

Microbiological Assay of Folic Acid Content in Some Selected Bangladeshi Food Stuffs

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Received: December 23, 2014 Accepted: January 6, 2015 Online Published: January 26, 2015

doi:10.5539/ijb.v7n2p35

URL: <http://dx.doi.org/10.5539/ijb.v7n2p35>

Abstract

Folic acid concentration in six common food samples of Bangladesh including lentil (*Lens culinaris Medik*) and bengal gram (*Cicer arietinum*), representatives of legumes; spinach (*Spinacia oleracea*) and basil (*Ocimum basilicum*), representatives of green leafy vegetables; milk, representative of a common animal source food and topa boro rice (*Oryza sativa*), a representative of cereal-grains were measured in the present study by microbiological assay using a new trienzyme folic acid extraction method. In this experiment, we estimated that, lentil, bengal gram, spinach, basil, milk and topa boro rice contained about 63 µg, 48 µg, 195 µg, 131 µg, 10 µg and 42 µg of folic acid per 100 g of food sample respectively. For the assay of this water-soluble vitamin, *Lactobacillus casei* (ATCC 7469) was employed as test organism. Trienzyme treatment was performed to release bound folic acid using protease, and α-amylase with chicken pancreases as the conjugase. The highest folic acid content was recorded for spinach (*Spinacia oleracea*), the well-known richest source of folic acid, followed by basil (*Ocimum basilicum*). The lowest value was recorded for milk. Topa boro rice, a kind of parboiled rice was also found to be a fair source of folic acid. For these six types of Bangladeshi foods studied, the content of folic acid ranged from 10 to 195 µg/100 g, indicating a very wide range of folic acid content in foods.

Keywords: folic acid, *Lactobacillus casei*, trienzyme, chicken pancrease

1. Introduction

Folic acid is an important member of the water-soluble B-group vitamins. Folic acid (PGA) is an orange-yellow crystal or powder with a molecular weight of 441.4; Hydrate, 477.4. At pH 7.0, absorption spectrum, λ_{\max} is 282-350 nm and at pH 13.0, λ_{\max} is 256-365 nm (Rex et al., 1986). It has limited solubility in water, but is soluble in acidic and alkaline solutions and insoluble in organic solvents (Dick et al., 1948). Folic acid is more stable in alkaline than in acidic conditions, thus standards for folic acid derivatives are prepared in basic solution (Keagy, 1986). It is involved in the formation of new cells, the metabolism of ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), essential for protein synthesis, formation of blood and transmission of genetic code. It is essential during pregnancy to reduce the risk of neural tube defects (birth defects affecting the brain and/or spinal cord) essential for the normal growth and development of the fetus. Folates, as various forms of tetrahydrofolate (THF), are substrates and coenzymes in the acquisition, transport, and enzymatic processing of one-carbon units for amino acid and nucleic acid metabolism and metabolic regulation (Cook et al., 2001). There is strong evidence for a relationship among inadequate folic acid status, elevated homocysteine concentration, and risk of coronary heart disease, venous thrombosis, carotid artery stenosis, and other forms of vascular disease (Rimm et al., 1998; Robinson et al., 1998). Mothers with inadequate folic acid status are at increased risk for having children with neural tube defects or other forms of birth defects (Botto et al., 1999). The risk of certain forms of cancer (e.g., colon, cervical, and breast) also increases when folic acid intake is inadequate (Kim, 1999; Choi & Manson, 2000). Folic acid deficiency can also contribute to depression (Fava et al., 1997; Green & Miller, 1997), impaired immune response, and neural and neurological damage (Houseton et al., 1988; Snowdon et al., 2000). The method of food folic acid measurement has improved over the years. As food, folic acid tables are notoriously unreliable for estimating accurate dietary intake, to improve the method of food folic acid analysis, the

tri-enzyme extraction method was developed about 15 years ago by researchers in Georgia, United States. For decades, the traditional food folic acid extraction method involved two steps including heat treatment, to release folic acid from its binding proteins, and folate conjugase treatment, to hydrolyze polyglutamyl folate to monoglutamyl folate. However, a trienzyme extraction method of food folic acid was developed in the mid 1990s. This method involves the use of α -amylase, protease and folic acid conjugase and allows for a more extraction of folic acid trapped in carbohydrate or protein matrices in food than the traditional method. In the last several years, this extraction method became widely used. In Bangladesh, it is the first attempt to determine food folic acid content by microbiological assay with the help of tri-enzyme extraction method. The method is based on the observation that certain organisms require specific vitamins for growth, using the basal medium containing all nutrients except that to be assayed. Growth responses of the organism are then compared quantitatively with standard of known concentration.

The microbiological assay of folic acid started with the findings of Stokstad (Stokstad, 1943) that growth of lactic acid bacteria such as *Lactobacillus rhamnosus* and *Streptococcus lactis* is influenced by liver and yeast extracts which are rich in folic acid (Bird et al., 1945). The most common detection method is a microbiological assay relying on the turbidimetric bacterial growth of *Lactobacillus casei* (ATCC 7469) (Hawkes et al., 1989). If anyone of the required growth factors is completely left out of the medium and then an unknown amount added, the final level of cell growth is directly related to the amount of the growth factor present in the medium. Bird et al reported that among the different methods, microbiological assay is the most reliable and accurate even at the fractions levels (Bird et al., 1969). Today the microbiological method is the only food folic acid method given Official Status by AOAC (AOAC, 2006) and AACC (AACC, 2000).

The biologically active compounds derived from folic acid and present in biological materials are folic acid, formyl-FAH₄ (tetra-hydrofolic acid), 5-formyl-FAH₄, 5,10-methenyl-FAH₄, 5,10-methylene-FAH₄, 5-formyl-FAH₄. All these compounds promote the growth of *Lactobacillus casei* but not *Streptococcus fecalis*. For this reason, *Lactobacillus casei* is the most suitable organism for determining the free folate activity of biological materials (Swaminathan, 1985) because it responds to the widest variety of folate derivatives (Krumdeick et al., 1983).

In the 1980s, a number of researchers reported that treatment with folic acid conjugase alone is usually not effective to liberate food-bound folate. The use of additional enzymes, proteolytic or amylolytic, was shown to liberate folic acid from the foods, thereby maximizing the folic acid activity in certain foods (Cerna & Kas, 1983). Later DE.Souza and colleagues reported a method of folic acid extraction where, in addition to the traditional treatment with folic acid conjugase, protease and α -amylase were also used (De Souza & Eitenmiller, 1990). The extraction method was named as the 'tri-enzyme treatment'. Although the order of enzyme addition was found to differ with investigators, the more common order appeared to be protease, α -amylase and finally conjugase (Tamura et al., 1997). It is now realized that conditions of the enzyme treatment might be different for each type of food and therefore, the identification of the optimum pH and a suitable incubation time for each food must be done prior to folic acid analysis (Aiso & Tamura, 1998). It is our hope that, the wide use of an appropriate procedure of the tri-enzyme extraction method, in combination with a reasonable detection method, help in establishing accurate and reliable food- folic acid tables, preparing a complete Bangladeshi Food Composition Table and this, in turn, makes it possible to accurately assess folic acid intake in the general population of Bangladesh. Then adequate folic acid nutritional status will be ensured among the mass people through consumption of folic acid -rich food items that will contribute for maintaining good health of people, especially for the adolescent girls and the pregnant and lactating women.

To determine folic acid content of some selected Bangladeshi Food stuffs employing the organism *Lactobacillus casei subsp. rhamnosus* (ATCC no. 7469) is used to determine the amount of folic acid present in these foods.

2. Materials and Methods

2.1 Collection of Sample

The present investigation was conducted on six types of food samples for the determination of folic acid content together with nutritive value. Here we used the following samples including lentil and bengal gram, representatives of legumes; spinach and basil, representatives of green leafy vegetables; topa boro rice, representatives of cereal grains and milk, a representative of animal source food from six different parts of Bangladesh. These six foods were mixed into three pairs of food. Each pair was mixed completely to make a composite mixture. Then folic acid concentration of the samples were estimated from each composite mixture using the method of Tamura et al. (1997).

2.2 Sample Preparation

2.2.1 Sample Homogenization

The collected samples were thoroughly washed for 3-4 times with distilled water to remove excess dirt, dust particles, unnecessary microbes and other extraneous materials (except milk). Then the samples, namely spinach and basil cut into small pieces and mixed homogenizely. For the samples, namely lentils, bengal gram, boro rice and milk, the above steps were not needed. Next a certain portion of each sample was put in mortar-pastel and was ground vigorously to make fine paste. The procedures of food folic acid assay generally begin with homogenization of a single food or food mixtures in a buffer with an appropriate pH, containing ascorbic acid or 2-mercaptoethanol. Here we used the working buffer with a pH of 6.1 containing ascorbic acid. About 100 ml of this buffer was added with each 1-2 g of sample and the solution was shaken properly in an earlenmeyer flask to make a homogenized sample solution.

2.2.2 Sample Extraction

2.2.2.1 Preparation of Stock Buffer:

For the convenient of this assay, we formerly prepared two types of stock buffer and stored them in a refrigerator for later use. Buffer A was prepared by dissolving 31.199 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 1000ml of de-ionized water and Buffer B was prepared by dissolving 71.59 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in another 1000ml of de-ionized water. These buffers were then used to prepare different types of working buffers having different pH.

2.2.2.2 Preparation of Working Buffer:

It was usually prepared freshly before use by diluting 212.5 ml of Buffer A and 35.5 ml of Buffer B. About 5 g of ascorbic acid was added with it. Then the pH was adjusted up to 6.1 by using Buffer B. Finally the volume was made up to 1000 ml.

2.2.2.3 Application of Working Buffer in Sample Extraction:

About 1 to 2 grams of test sample was mixed with 100 ml of working buffer, pH 6.1. Then the solution was autoclaved at 120°C and 15lbs pressure for 10 min. Then the extracted sample was cooled.

2.2.2.4 Enzymes-Sources of Enzymes:

Protease: It was prepared from the strains of *Streptomyces griseus* and sold from Sigma Chemical Co., USA.

α -Amylase: it was prepared from *Aspergillus oryzae* and also sold from Sigma Chemical co., USA.

Folate conjugase: It was prepared in laboratory by using the pancreas of the freshly slaughtered chicken.

2.2.2.5 Preparation of Enzyme Folate Conjugase

2.2.2.5.1 Preparation of Extraction Buffer, pH 7.8: 1.42 g of sodium phosphate dibasic and 1.0 g of ascorbic acid were dissolved in de-ionized water and diluted up to 100 ml. Then its pH was adjusted to 7.8 with 4N NaOH

2.2.2.5.2 Chicken Pancreases Solution (5 mg/ml): 0.5g of fresh chicken pancreases was weighed and washed with acetone. It was then chopped into small pieces using a sharp knife and was ground in a mortar-pastel. 100ml of pH 7.8 Phosphate buffer was added with it. Then the solution was stirred vigorously for 10 minutes at 3000 rpm capacity centrifuging machine and squeezed through glass wool.

2.3 Enzyme Treatment

After cooling, 1 ml of protease (2 mg/ml) was added per gram of sample and incubated at 37°C, for 3 hours. Then the enzyme was inactivated in water bath at 100°C for 5 min and cooled. Secondly, 20 mg of amylase powder per gram of sample was added and incubated at 37°C for 2 hours. Then the pH of the sample was adjusted up to 7.2. Finally, the enzyme folate conjugase was added in the sample along with 0.5 ml toluene and incubated at 37°C for 16 hours. The activity of this enzyme was then stopped by autoclaving at 120°C for 5 min (or boiling in a water bath at 100°C for 15 min) (Ashok et al., 2000)

2.4 Sample Dilution and Adjustment of pH

After cooling, the solution was diluted up to 200 ml and filtered. The pH of a portion of the clear filtrate was adjusted up to pH 6.2 and diluted to concentration of about 0.25-0.3 ng folic acid/ ml.

2.5 Preparation of Inoculums

2.5.1 Microorganism: Here we used *Lactobacillus casei*_ATCC 7469 for the assay method.

2.5.2 Stock Medium: Bacto-Micro Assay Culture Agar (MACA). Micro Vitamin Test Media were recommended for cultivation and maintenance of stock cultures of Lactobacilli used in microbiological assays of vitamins.

2.5.3 Culture Medium: Bacto-Micro Assay Culture Agar (MACA) for stock culture, Micro-Inoculums Broth Media for the preparation of inoculums and Folic acid Casei Media for Folic acid assay. All the three media were collected from Hi-Media Laboratories Limited, Mumbai, India.

2.5.4 Stock Culture: Stock cultures were prepared by stab inoculation in triplicates. One was used for the preparation of stock cultures while others were used for inoculums preparation for assays, followed by incubation at 35-37°C for 24-48 hours. Transfer of cultures should be made at weekly or bi-weekly intervals, culture (3 tubes) in MACA. The tubes were stored in refrigerator.

2.5.5 Preparation of Micro Vitamin Test Culture Agar for Stock culture: 52.1 gram of medium was suspended in 1000 ml of distilled water. 15 g of agar was added with it. Then it was heated if necessary to dissolve the medium completely. pH 6.7±0.2 was adjusted. Then it was dispensed and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2.5.6 Preparation of Micro Vitamin Test Inoculums Broth for Inocula: 37.1 gram of media was suspended in 1000 ml of distilled water. Then it was heated if necessary to dissolve the medium completely. pH 6.7±0.2 was adjusted. Then it was dispensed and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2.5.7 Inoculums: Subculture of *L casei* was used to form a stock culture for micro-inoculum broth. After 18-24 hours, the stock culture was incubated at 35-37°C, under aseptic condition. Then the cells were washed with 3×10 ml (5 ml) portions sterilized 0.9% NaCl solution (NSS). Then the last supernatant was decanted. The cells were diluted to an appropriate inoculums with NSS.

2.5.8 Assay Media: Bacto-Folic acid assay medium was used for the assay procedure. Its pH was adjusted up to 6.2 before use.

2.6 Preparation of Standard Folic acid Solution

2.6.1 Stock Standard: 25 mg of USP Folic acid was dissolved in 25% of ethyl alcohol and the pH of this solution was adjusted up to 7.0 by using 0.1N NaOH. The volume was made up to 250 ml with 25% of ethyl alcohol in a volumetric flask where the concentration of folic acid was 100 µg/ml.

2.6.2 Preparation of Phosphate Buffer, pH 7.0: 58.5 ml of Buffer A and 91.5 ml of Buffer B were mixed together and the pH of the solution was adjusted up to 7.0 by using 0.1N NaOH and 0.05N HCl. Then the volume was made up to 300 ml with de-ionized water.

2.6.3 Intermediate Standard I: 1 ml (100 µg/ml) of stock standard was diluted to 100 ml in a volumetric flask so that the concentration of Folic acid in this diluted solution had become 1000 ng/ml.

2.6.4 Intermediate Standard II: 1 ml (1000 ng/ml) of Intermediate Standard I solution was diluted to 100 ml in a volumetric flask so that the concentration of Folic acid in this diluted solution had become 10 ng/ml.

2.6.5 Preparation of Working Standard: 5 ml (10 ng/ml) of Intermediate Standard II solution was diluted to 100 ml in a volumetric flask so that the concentration of Folic acid in this diluted solution had become 0.5 ng/ml. then the pH of this Working Standard was adjusted up to 7.0 by using 0.1N NaOH.

2.7 Preparation of Control

About 100 ml of working buffer, pH 6.1 was taken to prepare 'control' for the samples we used in this assay method. After autoclaving this buffer, it was processed following all the steps which had been performed in case of the samples including enzyme treatment, enzyme inactivation, dilution and filtration. The purpose of preparing this 'control' was to eradicate the traces of folic acid that might come from the enzymes used rather than the sample itself and might be used by the test organism after inoculation and during incubation. After subtracting the amount of Folic acid, present in 'control' from the amount of Folic acid, present in sample, we may be able to get a more accurate value of folic acid content present in our selected food samples.

2.8 Folic Acid Determination Procedure From Food Samples

Food samples (approx 1.0 g) were homogenize in 100 ml buffer, pH 6.1 (with ascorbic acid) and autoclaved at 121°C and 15 lbs pressure for 10 minutes. Then samples were allowed for cooling and incubated with the enzyme, protease at 37°C for 3 hours. Inactivation of this enzyme was performed by boiling in water bath for 5 minutes and cooled and incubated with the enzyme, α -amylase at 37°C for 2 hours. The pH should be maintained at 7.2 Then it was incubated with Chicken pancreas powder and 0.5 ml toluene at 37°C for 16 hours and autoclaved at 121°C and 15 lbs pressure for 10 minutes to inactivate the action of enzymes. Cooling, dilution, filtration and adjustment of pH maintained to 6.2 to a portion of clear filtrate. Further dilution and pipetting in duplicate test tubes were done. Addition of working buffer (pH 6.1) and previously prepared media performed in the same test tubes and

autoclaved at 121°C and 15 lbs pressure for 10 min for sterilization. Then Inoculation and incubation at 35-37°C for 18-24 hrs were conducted. Boiling at 100°C was done to stop the growth of bacteria. Finally measurement of bacterial growth was recorded by taking the absorbance at 630 nm using UV Spectrophotometer.

2.9 Folic Acid Determination Procedure From Standard Folic Acid Solution

25 mg of Standard Folic acid was dissolved in 25% Ethyl alcohol and pH adjustment was conducted up to 7.0 with 0.1N NaOH. Stock Standard was formulated by making the volume up to 250 ml with 25% ethyl alcohol. Stock standard was then diluted to prepare working Standard and pipetting was done in triplicate test tubes. After adding working buffer (pH 6.1) and previously prepared media in the same test tubes, these were autoclaved at 121°C and 15 lbs pressure for 10 min for sterilization. Then Inoculation and incubation were performed at 35-37°C for 18-24 hours. Boiling at 100°C was done to stop the growth of bacteria. Finally measurement of bacterial growth was recorded by taking the absorbance at 630 nm using UV-Spectrophotometer.

3. Results

The present study has been carried out with a view to estimate the folate content of six types of selected food stuffs such as- lentil, bengal gram, spinach, basil, milk and topa boro rice. In this microbiological assay method, the organism, *L.casei* ATCC 7469 was used and it was observed that, the growth of this organism was increased with each additional amount of folic acid contained in sample.

3.1 Construction of Standard Curve for Estimation of Folate Content in Food Samples

A standard curve was generated by turbidity readings of spectrophotometer due to the growth of *L. casei* in the media containing 0.125, 0.25, 0.5, 0.75 and 1.0 ng per tube of triplicate tubes of standard solutions (Figure 1). Absorbance values were taken at 630 nm of wave length.

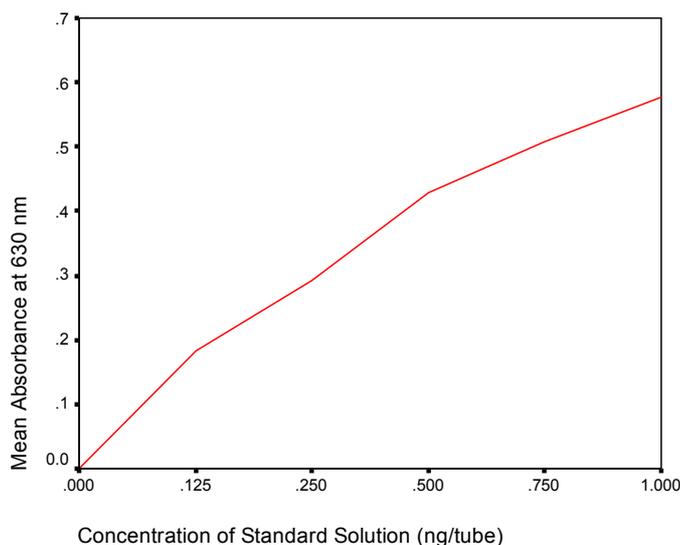


Figure 1. Standard Curve of Folic acid

3.2 Estimation of Folic Acid in Food Samples

The folic acid of test food samples was determined according to AOAC method (AOAC, 1990). After trienzyme treatment, the free-folates were extracted and diluted with basal medium containing all growth nutrients except folate, and the turbidity of the *L. casei* growth response for the samples was compared quantitatively to that of known standard solutions. Absorbance of control at 630 nm was 0.313 which was essential for determining the folic acid in control after dilution. After dilution, amount of folic acid in control (D.F.=20,000) were 3µg and 3000 ng.

Absorbance for food samples at 630 nm was recorded in Table 1 which was used later for determining folic acid concentration (Table 2) from standard curve. The folic acid concentration from different food stuffs after dilution obtained from table 2 expressed in µg was used to estimate actual folic acid content in food samples (Table 3).

Table 1. Absorbance for Food samples at 630 nm

Food Sample	Extract (ml)	Absorbance at 630 nm
Lentil	2.0	0.132
Bengal gram	2.0	0.149
Spinach	2.0	0.215
Basil	2.0	0.174
Milk	2.0	0.118
Topa Boro rice	2.0	0.126

Table 2. Determination of folic acid concentration in Food samples from standard curve

Food samples	Absorbance at 630 nm	Folic acid content in Food sample		After dilution	
		ng/tube	ng/ml	ng	µg
Lentil	0.132	0.092	0.046	3680	3.68
Bengal gram	0.149	0.100	0.050	4000	4.00
Spinach	0.215	0.160	0.080	6400	6.40
Basil	0.174	0.110	0.055	4400	4.40
Milk	0.118	0.080	0.040	3200	3.20
Topa Boro Rice	0.126	0.086	0.043	3440	3.44

Table 3. Determination of Actual Folic acid Content

Food samples	Folic acid, present in Food (µg)	Folic acid, present in Control (µg)	Amount of Folic acid, present only in Food (µg)	Weight of food sample (g/ml)	Actual folic acid content in Food samples	
					µg/g or ml	µg/100 g or 100 ml
Lentil	3.68		0.68	1.084	0.63	63
Bengal gram	4.00		1.00	2.054	0.48	48
Spinach	6.40		3.40	1.740	1.95	195
Basil	4.40	3.0	1.40	1.066	1.31	131
Milk	3.20		0.20	2.000	0.10	10
Topa Boro Rice	3.44		0.44	1.055	0.42	42

From the above tables, it would be seen that the study food samples, namely lentil, bengal gram, spinach, basil, milk and topa boro rice contained approx. 63 µg, 48 µg, 195 µg, 131 µg, 10 µg and 42 µg of folic acid per 100 g of Food samples respectively. These values also compared with reference values in Table 8.

Table 4. Comparison of experimental folic acid value with reference folic acid value of study food samples

Food samples	Experimental folic acid value in study food samples (µg/100g)	Reference folic acid value of study food samples (µg/100g)
Lentil	63	24
Bengal gram	48	34
Spinach	195	194
Basil	131	64
Milk	10	5.6
Topa Boro Rice	42	Not available

Source: USDA (www.USDA.gov).

4. Discussion

Folic acid assay is more challenging than many other micronutrients due to its sensitivity to physical environments and various forms in which it exists. It warrants a good knowledge of folic acid chemistry and appropriate extraction and detection techniques. The extraction techniques may differ with the type, nature, state, origin of foods as well as with the methods of detection. Besides, a careful selection of buffer type, antioxidant, heating condition, conjugate type, incubation conditions and purification methods is needed as each of these steps is likely to affect the final yield of the vitamin.

Although microbiological assay is the most commonly used method of folic acid assay, it is time-consuming, needs great care and skill and cannot differentiate the individual folic acid. HPLC is a better analytical technique but involves a complex extraction and purification procedure. The more recent methods using the LC/MS/MS techniques offer hope with accurate quantification and better specificity of the folic acid forms. Immunoassay techniques are rapid, easier and much cheaper but less suitable for determination of folic acid in food samples.

The information available on the effect of a particular analytical technique on folic acid content of a particular food is insufficient. Therefore, it is suggested that an optimization of the extraction and detection of folic acid in each food group should be carried out before actual analysis is carried out. It appears that no folic acid analytical method is perfect. The choice of a particular method is largely determined by the purpose of analysis, e.g. food composition, nutritional intervention, regulatory purpose, and to a lesser degree by the resources available, assay time and cost, and analysis themselves.

As summarized in Table 8, the values found in the present study are markedly higher than those published in different articles previously. The reason for these higher values is likely due to the newly developed method of folic acid extraction from foods called tri-enzyme treatment, which generally provides the highest detectable value of food folic acid concentration in a certain food items among the majority of the existing methods (Tamura, 1998).

We are not certain how much of these folic acids are bio-available for absorption and metabolism in humans. There are possibilities that, the previously estimated bioavailability and requirements of food folic acid are grossly underestimated (Tamura, 1998). It is now obvious that, we must evaluate what these higher values actually means in terms of maintaining adequate folic acid nurture for overall health and prevention of diseases. It is evident that, more studies are needed in the areas of food folic acid concentrations and bioavailability as well as appropriate dietary folic acid intake recommendations.

5. Conclusion

Folic acid content of six common Bangladeshi foods was analyzed with the microbiological assay, using the tri-enzyme extraction method. From this study it indicates that the Bangladeshi people have access to foods with high folic acid levels and it is possible to meet the recommended dietary intake for folic acid of 200-300 µg/day by the population. We hope that, these new values will serve as a useful means to calculate dietary intake of folic acid in the general population. It is our hope that, the wide use of an more sophisticated and appropriate procedure of the tri-enzyme extraction method, in combination with a reasonable detection method, help in establishing more accurate and reliable food- folic acid tables and then adequate folic acid nutritional status will be ensured among the mass people through consumption of folic acid -rich food items that will contribute for maintaining good health of people, especially for the adolescent girls and the pregnant and lactating women.

Acknowledgment

This study was conducted in the Institute of Nutrition and Food Science, University of Dhaka, Dhaka-1000, Bangladesh with a partial financial support from UN FAO (United Nation Food and Agricultural Organization) at preliminary stage. Later no fund was available to further continue this research study.

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