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Determination and evaluation of the pyridoxal, pyridoxine, and pyridoxamine forms of vitamin B_6 in plant-based foods in terms of healthy vegetarian nutrition

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Summary. *Background and Objective:* Vitamin B_6 deficiency is observed in vegetarians. In many studies, vitamin B_6 is determined as the sum of the pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM) forms. Because the bioavailability of the PL, PN and PM forms of vitamin B_6 are different, knowing the amounts of these forms in foods is important for healthy nutrition. The aim of this study was to determine the PL, PN and PM forms of vitamin B_6 in plant-derived foods and to evaluate these forms in terms of vegetarian and non-vegetarian nutrition. *Methodology:* In this study, a total of 61 foods from the vegetable (26), fruit (19), grain (10), legume (3) and nut (3) categories were examined using high-performance liquid chromatography (HPLC). *Results:* It was found that vegetables, fruits and grains contained more of the PN form along with the PL and PM forms, while the PL form was found in high levels in nuts. The PL form was found at an average rate of 29.6% in vegetables and 19.4% in fruits. The PN form was found predominantly in legumes and grains. *Conclusions:* When we evaluated the results, vegetables, fruits and grains contained more of the PN form along with the PL and PM forms, while the PL form was found in high amounts in nuts. Many vegetables contain low amounts of protein, but they contain high amounts of vitamin B_6 in the PLP form. These foods can be consumed together with foods that contain high levels of protein (legumes, grains) and low levels of vitamin B_6 in the context of vegetarian nutrition. This profile determination study will be an important source of information for the planning of various healthy diets for vegetarians as well as non-vegetarians.

Key words: Vitamin B_6, pyridoxal, pyridoxine, pyridoxamine, vegetarian nutrition, plant-based foods, HPLC

Introduction

A vegetarian diet is a primarily plant-based diet that occasionally includes meat, fish, dairy, eggs, and poultry in small quantities or not at all. Many types of diseases, such as cardiovascular disease, certain types of cancers, and obesity, are less common in individuals who follow this type of diet. However, vitamin B_{12} and vitamin D deficiency are more common in individuals who have adopted vegetarian diets because they do not consume animal-based foods (1). In addition, it has been reported that vitamin B_6 deficiency is observed in individuals who follow this type of diet (2-4).

Vitamin B_6 consists of 6 interconvertible forms, namely, PL, PN, PM, and the phosphate derivatives of these forms, pyridoxal 5'-phosphate (PLP), pyridoxine 5'-phosphate (PNP), pyridoxamine 5'-phosphate and pyridoxine-glucoside (PNG). The phosphate and glucoside forms of vitamin B_6 are hydrolyzed by the brush border enzymes into the PL, PN and PM forms prior to absorption in the small intestinal lumen. In the liver, the PL, PN and PM forms are phosphorylated by pyridoxal kinase enzymes and are converted into the PLP, PNP and PMP forms, respectively. The PNP and PMP forms are converted into the PLP form by pyridoxine (pyridoxamine) 5-phosphate oxidase (5). Vitamin B_6

functions as a cofactor in many biochemical reactions. In amino acid metabolism, the active form of vitamin B_6, PLP, works as a coenzyme for more than 100 known enzymes. Vitamin B_6 deficiency is associated with signs of skin aging, eczema, disorders of the digestive system, depression, confusion and visual disorders (6). Additionally, PLP is involved in the conversion of homocysteine into cysteine, a product of methionine metabolism. The increase in serum homocysteine levels associated with PLP deficiency leads to cardiovascular diseases (7).

It has been stated that the daily vitamin B_6 requirement is 16 µg per gram of protein consumed for both males and females. The vitamin B_6 requirement increases as the intake of protein increases because vitamin B_6 acts as a coenzyme in amino acid metabolism (6, 8, 9). The daily recommended intake of vitamin B_6 per day is between 1.5 and 1.7 mg for humans (10). Vitamin B_6 is found in a wide variety of foods, but some of the best sources are yeast extracts, wheat bran, meat, fish, grains, legumes and dark leafy vegetables (11). However, vegetarians obtain most of their vitamin B_6 from legumes, grains, cereals, vegetables, nuts and fruits, which contain high amounts of PNG form of vitamin B_6. PNG is known to be less bioavailable than the PLP form. Therefore, the vitamin B_6 status of vegetarians may be lower than that of non-vegetarians, which may lead to an increase in serum homocysteine levels (3).

It was reported that the bioavailability of vitamin B_6 was 58±13% when the PN-glucoside form of vitamin B_6 was orally administered to humans (12). In a clinical study, when the PLP and PN.HCl forms were orally administered to humans, serum PLP levels were reported to be approximately 50% higher in subjects who received PLP (13).

The HPLC method is preferred for the identification of vitamin B_6. Through this method, all forms of vitamin B_6 can be determined (14). Vitamin B_6 can be found in free forms as PL, PN and PM in foods or in the phosphorylated or glycoside forms. Usually, in the analysis, the phosphate and glycoside bonds of vitamin B_6 are liberated by enzymes and are shown as total PL, PN and PM (15). However, in many studies, due to the long duration of analysis and the effects of the matrix, the PL and PM forms are converted into the PN form by methods of derivation, and the total vitamin B_6 level is given.

The content of the PL, PN and PM forms of vitamin B_6 vary in plant-based foods. In many studies in the literature or in food composition databases, vitamin B_6 content is expressed as the sum of the PL, PN and PM forms or in the PN.HCl form. Very few studies have reported the PL, PN and PM forms of vitamin B_6. Therefore, the determination of the profile of vitamin B_6 will be important for human nutrition in terms of the bioavailability of vitamin B_6 in foods. The aim of this study was to identify the PL, PN and PM forms of vitamin B_6 in plant-derived foods and evaluate these forms in terms of vegetarian as well as nonvegetarian nutrition.

Materials and Methods

The standard vitamins (pyridoxine hydrochloride, pyridoxamine hydrochloride and pyridoxal hydrochloride), taka-diastase from *Aspergillus oryzae*, (100 U/mg), acid phosphatase, (potatoes, 0.5-3.0 U/mg), β-glucosidase (from lyophilized almond powder, 10-30 units/mg solid), acetonitrile (ACN), potassium dihydrogen phosphate (KH_2PO_4) and 1-octanesulfonic acid sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). In this study, all other chemicals used were of high purity.

Sampling

All foods examined in this study were purchased from local markets. A total of 61 foods were examined in the categories of vegetables (26), fruits (19), grains (10), legumes (3) and nuts (3).

Standard Preparation

A standard stock solution of each vitamin was prepared in a 0.1 N hydrochloric acid (HCl) solution. Each standard was freshly prepared daily. The three levels of working standards were prepared from the stock solution.

Extraction of B_6 vitamers from foods

The extraction method described by Kall (15) was used with some modifications. The samples were first homogenized, and 10 g of each sample was placed into a 100 ml Erlenmeyer flask. Next, 60 ml of 0.1 N hydrochloric acid solution was added before the mixture was transferred to an autoclave, where it was kept at 121°C for 30 minutes. The samples were cooled and then the pH was adjusted to 4.5 using a sodium acetate (2.5 mM) solution. Then, 100 mg taka-diastase, 10 mg acid phosphatase, and 10 mg β-glucosidase enzymes were added to the sample before it was incubated for 18 hours at 37°C in a shaking water bath. The samples were cooled until they reached room temperature, and a volume of 100 ml was reached using a 0.1 N HCl solution. Finally, the samples were filtered, first with filter paper and then with a 0.45 µm filter, before being transferred into the HPLC device. All analyses were performed in triplicate, and the average value was used.

HPLC determination of B_6 vitamers

The PL, PN and PM forms of vitamin B_6 were identified using the reversed-phase HPLC method. The HPLC conditions described by Ceylan *et al.* (16) were used with some modifications. The HPLC system that was used in the study included a Shimadzu Nexera-i device with a Shimadzu RF-20A fluorescence detector (Shimadzu Corporation, Kyoto, Japan). The mobile phase was prepared in the following manner. First, the buffer solution was prepared by dissolving 11 g of KH_2PO_4 and 0.5 g of 1-octanesulfonic acid in 950 mL of deionized water. Next, 40 ml of ACN was added, and the pH was adjusted to 2.4 with orthophosphoric acid. The fluorescence detector excitation and emission wavelengths were set at 290 and 395 nm, respectively. B_6 vitamers were separated with an ACE C18, 5 µm, 4.6x250 mm column (Agilent, Santa Clara, CA, USA) with a flow rate of 1 mL/min. The column oven temperature was set to 25°C.

Quantification and Quality Control

In the study, the certified reference material (Standard Reference Material 1849a: Infant Formula) was used to check the accuracy and performance of the method used. Moreover, we also participated in a proficiency test to analyze breakfast cereal test material, which was organized by Food Analysis Performance Assessment Scheme in UK, 2018.

Results

The amount of vitamin B_6 in the reference material was found to be 13.21 mg/kg (assigned value 13.46±0.93), and the recovery rate was 98%. The FAPAS test result was found to be in the acceptable range (–2 ≤ Z score ≤ +2).

The sample HPLC chromatogram of the vitamin B_6 content in pineapple is shown in Figure 1. The amounts of PL, PN, and PM and the total vitamin B_6 content of the vegetables are shown in Table 1. The total vitamin B_6 content was found to be highest in dried garlic (957.9 µg /100 g) and lowest in cucumber (28.3 µg /100 g). The level of the PL form of vitamin

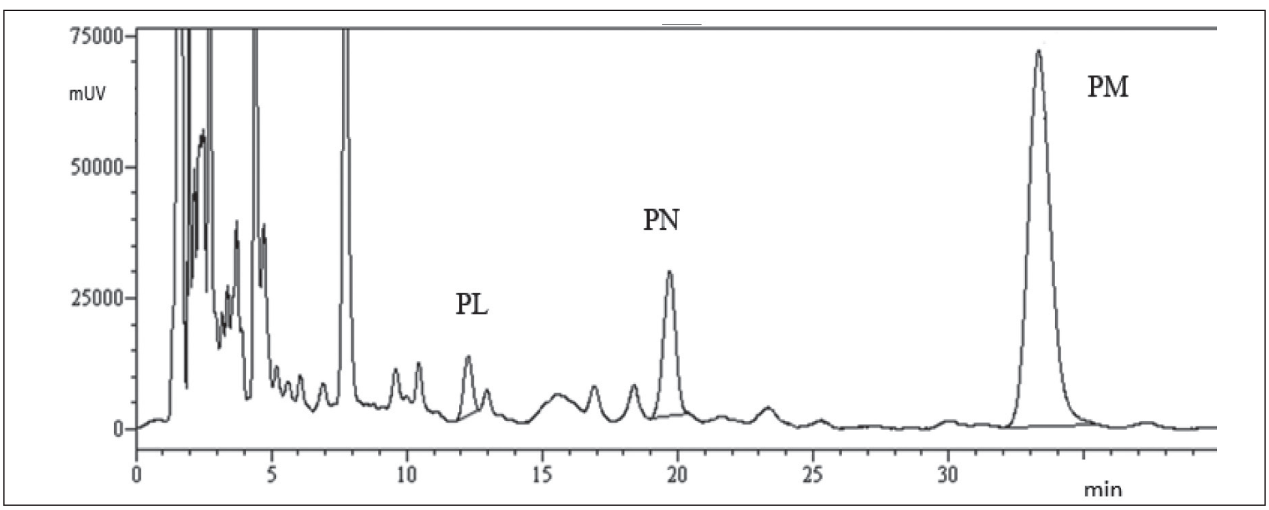

Figure 1. HPLC chromatogram of vitamin B_6 in pineapple

Table 1. Amounts of the PL, PN and PM forms and total vitamin B_6 in vegetables

Sample name	PL µg/100 g	PN µg/100 g	PM µg/100 g	Vitamin B_6, total µg/100 g
Dried garlic	571.4±28.6	366.2±23.2	20.2±1.1	957.9
Peppermint	61.6±3.5	381.9±28.4	14.0±1.0	457.4
Sweet corn	254.5±18.2	34.6±3.0	81.7±3.5	370.7
Cress	46.0±2.4	216.4±15.5	47.5±2.4	309.9
Dill	40.2±3.0	188.5±12.0	9.1±0.6	237.8
Spinach	68.1±4.1	115.2±8.7	44.0±1.8	227.3
Broccoli	103.4±5.8	65.8±2.9	20.2±0.7	189.5
Yellow pepper	19.7±1.0	97.1±5.4	67.7±4.2	184.5
Brussels sprouts	44.3±2.3	129.2±7.2	8.4±0.6	181.9
Cauliflower	84.6±5.1	49.4±2.1	36.3±1.3	170.2
White potato	30.4±2.0	114.4±6.3	25.1±1.5	169.9
Fennel leaves	21.3±1.2	142.4±9.6	4.2±0.3	167.9
Beans	66.5±4.2	62.5±3.0	9.1±0.4	138.1
Chard	48.4±3.5	60.1±2.8	26.5±2	135.0
Carrots	14.8±1.0	98.8±6.2	4.9±0.5	118.4
Rocket	15.6±0.8	71.6±4.8	22.3±1	109.5
Green onion	24.6±1.3	60.1±4.2	9.1±0.6	93.8
Black radish	27.1±1.2	63.4±4.0	2.1±0.1	92.6
Purslane	45.2±3.3	12.3±3.4	31.4±1.8	88.9
Leek	34.5±2.4	41.2±2.1	11.2±1.0	86.8
Green beans	4.9±0.6	64.2±3.2	11.2±0.6	80.3
Peas	14.8±1.1	31.3±2.0	30.0±1.4	76.1
Cabbage	25.5±2.0	19.8±1.5	20.2±1.3	65.4
Tomato	5.7±0.4	31.3±3.0	27.9±2	64.9
Crisp lettuce	19.7±1.2	22.2±1.3	4.9±0.5	46.8
Cucumber	6.6±0.6	18.9±1.5	2.8±0.1	28.3

Average value was used (n=3). nd, not detected; pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM)

B_6 was found to be highest in sweet corn (68.6%), dried garlic (59.7%), broccoli (54.6%), purslane (50.8%), and cauliflower (49.7%) and lowest in green beans (6.1%). The level of the PN form was found to be highest in fennel (84.8%) and lowest in sweet corn (9.3%), while the level of the PM form was found to be highest in tomato (43.0%) and lowest in dried garlic (2.1%). The average rates of the PL, PN and PM forms in the vegetables were 29.6%, 54.5% and 16.0%, respectively (Fig. 2a). The PN form of vitamin B_6 was predominant in the vegetables. When green and non-green vegetables were compared, it was observed that the PN form was predominant at similar rates in the two groups (56% green, 52% nongreen).

The results in fruits are shown in Table 2. The total vitamin B_6 content was found to be highest in figs (374.7 µg/100 g) and bananas (288.9 µg/100 g). As shown in the table, the levels of vitamin B_6 was low in the oth-

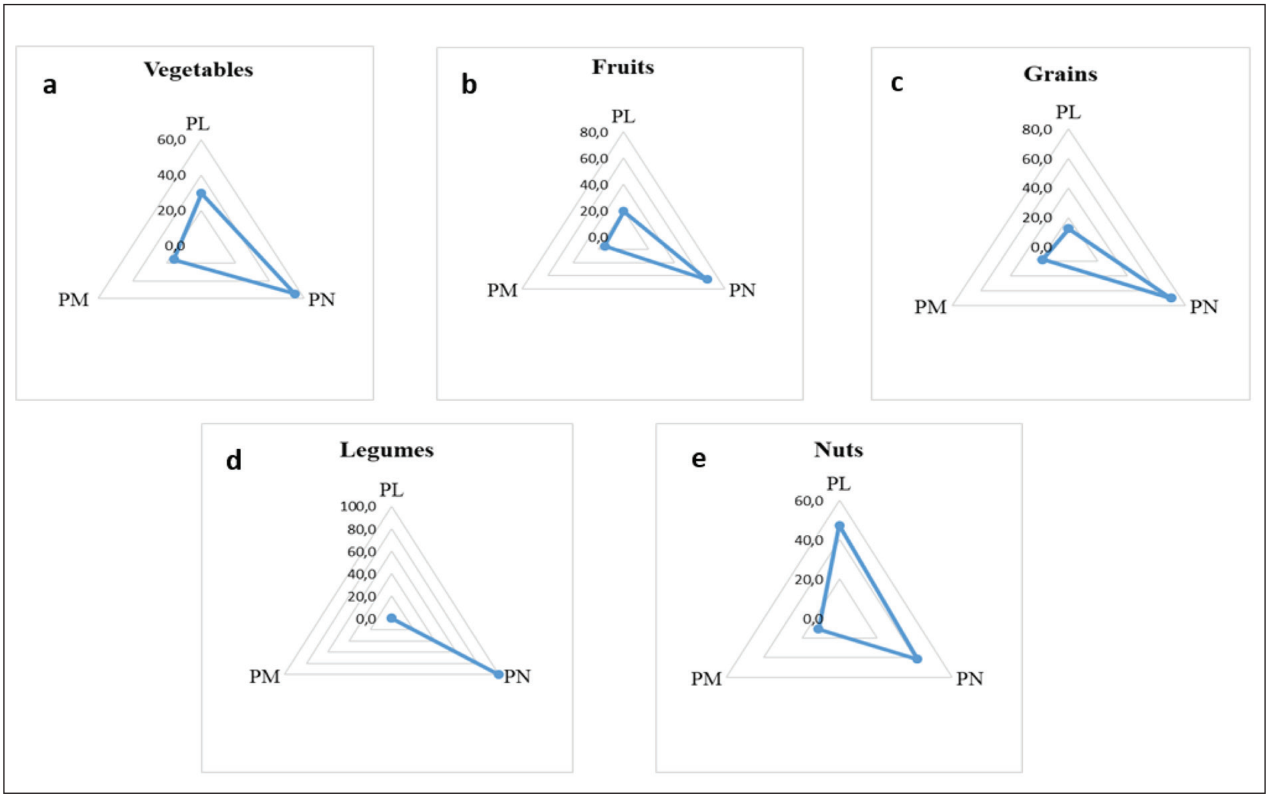

Figure 2. Rates of the PL, PN and PM forms of vitamin B_6 in vegetables, fruits, grains, legumes and nuts

er fruits. The level of the PL form was determined to be the highest in oranges (54.6%), kiwi (44.1%), and white grapes (41.0%) and lowest in pineapple (24.4%) and cherries (1.7%). The level of the PN form was determined to be the highest in cherries (92.7%), figs (89.3%), nectarines (92.3%), and apples (91.6%) and lowest in bananas (8.3%), while the level of the PM form was found to be the highest in pineapple (69.2%) and was not detected at all in plums, quince or kiwi. The average rates of the PL, PN, and PM forms in the fruit samples were 19.4%, 65.9%, and 14.7%, respectively (Fig. 2b). The PN form was predominant in the fruits.

The results in grains, legumes and nuts are shown in Table 3. The total vitamin B_6 content was highest in wheat germ (564.1 µg/100 g) and lowest in rice (60.9 µg/100 g) and wheat flour (30.1 µg/100 g). The level of the PL form was found to be the highest in wheat germ (37.7%), while it was not detected in rice or wheat flour. The PN form was detected at the highest level in rice (100.0%) and at the lowest level in wheat bread (37.7%), while the PM form was detected at the highest levels in wheat bread (32.2%) and was not detected in rice or wheat germ. As seen in the table, the PN form was predominant in the grain samples, and the average rate of PN was 70.0% (Fig. 2c). In the legume samples, despite containing high amounts of vitamin B_6 (399.2-464.2 µg/100 g), none of the PL or PM forms were detected, but the PN form was present (Fig. 2d).

Discussion

The PN and PNP forms of vitamin B_6 were predominant in plant-based foods. PN-glucoside was found at a rate of 5% in bananas, 58% in green beans, 70% in carrots, and 69% in orange juice (17). In our study, the PN form was found at a rate of 8.3% in bananas, 80.0% in green beans, 83.4% in carrots, and 35.2% in oranges. In the study conducted by Kall (15), the PN, PL and PM forms were found in broccoli at rates of 40%, 49%, and 11%, respectively. In the same study, it was observed that the PN form was predomi-

Table 2. Amounts of the PL, PN and PM forms and total vitamin B_6 in fruits

Sample name	PL µg/100 g	PN µg/100 g	PM µg/100 g	Vitamin B_6, total µg/100 g
Figs	39.4±2.4	334.5±25.2	0.7±0.1	374.7
Bananas	76.4±5.2	24.1±1.8	188.5±12.5	288.9
Kiwi	50.1±3.8	63.5±5.4	nd	113.6
Mandarin	5.7±0.4	61.0±4.4	34.9±2.2	101.6
Oranges	47.6±2.8	30.8±2.8	9.1±0.6	87.5
Pineapple	4.9±0.3	19.0±1.2	53.7±4.0	77.7
Apricots	1.6±0.1	54.1±3.8	7.0±0.4	62.7
Watermelon	3.3±0.2	38.7±2.4	18.8±1.0	60.8
Pink-red grapes	10.7±0.5	56.0±5.0	0.7±0.1	67.3
Lemons	16.4±0.5	30.0±2.6	11.2±0.4	57.6
Plums	11.5±0.6	45.0±3.8	nd	56.5
Apples	3.3±0.2	51.5±4.6	1.4±0.1	56.2
Raspberries	19.7±0.8	27.4±2.3	3.5±0.2	50.6
Sweet red cherries	0.8±0.1	45.8±2.4	2.8±0.2	49.4
Quince	14.8±0.7	27.2±2.2	nd	41.9
White grapes	14.0±0.4	17.7±1.5	2.8±0.1	34.4
Nectarines	1.6±0.2	28.1±1.8	0.7±0.1	30.4
Strawberries	2.5±0.2	26.4±2.0	0.7±0.1	29.6
Pears	1.6±0.1	10.8±0.4	1.4±0.2	13.9

Average value was used (n=3). nd, not detected; pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM).

nant in other fruits and vegetables. In our study, the PN, PL and PM forms were found in broccoli at rates of 34.7%, 54.6%, and 10.7%, respectively, and the PN form was generally more predominant in other fruits and vegetables. Additionally, the total vitamin B_6 results in this study were consistent with the figures in food composition databases (11). As seen in the table, vegetables such as broccoli, purslane, cauliflower and beans contain high amounts of vitamin B_6, especially the PL form.

It was reported that the PN form of vitamin B_6 is more predominant in cereals and legumes (18). During milling processes, 75-90% of vitamin B_6 in whole grains is lost. Therefore, there was less vitamin B_6 in the milled grains (19). Considering our results, the PN form was found in higher amounts in grains. As seen in the table, there was 321.2 µg/100 g of vitamin B_6 in wheat bread but only 30.1 µg/100 g of vitamin B_6 in flour. There was a significant loss of vitamin B_6 in flour as a result of the grinding stage. In addition, the PL form was detected at certain amounts in wheat bread but was completely absent in flour. In nuts, high amounts of vitamin B_6 were observed in pistachios (1252.5 µg/100 g), hazelnuts (377.9 µg/100 g) and walnuts (442.9 µg/100 g). In particular, hazelnuts (72.6%) and walnuts (53.4%) contain high levels of the PL form. In pistachios, a high level of the PN form (70.8%) was observed (Fig. 2e).

When we evaluated our results, vegetables, fruits, grains and dried legumes generally contained high levels of the PN form. The bioavailability of the PL and PM forms of vitamin B_6 is higher than that of the PN form. In previous studies, it was reported that the bioavailability of vitamin B_6 of animal origin

Table 3. Amounts of the PL, PN and PM forms and total vitamin B_6 in grains, legumes and nuts

Grains	**PL µg/100 g**	**PN µg/100 g**	**PM µg/100 g**	**Vitamin B_6 total µg/100 g**
Wheat germ	212.6±12.2	351.4±22.2	nd	564.1
Durum wheat	22.2±1.5	242.0±13.1	89.3±4.2	353.5
Wheat bread	62.4±5.4	155.5±10.0	103.3±6.8	321.2
Dried corn	79+.6±5.0	163.0±13.5	64.2±4.1	306.8
Barley	15.6±1.1	158.8±14.4	56.5±3.3	231.0
Rye	18.9±1.3	153.1±7.4	30.0±1.4	202.0
Oat flour	6.6±0.3	132.5±4.8	40.5±2.6	179.6
Oat	24.6±1.2	127.6±9.5	28.6±1.6	180.8
Rice	nd	60.9±4.6	nd	60.9
Wheat flour	nd	23.9±2.0	6.3±0.2	30.1
Legumes				
Dried beans	nd	464.2±22.2	nd	464.2
Red lentils	nd	440.3±16.8	nd	440.3
Chickpeas	nd	399.2±20.1	nd	399.2
Nuts				
Hazelnuts	274.2±11.1	103.7±6.4	nd	377.9
Dried walnuts	236.4±16.5	118.5±8.4	87.9±4.8	442.9
Pistachios	197.9±13.0	886.4±26.6	168.2±11.1	1252.5

Average value was used (n=3). nd, not detected; pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM).

reached 100%, and fiber-containing foods reduced the bioavailability of B_6 by 5-10% (6). It was emphasized that the bioavailability of foods containing the PN-glucoside form is very low. In another study, it was found that serum PLP levels were very low during the lactation period among vegetarian women (20). It is known that vitamin B_6 has many positive health effects. The most important of these is that it reduces homocysteine formation (21), especially the PLP form of vitamin B_6, which has a more active role in the related mechanisms. It is recommended to consume 16 µg of vitamin B_6 per gram of protein in the diet (6, 9).

There are significant losses of vitamin B_6 in grains due to the milling process. Although milled flour contains high amounts of protein, it contains low amounts of vitamin B_6. Likewise, legumes contain high amounts of protein and vitamin B_6. However, the bioavailability of vitamin B_6 may be lower because it contains only the PN form along with dietary fiber. It is thought that vegetarians have higher fiber intake than non-vegetarians, and this may reduce the bioavailability of vitamin B_6 (3). In particular, vitamin B_6 converts the homocysteine resulting from methionine metabolism into cysteine. Thus, serum homocysteine levels may increase in individuals with vitamin B_6 deficiency. In a study of vegetarians in Austria, vitamin B_6 deficiency was found in one third of all subjects (n=128), and a high plasma homocysteine concentration was detected in 66% of vegans (4). Therefore, other foods rich in the PL form of vitamin B_6 (broccoli, purslane, cauliflower, bean, etc.) may be consumed together with grains and legumes.

Conclusion

In many studies in the literature and in food composition databases, vitamin B_6 is generally discussed as a single component. Vitamin B_6 plays an important role in the metabolism of proteins, carbohydrates and lipids as well as in the reduction of the levels of serum homocysteine. The contents of the PL, PN and PM forms of vitamin B_6 vary among food groups. Therefore, their bioavailabilities may be different. This study is the most comprehensive profile-specification study of vitamin B_6 that has been conducted thus far. When we evaluated the results, it was shown that vegetables, fruits and grains contain more of the PN form along with the PL and PM forms, while the PL form is found at high rates in nuts. Many vegetables contain low amounts of protein, but they contain high amounts of vitamin B_6, especially the PLP form. Vegetarians can consume these foods together with foods that contain high protein (legumes, grains) and low vitamin B_6. This profile determination study will be an important source of information for the development of various healthy diets for vegetarians as well as non-vegetarians.

Acknowledgments

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