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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

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Abstract
The prevalence estimate of neural tube defects in India is restricted to some village clusters and has been reported to be 6.57–8.21 per 1,000 live births, one of the highest in the world. Dietary intake data for folate are scant and do not provide an accurate estimate because of the lack of analysed data in commonly consumed cooked/processed foods. A 24-h dietary recall survey of 200 respondents aged 17–24 years in one of the Southern states of India was used to prioritize the common foods consumed. Forty-three foods/preparations were analysed for their total folate content using the tri-enzyme extraction technique and microbiological assay using *Lactobacillus casei*, subsp. *Rhamnosus*. The mean dietary intake of the selected population was estimated to be 277 μg/day based on the analytical results obtained through this study. This value is relatively higher (2.8 times) than the calculated values reported on the intakes of the rural population (98 μg/day) reported by the National Nutrition Monitoring Bureau, India.

Keywords: *Microbiological assay, neural tube defects, tri-enzyme extraction, total folate*

Introduction

Folate is a water-soluble B vitamin with several health benefits, such as prevention of neural tube defects (NTDs) in the newborn, cardiovascular diseases and certain forms of cancer (Selhub 2002). In India, the prevalence estimate of NTDs in the village clusters has been reported to be 6.57–8.21 per 1,000 live births (Salvi and Damania 2005).

The National Nutrition Monitoring Bureau (2000) from the National Institute of Nutrition in India has reported that the folate intake of the rural population was only 98 μg/day.

Folate intake recommendations in India vary under different physiological conditions. An intake of 400 μg is required for women planning to become pregnant, 4000 μg for those with a history of NTD-affected pregnancy; 350 μg is required to maintain plasma homocysteine levels, and 650 μg for those with elevated plasma

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homocysteine (Krishnaswamy and Nair 2001). The Indian Council of Medical Research committee has recommended 100 μg folic acid as the Recommended Dietary Intake for adults. Whether the folate intake is adequate in the general population as well as for the vulnerable group remains unanswered as these recommendations are based on literature evidence, which have been criticized to underestimate the folate content (Life Sciences Research Office 1995).

Folate fortification in several western countries is being practiced either on a voluntary or mandatory basis. India is currently considering fortification of foods with folate, with a concern that this should be at a level high enough to improve the nutritional status of the population (Salvi and Damania 2005). The folate fortification approach needs careful consideration for feasibility, sustainability and effectiveness in India. The current folate intake needs to be re-assessed to gauge the level of folate deficiency prior to fortification. In order to implement folate fortification effectively, short-term studies on the effects of consuming a single fortified food on folate status is insufficient since they do not take into account the amount of folate intake through the consumption of a wide range of foods on a long-term basis. Therefore there is a need for a critical evaluation of all dietary folate sources, which are the predominant contributors of folate intake in India. To date, there are no analytical data for folate contents in cooked/processed foods as consumed by the population in any food composition database that is currently available in India. Improved methods for total folate determination using the microbiological assay include a tri-enzyme technique (AOAC 2007; AACC 2008).

This study was aimed at developing a folate composition database of traditional Indian foods/preparations using the tri-enzyme technique and the microbiological assay, with cooked foods as the priority since cooking is one of the identified steps that can cause significant loss of folate (Vahteristo et al. 1996). Although economic growth has influenced the traditional eating patterns, a majority of the population still spend more than one-half of their income on foods consumed at home. Folate data for a total of 42 traditional South Indian food preparations, including six milk-based beverages and preparations, are reported in this study.

Materials and methods

Selection of foods

Foods were prioritized for the analysis based on a 24-h dietary recall completed by 200 respondents (data not shown). Forty-three foods/preparations were selected and categorized under different food groups. The ingredients for the food samples analysed were purchased from a local supermarket, and the food preparation was carried out in the Food Science laboratory at the Department of Nutrition, PSG Arts and Science College, Coimbatore, India. All of the recipes were standardized in the laboratory and prepared based on traditional methods followed by consumers in duplicate (preparation methods not shown here).

The following were analysed for their total folate contents using the microbiological assay.

- *Rice-based preparations*: cooked plain rice, lemon rice (cooked rice mixed with lemon juice), tomato rice (rice cooked with tomatoes and spices), vegetable *biriyani* (rice cooked with seasonal vegetables and spices), *Bhelpuri* (spicy puffed

rice-based preparation), fermented foods made from black gram *dhal* and rice in the ratio of 1:4, *idli* (steamed rice cakes), *dosa* and onion *dosa* (thin rice crepe), *pongal* (soft food made from roasted split mung bean and raw rice), and *uppuma* (semolina cooked with spices and selected vegetables).

- *Wheat-based preparations*: *chapathi* (cooked flatbread), *poori* (deep fried wheat bread), and noodles.
- *Legume-based preparations*: *vada* (a fried savory made from *split chickpea*), *adai* (thick south Indian crepe made from flour of split pigeon pea, split chickpea and rice), onion *sambhar* (thick soup made of split pigeon pea, onion and spices), thick *dhal* (thick soup made out of split pigeon pea), split chickpea and sprouted mung bean *sundal* (tea-time snack prepared by shallow frying sprouted mung bean, seasoned with added salt), *thuvayal* (roasted split pigeon pea ground to a paste with tamarind, salt and spices), tomato *rasam* (thin soup made out of split pigeon pea, tamarind and spices), and *pappad* (preserved black gram *dhal* crisp, deep fried).
- *Vegetable-based preparations*: *porriyal* (vegetables—carrot, cabbage, bitter gourd, boiled potato and beetroot—sauteed in oil, salt and spices individually for analysis), chutney (smooth vegetable-based paste), and *snake gourd kootu* (a vegetable stew cooked with split mung bean).
- *Milk-based preparations/beverages*: coffee (made with milk, sugar and filtered coffee), tea (made by steeping tea leaves in boiling water and adding milk and sugar to it), home-made yoghurt (made by adding a tablespoon of butter milk to 1 cup of milk and fermented at room temperature, 25–30°C), *Morkozhambu* (buttermilk preparation cooked with vegetable and coconut), vermicelli *payasam* (a milk-based sweet preparation made with vermicelli, sugar and nuts and raisins), and whole milk.

All recipes were prepared according to standard traditional recipes (not shown here) used by consumers.

Sample preparation and extraction

The foods were prepared according to the standardized recipes in duplicate. Prepared foods were homogenized individually in a domestic kitchen blender and composite samples were prepared and stored at −20°C until further analysis. Sample extraction was carried out in subdued light and all the glassware were wrapped with aluminium foil. Five grams of each homogenate was mixed with 100 ml extraction buffer (0.1 M potassium phosphate, 1% ascorbic acid and pH 6.1) and heated at 100°C for 10 min.

Moisture determination

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

Tri-enzyme treatment and deconjugation

The sample from the heat treatment step was treated with three enzymes, protease, α-amylase and chicken pancreas, and the extraction method was based on Tamura et al. (1997) and AOAC (2007) with slight modification where the centrifugation step

was eliminated before the tri-enzyme treatment and the final enzyme-treated samples were centrifuged (Beckman J2-MC with JA 14 fixed angle rotator; Beckman Instruments, Inc., Paulo Alto, CA, USA) at 10,000 rpm for 15 min at 4°C and filtered, if necessary. This is based on the rationale that the removal of undigested food residues by centrifugation before enzyme treatment would lead to entrapment of folates resulting in underestimation of the vitamin content.

The supernatant was transferred into small brown bottles and stored at −18°C. The extract was directly used for determination of total folate content of foods.

Innoculum preparation

Glycerol cryoprotected *Lactobacillus casei* subsp Rhamnosus (ATCC 7469) was prepared according to Shrestha et al. (2000).

Folate assay

Folic acid (F-7876; Schircks Laboratories, Jona, Switzerland) standard solutions, preparation of standards and samples and the assay were carried out as previously described. The samples were analysed in triplicate.

Quality control

The certified reference materials (CRM 121, wholemeal flour; and CRM 485, lyophilized vegetable mix) obtained from Schircks Laboratories (Jona, Switzerland) were analysed for quality control. Recovery studies were also carried out by spiking 0.2 ng folic acid into the sample tubes. Recovery of added folic acid was calculated as:

$$\% \text{ recovery} = [(\text{ng folic acid in spiked sample tube} - \text{ng folic acid in unspiked sample tube}) / \text{ng folic acid in spiked sample}] \times 100$$

The assays with a percentage recovery of added folic acid beyond the range of 95–105% were not accepted and the values were discarded.

Results and discussion

Total folate content of foods

The final folate content of foods seemed largely dependent on the conditions of food preparation. Tables I, II, III and IV indicate the folate content in various Indian food preparations using the microbiological assay with tri-enzyme extraction.

Table I presents the total folate contents in 16 common cereal-based preparations using tri-enzyme extraction. The total folate content of cereal-based preparations was found to vary from 1 to 201 μg/100 g on a fresh weight basis. *Dosa* prepared from home-made batter was found to contain the highest amount of total folate, whereas *dosa* prepared from commercial batter contained the least. The same trend was observed with *idli*. In contrast to other processing conditions, fermentation showed a positive impact on concentration of folate in foods. Traditionally, black gram *dhal* (*Phaseolus mungoroxb*) and rice are soaked for a couple of hours, and these two ingredients are ground to a fine paste and left to ferment overnight to make the batter

Table I. Folate content (μg/100 g) in cereal-based preparations.

Food item	Moisture (%)	Total folate
Cooked rice (raw rice, boiled)	78	0
Cooked rice (parboiled rice, pressure cooked)	75	35±3 (140)
Lemon rice	57	26±4 (60)
Tomato rice	72	29±3 (68)
Idli (commercial batter)	73	1±4 (5)
Idli (home-made batter)	65	24±2 (68)
Dosa (commercial batter)	58	1±1(3)
Dosa (home-made batter)	45	201±3 (365)
Onion *dosa*	59	16±9 (38)
Pongal	74	0
Uppuma	63	97±59 (262)
Chapathi	31	29±2 (42)
Poori	21	13±4 (16)
Vegetable *biriyani*	77	12±12 (54)
Noodles	78	13±15 (54)
Bhelpuri	73	6±4 (20)

Data presented as mean ± standard deviation of triplicate determinations expressed on a fresh weight basis. Data in parentheses indicate the total folate on a dry weight basis.

for *idli* and *dosa*. The fermentation is caused by air-borne wild yeast extremely rich in folate, contributing to the increase (Desikachar et al. 1960). Similar studies with breads and *tempe* have reported twofold to fourfold higher folate values due to the fermentation process (Murata et al. 1970; Hawkes and Villota 1989; Arcot et al. 2002). Black gram *dhal*, an important component of both *idli* and *dosa* batters, is a good source of folate (132 μg/100 g in the raw) (Gopalan et al. 2002). The home-made batters contain rice and black gram *dhal* in the proportion of 2:1 for *idli* and 4:1 for *dosa*. The commercial batters contained no black gram *dhal* or was present in lower proportions than usual, contributing to the differences in the total folate levels.

Cooked polished white rice (*Oryza sativa*), which is the staple cereal in South India, did not contain any folate—possibly due to leaching when washed and cooked in water. Comparatively higher values were seen for lemon rice and tomato rice, which can be attributed to the antioxidant effect of ascorbic acid (from lemons and tomatoes) in the foods analysed and their natural folate contents. Similar reports on other foods containing ascorbic acid have been reported (Day and Gregory 1983). Parboiled rice analysed was found to have a value of 35±3 μg on a fresh weight basis/

Table II. Folate content (μg/100 g) in legume-based preparations.

Food item	Moisture (%)	Total folate
Vada	40	25±2 (42)
Adai	53	38±12 (81)
Onion *sambhar*	84	15±2 (95)
Thick *dhal*	68	7±8 (23)
Chickpea *sundal* (*Cicer arietinum*)	59	55±4 (135)
Sprouted mung bean *sundal* (*Vigna radiata*)	41	89±15 (151)
Thovayal	49	35±16 (68)

Data presented as mean ± standard deviation of triplicate determinations expressed on a fresh weight basis. Data in parentheses indicate the total folate on a dry weight basis.

Table III. Folate content (μg/100 g) in vegetable-based preparations and fruit.

Food item	Moisture (%)	Total folate
Amaranth *Porriyal* (*Amaranthus gangeticus*)	89	49±2 (442)
Agathi *Porriyal* (*Sesbania grandiflora*)	81	58±19 (305)
Green beans (*Phaseolus vulgaris*)	79	45±10 (212)
Drumstick leaves porriyal (*Moringa oleifera*)	62	86±1 (225)
Cabbage *Porriyal* (*Brassica Oleracea*)	74	37±1 (143)
Curried Potato (*Solanum tuberosum*)	77	23±2 (98)
Carrot *Porriyal* (*Daucus carota*)	78	20±1(92)
Fried Bittergourd (*Momordica charantia*)	75	38±5 (152)
Coriander chutney (*Coriandrum sativum*)	79	65±1 (310)
Coconut chutney (*cocos nucifera*)	66	35±6 (104)
Tomato chutney (*Lycopersicon esculentum*)	78	36±3 (164)
Tomato *rasam* (*Lycopersicon esculentum*)	97	11±1 (357)
Snakegourd *kootu* (*Trichosanthes anguina*)	77	38±39 (166)
Banana (Morris variety)	79	13±8 (60)

Data presented as mean ± standard deviation of triplicate determinations expressed on a fresh weight basis. Data in parentheses indicate the total folate on a dry weight basis.

100 g due to better retention of the vitamin during the parboiling process. The total folate value for *uppuma* was 97±59 μg on a fresh weight basis, the main ingredient being semolina—which has been reported to contain 72 μg/100 g when raw (USDA 2007). The two wheat-based preparations analysed were *chapathi* and *poori*. The former involved the use of dry heat whereas the latter is a deep-fried product. The lower value for *poori* could be due to the interference of fat during folate extraction, resulting in underestimation of folate that needs further investigation.

Consumption of legumes is very high in India with an apparent consumption level of 8.4 kg/capita/year, the highest among the Asian countries (China, Indonesia and Japan). In general, China and India contribute about 50% of the total legume production in the world (FAO 1998). The folate content in various legume-based preparations is presented in Table II. This study revealed the highest value for sprouted mung bean (89±15 μg/100 g on a fresh weight basis) followed by chickpea *sundal* (55±4 μg/100 g on a fresh weight basis). The highest value in the legume-based preparations may be accounted for by the inherently high levels of natural folates released during enzyme treatment. Legume sprouts are widely consumed in India. The sprouted mung bean *sundal* analysed had a total folate content of 89± 15 μg/100 g, although Gopalan et al. (2002) revealed a zero value for raw whole mung

Table IV. Folate content (μg/100 g) in milk-based beverages and preparations.

Food item	Moisture (%)	Total folate
Milk	95	8±0.5 (160)
Coffee	91	22±2 (239)
Tea	92	13±3 (161)
Home-made yoghurt	86	20±7 (139)
Morkozhambu	85	14±8 (95)
Vermicelli *payasam*	72	23±8 (81)

Data presented as mean ± standard deviation of triplicate determinations expressed on a fresh weight basis. Data in parentheses indicate the total folate on a dry weight basis.

bean. This could possibly be justified by the considerable changes in chemical composition of legumes during germination, a process reported to improve the nutritional quality of legumes by increasing the ascorbic acid and folate content (Augustin and Klein 1989) and the improvements in the extraction methods today. Gopalan et al. (2002) have reported a total folate value of 147.5 μg/100 g for raw dehulled split chickpea.

Table III indicates the total folate contents in 13 common Indian vegetable-based preparations and the most commonly consumed fruit, namely banana, using the tri-enzyme technique. The total folate content of the analysed vegetables ranged from 11 to 86 μg folate/100 g on a fresh weight basis. In addition, the results confirm that the green leafy vegetables are good sources of folate. Although it is justifiable that these vegetables contain negligible amounts of starch and protein and that further treatment with protease and amylase is not needed, as reported by others (Tamura et al. 1997; Iwatani et al. 2003), an increase of 51% and 22% folate, respectively, was seen in the spinach extract treated with tri-enzymes compared with single enzyme (Martin et al. 1990; Aiso and Tamura 1998). Based on this, tri-enzyme treatment was chosen as an extraction method for the vegetable preparations.

Sixty to 100% folate was retained in vegetables after cooking (Lin and Lin 1999). Besides the cooking conditions, the ascorbic acid concentration can play an important role in the folate value of particular foods (Kirsch and Chen 1984; Pederson 1988; Gregory 1989; Tamura 1998). This is evident from the values obtained for tomato-based preparations such as tomato chutney and *rasam* in Table III. Banana was found to contain 13 ± 8 μg folate/100 g on a fresh weight basis.

Table IV presents the values for milk-based beverages and two milk-based preparations along with yoghurt, a product commonly eaten by the population. Coffee, which is the traditional beverage in India, has been reported to have a folate value of 22 ± 2 μg/100 g—this is contributed mainly by the folate present in the coffee bean, which is already a fermented product, and not milk, which is a poor source of folate (8.5 μg/100 g) as reported earlier by Gopalan et al. (2002) and in this study (8 ± 0.5 μg/100 g).

The total folate value obtained in this study for the home-made yoghurt (20 ± 7 μg/100 g on a fresh weight basis) is due to the significant increase in total folate due to fermentation. This phenomenon is well documented in similar products (yoghurt and cheese) due to the presence of active folate-producing bacteria (Rao et al. 1984; Drewek and Czarnocka-Roczniakowa 1987). Folate values obtained for yoghurt are higher than the value reported earlier (12.5 μg/100 g) by Gopalan et al. (2002). This observation may be probably due to the tri-enzyme treatment employed in the present study yielding more extractable folate compared with the single enzyme treatment employed in the earlier study. Also, seasonal variation in dairy folates may seem logical considering the fact that folate is unstable with higher concentrations present in fresh green plants fed to the cows in summer compared with the long stored winter cattle feed. Milk had a value of 8 ± 0.5 μg/100 g in this study. The folate content of milk has been reported to range between 4 and 10 μg/100 g, with the higher folate levels reported during summer when compared with winter, as reviewed by Forssen et al. (2000).

Estimates of folate intake in the selected population

Folate intakes based on the 24-h recall survey from 200 respondents within the age group 18–24 years has been estimated to be 277 μg/day on average for the urban population in this study based on the analytical results using the tri-enzyme technique. The value from the current study is about 2.8 times higher than the values (98 μg/day) reported by the National Nutrition Monitoring Bureau (2002), which reported intakes for the rural population only, and 1.6 times higher than the values reported by the National Pilot Program in India (Figure 1) (167 μg/day) (Salvi and Damania 2005). The decreased intakes obtained in previous studies can be attributed to a few reasons, namely: that only raw food data based on the single enzyme treatment are available (Gopalan et al. 2002); this study used a tri-enzyme extraction, which is known to extract more folate from foods of varying matrices; and the differences in agricultural practices, soil composition, maturity and the chemical stability of folate during the processing conditions (heat, exposure to oxygen, light sensitivity and leaching into the cooking medium) before consumption could also contribute to several differences in total folates, particularly in vegetables (Scott et al. 2000; Iwatani et al. 2003).

The mean dietary folate intakes from unfortified sources were also reported to be 200–280 μg/day in the USA, Netherlands, Denmark, Finland, Spain, and Costa Rica (Hun et al. 2005). From the values currently obtained on food folate and the re-calculation of food folate intakes in the selected population, it seems that the current recommendations in India by the Indian Council of Medical Research of 100 μg/day for the adult population may need to be revised. However, similar studies in other parts of the country, both urban and rural, should be conducted and intake data should be revised to provide a more reliable recommendation for folate intake given that previous studies have indicated a prevalence estimate of neural tube defects in the village clusters to be 6.57–8.21 per 1,000 live births (Salvi and Damania 2005).

The analysed values showed that foods normally consumed for breakfast contributed to the major percentage of daily total folate intake (Figure 2). This is probably due to the high consumption of fermented cereal foods like *dosa* with a legume-based side dish (*Sambhar*). Although the meal consumed for lunch had a similar combination of cereals and legumes in addition to folate-rich vegetables, the folate intake seemed to be less (67 μg) when compared with the breakfast foods

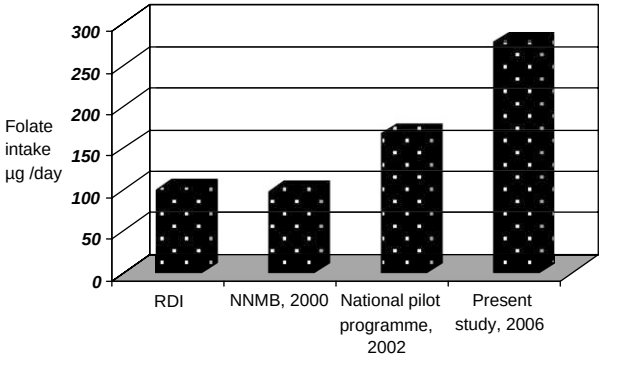

Figure 1. Estimated intake (μg/day) based on the 24-h recall compared with the Recommended Dietary Intake, National Nutrition Monitoring Bureau data and National Pilot Program.

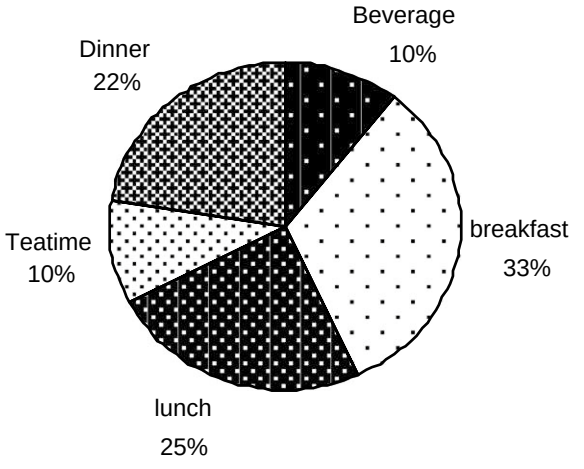

Figure 2. Percentage folate distributions from meals.

(90 μg)—probably due to the consumption of substantial quantities of rice, which is the staple cereal in the South Indian Diets. Polished white cooked rice was analysed to have 0 μg/100 g, whereas the quantity of vegetables consumed was on an average 50 g (one serving) that had an analysed value of 21 μg/serving.

The 24-h recall survey revealed that only less than 10% of the respondents included fruit daily, although fruits are considered one of the good sources of folate.

Quality control

Commercially available certified materials (wholemeal flour, and a vegetable matrix) were chosen to check for the accuracy of the method. The results are presented in Table V, which indicate the accuracy of the results obtained using the microbiological assay.

The present study provides valuable information on the folate content in various traditional foods/preparations consumed in South India. A similar approach should be taken to estimate the actual folate intakes of populations in other parts of the country given the diversity in foods eaten across the country. Such studies will confirm the need to revise the dietary intake recommendations, which is currently set at only 100 μg/100 g for adults. The data can also be used as a guideline for promoting foods high in folates that might be beneficial in reducing folic acid deficiency in the population, until such time that fortification of foods is mandated. However, good folate sources cannot be solely identified from high folate sources alone as the bioavailability of folates in these foods should be considered a priority for future studies.

Table V. Comparison of total folate content (μg/100 g) generated using the microbiological assay method versus certified values based on triplicate determinations.

Certified material	Analysed value	Certified value
CRM-121 (wholemeal flour)	56 ± 6	50 ± 11
CRM-485 (vegetable matrix)	287 ± 19	315 ± 44

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