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ORIGINAL ARTICLE

Naturally occurring folates in selected traditionally prepared foods in Southern India

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Abstract A wide range of Indian foods (cereals, pulses, vegetables and milk based preparations) were analysed for five folate vitamers naturally present in the foods (n = 44). A liquid chromatography–mass spectrometry (LC–MS/MS) method using reversed phase chromatography and tandem mass spectrometry, coupled via positive mode electrospray ionization was used for the detection and quantification of the vitamers. The optimized LC–MS/MS method was capable of analysing the five most commonly-occurring folates (folic acid, 5-methyl tetrahydrofolic acid, tetrahydrofolic acid, 10-formyl folic acid and 5-formyl tetrahydrofolic acid) in 20 min. Quantification of folates was performed using ^{13}C labelled internal standards. 5-methyl tetrahydrofolate was predominant in cereals, pulses and vegetable preparations. Fermented cereal preparations, beverages (coffee and tea) and green leafy vegetables were the main sources contributing to 5-formyl THF. Folic acid was identified in home-made yoghurt. All the values obtained in the present study using LC–MS/MS were compared to the total folate analysed using the microbiological assay in 2010 to generate data on the same foods. Findings suggest that the data obtained using both techniques showed agreement in the values (total folate calculated by adding the individual vitamers in the case of the LC–MS/MS values) particularly when foods were predominant in 5 methyl tetrahydrofolate.

Keywords Folates · Liquid chromatography–mass spectrometry · Stable isotopes · Internal standards · Microbiological assay

Abbreviations

LC–MS/MS	Liquid chromatography–tandem mass spectrometry
SRM	Selected reaction monitoring
HPLC	High pressure liquid chromatography
GCMS	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

Introduction

Folates are one of the most nutritionally significant vitamins. Public interest in folate has intensified in recent years due to its function in the maintenance of health and prevention of disease. Folate deficiency has been linked to neural tube defects (NTD) (Czeizel and Dudas 1992), dementia and Alzheimer's disease (Seshadri et al. 2002), Osteoporosis (Gjesdal et al. 2006) depression, Cleft lip/-palate (Krapels et al. 2004), heart disease (Christopherson 1996), breast and colon cancers (Lucock 2000). However,

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the bioavailability of naturally occurring dietary folate is still a matter of nutritional concern and debate since the classic study of Tamura and Stokstad (1973). Assessing folate intake accurately becomes vital because quantity of folates in foods must be known in order to plan diets and study the relationship between bioavailability and nutritional status.

In India, the prevalence estimate of neural tube defects (NTD) in village clusters has been reported to be 6.57–8.21 per 1000 live births, one of the highest in the world (Salvi and Damania 2005) and the population depends solely on their diet to meet their requirements. So the extent to which the naturally occurring folates are absorbed in the general population remains questionable because 1. the values for the folate content of foods in the national food table in India do not reflect the current methods 2. lack of data on processed foods and 3. lack of information on the individual forms of folate naturally present in the foods and therefore the diets. This study was also part of a larger study concerning the bioavailability of 5-methyl tetrahydrofolate from traditional plant based foods consumed by the Indian population which demanded the identification and quantitation of other forms of folate to design control and experimental diets and to limit the effect of other forms on the absorption of the target form (5-MTHF). This is because natural folates differ in their bioavailability and therefore generation of more accurate databases on the folate content of foods with information on the individual folate profile was seen as a high priority to study bioavailability.

Quantification of folate is not straight forward and several methods of folate analysis are currently in use (Arcot and Shrestha 2005; Ringling and Rychlik 2013). The most common method is the microbiological assay method which takes 48–72 h for total folate analyses (Arcot and Shrestha 2005). Coupling HPLC with mass spectrometry is the method used for quantifying individual folate forms in foods (Friesleben et al. 2003). The LC–MS study published on folates in foods by Stokes and Webb (1999) laid the groundwork for all the other LC–MS and LC–MS/MS based studies in folate research. Their study demonstrated the separation of folic acid (FA), tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MTHF) and 5-formyl tetrahydrofolate (5-formyl THF) using the LC–MS. However, the study did not control for losses of more labile folate forms during sample processing, as they did not use internal standards. Consequently only folic acid was detected and was successfully quantified in multivitamin tablets, breakfast cereals and beef/vegetable extracts. Pawlosky and Flanagan (2001) were subsequently able to quantify both FA and/or 5-MTHF in fortified cereals, fortified breads, citrus juices, vegetable, fruits and reference materials using internal standards ($^{13}C_5$-FA and $^{13}C_5$-5MTHF) stable isotope LC–MS technique. Friesleben et al. (2003) used deuterated standards synthesized in-house and demonstrated the determination of THF, 5-formyl THF and 10 formyl FA in addition to FA and 5-MTHF in a variety of vegetables, cereals, meats, juices, breads, rice and very recently legumes. Several investigators subsequently reported using the LC–MS/MS method for the determination of FA in fortified and non-fortified pasta; 5-MTHF, 5-formyl THF and 10 formyl FA in green and red pepper extracts (Leporati et al. 2005; Phillips et al. 2006) developed a non-isotope dilution LC–MS/MS technique to detect and quantitate 5-MTHF, 5-formyl THF and 10 formyl FA in green and red pepper extracts. Ringling and Rychlik (2013) demonstrated an LC–MS/MS method using deuterated internal standards to include 10-formyl dihydrofolate and 5,10-methenyl tetrahydrofolate in the analysis.

Method comparisons reported so far are mixed when concerning concentrations determined using LC–MS/MS and microbiological assays (Rychlik et al. 2003; Rychlik 2004). The microbiological assay (MA) values in such studies were derived from literature. This has many limitations, such as differences between samples, sample treatments, deconjugation steps and extraction techniques. This approach has raised a serious question—are these discrepancies in values due to sample variability or due to methodological bias? Despite the fact that the microbiological assay is extremely laborious and provides only a sum of the individual folate forms, it remains the method of choice for determination of food folates due to its high sensitivity.

However, comparison to an accepted standard reference method is crucial to assess the performance of these methodologies.

The application of LC–MS/MS on complex food matrices which were processed as found in Indian foods was attempted in the present study. This study reports folate profile (quantified) of cooked/processed foods (using standardized recipes) of Indian origin with a wide variety of matrices using the developed LC–MS/MS method published earlier (Vishnumohan et al. 2011). This is the first time that individual vitamers for processed Indian foods is being analysed and reported.

In addition, in this study, the analysis was performed by LC–MS/MS and microbiological assay on the same samples using the same folate extraction techniques, permitting a more meaningful comparison of methodologies.

The overall objective of the present study was to quantify the different forms of folate within 20 min using an LC–MS/MS method in a wide variety of complex food matrices covering a wide range of cereal, legume, vegetable and milk based preparations.

Materials and methods

Folate compounds

Folic acid, 5-methyl tetrahydrofolic acid, 5-formyl tetrahydrofolic acid, 10-formyl folic acid and tetrahydrofolic acid were obtained from Schircks Laboratories, Jona, Switzerland.

All reagents were of analytical grade. MilliQ water used had a conductivity of 0.1 Ω.

Folate standard solutions

Stock solutions (1 mg/mL) were prepared by dissolving the folate standards in 0.05 M HEPES-CHES buffer, pH 7.85 [containing 2% (w/w) sodium ascorbate and 0.01 M 2-mercaptoethanol] (Friesleben et al. 2003). 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 the analysis to minimize the freeze thaw cycles. All samples were extracted fresh on the day of analysis and stored at −18 °C for a maximum period of 1 week. If re-analysis of the sample was required after 1 week storage, fresh sample extraction was performed.

Isotopically labelled internal standards

The LC–MS/MS method described in this paper involves the use of commercially-available carbon (C^{13}) labelled isotopomers of the folates as internal standards purchased from Eprova, Switzerland. The only commercially-unavailable labelled vitamer (10-formyl folic acid) 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. Purity was confirmed using LC–MS/MS method published by Vishnumohan et al. (2011). Isotopically-labeled standards (10 µg/mL) were prepared in 0.05 M HEPES-CHES buffer, pH 7.85 [containing 2% (by mass) sodium ascorbate and 0.01 M 2-mercaptoethanol] and were stored under nitrogen at −80 °C. Internal standards used in this study were labelled by incorporating ^{13}C atoms in the folate molecule. The ^{13}C atoms occupied each of the carbons on the glutamic acid portion of the molecule, resulting in a molecular weight of five Daltons greater than the unlabelled counterpart.

Food samples

Indian cuisine varies widely across the country according to the region, culture and tradition, characterized by the use of different spices, vegetables, grains, fruits and a variety of animal source foods. Analyzing all the foods that are consumed in the country is not feasible due to the prohibitive cost involved and thus it was essential to prioritize foods for folate analysis. Foods were chosen for the analysis based on a 24-h dietary recall survey and a food frequency questionnaire filled by 200 respondents Forty-four foods/preparations were selected and categorized under different food groups. The ingredients for the food samples analyzed were purchased from a local supermarket in the city of Coimbatore, India. Food preparation was carried out in the Food Science laboratory at the Department of Nutrition, PSG Arts and Science College, Coimbatore, India. All the recipes were standardized and reproduced at least twice. The following were analyzed for their total folate contents (Vishnumohan et al. 2009) and naturally occurring folates using the LC–MS method (Vishnumohan et al. 2011).

Rice based preparations: cooked rice, lemon rice, tomato rice, vegetable *biriyani*, *Bhelpuri, dosa* and onion *dosa*, *pongal*, *uppuma*, wheat based preparations: *chapathi*, *poori*, noodles; legume based preparations: *vada*, *adai*, onion *sambhar*, thick *dhal*, split chickpea and sprouted mungbean *sundal*, *thuvayal*, tomato *rasam*, *pappad;* vegetable based preparations: sauted vegetables (carrot, cabbage, bitter gourd, boiled potato and beetroot sauteed in oil and spices individually for analysis), chutneys (savoury), *snake gourd kootu;* milk based preparations/beverages: coffee, tea, yoghurt, *Morkolambu*, vermicelli *payasam* and milk.

All recipes were prepared in duplicate according to the traditional methods followed by consumers and described in detail in Vishnumohan et al. (2009).

Prepared foods were individually homogenized in a domestic kitchen blender. Composite samples of the same foods were prepared and all food samples were freeze dried (for transportation purposes) immediately and kept in sealed moisture proof aluminium coated bags. Samples were then transported to Sydney, Australia by air to perform the LC–MS/MS analysis.

Sample extraction

Sample extraction was carried out in subdued light, with all glassware wrapped in aluminium foil. To 0.5–1 g of freeze-dried sample were 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 until the sample was completely

dispersed in the buffer. The homogenate was placed in a water bath at 100 °C for 10 min, then immediately cooled.

Tri-enzyme treatment and deconjugation

The food extracts were treated with three enzymes: protease, α-amylase and conjugase purchased as follows: α-amylase (A-3176, Sigma Chemical Co., St. Louis, MO 63178) (20 mg/mL), protease (Megazyme, subtilisin A from *B. licheniformis*) (2 mg/mL) and human plasma (Red cross society, Prince of Wales Hospital, Sydney Australia) for LC–MS/MS analysis. The enzymes were prepared following the method by Rader et al. (1998). The sample mixture was treated with enzymes sequentially according to Shrestha et al. (2000). 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 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 and further treated with 1.6 mL of α-amylase for 4 h at 37 °C. The pH of the enzyme-hydrolyzed extract was adjusted to 7.2 using 1 M NaOH. 10 mL of the hydrolyzed extract was treated with 1.0 mL of conjugase and incubated for 3 h at 37 °C. The deconjugated mixture 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 −18 °C until further analysis.

Sample purification for LC–MS/MS analysis

Purification was carried out using a reversed phase C18 solid phase extraction with a strong anion exchange cartridge (SAX) (3 mL/500 mg of quaternary amine) as suggested by Freisleben et al. (2003) 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 phosphate ascorbate buffer (conditioning solution): (pH 7.0, 0.01 mol/L, 1.36 g of anhydrous dipotassium hydrogen orthophosphate, 0.4 g anhydrous potassium dihydrogen orthophosphate, 1 g ascorbic acid and 0.1 mL mercaptoethanol were dissolved in 1 L milliQ water). 3 mL 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 in water). The eluate was reduced to dryness using a vacuum centrifuge and re-suspended in 100 µL of 0.1% formic acid for analysis with LC–MS/MS.

Folate analysis

LC–MS/MS instrumentation and analysis of folates

The LC–MS/system used in this study consisted of a ThermoFisher Scientific Surveyor LC and autosampler coupled directly to a ThermoFisher Scientific LCQ Deca XP Plus ion trap mass spectrometer via an electrospray interface. LC was performed on a C18 reversed phase column (Zorbax Eclipse, 5 micron, 2.1 mm by 150 mm). An earlier publication by Vishnumohan et al. (2011) describes the details for folate analysis using LC–MS/MS and quality control measures.

Data calculation

Quantification of the food folates was performed using the responses (peak areas) obtained from the analytes and the internal standards. The standards and samples were spiked with isotopically-labelled standards (100 ng/injection) before the purification procedure and were taken through the described procedure for quantitation. The mass of the analyte (a) in the sample was calculated using the formula below:

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

Results and discussion

Folate content of food samples

The folate content of the food preparations analysed using LC–MS/MS are presented in Table 1. The results obtained for the individual vitamers using LC–MS/MS were summed up and compared with the analyzed total folate data generated using the microbiological assay published by Vishnumohan et al. (2009).

Analysed folate forms in foods using LC–MS/MS

The food preparations were analysed using LC–MS/MS to obtain data on the individual folate forms. It has been shown previously, using HPLC, that 5-methyl THF was the major form in fruits and vegetables (Vahteristo et al. 1997a, b). Our study showed similar results. Green leafy vegetables analyzed in the study [Amaranth (*Amaranthus*) and Agathi (*Sesbania grandiflora*) leafy vegetable preparations] were an exception, containing 5-formyl THF as the predominant form in addition to 5-methyl tetrahydrofolate (5-MTHF). 5-formyl THF

Table 1 Analysed mean individual folate vitamers and total folate content (μg/100 g) in Indian traditional foods using the LC–MS/MS method

Food item	Moisture (%)	Folic acid (μg/100 g)	THF (μg/100 g)	5-Methyl THF (μg/100 g)	5-Formyl THF (μg/100 g)	Total folate using LCMS (μg/100 g)[d]
Cereal based preparations						
Cooked rice (raw rice, boiled)	78	nd	nd	nd	nd	0(0)
Cooked rice (parboiled rice, pressure cooked)[f]	75	nd	nd	nq	nd	nq
Lemon rice[f]	57	nd	nd	nq	nd	nq
Tomato rice[f]	72	nd	nd	nq	nd	nq
Idli (commercial batter)[f]	73	nd	nd	nq	nd	nq
Idli (home made batter)[b]	65	nd	nd	nq	221 ± 5	221 ± 5 (77)
Dosa (commercial batter)[f]	58	nd	nd	nq	nd	nq
Dosa (home made batter)[b]	45	nd	nd	nq	816 ± 5	816 ± 5 (449)
Onion dosa[b]	59	nd	nd	nq	253 ± 4	253 ± 4 (104)
Pongal	74	nd	nd	nd	nd	0 (0)
Uppuma[a]	63	nd	nd	345 ± 7	nd	345 ± 7(128)
Chappathi[a]	31	nd	nd	44 ± 5	nd	44 ± 5 (30)
Poori[e]	21	nd	nd	81 ± 4	nd	81 ± 4 (64)
Vegetable biriyani[a]	77	nd	nd	45 ± 13	nd	45 ± 13 (10)
Noodles[a]	78	nd	nd	57 ± 20	nd	57 ± 20 (13)
Bhelpuri[a]	73	nd	nd	37 ± 6	nd	37 ± 6 (10)
Pulse based preparations						
Vada[e]	40	nd	nd	211 ± 11	nd	211 ± 11 (127)
Adai[a]	53	nd	nd	92 ± 10	nd	92 ± 10 (43)
Onion sambhar[a]	84	nd	nd	116 ± 8	nd	116 ± 8 (19)
Thick dhal[a]	68	nd	nd	32 ± 11	nd	32 ± 11 (10)
Bengal gram sundal[a]	59	nd	nd	42 ± 5	86 ± 18	128 ± 15 (52)
Sprouted greengram[b]	41	nd	nd	141 + 9	109 ± 7	250 + 9 (148)
Thovayal[a]	49	nd	nd	58 ± 4	nd	58 ± 4 (30)
Pappad[e]	5	nd	nd	250 ± 21	nd	250 ± 21 (237)
Vegetable based preparations						
Amaranth porriyal[b]	89	nd	nd	185 ± 5	692 ± 5	877 ± 4 (96)
Agathi porriyal[b]	81	nd	nd	167 ± 3	548 ± 9	715 ± 7 (136)
Green beans porriyal[b]	79	nd	nd	81 ± 4	nq	81 ± 4 (17)
Drumstick leaves porriyal[b]	62	nd	nd	72 ± 4	nq	72 ± 4 (27)
Cabbage porriyal[a]	74	94 ± 7	nd	83 ± 6	nd	177 ± 1 (46)
Potato masala[a]	77	nd	nd	74 ± 10	nd	74 ± 10 (17)
Carrot porriyal[a]	78	nd	nd	88 ± 14	nd	88 ± 14 (19)
Fried bittergourd[a]	75	nd	nd	130 ± 1	nd	130 ± 1 (33)
Corriander chutney[b]	79	nd	nd	261 ± 3	356 ± 8	617 ± 3 (130)
Coconut chutney[b]	66	nd	nd	70 ± 11	290 ± 17	360 ± 12 (122)
Tomato chutney[a]	78	nd	nd	156 ± 6	nd	156 ± 6 (34)
Tomato rasam[f]	97	nd	nd	nq	nd	nq
Snake gourd kootu[a]	77	66 ± 1	nd	172 ± 1	nd	238 ± 1 (55)
Beetroot porriyal[a]	76	nd	nd	57 ± 12	nd	57 ± 12 (14)
Milk and milk products						
Milk[a]	96	nd	nd	50 ± 7	nd	50 ± 7(2)
Coffee[b]	91	nd	nd	nd	325 ± 1	325 ± 1 (29)
Tea[f]	92	nd	nd	nd	nq	nq
Home-made yoghurt[c]	86	25 ± 15	nd	nd	164 ± 17	189 ± 6 (26)
Morkozhambu[b]	85	nd	nd	nd	177 ± 6	177 ± 6 (27)

Table 1 continued

Food item	Moisture (%)	Folic acid (μg/100 g)	THF (μg/100 g)	5-Methyl THF (μg/100 g)	5-Formyl THF (μg/100 g)	Total folate using LCMS (μg/100 g)[d]
Vermicellipayasam[a]	72	nd	70 ± 5	nd	nd	70 ± 5 (20)

Values are mean ± SD of triplicate determinations expressed on a dry weight basis

10 Formyl Folic acid not detected in the samples

The values in parentheses indicate the total folate on a fresh weight basis

nd not detected, *nq* non quantifiable

[a]Foods predominant in 5-methyl tetrahydrofolate

[b]Foods predominant in 5-formyl tetrahydrofolate

[c]Foods predominant in folic acid

[d]Individual vitamers added to provide one single total value

[e]Foods rich in fat

[f]Not detectable

together with 5-MTHF, 10-formyl folic acid and folic acid were identified in cereals (Pfeiffer et al. 1997) whereas our study found only 5-MTHF in most of the cereal preparations (cooked parboiled rice, *chapatti*, *poori*, *uppuma*, noodles, *bhelpuri*, vegetable *biriyani*) with exceptions being home-made *dosa* and *idli* showing 5-formyl THF to be the predominant form, probably contributed by the black gram (*Vigna mungo*) dhal added to the preparation. Earlier studies on legumes have shown that 5-MTHF was the predominant form in soyabeans (Shin et al. 1975) and lima beans (Seyoum and Selhub 1993). Differences in the methodology for analyzing the native folates, detection and quantitation limits can contribute to differences in the folate forms identified in the same samples. For example, different forms of folate have been reported in fermented milk products. Muller (1993) found yoghurt and buttermilk to contain approximately 80–90% of the total folate appearing in the form of 5-formyl THF using HPLC. Vahteristo et al. (1997a, b) reported THF, 5-methyl THF and 5-Formyl THF in buttermilk and plain yoghurt with 5-formyl THF as the predominant form. This is likely due to the differences in the preparation of yoghurt. Wigertz and Jaegerstad (1995) found no 5-formyl THF using HPLC-FD, as the quantification was limited by the low fluorescence activity of this form. In the present study 5-formyl THF is reported to be the predominant form in the homemade natural yoghurt analyzed. The difference in the folate forms might also be due to use of different bacterial cultures during fermentation.

Comparison of total folate values obtained using the LC–MS/MS method with those generated using a microbiological assay

The original samples were prepared in duplicates to reduce the chance of sample variation and any variation in the data obtained was directly accounted for by methodological issues only unlike other studies that compared existing data. To obtain accurate food folate data, the foods were analysed using the optimized LC–MS/MS method as described by Vishnumohan et al. (2011). The results obtained for the individual vitamers using LC–MS/MS were summed up and compared with the analysed total folate data generated using microbiological assay. One half was assayed using the microbiological assay. The sum of individual vitamers obtained using the LC–MS/MS method was compared against the total folate values generated using microbiological assay. Comparable quantitative results were obtained between the techniques particularly for foods predominant in 5-MTHF as seen in Table 1 supporting the assumption that the microorganism responds to 5-MTHF present in food samples efficiently. This study comparing the folate values determined chromatographically and microbiologically revealed a remarkable increase in folate content up to 80% in fried food preparations like poori, vada and Pappad (refer to Table 1 for samples with superscript e). This study suggests that fat might interfere with the uptake of food folates by the microorganism providing leads for further study. The LC–MS/MS method showed higher total folate data for foods predominant in 5-formyl THF (refer to Table 1 for samples with superscript b). The percentage increase in the total folate content of 5-formyl THF predominant foods was between 55 and 70% for the cereal based preparations (homemade *idli*, *dosa* and onion *dosa*). Interestingly the above cereal preparations did not show the presence of 5-formyl THF when prepared using the commercially available batter. The reason could be that the commercial preparations may contain less amount of the folate rich black gram dhal (more expensive) for increasing the profit margin. A 40% increase in the total folate content was obtained for the pulse based preparation (sprouted mung bean (*Vigna radiata*) sundal), 50–57% for green leafy vegetables (coriander chutney, amaranth and *agathi*) with the exception

being drumstick (*Moringa oleifera*) leaves *preparation* as the 5-methyl THF could not be quantified due to matrix interference and 70% for coconut chutney. 26% increase in total folate content was seen in milk-based products (coffee and curd) showing a 46% increase for *morkolambu* (buttermilk based preparation) when analysed using LC–MS/MS. The total folate content obtained from LC–MS/MS was 1.4–3.5 times higher in foods predominant in 5-formyl tetrahydrofolate than the values obtained from microbiological assay with a maximal percentage increase up to 70% in their total folate contents. To date there is no study supporting the use of folic acid as a calibrant for foods predominant in 5-formyl THF. O'Broin et al. (1975) reported that *L. casei* gave essentially the same response to 5-methyl THF (5-MTHF) and 5-formyl THF, while Newman and Tsai (1986) reported slight differences in the molar response of the *L. casei* to folic acid, 5-methyl THF and 5-formyl THF. The implications of the choice of the calibrant in Newman and Tsai's study used in the microbiological assay are unclear and contradictory to date.

Conclusion

The LC–MS/MS method optimized and used in the present study was specific enough to quantify the different forms of folates in individual food preparations covering varied matrices—cereal, pulse, vegetable and milk based preparations. The National Institute of Nutrition in India has recently released the updated Indian Food Composition database incorporating the data on total folate using trienzyme procedure and liquid chromatography for raw foods. The present study makes a significant contribution to the Indian food folate composition database by complementing it with the folate data for cooked Indian foods. The study reveals that Indian diets are predominant in 5-MTHF and the important sources being cereals, pulses and vegetables. Fermented cereal preparations (Idli and Dosa), beverages like coffee and tea and green leafy vegetables were the main sources contributing to 5-formyl THF. Folic acid was indentified in the fermented milk products (Homemade yoghurt). However, in order to judge the good and poor sources of folates, we need to have validated figures on the absorption and bioavailability of food folates. Increasing the knowledge base around the different forms of folate in mixed traditional diets such as in this study is necessary to study bioavailability in populations which solely depend on food folate but also to establish recommendations of folate intake for the respective populations.

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References

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

Christopherson RI (1996) How folate functions inside the body. Aust J Nutr Diet 53:s8–s10

Czeizel JF, Dudas J (1992) Prevention of first occurence of neural tube defects by periconceptional vitamin supplementation. N Engl J Med 327:1832–1835

Friesleben A, Schieberle P, Rychlik M (2003) Specific and sensitive quantification of folate vitamers in foods by stable isotope dilution assays using high performance liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 376:149–156

Gjesdal C, Vollset S, Ueland P, Refsum H, Drevon C, Gjessing H, Tell G (2006) Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 166:88–94

Krapels IPC, van Rooij IALM, Ocke MC, West CE, van der Horst CMAM, Steegers-Theunissen RPM (2004) Maternal nutritional status and the risk of orofacial cleft offspring in humans. J Nutr 134:3106–3113

Leporati A, Catellani D, Suman M, Andreoli R, Manini P, Niessen WM (2005) Application of a liquid chromatography tandem mass spectrometry method to the analysis of water-soluble vitamins in Italian pasta. Anal Chim Acta 531:87–95

Lucock M (2000) Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab 71:121–138

Muller H (1993) The determination of the folic acid content of foods of animal origin using high performance liquid chromatography (HPLC). Z Lebensm-Unters Forsch 196:518–521

Newman EM, Tsai JF (1986) Microbiological analysis of 5-formyl-tetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem 154:509–515

O'Broin JD, Temperley IJ, Brown JP, Scott JM (1975) Nutritional stability of various naturally occurring monoglutamate derivatives of folic acid. Am J Clin Nutr 28:438–444

Pawlosky R, Flanagan V (2001) A quantitative stable-isotope LC–MS method for the determination of folic acid in fortified foods. J Agric Food Chem 49:1282–1286

Pfeiffer CM, Rogers LM, Gregory JF III (1997) Determination of folate in cereal-grain food products using tri-enzyme extraction and combined affinity and reverse-phase liquid chromatography. J Agric Food Chem 45:407–413

Phillips K, Ruggio D, Ashraf-Khorasanni M, Haytowitz D (2006) Development of accurate and representative food composition data for the US food supply. J Agric Food Chem 54:9998

Rader JI, Weaver CM, Angyal G (1998) Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products. Food Chem 62:451–465

Ringling C, Rychlik M (2013) Analysis of seven folates in food by LC–MS/MS to improve accuracy of total folate data. Eur Food Res Technol 236:17–28

Rychlik M (2004) Revised folate content of foods determined by stable isotope dilution assays. J Food Compos Anal 17(3–4):475–483

Rychlik M, Netzel M, Pfannebecker I, Frank T, Bitsch I (2003) Application of stable isotope dilution assays based on liquid chromatography–tandem mass spectrometry for the assessment

of folate bioavailability. J Chromatogr B Analyt Technol Biomed Life Sci 792(2):167–176

Salvi V, Damania K (2005) Neural tube defects in India—time for action. Lancet 366:871–872

Seshadri S, Beiser A, Selhub J, Jaques PF, Rosenberg IH, D'Agostino RB, Wilson PWF, Wolf PA (2002) Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 346:476–483

Seyoum E, Selhub J (1993) Combined affinity and ion pair column chromatography for the analysis of food folate. J Nutr Biochem 4:488–494

Shin YS, Kim ES, Watson JE, Stokstad ELR (1975) Folic acid compounds in nature. IV. Folic acid compounds in soybeans and cow milk. Can J Biochem 53:338–343

Shrestha AK, Arcot J, Paterson J (2000) Folate assay of foods by traditional and tri-enzyme treatments using cryoprotected *Lactobacillus casei*. Food Chem 71:545–552

Stokes P, Webb K (1999) Analysis of some folate monoglutamates by high-performance liquid chromatography–mass spectrometry. I. J Chromatogr A 864:59–67

Tamura T, Stokstad ELR (1973) The availability of food folate in man. Br J Haematol 25:513–532

Vahteristo L, Lehikoinen K, Ollilainen V, Varo P (1997a) 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

Vahteristo LT, Ollilainen V, Varo P (1997b) Liquid chromatographic determination of folate monoglutamates in fish, meat, egg, and dairy products consumed in Finland. J AOAC Int 80:373–378

Vishnumohan S, Arcot J, Sini S, Uthira L, Ramachandran S (2009) Determination of folate contents in selected Indian foods using the tri-enzyme extraction and estimated folate intakes of the population based on 24-h recall. Int J Food Sci Nutr 60:170–180

Vishnumohan S, Arcot J, Pickford R (2011) Naturally-occurring folates in foods: method development and analysis using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Food Chem 125:736–742

Wigertz K, Jaegerstad M (1995) Comparison of a HPLC and radioprotein-binding assay for the determination of folates in milk and blood samples. Food Chem 54:429–436