 447–295), (g) (6S)-H_4Pte[$^{13}C_5$]Glu (m/z 451–317), (h) (6S)-5-CH_3-H_4Pte[$^{13}C_5$]Glu (m/z 465–313), (i) 10-CHO Pte[$^{13}C_5$]Glu (m/z 475–457) and (j) (6S)-5-CHO-H_4Pte[$^{13}C_5$]Glu (m/z 479–327).

The isolation width of the precursor ion was adjusted to 3 Da and the isolation width of the product ion was set to 2 Da.

The precursor ions of the internal standards had a mass difference of five in the MS mode and the product ions chosen for detection in SRM showed no mass difference with those of the unlabelled analyte. This is simply because the labelled glutamic acid portion of the molecule is lost as a neutral during collisional activation. The only vitamer which maintained a mass difference in the product ions was 10-CHO Pte[$^{13}C_5$]Glu, as the preferred (most intense) fragmentation involved the loss of a water molecule and not the labelled glutamic acid.

2.10. Determination of relative response factors for LC–MS/MS

Equal masses of unlabelled folate vitamers (v), together with the isotopically-labelled internal standards were dissolved in HEPES–CHES buffer. Subsequently the mixtures were purified and subjected to LC–MS/MS as outlined before. Relative response factor (RRF) was calculated using the formula:

$$RRF = \left(\frac{Mass_v}{Area_v}\right) \div \left(\frac{Mass_{InternalStd}}{Area_{InternalStd}}\right)$$

The calculated relative response factor was 0.99 for the folate vitamers which demonstrated that the internal standards behaved similarly to their respective analytes.

2.11. Data calculation

Quantification of the food folates was based on the internal standard method.

The standards and samples were spiked with internal standards (100 ng/injection) before the clean-up procedure and were taken through the described procedure for quantitation. The mass of the analyte (a) in the sample is calculated using the formula below:

$$(Mass_a)_{sample} = (Area_a)_{sample} \times RRF \times \left(\frac{Mass_{Internal\,Std}}{Area_{Internal\,Std}}\right)$$

2.12. Quality control

A food sample that is known to have very little or no folate such as raw rice sample was chosen initially to be spiked with a known amount of the different folate compounds to test for any matrix interference. Raw rice was cooked using the absorption technique (cooked in excess water) and freeze-dried. To 1 g of this folate free matrix 100 ng of the five carbon labelled and unlabelled standards were added by spiking the matrix with 10 μL (10 μg/mL) of the standards. Extraction and sample clean-up was continued as described earlier. LC–MS/MS analysis was conducted as outlined above. The assay was performed in triplicates. Certified reference materials to represent different matrices were chosen to be

analysed using the established method such as CRM 121 (Wholemeal flour); CRM 485 (Lyophilised mixed vegetables); SRM 1846-Infant Formula and CRM 487, Lyophilised Pig Liver to ensure accuracy of data.

3. Results and discussion

3.1. Separation and identification of folate compounds using LC–MS and LC–MS/MS

The different folate vitamers exhibit small differences in their ionic character and are thus well suited to be analysed by high-performance liquid chromatography.

Initial work was performed using a 15 min LC gradient. However, it was found that an extra 5 min equilibration time was required, resulting in a total runtime of 20 min. The gradient elution using C18 reversed phase column showed a good separation of folate compounds within 15 min. Solid phase extraction using a strong anion exchange stationary phase (SPE-SAX), which has previously been shown to be effective for purification of folate standards (Ginting & Arcot, 2004), helped to protect the mass spectrometer from the buffer and matrix components which might otherwise interfere in the analysis resulting in reduced sensitivity. For example, buffer ions could clearly be seen eluting in the first 3 min of analysis when the sample was not purified using SPE (Fig. 1).

Sample clean-up can be achieved by affinity chromatography using folate binding proteins, which offers the potential to remove more matrix interferences than the SPE approach. However these methods are more expensive requiring detailed column preparation (Seyoum & Selhub, 1993), and they have little affinity for formylated folates (Gregory, 1989) and possess a limited number of extract applications (Kariluoto et al., 2001). The analytical method presented in this paper is powerful enough to detect the folates in foods purified using the convenient SPE-SAX columns.

Initial experiments revealed an excess of co-eluting impurity isobaric with folic acid. This prevented the detection of folic acid when performing SRM experiments. It is proposed that the ion trap is filled with the interfering ions, therefore reducing the amount of folic acid that can enter the trap and be detected (Fig. 2A). The problem was solved by altering the HPLC gradient so that the folic acid and this impurity were separated on the column, therefore removing the interference and allowing the measurement to proceed (Fig. 2B). Confirmation of the identity of the compounds was obtained by performing tandem mass spectrometry (MS/MS) experiments where fragmentation of the protonated molecule was achieved by collision induced dissociation (CID). Fig. 3 depicts the chromatographic profile of the selected ion for the analytes and labelled internal standards. The use of tandem mass spectrometry also allows highly specific detection of the folate vitamers when employing Selective Reaction Monitoring (SRM).

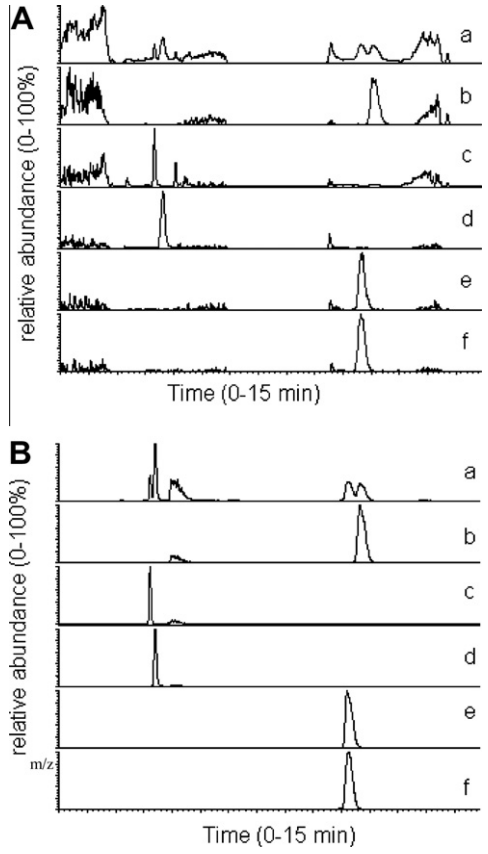

Fig. 1. (A) LC–MS chromatogram obtained from analysing 10 ng/injection of folate standards without SPE purification. (a) Total ion chromatogram, (b) pteroylglutamic acid (m/z 442), (c) tetrahydrofolate (m/z 446), (d) 5-methyltetrahydrofolate (m/z 460), (e) 10-formylfolate (m/z 470) and (f) 5-formyltetrahydrofolate (m/z 474). (B) LC–MS chromatogram obtained from analysis of SPE-SAX purified folate standards (10 ng). The mass spectrometric signals obtained from the protonated vitamers are plotted against time. (a) Total ion chromatogram, (b) pteroylglutamic acid (m/z 442), (c) tetrahydrofolate (m/z 446), (d) 5-methyltetrahydrofolate (m/z 460), (e) 10-formylfolate (m/z 470) and (f) 5-formyltetrahydrofolate (m/z 474).

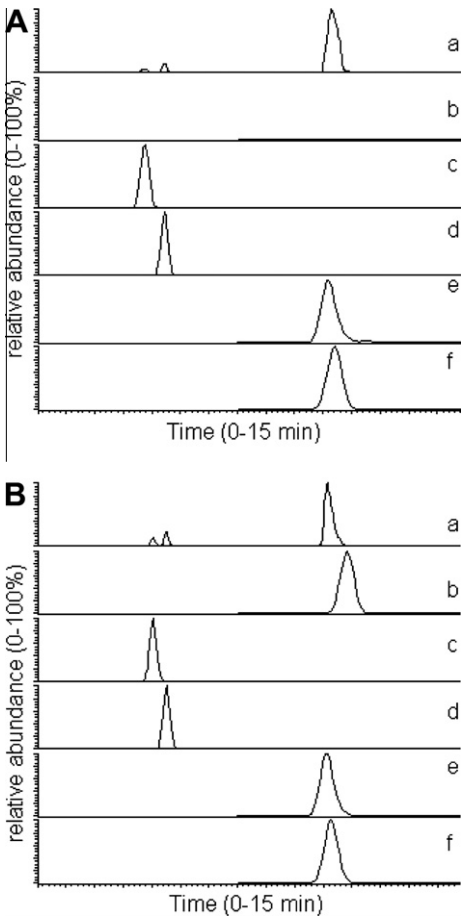

Fig. 2. LC–SRM chromatogram obtained from analysing 10 ng/injection of folate standards. (A) Initial chromatographic conditions. (B) Optimised chromatographic conditions. (a) Total ion chromatogram, (b) pteroylglutamic acid (SRM transition m/z 442–295) (c) tetrahydrofolate (SRM transition m/z 446–317), (d) 5-methyl tetrahydrofolate (SRM transition m/z 460–313), (e) 10-formylfolate (SRM transition m/z 470–452) and (f) 5-formyltetrahydrofolate (SRM transition m/z 474–327).

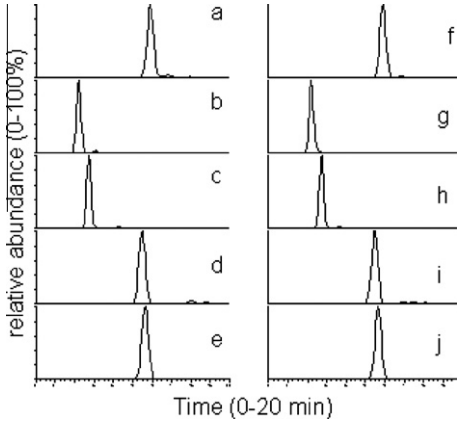

Fig. 3. LC–SRM chromatograms obtained from 10 ng/injection of folate standards and their ^{13}C5-labelled counterparts. Unlabelled – (a) pteroylglutamic (SRM transition *m*/*z* 442–295) (b) tetrahydrofolate (SRM transition *m*/*z* 446–317), (c) 5-methyltetrahydrofolate (SRM transition *m*/*z* 460–313), (d) 10-formylfolate (SRM transition *m*/*z* 470–452) and (e) 5-formyltetrahydrofolate (SRM transition *m*/*z* 474–327). Labelled – (f) Pte[$^{13}C_5$]Glu (SRM transition *m*/*z* 447–295) (g) H_4Pte[$^{13}C_5$]Glu (SRM transition *m*/*z* 451–317), (h) 5-CH_3-H_4Pte[$^{13}C_5$]Glu (SRM transition *m*/*z* 465–313), (i) 10-CHO Pte[$^{13}C_5$]Glu (SRM transition *m*/*z* 475–457) and (j) 5-CHO-H_4Pte[$^{13}C_5$]Glu (SRM transition *m*/*z* 479–327).

3.2. In-house synthesis of internal standard

The commercially unavailable internal standard 10-CHO Pte[$^{13}C_5$]Glu synthesised in-house by reacting Pte[$^{13}C_5$]Glu with formic acid at 50–60 °C for 2 h was successful yielding 98% purity.

3.3. Calibration and linearity data for LC–MS/MS folate analysis

The response of the method was tested over a range of concentrations (0–1000 ng/injection). At the higher ng levels the response of the instrument to the folate lost linearity, and the upper limit of quantitation had been passed. At lower ng levels the response of the instrument to the folate retained linearity. About 20–100 ng/injection was used for the analysis of folates in foods. A wide range of standard concentrations were used to cover the wide range of matrices used in the study and the on column detection limits (LOD) and quantification limits (LOQ) were estimated were estimated to be 10 and 20 ng, respectively. Under normal circumstances the detection and quantification limits would have been established with samples. The samples used in the present study were so varied that it was not possible to choose a representative matrix blank to spike into. So we decided to evaluate the detection and quantification limits of the instrument using standards and not the method itself. This is a limitation of the study.

Table 2
Quality control data generated by the LC–MS/MS method.

Folate vitamer	Linearity data[a]	Mean recovery (%)[b]
PteGlu	0.95	92
H_4folate	0.94	77
5-CH_3-H_4folate	0.99	92
10-CHO-PteGlu	0.98	92
5-CHO-H_4folate	0.98	99

[a] All values are means of six replicate analyses.
[b] All values are means of triplicate analyses.

3.4. Application of the method to certified reference materials

Commercially available certified materials, wholemeal flour (CRM 121), infant milk formula (SRM 1846) and a vegetable matrix (CRM 485) containing 50, 129 and 315 μg/100 g total folate respectively was chosen to check the accuracy of the method. These samples were spiked with internal standards (100 ng/injection) before the clean-up procedure and were taken through the described procedure for quantitation. Table 1 gives the comparison of individual and total folate values obtained using the newer LC–MS/MS method with those generated using a HPLC method (Finglas et al., 1999) and certified values generated using a microbiological assay method. Values obtained in this study were in agreement with the certified values. The value for 5-methyl tetrahydrofolate reported for CRM 121 (whole meal flour) was well within the range reported by Finglas et al. (1999). However the present study yielded higher values for 5-methyltetrahydrofolate quantified in mixed vegetables (CRM 485) and pig liver (CRM 487) when compared to the indicative HPLC data reported by Finglas et al. (1999). Although the forms identified in the reference materials in the present study correlated with the forms identified by Finglas et al. (1999), a direct comparison could not be drawn between the studies because of the following reasons: variation in the enzyme treatment during extraction; 10-formyl tetrahydrofolate was not analysed in the present study and though the milk matrices were comparable, SRM 1846 (infant milk formula) was analysed in the present study but CRM 421 (milk powder) was analysed by Finglas et al. (1999).

Table 1
Comparison of mean values generated by LC–MS/MS method (present study) and Finglas et al. (1999).

Reference	Sample	PteGlu (μg/100 g)	H_4folate (μg/100 g)	5-CH_3-H_4folate (μg/100 g)	10-CHO-PteGlu (μg/100 g)	5-CHO-H_4folate (μg/100 g)	Sum of folate[a] (μg/100 g)	Certified[a] value (μg/100 g)
Present study	CRM 121	ND	15	17	NA	34	66 ± 6	50 ± 11
Finglas et al. (1999)	CRM 121	ND	5–10	7–19	0–6	427	NA	50 ± 11
Present study	CRM 485	ND	ND	375	NA	ND	375 ± 16	315 ± 44
Finglas et al. (1999)	CRM 485	ND	5	202-294	ND	ND	NA	315 ± 44
Present study	CRM 487	275	395	482	NA	291	1443 ± 13	1335 ± 198
Finglas et al. (1999)	CRM 487	139	328–467	203–309	36–37	146–400	NA	1335 ± 198

All values in the present study are means of six replicate analyses. Finglas et al. (1999) obtained individual folate values using HPLC using hog kidney as conjugase. Present study obtained individual values using LC–MS/MS and the use of human plasma as conjugase. CRM 121 – wholemeal flour; CRM 485 – lyophilised mixed vegetables; CRM 487 – lyophilised pig liver. NA – Not analysed; ND – not detected.
[a] All values are means ± SD where indicated.

Table 3
Analysed individual folate profile and total folate content (μg/100 g) expressed as mean ± SD in dry weight basis in Indian traditional foods.

Foods	Moisture (%)	Folic acid (μg/100 g)	THF (μg/100 g)	5-MTHF (μg/100 g)	10-Formyl THF (μg/100 g)	5-Formyl THF (μg/100 g)	Total folate[a] (LC–MS/MS) (μg/100 g)	Total folate[b] (MA) (μg/100 g)
Semolina (cooked with selected spices and vegetables) ($n = 3$)	63	ND	ND	345 ± 7	ND	ND	345 ± 7 (128)	262 ± 59 (97)
Cooked legume (cajanus cajun) ($n = 3$)	68	ND	ND	32 ± 11	ND	ND	32 ± 11 (10)	23 ± 8 (7)
Carrot (vegetable matrix) ($n = 3$)	78	ND	ND	88 ± 14	ND	ND	88 ± 14 (19)	92 ± 1 (20)
Milk ($n = 3$)	96	ND	ND	50 ± 7	ND	ND	50 ± 7 (2)	59 ± 1 (2)
Banana (fruit matrix) ($n = 3$)	79	29 ± 19	ND	50 ± 6	ND	ND	79 ± 19 (17)	60 ± 8 (13)

Values in parenthesis indicate total folate content on a fresh weight basis. ND – Not detected; MA – microbiological assay.
[a] Folate values are based on LC–MS/MS data.
[b] Total folate values generated using microbiological assay (Vishnumohan et al., 2009) have been included for comparison.

3.5. Precision and recovery

For examining precision, a vegetable matrix (CRM 485) was analysed which revealed an intra-assay and inter-assay coefficient of variation.

In order to evaluate inter-sample precision, a sample of vegetable matrix was extracted and three repetitive analyses were performed within a time frame of 3 weeks. The sample extract was stored at −80 °C under nitrogen until analysis. The coefficient of variation (CV) was 10.9% which was considered acceptable.

Intra-assay precision was determined repeatedly by extracting aliquots of another vegetable matrix and analysing the extracts as detailed before. For three determinations, the CV was 4.4% which was again acceptable.

Rice was cooked in excess water for an extended period of time to allow for the destruction of any folate that may be present and was analysed to confirm the absence of any folate. The same rice sample was spiked with the five forms of folates before extraction and analysed using the LC–MS/MS. The recoveries obtained for each of the five folates are presented in Table 2.

3.6. Folate content in foods

A number of foods as described in Section 2.3 were chosen to be analysed using the newly developed LC–MS/MS method and compared with the microbiological methods. Table 3 gives the comparison of total folate values obtained using the newer LC–MS/MS method with those generated using a microbiological assay published recently (Vishnumohan et al., 2009). Freisleben et al. (2003b) has reported tetrahydrofolate (4.9–6.7 μg/100 g fresh weight) in raw carrots. This is in contrast to the present study, as we did not detect any traces of tetrahydrofolate in carrots. A possible explanation for this might be that the present study has analysed cooked carrots (sautéed) and cooking could have caused the destruction of the highly unstable tetrahydrofolate. This is supported by the reports of Vahteristo et al. (1997), who found contents as low as 1 μg/100 g fresh weight in cooked carrots (boiled). In contrast to Vahteristo et al. (1997), we could not detect any 5-formyl tetrahydrofolate in the sautéed carrots. However this was in agreement with Freisleben et al. (2003b). It is interesting to note the general dominance of 5-methyl tetrahydrofolate in all the food preparations analysed except banana. These Indian foods were analysed for their folate content for the first time to reflect cooking methods commonly followed by the Indian population. Data from this study were used for a bioavailability study of 5-methyl tetrahydrofolate from typical diets. However differences in the methodology for analysing the native folates, detection and quantitation limits, folate interconversions and method of cooking can contribute to differences in the folate forms identified in the same or similar samples.

A method comparison between LC–MS/MS and microbiological assay (the only AOAC official method for folate analysis) based on identical food samples and identical extracts have never been done before. The strength of the present study is that it has attempted to elucidate the differences between the two methods based on identical extracts. This entailed treating the food extracts with enzymes (tri-enzyme treatment) without the addition of internal standards. The reason for this being, the microbiological assay cannot differentiate between internal standards and the analytes. Therefore the internal standards were added to food extracts prior to purification. However this approach may not have accounted for the losses during the sample preparatory phase prior to purification which is considered to be a limitation in this study.

4. Conclusion

An LC–MS/MS system capable of separating the five main folates was developed and suitable confirmation ions for each vitamer were identified. This new method provides enhanced sample throughput of 36 samples within 12 h when compared to the existing mass spectrometry methods. The method is ideal to quantify simultaneously the different forms of folates in foods in spite of their structural variety and instability. Because of the high selectivity of the SRM mode and the complete correction of losses during sample purification by using isotopically-labelled internal standards, the data generated should be more accurate than the results published previously in literature and will be more useful for bioavailability studies. The method described is a suitable analytical method for determination of food folates.

Safety considerations

General guidelines for work with organic solvents and acids were respected. Mercaptoethanol has been indicated to be toxic by European Union regulatory information.

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