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Estimation of Vitamins B-Complex (B_2, B_3, B_5 and B_6) of Some Leafy Vegetables Indigenous to Bangladesh by HPLC Method

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ABSTRACT

The current experiment was conducted for the simultaneous determination of several water-soluble vitamins like riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5) and pyridoxine (vitamin B_6) in five highly consumed local leafy vegetables named as bottle gourd leaves (*Lagenaria vulgaris*) (local name Lau shak), green amaranth leaves (*Amaranthus viridis*) (local name Data shak), red amaranth leaves (*Amaranthus gangeticuss*) (local name Lal shak), Indian spinach (*Basella alba*) (local name Pui shak) and bitter gourd leaves (*Momordica charantia*) (local name Korola shak). The analyses were performed by HPLC using an analytical reversed phase C-18 (ODS column, 250 × 4.6 mm, 5 µm, Phenomenex, Inc.) column with the mobile phase consisting of a mixture of buffer (hexane sulphonic acid sodium, potassium dihydrogen phosphate and triethylamine, pH 3.0) and methanol in the ratio of 96:4 (v/v) at a flow rate of 1 mL/min with UV detection at 210 nm. The retention times for the vitamins were obtained as 3.61 min, 6.37 min, 9.51 min and 11.51 min for Vitamins B_2, B_3, B_5 and B_6, respectively. These obtained values of the vitamins were compared with the values available in published literatures of Deshio Khaddar Pustiman (DKPM), Indian food value (IFV) and United States Department of Agriculture (USDA).

Keywords: HPLC Analysis; Vitamin B_2; Vitamin B_3; Vitamin B_5; Vitamin B_6; Leafy Vegetables

1. Introduction

Leafy vegetables are those plants whose leaves or aerial parts are used as food [1]. Vegetables constitute essential components of the diet such as protein, vitamins, iron, calcium and other nutrients which are usually in very small quantity [2]. Increased awareness of the usefulness of vegetable inclusions in human food has enhanced their consumption as part of the daily diet. The therapeutic potential of the vegetables is usually attributed to the high content of vital vitamins as well as micronutrients [3]. Vegetables are highly recommended because of the relatively high nutritional value and their consumption gives diversity to daily food intake, adding flavor and taste to the diet [4]. Leafy vegetables are good source of vitamins and minerals. Even though vitamin is required a small amount per day in health, it plays a vital role in our health [5]. Vitamins are reported to reduce the damage by free radicals and check degenerative disease [6]. The vitamins B are a group of water soluble vitamins that play important roles in cell metabolism. The term B-group vitamins usually refer to thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, cyanocobalamine and folic acid. The deficiency syndromes of some B vitamins are beri-beri (cardiac and dry), peripheral neuropathies, pellagra, and oral and genital lesions (related to riboflavin deficiency)—which were once major public health problems in parts of the world [7]. Several vitamins of the B-group act mainly as coenzymes in the metabolism of foodstuffs to produce energy [8].

As their chemicals structures are not related, a considerable number of papers have been published describing different physical, chemical and biological methods to analyze the vitamins [9]. The simultaneous determination of several water-soluble vitamins is difficult and often many different analyses have to be performed. Different instrumental methods have been used for the determina-

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tion of B-group vitamins, including electrochemical methods, *spectrophotometry*, derivative UV spectrophotometry, spectrofluorimetry, normal phase and reversed phase TLC and HPLC procedures as well as capillary electrophoresis. The determination of B-complex mainly in tablets using HPLC methods has been extensively described. The most widely used methods for the determination of B-group vitamins are reversed-phase HPLC, using a C18 column and aqueous–organic mobile phases, in acidic media [9]. However, this study investigated the B-vitamin profile namely riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5) and pyridoxine (vitamin B_6) in five highly consumed local leafy vegetables named as bottle gourd leaves (*Lagenaria vulgaris*) (local name Lau shak), green amaranth leaves (*Amaranthus viridis*) (local name Data shak), red amaranth leaves (*Amaranthus gangeticuss*) (local name Lal shak), Indian spinach (*Basella alba*) (local name Pui shak) and bitter gourd leaves (*Momordica charantia*) (local name Korola shak). These B vitamins were ana- lyzed with a reversed phase HPLC method equipped with UV detector and auto-injector using the modified meth- ods described by Aslam *et al.* [10]. To the best of our knowledge, there is no available data published in the literature regarding the vitamins content in these leafy vegetables grown in Bangladesh.

2. Materials and Methods

2.1. Collection of Samples

The current experiment was conducted on five leafy vegetable samples for the determination of their B vitamins content. The vegetables were bottle gourd leaves (*Lagenaria vulgaris*) (local name Lau shak), green amaranth leaves (*Amaranthus viridis*) (local name Data shak), red amaranth leaves (*Amaranthus gangeticuss*) (local name Lal shak), Indian spinach (*Basella alba*) (local name Pui shak) and bitter gourd leaves (*Momordica charantia*) (local name Korola shak).

Each of leafy vegetables was collected from nine regions of Bangladesh during the months of December 2011 to January 2012. The specimen samples were deposited in the laboratory of Institute of Nutrition and Food Science, University of Dhaka, Dhaka-1000, Bangladesh.

Samples were collected from different central markets of Dhaka city where the vegetables come from the different district of Bangladesh. Procedure for collection of food sample is important to get reliable representative nutrient values. Care was taken to avoid risk of inadvertent damage and deterioration of nutrients during transport from collection point to the laboratory. The vegetable samples were water sprayed to keep moisten, well packed in clean dark plastic poly bags to prevent water loss and damage by light, and then transported to lab within the shortest time span.

In the laboratory samples were prepared for analysis. The samples were washed well with double distilled water and the water was removed by blotting paper. The edible portion of the samples were then separated and weighted. These were chopped out into small pieces and homogenized. About 10 g sample from the chopped weight were taken for the estimation of B-vitamins. Some freshly chopped samples were preserved in refrigerator.

2.2. Reagents and Solvents

Standards of vitamins were purchased from Sigma-Aldrich, Taufkirchen, Germany. HPLC grade acetonitrile and methanol were purchased from Active Fine Chemicals Ltd., Dhaka, Bangladesh. Glacial acetic acid and orthophosphoric acid were purchased from BDH, Bengaluru-560025, India; triethylamine and hexane sulphonic acid sodium salt were purchased from Merck, Germany.

2.3. Instrumentation

High Performance Liquid Chromatographic system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model-SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used for the analysis. The data were recorded using LC-solutions software.

2.4. Preparation of Extraction Solutions

Extraction solution was made by mixing 50 mL of acetonitrile with 10 mL of glacial acetic acid and the volume was finally made up to 1000 mL with double distilled water.

2.5. Preparation of Buffer and Mobile Phase

To prepare buffer, 1.08 g of hexane sulphonic acid sodium salt and 1.36 g of potasium dihydrogen phosphate were dissolved in 940 mL HPLC water and 5 mL of triethylamine was added to it and the pH was adjusted to 3.0 with orthophosphoric acid. For the preparation of mobile phase, buffer and methanol were mixed in a ratio of 96:4 and filtered through 0.22 µm membrane filter and sonicated for degassing in an ultrasonic bath.

2.6. Standard Preparation

Standard stock solution for vitamin B_2 (riboflavin) was prepared by dissolving 6.9 mg of riboflavin in 100 mL of extraction solution. Standard stock solution for vitamin B_3 (niacin) was prepared by dissolving 41.5 mg of niacin in 25 mL of double distilled water. Standard stock solution for vitamin B_5 (calcium salt of pantothenic acid) was prepared by dissolving 21.4 mg of calcium d-pantothenate in 25 mL of double distilled water. Standard stock solution for vitamin B_6 (pyridoxine) was prepared by dissolving 20.8 mg of pyridoxine hydrochloride in 25 mL of double distilled water.

2.7. Preparation of Samples

10 g of each sample was weighed and made into homogenized in mortar with pestle and transferred into conical flask and 25 mL of extraction solution was added, kept on shaking water bath at 70˚C for 40 min. Thereafter, the sample was cooled down, filtered and finally the volume was made up to 50 mL with extraction solution. Then sample filtered through 0.45 µm filter tips and aliquots of 20 µL from this solution was injected into the HPLC by using auto-sampler.

2.8. Chromatographic Conditions

Analytical reversed phase C-18 column (ODS column, 250 × 4.6 mm, 5 µm, Phenomenex, Inc.) was used for the separation. Mobile phase consisting of a mixture of buffer and methanol in the ratio of 96:4 (v/v) was delivered at a flow rate of 1 mL/min with UV detection at 210 nm. The mobile phase was filtered through 0.22 µm membrane filter, sonicated and degassed before use. Analysis was performed at room temperature (~26˚C) temperature. All the prepared sample solutions were first chromatographed to ensure that interfering peaks were not present. 20 µL aliquots of the standard solutions and sample solutions were injected.

3. Results and Discussion

HPLC is one of the most convenient and accurate analytical techniques. To analyze the vitamin B complex from leafy vegetables, a RP-HPLC method was used which was described in the literature [10], by using the mobile phase comprising of buffer and methanol in the ratio of 96:4 (v/v) over C-18 column (250 × 4.6 mm, 5 µm, Phenomenex, Inc.) at ambient temperature. The flow rate was at 1 mL/min and the eluent was monitored by UV detector at 210 nm. The retention times of the vitamins were obtained as described in **Table 1**.

Table 1. Retention times of B-vitamins at 210 nm.

Name	Rt (minutes)
Vitamin B_2	3.61
Vitamin B_3	6.37
Vitamin B_5	9.51
Vitamin B_6	11.51

The method was found to be linear for the concentration range described in **Table 2**. When average peak areas were plotted against concentration levels a good correlation coefficients (r^2) were obtained as 0.99978, 0.99987, 0.99863 and 0.99996 for riboflavin, niacin, pantothenic acid and pyridoxine, respectively (**Table 2**). The slopes (m) of the calibration curve were found to be as 513339.785, 71752.58, 6583.039 and 67668.38774 for riboflavin, niacin, pantothenic acid and pyridoxine, respectively. The intercepts (c) of the calibration curve also were obtained to be as 119358.225, 96524.8, 24465.6 and 51841.6 for riboflavin, niacin, pantothenic acid and pyridoxine, respectively (**Table 2**, **Figure 1**).

The HPLC method was successfully performed for the estimation of riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), and pyridoxine (vitamin B_6) in leafy vegetables.

The amount of vitamin B_2 (riboflavin) in five leafy vegetables samples ranged from 0 to 0.523 mg/100 g (**Table 3**). The content of vitamin B_2 was found highest in green amaranth leaves (Data shak) (0.523 mg/100 g). Red amaranth leaves (Lal shak) contained second highest amount of vitamin B_2 (0.442 mg/100 g). Indian spinach (Pui shak) and bottle gourd leaves (Lau shak) have 0.397 mg/100 g and 0.321 mg/100 g vitamin B_2, respectively. Among the five leafy vegetables vitamin B_2 was lowest in bitter gourd leaves (Korola shak) (0.137 mg/100 g).

The highest amount of vitamin B_3 0.512 mg/100 g was estimated in bitter gourd leaves (Korola shak). Red amaranth leaves (Lal shak) contained 0.016 mg/100g; it was second the highest position of vitamin B_3. Indian spinach (Pui shak) and green amaranth leaves (Data shak) contained slight amount of niacin. Vitamin B_3 was not found in bottle gourd leaves (Lau shak).

The content of vitamin B_5 was not identified in green amaranth leaves (Data shak), red amaranth leaves (Lal shak), Indian spinach (Pui shak), bitter gourd leaves (Korola shak) and bottle gourd leaves (Lau shak), respectably (**Table 3**).

Availability of vitamin B_6 is comparatively less/absent in the leafy vegetables. The highest content of vitamin B_6 was present in bottle gourd leaves (Lau shak) which were 0.755 mg/100 g. Red amaranth leaves (Lal shak) contained 0.152 mg/100 g of vitamin B_6. Green amaranth leaves (Data shak) contained slight amount of pyridoxine (vitamin B_6), where it could not identify in bitter gourd leaves (Korola shak) and Indian spinach (Pui shak).

Comparison of B vitamin values between the present studies and the literature data is presented in **Table 4**. Literature data for vitamin B_5 and B_6 in selected leafy vegetables are unavailable. In the present study vitamin B_2 was detected in bottle gourd leaves 0.321 mg/100 g where Deshio Khaddar Pustiman (DKPM) [11], Indian

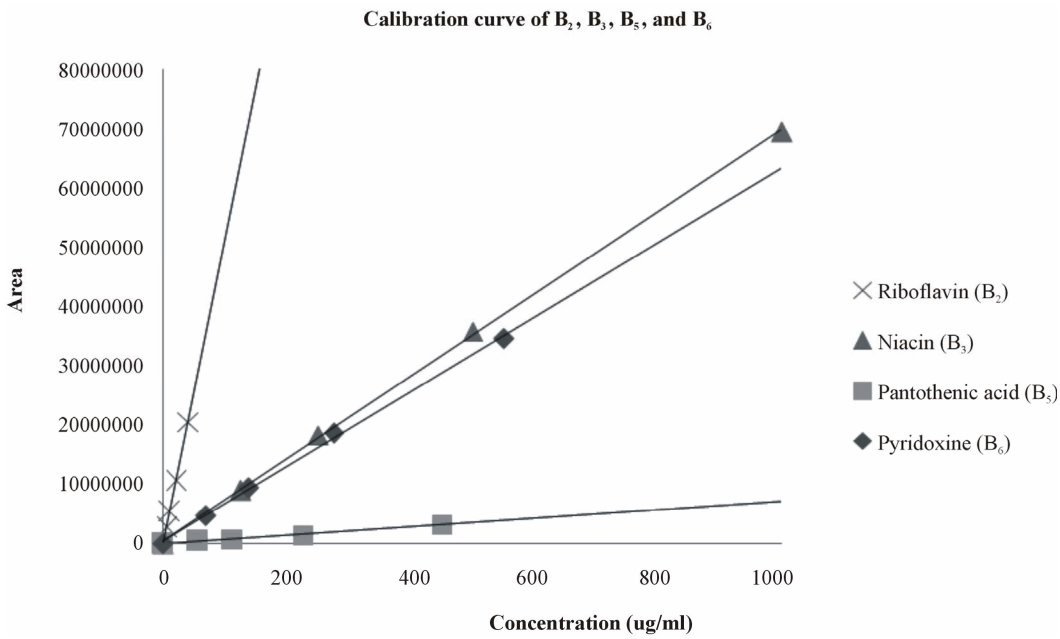

Figure 1. Calibration curve of vitamin B_2 (riboflavin), vitamin B_3 (niacin), vitamin B_5 (pantothenic acid) and vitamin B_6 (pyridoxine).

Table 2. The important parameters of the linearity curves for the standards.

Vitamin		*y = mx + c*	r^2	Conc. range (µg/mL)
B_2	Riboflavin	*y* = 513339.785*x* + 119358.225	0.99978	5 - 40
B_3	Niacin	*y* = 71752.58*x* + 96524.8	0.99987	125 - 500
B_5	Pantothenic acid	*y* = 6583.039*x* + 24465.6	0.99863	56.25 - 225
B_6	Pyridoxine	*y* = 67668.38774*x* + 51841.6	0.99996	68.75 - 275

m = slope; *c* = intercept; *r* = correlation coefficient.

Table 3. Vitamin B content of leafy vegetables.

English name	Scientific name	Bengali or local name	Average content of vitamin B (mg/100g of edible portion)			
			$B_2 \pm$ SD	$B_3 \pm$ SD	$B_5 \pm$ SD	$B_6 \pm$ SD
Bottle gourd leaves	*Lagenaria vulgaris*	Lau shak	0.321 ± 0.008	0.000	0.000	0.755 ± 0.116
Green amaranth leaves	*Amaranthus viridis*	Data shak	0.523 ± 0.003	0.051 ± 0.037	0.000	0.07 ± 0.002
Red amaranth leaves	*Amaranthus gangeticuss*	Lal shak	0.442 ± 0.009	0.016 ± 0.002	0.000	0.152 ± 0.006
Indian spinach	*Basella alba*	Pui shak	0.397 ± 0.007	0.023 ± 0.002	0.000	0.000
Bitter gourd leaves	*Momordica charantia*	Korola shak	0.137 ± 0.019	0.512 ± 0.103	0.000	0.000

"SD = Standard deviation".

food value (IFV) [12] and United States Department of Agriculture (USDA) [13] did not show the result. Bitter gourd leaves contain 0.137 mg/100 g B_2, which was not done in DKPM, IFV and USDA, respectively. Indian spinach contains 0.397 mg/100g of vitamin B_2, while the Indian study reported that Indian spinach contains 0.26 mg/100 g, the value was not found in DKPM and USDA. In the present study the content of B_2 was almost double in green amaranth leaves (0.523 mg/100 g) compare to the IFV and DKPM (0.18 mg/100 g). On the other hand, the value obtained from red amaranth Leaves 0.442 mg/ 100g was nearer to values of IFV but there was little difference with DKPM (**Table 4**).

The content of vitamin B_3 was investigated for all of selected leafy vegetable samples. Vitamin B_3 content of bitter gourd leaves was found 0.512 mg/100g whereas the value was not found IFV, DKPM and USDA, respectively. In this study vitamin B_3 was not found in bottle

Table 4. Comparison with published data.

Sample	Reference	Vitamin B_2 (riboflavin)	Vitamin B_3 (niacin)	Vitamin B_5 (pantothenic acid)	Vitamin B_6 (pyridoxine)
Bottle gourd leaves	A.V	0.321	0	0	0.755
	DKPM	-	-	-	-
	IFV	-	-	-	-
	USDA	-	-	-	-
Green amaranth leaves	A. V	0.523	0.051	0	0.07
	DKPM	0.18	0.18	-	-
	IFV	0.18	0.18	-	-
	USDA	0.158	0.658	-	0.192
Red amaranth leaves	A. V	0.442	0.0162	0	0.152
	DKPM	0.13	0.13	-	-
	IFV	0.30	0.30	-	-
	USDA	-	-	-	-
Indian spinach	A. V	0.397	0.023	0	0
	DKPM	-	-	-	-
	IFV	0.26	0.26	-	-
	USDA	-	-	-	-
Bitter gourd leaves	A. V	0.137	0.512	0	0
	DKPM	-	-	-	-
	IFV	-	-	-	-
	USDA	-	-	-	-

DKPM = Deshio Khaddar Pustiman [11], IFV = Indian Food Value [12], USDA = United States Department of Agriculture [13], AV = Average Value, and "-" = Not Done.

gourd leaves, where IFV, DKPM and USDA were not done the estimation before. Beside these the content of vitamin B_3 of red amaranth leaves was found 0.0162 mg/100 g, where DKPM and IFV got 0.13 and 0.30 mg/ 100 g, respectively. Green amaranth leaves were found 0.051 mg/100 g of vitamin B_3 where DKPM, IFV and USDA were found 0.18, 0.18 and 0.658 mg/100 g, respectively. In Indian spinach, the value was 0.023 mg/ 100 g where IFV was got 0.26 mg/100 g but DKPM and USDA were not done the estimation (**Table 4**).

The five leafy vegetable samples was not contained any vitamin B_5. Literature data for vitamin B_5 in selected leafy vegetables are unavailable (**Table 4**).

Literature data for vitamin B_6 in selected leafy vegetables are also unavailable. We found that bottle gourd leaves and red amaranth leaves contained vitamin B_6 as 0.755 mg/100g and 0.152 mg/100g, respectively, where green amaranth leaves contained slight amount and it was absent in Indian spinach and bitter gourd leaves (**Table 4**). Some values obtained from the present study were found to be different for different samples than the previous reports. This variation may occur due to various factors such as the environmental aspects like season, rainfall, climate, geographical and geological. Maturity of vegetables may also be partly responsible for this variation.

4. Conclusions

Health is very important to survival. A healthy and balanced diet is required to maintain a good health. To have a healthy diet everybody should know the nutrient composition of the foods they eat as well as the important nutrient content and amount of vitamin on their foods. In Bangladesh no recent data is available about the composition of foods that are eaten by the local and ethnic people. Moreover, Bangladeshi and Indian frequently suffer from lack of B vitamins in their daily diets.

Vitamin B deficiency is one of the major dietary problems in developing countries like Bangladesh. Poor people cannot intake vitamin B from rich foods like liver, egg, meat, fish etc. They depend on vegetables and fruits for vitamins and minerals. The study will provide the vitamins B composition of some leafy vegetables. Here we reported of vitamins B in a limited number of samples. We hope that, these new values will serve as a useful means to calculate dietary intake of B vitamins in the general population. These data will also be helpful in the preparation of a complete food composition table which will then be used everywhere, e.g. for the preparation of diet therapy, for food based dietary guidelines, for nutrition education, for food security, safety and regulation, for the labeling of food in food industry, for nutritional survey and also for other research purposes. Encouraging regular intake of those leafy vegetables containing B vitamins would remove vitamin B deficiency in Bangladesh. There is worldwide call for food composition database. With the increasing concern of the relationships between diet, food habits and degenerative diseases, development of FCD/FCT is a time demand issue. FCD provides support to variety of research activities in the food security mapping [14]. The third world countries are far behind to address this issue. South Asian countries, Particularly SAARC countries appear to be still in the initial stage of development of food composition database (FCD) as well as of food composition tables.

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