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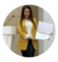

Jale Çatak
Istanbul Sabahattin Zaim University

Merve Nur Gizlici
Istanbul Sabahattin Zaim University

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Research article

The effect of *in vitro* simulated gastrointestinal digestive system on the biodegradation of B group vitamins in bread

Jale Çatak [*], Merve Nur Gizlici

Department of Nutrition and Dietetics, Faculty of Health Sciences, İstanbul Sabahattin Zaim University, 34303, Küçükçekmece, Halkalı, Istanbul, Turkey

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ABSTRACT

Today, there is a growing interest in the consumption of whole grain products and the development of bread enriched with vitamins that have functional properties. Considerable losses arise in naturally found vitamins with food processing. Therefore, it is recommended to add vitamins to bread to obtain a satisfactory level. The aim of the current research was to investigate and assess the bioaccessibilities of the vitamins B_1, B_2, B_3, and B_6 in enriched commercial whole wheat breads by an *in vitro* digestion model. The average bioaccessibility of vitamin B_1, B_2, B_3, and B_6 in enriched breads after digestion was 80%, 64%, 79%, and 64%, respectively. After digestion, the bioaccessibilities of vitamins were affected. Mainly, vitamins B_2 and B_6 had the lowest bioaccessibility than vitamins B_1 and B_3. *In vitro* bioaccessibility was 70.9–90.2%, 54.2–89.7%, 42.1–94.9%, and 44.1–92.5% for vitamins B_1, B_2, B_3, and B_6, respectively in enriched commercial whole wheat bread. Vitamin B_3 was seen with predominantly higher levels among the breads. Knowing the content of these vitamins in breads after digestion is necessary for the healthy nutrition of the population and for determining daily intake.

1. Introduction

Cereals naturally contain high amounts of water-soluble vitamins [USDA, 2022]. Bread mainly consist of processed cereals. Vitamins B_1, B_2, B_3, and B_6 are the leading constituents of bread. Significant losses occur in vitamin content during the processing of cereals [Sauberlich, 1985]. Therefore, the amount of vitamins B_1, B_2, B_3, and B_6 is expected to be low in bread. So, these vitamins are usually added to commercial bread to guarantee an adequate intake of persons. In Turkey, the national staple food is bread with an average daily intake of 319 g [TMO, 2013], and it can provide the required supplements of vitamin B_1, B_2, B_3, and B_6 to the population.

Bread is popular across the world as a staple food in many populations. Nowadays, replacing refined flour with high-fiber constituents is gaining additional attention in healthy nutrition, aiming to slow down its digestion among several health benefits. Also, it is well known that vitamins have health-promoting potency. The vitamin-fortified whole wheat bread can provide health benefits to consumers who demand a healthier alternative to the usual type of bread. Therefore, there is a growing interest in the consumption of whole-grain products and the development of products, enriched by vitamin [Blandino et al., 2013].

Vitamin B_1 (thiamine) functions in the pyruvate's conversion to the acetyl CoA in energy metabolism as a coenzyme, and vitamin B_2 (riboflavin) is essential in tricarboxylic and electron transport chains. Vitamin B_3 (niacin) is important in energy metabolism, mainly oxidative phosphorylation, which is also critical for the metabolism of protein, carbohydrates, and fat. Vitamin B_6 is primarily involved in protein metabolism, transamination, and deamination metabolism. Vitamin B_6 is also essential in the prevention of homocysteine-associated vascular diseases [Ball, 2004]. Vitamin B_1's average requirement is approximately 0.072 mg/MJ for adult men and women, vitamin B_2 is 1.3 mg/d, niacin is 1.3 mg NE/MJ, and vitamin B_6 is 1.5 and 1.3 mg/d [European Food Safety Authority, 2017].

Vitamin B_1 and vitamin B_2 exist in their free forms such as thiamine and riboflavin or in their phosphorylated forms such as TMP (thiamin monophosphate), TPP (thiamin pyrophosphate), FMN (flavin mononucleotide), and FAD (flavin adenine dinucleotide) in foods and living tissues. Vitamin B_3 (niacin), can be exist in the forms of nicotinic acid and nicotinamide. The commercially available and widely used form in foodstuffs is nicotinamide. In biologicals, nicotinamide is present as NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) [Eittenmiller et al., 2008]. TPP plays a role in the pyruvate's conversion to acetyl CoA and as a coenzyme in the

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[*] Corresponding author
E-mail address: jalecatak@gmail.com (J. Çatak).

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pathway of pentose phosphate. In contrast, FMN, FAD, and NAD play a role in energy metabolism, mainly in the electron transport chain and in the TCA (tricarboxylic acid cycle). The phosphate forms of vitamin B_1 and vitamin B_2 are hydrolyzed into free forms by the alkaline phosphatase enzyme in intestinal absorption [Ball, 2006]. Vitamin B_6 found in foodstuffs in the forms of; PL (pyridoxal), PN (pyridoxine), PM (pyridoxamine), PLP (PL-5′-phosphate), PNP (PN-5′-phosphate), PM-5′-phosphate, PNG (pyridoxine-glucoside), and PN.HCI (pyridoxine hydrochloride) [Çatak and Çaman, 2020; Çatak et al., 2020]. The commercially available and widely used form in foodstuffs is PN.HCI. PLP, the bioactive form, acts as a coenzyme in living organisms in more than 100 identified enzymes [Eittenmiller et al., 2008; Yaman et al., 2021].

Functional foods, like high vitamin and fiber-containing whole wheat breads, should include bioactive substances, but they should also be easily digested and absorbed by the human metabolism. Thus, investigations that measure the bioaccessibility of micronutrients are crucial to reflect the true nutritional value of foodstuff. Bioaccessibility is the total amount of an ingested nutrient that is potentially available for absorption in the gastrointestinal system. Thus, bioaccessibility is utilized to estimate the bioavailability of foodstuffs [Benito and Miller, 1998]. Nowadays, it is critical to know the bioavailability of micronutrients, such as vitamins in a diet, in terms of creating healthy diets [Van den Berg et al., 2002]. But, there is not enough data on the vitamin digestibility in humans since the bioavailability investigations require clinical studies which have disadvantages due to the necessity of ethical procedures, time, and cost. Therefore, *in vitro* procedures are used to investigate the bioaccessibility of nutrients. *In vitro* methods present many benefits compared to *in vivo* investigations since there are cost-effective, less time required, provide better controls of test variables than animal or human research, and allow rapidly monitoring of digestion, the release of nutrients, and structural changes [Hur et al., 2011; Yaman et al., 2019; Uğur et al., 2020].

In the literature, limited studies are available on the bioaccessibilities of B vitamins in foods, but no work is available in different kinds of fortified commercial whole wheat breads. Therefore, the study's objective is to investigate and assess the effect of *in vitro* gastrointestinal digestive system model on the bioaccessibilities of vitamins B_1, B_2, B_3, and B_6 in enriched commercial whole wheat breads.

2. Materials and methods

2.1. Chemicals and materials

The standards of vitamin (thiamine, riboflavin, nicotinamide, pyridoxal hydrochloride), pepsin from porcine gastric mucosa (lyophilized powder, ≥250 U/mg solid), taka diastase from *Aspergillus oryzae* (powder, 100 U/mg), beta-glucosidase from almonds (lyophilized powder, 10–30 U/mg solid), acid phosphatase from potatoes (lyophilized powder, 0.5–3.0 U/mg), alpha-amylase from *Aspergillus oryzae* (powder, 1.5 U/mg), pancreatin (from porcine pancreas 8xUSP specifications), lipase (from porcine pancreas Type II, 100–500 U/mg protein), NaCl, $CaCl_2.2H_2O$, hydrochloric acid (HCl, 37%), urea, uric acid, mucin, methanol (MeOH), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$), 1-heptanesulfonic acid sodium salt, potassium ferricyanide (III) ($K_3Fe(CN)_6$), hydrogen peroxide (H_2O_2) (30%), KCl, ortho-phosphoric acid, trichloroacetic acid (TCA), potassium dihydrogen phosphate (KH_2PO_4), $NaHCO_3$, acetonitrile (ACN), bovine serum albumin, bile salts mixture, and a Teflon tube with the length of 20 m and diameter of 0.5 mm were provided from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Standard preparation

Three levels of working standards are prepared fresh daily from a stock solution in 0.1N HCl solution.

2.3. Sampling

In the investigation, 8 types of marketable whole wheat breads enriched with B complex vitamins (B_1, B_2, B_3, B_6) were bought from different market places in Istanbul, Turkey. The product list and the main constituents of fortified breads are shown in Table 1. For the prioritization of whole wheat bread, we considered criteria such as; the lack of nutrient data, the economic importance of bread, bread diversity, and the frequent consumption of the bread and its marketing potential. A total of 8 commercial whole wheat bread fortified with B group vitamins were included in this study. The bread samples used in this study were collected from at least eight different locations in Istanbul, Turkey. The supermarkets have been chosen among those that appeal to large populations, have multi chains, and have high sales rates.

2.4. Vitamin B_3 extraction in fortified breads

Çatak [2019] described the extraction and HPLC determination methods for vitamin B_3 were performed with minor modifications. First, a five g homogenized bread sample was put into a 100 mL Erlenmeyer flask. After the addition of the 60 mL 0.1 N HCl solution, the test sample was autoclaved at 121 °C for 30 min. Enzymatic extraction is not required for vitamin B_3. Then the bread samples were cooled and using deionized water, the volume was adjusted. At that point, the samples were filtered through a CA (cellulose acetate) filter (0.45 μm) and injected into the HPLC.

2.5. Vitamins B_1 and B_2 extraction in fortified breads

The extraction procedure and HPLC determination method about vitamins B_1 and B_2 described by Akça et al. [2019] were performed with minor adaptations. The initial step of the extraction process was the same as mentioned above until the end of autoclaving. Then, an enzymatic process was achieved for releasing the phosphorylated vitamers of thiamine (TDP, TTP, and TMP) and riboflavin (FMN and FAD). The mixture was cooled; then, the pH was adjusted to 4.5 using a sodium acetate solution (2.5 mM). The enzymatic extraction stage as follows; 10 mg acid phosphatase and 100 mg taka diastase were added and incubated in a

Table 1. Characteristics of the fortified commercial whole wheat breads, bread types with their main contents.

Sample	Bread type	Main bread content including added vitamins B	Dietary Fiber (g/100 g)
1	Whole wheat bread	Whole wheat flour, sour dough, vitamin B_1 and B_3	6.98
2	Buckwheat rye bread	Whole wheat flour, wheat flour, rye flour, buckwheat flour, vitamin B_1, B_2, B_3, and B_6	6.5
3	Whole wheat bread with chia seeds	Whole wheat flour, wheat flour, chia seed, sunflower seed, flax seed, vitamin B_1, B_2, B_3 and B_6	6.6
4	Whole wheat bran bread	Whole wheat flour, wheat flour, wheat bran, vitamin B_1, B_2, and B_6	7.22
5	Traditional bread	Whole wheat flour, wheat flour bran, rye flour, rye bran, vitamin B_1, B_2, and B_6	7.93
6	Rye bread	Rye flour, wheat flour, whole wheat flour, rye, vitamin B_1, B_2, B_3, and B_6	9.40
7	Light bread	Whole wheat flour, wheat bran, vitamin B_1, B_2, and B_6	13.48
8	Multigrain bread	Wheat flour, whole wheat flour, wheat-rye-oat grains, sunflower seed, flax seed, poppy seed, millet, black cumin seed, vitamin B_1, B_2, and B_6	10.5

shaking water bath for 3 h at 37 °C. In the last stage of in vitro gastrointestinal digestion, just acid phosphatase was utilized in breads. Following, the samples were cooled and filtered using a CA filter (0.45 μm). Lastly, for the determination of vitamin B_2, the solution was injected into the HPLC.

2.6. Derivatization process of thiamine

Pre-column derivatization was done in the analysis of thiamine. Thiamine, which is the free form of vitamin B_1, cannot be determined in the fluorescence detector because of its molecular configuration. Thus, thiamine is initially converted to the thiochrome, fluorescence derivative, using a potassium ferricyanide solution. First, 1.5 mL $K_3Fe(CN)_6$ solution was prepared with 0.25 g in 25 mL NaOH solution (15%) and added to 20 mL of filtrate, which was delivered from the above solution. Then, the pH was adjusted to 7.1 ± 0.1 using ortho-phosphoric acid. Finally, the derivatized solution was filtered using a CA filter (0.45 μm) and injected into the HPLC for vitamin B_1 analysis. The standard of thiamine was also derivatized using $K_3Fe(CN)_6$ solution and adjusted to pH 7.1 ± 0.1 with ortho-phosphoric acid.

2.7. Vitamin B_6 extraction in fortified breads

Kall [2003] extraction technique for vitamin B_6 was implemented with minor modifications. First, after homogenizing the breads, 5 g of the sample was taken into a 500 mL Erlenmeyer flask. Afterward, 60 mL of 0.1 N HCl solution was added, then this mixture was autoclaved at 121 °C for 30 min. Then, the enzymatic process was achieved to release the phosphorylated and glycoside forms of vitamin B_6 (PN). After cooling using a sodium acetate solution (2.5 mM), the samples' pH was adjusted to 4.5. Next, the enzymes of 100 mg taka-diastase, 10 mg acid phosphatase, and 10 mg beta-glucosidase were added to the samples. Then, the samples were incubated in a shaking water bath for 18 h at 37 °C. In the last part of the *in vitro* method, only the acid phosphatase and β-glucosidase were utilized in samples. After cooling the test samples, the volumes were completed with 0.1 N HCI, filtered through a 0.45 μm CA filter, and then injected into the HPLC.

2.8. HPLC analysis

The Shimadzu Nexera-İ LC-2040C 3D pump by a Shimadzu RF-20A fluorescence detector (Shimadzu, Kyoto, Japan) was performed to separate vitamins.

2.9. Vitamin B_1

The mobile phase was prepared in a 75% buffer solution (0.033M KH_2PO_4) and 25% methanol. Using ortho-phosphoric acid, the pH was adjusted to 7.1 ± 0.1 and with a 0.22 μm CA, filtered under vacuum. Vitamin B_1 separation, which converted to thiochrome form, was accomplished using an Eclipse X08-C18 column (4.6 × 150 mm, 5 μm) (Agilent Technologies, USA). The temperature of the column was set at 25 °C and the flow rate was 1 mL/min. The excitation wavelength of the fluorescence detector was 366 nm, and the emission wavelength was 445 nm.

2.10. Vitamin B_2

The mobile phase was prepared with 250 mL of methanol and 750 mL of distilled water. Utilizing an Eclipse X08-C18 column (4.6 × 150 mm, 5 μm) (Agilent Technologies, USA) the separation of riboflavin, the free form of vitamin B_2, was accomplished. The excitation wavelength of the fluorescence detector was 445 nm, and the emission wavelength was 525 nm. The temperature of the column was set at 25 °C and the flow rate was 1 mL/min.

2.11. Vitamin B_3

For the detection of the vitamers of niacin (nicotinic acid and nicotinamide), post-column derivatization is required. Çatak [2019], described the determination technique for niacin was performed with minor modifications. For post-column derivatization of nicotinamide, a photochemical derivatization system was performed via a Teflon tube (length: 20 m; diameter: 0.5 mm) on a UV-A lamp (60 cm). Using an aluminum foil, the entire system was clothed. Following, the set system was connected between the fluorescence detector and the HPLC column. The mobile phase was composed daily as: 9.5 g KH_2PO_4 was dissolved in 500 mL distilled water. Then, 2 mL of $CuSO_4.5H_2O$ solution (0.12 g dissolved in 100 mL deionized water) and 7.5 mL of H_2O_2 solution (31%) were added, and the volume was finished using deionized water. Finally, employing a 0.22 μm CA filter, the mobile phase was filtered under a vacuum. The Eclipse X08-C18 column (4.6 × 150 mm, 5 μm) (Agilent Technologies, USA), was utilized to separate nicotinamide. The column temperature was set at 25 °C (excitation wavelength: 322 nm, emission wavelength: 380 nm, flow rate: 1 mL/min).

2.12. Vitamin B_6

The PN vitamer of vitamin B_6 was determined by the HPLC device according to the methodology defined in Çatak2020], together with minor modifications. The mobile phase freshly prepared daily, which contains 95% buffer solution (11 g KH_2PO_4, 0.5 g 1-heptane sulfonic acid) and 5% acetonitrile. Next, using ortho-phosphoric acid, the pH was adjusted to 2.4, and using a 0.20 μm CA filter, the solution filtered under a vacuum. Using an Eclipse X08-C18 column (4.6 × 150 mm, 5 μm) (Agilent Technologies, USA), the PL vitamer of vitamin B_6 were separated. The column temperature was maintained at 25 °C. The flow rate was 0.8 mL/min (excitation wavelength: 290 nm, emission wavelength: 395 nm, flow rate: 0.8 mL/min).

2.13. Bioaccessibilities of vitamins B_1, B_2, B_3, and B_6

Using an *in vitro* human digestion model, the bioaccessibilities of vitamins B_1, B_2, B_3, and B_6 in enriched commercial whole wheat breads were investigated. This *in vitro* system involved the mouth, stomach, and small intestine. *In vitro* investigation was performed through the method represented by Uğur et al. [2020] with minor modifications.

2.14. Digestion enzymes and solutions employed in the human digestion model

The solutions employed in the human digestion model were prepared as shown in Figure 1 (saliva solution, gastric, duodenal, and bile juices). The organic and inorganic components were prepared with 500 mL of deionized water for each digestive enzyme. Then, each enzyme was mixed in that solution. The pH was adjusted to the suitable value for each solution with 1M HCl or 0.2M NaOH (given in Figure 1).

2.15. In vitro digestion

In the mouth stage, five g of homogenized bread was taken into a 50 mL falcon tube and mixed by 5 mL of saliva with a vortex for 20 s. This mixture was incubated in a shaking water bath for 5 min at 37 °C. After this step, in the gastric stage, 12 mL of gastric juice was added to the test sample delivered from the mouth step, and this mixture was incubated for 2 h at 37 °C once again in a shaking water bath. Then, 10 mL of duodenal juice and 5 mL of bile juice were added to the test sample delivered from the gastric step. The solution's pH was at 8.0 ± 0.2 that adjusted using NaOH if required. The obtained mixture was incubated in a shaking water bath for 2 h at 37 °C. The final proportion of bread to the digestive solution was 5 g in 32 mL.

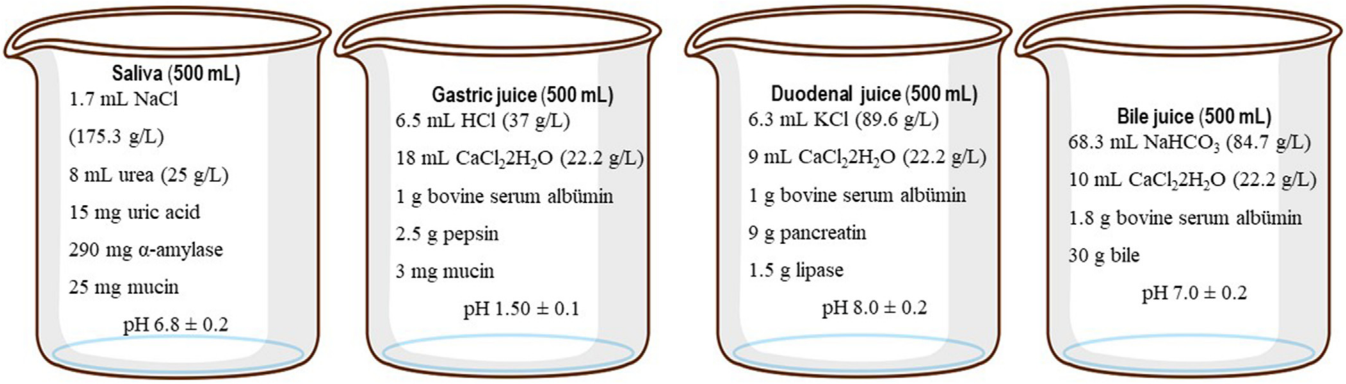

Figure 1. Concentrations and constituents of the *in vitro* human gastrointestinal model.

The pH of the solution was adjusted to 4.5 after the digestion process was finished using trichloroacetic acid. By deionized water, the finishing volume was diluted to 50 mL. Next, centrifuged at 8000 rpm for 10 min. The resulting solution was used in vitamins B_1, B_2, B_3, and B_6 detection. The calculations of the bioaccessibilities were performed by dividing the concentrations of the vitamins in the digesta by the total vitamin concentrations in the original nondigested samples and expressed as a percentage (%).

2.16. *Method validation and quantification*

Method validation of vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and PN analysis was verified using AOAC guidelines (AOAC, 2002). In Table 2, the method validation parameters are given. Linearity was found from 0.05 to 0.2 μg/mL for vitamin B_1 and vitamin B_2, from 1 to 10 μg/mL for nicotinic acid and nicotinamide, and between 0.01 and 0.5 μg/mL for PN using 5 levels of calibration in triplicate. Limit of detection (LOD) and limit of quantification (LOQ) were found as 3 and 10, respectively, according to the signal-to-noise (S/N) ratio. The study's quantification was performed by measuring the peak area, which was plotted against the concentration. Precision of vitamins was assessed for repeatability and reproducibility by analyzing bread ten times on the same day and three times on the other three days. In addition, 0.1 μg/mL of vitamin B_1 and vitamin B_2, 2 μg/mL of nicotinic acid and nicotinamide, and 0.1 μg/mL of PN were spiked to the bread to verify the recovery of the methodology. All analyses were performed in triplicate ($n = 3$).

The assessment of the accuracy and the performance of the analytical technique was performed by examining the Standard Reference Material (SRM 1849a), certified reference material, which was supplied from the National Institute of Standards & Technology (Gaithersburg, MD, USA) and was proceeded similarly to the unknown samples. At the same time, we also have partaken in a proficiency test controlled by FAPAS (Food Analysis Performance Assessment Scheme, UK, 2018). The quality procedures of the method were based on ISO/IEC 17025 requirements.

2.17. *Statistical analysis*

Each bread was analyzed at least three times in each separate experiment, and the mean value was used. Significant differences within groups were statistically determined by one-way analysis of variance (ANOVA; $p < 0.05$, Tukey' s test). The results were expressed as mean ± standard deviation.

3. Results and Discussion

In Figure 2, the chromatogram of vitamin B_1 in whole wheat bread is depicted. As can be seen from HPLC chromatogram, vitamins were well separated by the HPLC technique, one of the most preferred and precise analytical techniques for determining vitamins in foods.

In this research, the bioaccessibility of B vitamins in 8 kinds of fortified commercial whole wheat bread samples was studied. Fortified breads selected for the study were whole wheat bread, buckwheat rye bread, whole wheat bread with chia seeds, whole wheat bran bread, traditional bread, rye bread, light bread, and multigrain bread. In the samples, all breads are fortified with vitamin B_1. However, only one sample (whole wheat bread) is not fortified with vitamins B_2 and B_6, and the other seven bread samples are fortified with vitamins B_2 and B_6. Besides, only half of the samples are fortified with vitamin B_3.

The concentrations of vitamins B_1, B_2, B_3, and B_6 in enriched bread samples before and after *in vitro* digestion and the bioaccessibilities (%) of these vitamins after *in vitro* digestion are given in Tables 3 and 4, respectively.

3.1. *Method validation and quantification*

In Table 2, The method validation result values of the vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and PN are summarized. As seen from the table, the calculated LOQ was 0.018, 0.017, 0.020, 0.029, and 0.004 μg/mL for vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and PN, respectively. The reproducibility limits (%) of vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and PN were 2.3, 1.5, 2.6, 3.2, and 4.8,

Table 2. Validation parameters of vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and pyridoxine.

Analytical parameters	Vitamin B_1	Vitamin B_2	Nicotinic acid	Nicotinamide	PN
Linear range (μg/mL)	0.05–0.2	0.05–0.2	1–10	1–10	0.01–0.5
Correlation coefficient (r^2)	0.998	0.998	0.999	0.998	0.9998
LOD (μg/mL)	0.005	0.004	0.006	0.010	0.0011
LOQ (μg/mL)	0.018	0.017	0.020	0.029	0.004
Repeatability limit (%)	2.0	1.2	2.3	2.9	2.8
Reproducibility limit (%)	2.3	1.5	2.6	3.2	4.8
Recovery (%)	96.80–99.30	97.30–101.20	98.60–100.30	97.80–102.50	93.20–96.80

PN, pyridoxine; LOD, limit of detection; LOQ, limit of quantification.

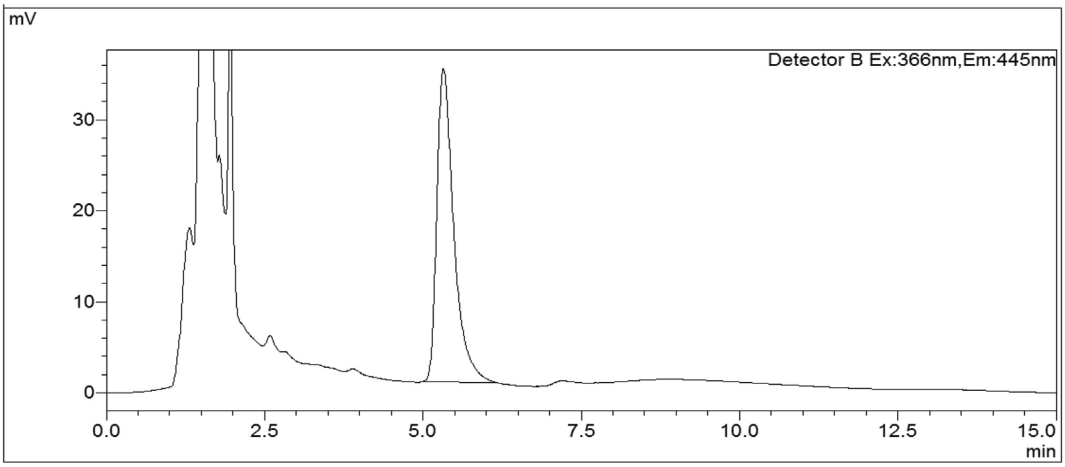

Figure 2. HPLC chromatogram of vitamin B_1 in whole wheat bread.

respectively, and these results show good reproducibility for each vitamin. Recovery rates for vitamin B_1, vitamin B_2, nicotinic acid, nicotinamide, and PN ranged from 96.80% to 99.30%, 97.20%–101.20%, 98.60%–100.30%, 97.80%–102.50%, and from 93.20% to 96.80%, respectively.

Using quality control material for the accuracy of the analysis is always suggested. In the current work, the FAPAS test outcomes for vitamins B_1, B_2, B_3, and B_6 were determined in an appropriate range ($-2 \leqslant$ Z score $\leqslant +2$).

3.2. Vitamin B_1, B_2, B_3, and B_6 concentration in fortified breads

The contents of vitamins B_1, B_2, B_3, and B_6 in enriched bread samples were measured. As seen in Table 3, the concentration of analysed vitamin B_1 varied between 0.622 and 3.324 mg/100 g at initial, and from 0.502 to 2.698 mg/100 g after digestion in breads. The highest vitamin B_1 was determined in buckwheat rye bread, while the lowest was in whole wheat bran bread both at initial and after digestion. Although the amounts of vitamin B_1 decreased in all bread samples after digestion, the significant decrease was observed in six samples ($p < 0.05$). In two fortified breads, there was no significant difference compared to the initial value. In the American Food Composition Database [USDA, 2022], in some enriched breads, the total vitamin B_1 amount ranges from 0 to 1 mg/100 g. Our results of measured vitamin B_1 are congruent with the findings determined by the USDA by 5 out of 8 fortified breads. The obtained values of total vitamin B_1 were compared with the values available in the published literature of the Turkish Food Composition Database (TURCOMP) [TURCOMP, 2022]. In TURCOMP, only the data of 3 types of breads (whole wheat bread, bread with bran, and rye bread) are available, and these breads were consistent with the samples of our study, but these breads are not fortified. Naturally available vitamin B_1 in these breads reported with the lower total amounts. According to the TURCOMP, the total naturally available vitamin B_1 level of breads (not fortified) include: whole wheat bread (0.148 mg/100 g), bread with bran (0.204 mg/100 g) and rye bread (0.152 mg/100 g). In our study, the amount of total vitamin B_1 in fortified whole wheat bread, whole wheat bran bread, and rye bread were remarkably higher than the values in TURCOMP. The fortified breads contained 3–12 times more vitamin B_1 than the not-fortified-breads compared to the data in TURCOMP. Also, there is no maximum allowable daily limit available about added vitamin B_1 in the Turkish Food Codex.

Vitamin B_2 varied between 0.369 and 1.927 mg/100 g at initial, and between 0.235 and 1.261 mg/100 g after digestion in breads (Table 3). The highest vitamin B_2 was found in buckwheat rye bread with 1.927 mg/100 g, while the lowest was determined in traditional bread and whole wheat bread with similar values in the initial. After digestion, again, the buckwheat rye bread contained the highest vitamin B_2 (1.261 mg/100 g), but the lowest was seen in rye bread with 0.235 mg/100 g. Among the samples, only the whole wheat bread is not fortified with vitamin B_2. The amounts of vitamin B_2 decreased in all samples after digestion, but the significant decrease was observed in 5 samples ($p < 0.05$). In three breads, there was no significant difference compared to the initial value. Total vitamin B_2 content varies between 0.1 and 0.7 mg/100 g in some enriched breads in the American Food Composition Database [USDA, 2022]. Our measured results of vitamin B_2 are congruent with the findings reported by the USDA except for two samples. Besides, the obtained vitamin B_2 values were compared with the values obtainable from the declared publications of TURCOMP [TURCOMP, 2022], which are not fortified breads (whole wheat bread, bread with bran, and rye bread). Naturally available vitamin B_2 in these breads reported with the lower total amounts. According to the TURCOMP, the total naturally available vitamin B_2 content of not-fortified-breads include: whole wheat bread (0.06 mg/100 g), bread with bran (0.075 mg/100 g) and rye bread (0.053 mg/100 g). In our investigation, the amount of total vitamin B_2 in these breads was remarkably higher than the values in TURCOMP. The fortified whole wheat bran bread and rye bread samples contained 6–8 times more vitamin B_2 than the not-fortified-breads compared to the data in TURCOMP. Also, there is no maximum allowable daily limit available about added vitamin B_2 in the Turkish Food Codex.

In the samples, only half of the samples were fortified with vitamin B_3, and all fortified breads had higher amounts of vitamin B_3. The levels of measured vitamin B_3 ranged from 1.025 to 9.122 mg/100 g in the initial, and from 0.971 to 7.657 mg/100 g after digestion in breads (Table 3). The highest vitamin B_3 was found in buckwheat rye bread with 9.122 mg/100 g while the lowest vitamin B_3 was in traditional bread (not-fortified sample) with 1.025 mg/100 g in the initial. After digestion again, the highest vitamin B_3 was in buckwheat rye bread (7.657 mg/100 g), and the lowest was in traditional bread (0.971 mg/100 g). Although the amounts of vitamin B_3 decreased in all fortified bread samples after digestion, the significant decrease was observed in 5 samples ($p < 0.05$). In three breads, there was no significant difference compared to the initial value. However, the samples of not-fortified breads (whole wheat bran bread, traditional bread, light bread, and multigrain bread) contained 0.971–1.457 mg/100 g vitamin B_3 after digestion. Vitamin B_3 was determined predominantly higher levels among the samples. Cereals are known as good sources of niacin in the literature. Çatak [2019], reported total vitamin B_3 content in wheat (bread) and rye as 5.483 and 4.168 mg/100 g. Turkish Food Codex has been reported the maximum tolerable daily limit about added vitamin B_3 in foodstuffs is 250 mg/day for 4–10 years, and 500 mg/day for 11 years and older. In all breads, the content

Table 3. The amount of vitamin B_1, vitamin B_2, vitamin B_3 (nicotinamide) in fortified commercial whole wheat breads before and after digestion and the *in vitro* bioaccessibility.

Sample	Vitamin B_1			Vitamin B_2			Vitamin B_3		
	Initial value (mg/100 g)	After digestion (mg/100 g)	Bioaccessibility (%)	Initial value (mg/100 g)	After digestion (mg/100 g)	Bioaccessibility (%)	Initial value (mg/100 g)	After digestion (mg/100 g)	Bioaccessibility (%)
1	1.723 ± 0.078^{b}	1.220 ± 0.055^{b}	70.9 ± 5.7	0.370 ± 0.017^{c}	0.331 ± 0.015^{c}	89.7 ± 7.2	1.386 ± 0.063^{def}	1.200 ± 0.054^{cd}	86.7 ± 7.0
2	3.324 ± 0.150^{a}	2.698 ± 0.122^{a}	81.3 ± 6.5	1.927 ± 0.087^{a}	1.261 ± 0.057^{a}	65.6 ± 5.3	9.122 ± 0.413^{a}	7.657 ± 0.346^{a}	84.1 ± 6.8
3	1.509 ± 0.068^{c}	1.298 ± 0.059^{b}	86.2 ± 6.9	1.534 ± 0.069^{b}	0.929 ± 0.042^{b}	60.7 ± 4.9	7.055 ± 0.319^{b}	6.077 ± 0.275^{b}	86.3 ± 6.9
4	0.622 ± 0.028^{e}	0.502 ± 0.023^{d}	80.9 ± 6.5	0.418 ± 0.019^{c}	0.249 ± 0.011^{d}	59.8 ± 4.8	1.729 ± 0.078^{de}	1.413 ± 0.064^{cd}	81.9 ± 6.6
5	0.742 ± 0.034^{de}	0.595 ± 0.027^{cd}	80.4 ± 6.5	0.369 ± 0.017^{c}	0.246 ± 0.011^{d}	66.9 ± 5.4	1.025 ± 0.046^{f}	0.971 ± 0.044^{d}	94.9 ± 7.6
6	0.910 ± 0.041^{d}	0.687 ± 0.031^{c}	75.6 ± 6.1	0.435 ± 0.020^{c}	0.235 ± 0.011^{d}	54.2 ± 4.4	1.218 ± 0.055^{ef}	1.123 ± 0.051^{cd}	92.4 ± 7.4
7	0.935 ± 0.042^{d}	0.676 ± 0.031^{c}	72.4 ± 5.8	0.428 ± 0.019^{c}	0.244 ± 0.011^{d}	57.2 ± 4.6	1.875 ± 0.085^{d}	1.229 ± 0.056^{cd}	65.7 ± 5.3
8	0.812 ± 0.037^{de}	0.732 ± 0.033^{c}	90.2 ± 7.2	0.474 ± 0.02^{c}	0.278 ± 0.013^{cd}	58.7 ± 4.7	3.471 ± 0.157^{c}	1.457 ± 0.066^{c}	42.1 ± 3.4

Values are mean ± SD, n = 3. Different letters (a-f alphabets) in the same lines show statistical differences between the groups (ANOVA; $p < 0.05$, Tukey's test).

of vitamin B_3 is below the maximum allowable daily limit and is appropriate for the Turkish Food Codex [TFC, 2022].

The levels of measured vitamin B_6 varied from 0.065 to 2.47 mg/100 g in the initial, and from 0.06 to 1.725 mg/100 g after digestion in breads (Table 4). The level of vitamin B_6 was found in buckwheat rye bread the highest with 2.47 mg/100 g while the lowest quantity was in rye bread with 0.065 mg/100 g at initial. After digestion, again, the same samples had both higher and lower amounts. Among the samples, only the whole wheat bread is not fortified with vitamin B_6. Although the amounts of vitamin B_6 decreased in all samples after digestion, the significant decrease was observed in four samples ($p < 0.05$). There was no significant difference in the other four samples compared to the initial value. The obtained vitamin B_6 values were compared with the values obtainable from the declared publications of TURCOMP [TURCOMP, 2022], which are not-fortified-breads. Naturally available vitamin B_6 in whole wheat bread, bread with bran, and rye bread reported with the lower total amounts. According to the TURCOMP, the total naturally available vitamin B_6 level of breads (not fortified) include: whole wheat bread (0.061 mg/100 g), bread with bran (0.061 mg/100 g) and rye bread (0.036 mg/100 g). In our study, the concentration of vitamin B_6 in whole wheat bread, whole wheat bran bread, and rye bread was higher than the reported amounts in the Turkish Food Composition Database. The two fortified breads contained about two times more vitamin B_6 than the not-fortified-breads compared to the data in TURCOMP. Turkish Food Codex has been reported the maximum tolerable daily limit for added vitamin B_6 in foodstuffs is 5 mg/day for 4–10 years, and 10 mg/day for 11 years and older [TFC, 2022]. In all breads, the content of vitamin B_6 is below the maximum daily limit and is appropriate for the Turkish Food Codex.

Our results were compared with the Food Data Central of US Department of Agriculture. According to the USDA, the total quantity of vitamin B_1, B_2, and B_3 is 1, 0.327, and 5.769 mg/100 g, respectively, in a multi whole grain wheat bread which fortified with vitamins B_1, B_2, and B_3. In the present work, the content of vitamins B_1 and B_3 (0.812 and 3.471 mg/100 g) was found lower than the data of the USDA. In contrast, the vitamin B_2 content was higher (0.474 mg/100 g) in the multigrain bread sample, fortified with vitamin B_1 and B_2 [USDA, 2022].

Another remarkable point is that the values of buckwheat rye bread and whole wheat bread with chia seeds found with predominantly higher amounts in all studied vitamins. It is thought to be due to the content of buckwheat and chia seeds.

3.3. *In vitro bioaccessibility*

In Tables 3 and 4, the bioaccessible amount results for vitamins B_1, B_2, B_3, and B_6, and the percentage (%) of bioaccessibilities are given. As shown in Table 3, the bioaccessibilities of vitamin B_1 in enriched breads ranged between 70.9 and 90.2% after digestion. The amount of vitamin B_1 present in the bread samples are not totally bioaccessible. However, the lowest bioaccessibility was observed in whole wheat bread. Besides, the highest vitamin B_1 bioaccessibility was observed in multigrain bread.

According to Table 3, vitamin B_2 bioaccessibility in fortified breads ranged between 54.2 and 89.7% after digestion. The amount of vitamin B_2 present in the bread samples are not totally bioaccessible, but, the lowest bioaccessibility was observed in rye bread (54.2%). In one sample, whole wheat bread, the bioaccessibility of vitamin B_2 was surprisingly high among the samples (89.7%), not fortified with vitamin B_2.

The bioaccessibility of vitamin B_3 in fortified breads ranged between 42.1 and 94.9% after digestion (Table 3). The amount of vitamin B_3 present in the bread samples are not totally bioaccessible. However, the remarkably lowest bioaccessibility was observed in multigrain bread with 42.1%. The highest vitamin B_3 bioaccessibility was determined in traditional bread with approximately 95%. The most remarkable point here that these two bread samples (multigrain bread and traditional bread) are not-fortified with vitamin B_3.

As seen in Table 4, vitamin B_6 bioaccessibility in fortified breads ranged between 44.1 and 92.5% after digestion. The amount of vitamin B_6 present in the bread samples are not totally bioaccessible. However, the lowest bioaccessibility was detected in whole wheat bran bread with 44.1%. Also, the highest vitamin B_6 bioaccessibility was detected in rye bread.

We investigated the bioaccessible concentrations of vitamins B_1, B_2, B_3, and B_6 in enriched bread samples commonly consumed within all ages in the population. The mean bioaccessibility of vitamins B_1, B_2, B_3, and B_6 in enriched breads after digestion was 80%, 64%, 79%, and 64%, respectively. In this *in vitro* gastrointestinal digestion system, the bioaccessibility of B vitamins was low. Notably, the bioaccessibilities of vitamins B_6 and B_2 were lower than vitamins B_2 and B_3 after digestion. As can be seen from the findings, the highest bioaccessibility value was detected in vitamin B_3 by 95% in traditional bread. The lowest bioaccessibility was in vitamin B_3 by 42% in multigrain bread among the samples.

Kurek et al. [2017] stated the bioaccessibilities of vitamins B_1, B_2, B_3, and B_6, were 69.1–91.2%, 40.9–50.2%, 60.2–70.2%, and 27.52–34% respectively, in fortified wheat bread. The findings in our work were parallel with the study performed by Kurek et al. [2017]. In this work, the bioaccessibilities of vitamins B_1, B_2, $B_{3,}$ and $B_{6,}$ are 70.9–90.2%, 54.2–89.7%, 42.1–94.9%, and 44.1–92.5% respectively, in enriched breads. In particular, the bioaccessibility of vitamin B_1 was very close to our results. Consistent with the results of our study, Kurek et al., [2017] has determined the highest bioaccessibility in vitamin B_1 while the lowest bioaccessibility in vitamin B_2 and B_6.

The bioaccessibilities of naturally existing vitamin B_1 in wheat (durum) and maize bran varies between 75 and 95% [Yu and Kies, 1993; Zaupa et al., 2014]. Our findings are congruent with these mentioned values in terms of vitamin B_1 bioaccessibility in fortified breads (70.9 and 90.2%). The stability of vitamin B_1 is particularly influenced by heat

Table 4. The amount of vitamin B_6 (pyridoxine) in fortified commercial whole wheat breads before and after digestion and the *in vitro* bioaccessibility.

Sample	Vitamin B_6		Vitamin B_6
	Initial value (mg/100 g)	After digestion (mg/100 g)	Bioaccessibility (%)
1	0.989 ± 0.045^{c}	0.527 ± 0.024^{c}	53.4 ± 4.3
2	2.470 ± 0.112^{a}	1.725 ± 0.078^{a}	70.0 ± 5.6
3	1.837 ± 0.083^{b}	1.029 ± 0.047^{b}	56.1 ± 4.5
4	0.143 ± 0.006^{ef}	0.063 ± 0.003^{e}	44.1 ± 3.5
5	0.246 ± 0.011^{de}	0.126 ± 0.006^{e}	51.1 ± 4.1
6	0.065 ± 0.003^{f}	0.060 ± 0.003^{e}	92.5 ± 7.4
7	0.316 ± 0.014^{d}	0.259 ± 0.012^{d}	82.2 ± 6.6
8	0.139 ± 0.006^{ef}	0.084 ± 0.004^{e}	60.6 ± 4.9

Values are mean ± SD, $n = 3$. Different letters (a-f alphabets) in the same lines show statistical differences between the groups (ANOVA; $p < 0.05$, Tukey's test).

treatments [Eittenmiller et al., 2008]. Since the temperature of the *in vitro* environment is 37 °C, it is supposed that the bioaccessibility of vitamin B_1 affected.

The maximum stability of vitamins B_1 and B_2 is between pH 2 and 4 [Eittenmiller et al., 2008]. Since the pH of the *in vitro* small intestine medium is 7, it is thought that both vitamins are affected by this pH.

Çatak [2019] investigated the profiles of some plant-based foods and reported that cereals (including wheat (bread), wheat (durum), rye and oat) contain high-level nicotinic acid while they do not contain any nicotinamide. The bioavailability and bioaccessibility of nicotinic acid form and nicotinamide form are not the same. Nicotinamide is most bioavailable than nicotinic acid since nicotinic acid is chemically bound to polypeptides and polysaccharides. Owing to the breakdown of proteins and starch in the human gastrointestinal system, most of the nicotinic acid was not liberated from the polypeptides and polysaccharides. Therefore, half of the nicotinic acid is inaccessible [Ball, 2006]. It is well-known that nicotinic acid form is naturally found in these breads leading to lower bioaccessibility. Our results indicate that fortifying breads with vitamin B_3 leads to high bioaccessibility. The multigrain bread's bioaccessibility is remarkably low (42.1%) among the samples as expected, which is not fortified with B_3. The bioaccessibility was found to be low in 3 of 4 samples that were not fortified with vitamin B_3 as expected, but remarkably high in 1 sample (traditional bread).

The study of Sauberlich [1985] stated that vitamin B_6, naturally found in cereal-based foods, is lost between 75 and 90% during food processing. In addition to the PN.HCI (synthetic form), breads include naturally existing PN, which is the predominant form in cereals. The PN is less affected during processing stages and more stable compared to the PL and PM. It is thought that most of the losses occurring in PL and PM. Due to the pH and heat increase, the stability of PL and PM declines [Ball, 2006]. Animal-sourced foods include PLP with high amounts, while plant-sourced foods include PNG with high amounts. The PNG bioavailability is very little than the PLP [Gregory et al., 1991]. The study of Gregory et al. [1991] resulted the bioavailability of PL as 50% higher compared to the PN. The PL is more easily converted to the PLP than the PN and PM in humans. The PNG binds to sugars by conjugated bonds in plant-sourced foodstuffs. Besides, FAD is the bioactive form of vitamin B_2, and the PNG is also covalently bound to FAD [Ball, 2006]. Thus, it is estimated that the bioaccessibility of vitamin B_6 will be low. Our *in vitro* findings showed that the bioaccessible amounts of vitamin B_6 were lower. Besides, vitamin B_6 is also noncovalently bound to proteins, and the liberation of vitamin B_6 depends on the pH value [Ball, 2006]. In acidic conditions, all vitamers of vitamin B_6 are stable [Eittenmiller et al., 2008]. The stability of vitamin B_6 diminishes as pH rises to the stages above neutral conditions [Saidi and Warthensen, 1983]. The stability of vitamin B_6 in higher-level pH (duodenal juice, pH 8) may be less, resulting in low bioaccessibility. Thus, the available amount may differ related to the gastric acidity and small intestine acidity, and the bioaccessibility is most likely affected.

Dietary fiber may decrease the bioaccessibility of vitamins [Palafox-Carlos et al., 2011] since hydroxyl groups inside dietary fiber react with the B vitamins, reducing the bioaccessibility [Kurek et al., 2017]. Breads contain a certain amount of dietary fiber. Especially whole wheat bread, whole wheat bran bread, and oat bread has a high amount of dietary fiber. Kurek et al. [2017] indicated that the addition of dietary fiber in breads reduces the bioaccessibility of vitamin B_6 (27–34%). In our samples, the declared amounts of dietary fiber range from 6.5 to 13.48 g/100 g in breads (Table 1). According to our results, as the amount of dietary fiber increased, the bioaccessibilities in vitamins were found to be lower. As the amount of dietary fiber increased, the bioaccessibility in vitamins B_2 and B_3 was low in all samples. Although multigrain bread contains a high amount of fibers, the vitamin B_1 bioaccessibility was higher than expected. A similar finding has been observed in light bread and whole wheat bread with chia seeds for vitamin B_6. It is thought that this may be due to the variations in the amount of vitamins added.

Water-soluble vitamins are affected by the environment's heat, light, and pH, and there are considerable losses in the contents of these vitamins (Çatak et al., 2022a). Besides, water-soluble vitamins may also be linked by non-covalent bonds to polysaccharides and polypeptides in foods. Therefore, these vitamins may not be released depending on the gastrointestinal tract's pH. So, the intake level of these vitamins in the gastrointestinal tract may be affected [Ball, 2006].

In vitro, bioaccessibility methods are beneficial to obtain information on potential interactions between nutrients and food constituents, the effects of gastrointestinal dynamics, pH and enzymes, and food processing. *In vitro* digestion is a low cost and useful practice for monitoring micronutrients to define whether they are affected by digestion conditions or whether there are interactions with other food constituents that may affect the efficiency of digestion. Our *in vitro* digestion model provided highly accurate results on the digestibility and bioaccessibility of various whole wheat breads.

Additionally, some literature shows novel techniques for the determination of B-group vitamins. Ostovan et al. (2018) proposed a novel green synthesis strategy for the separation of B-group vitamins in juices. In a study by Arabi et al. (2020), the recent advances in molecular imprinting concerning novel preparation strategies of molecularly imprinted polymers (MIPs) and typical applications of molecular imprinting-based solid-phase extraction (MI-SPE) has reviewed. The authors reported the potential of MIPs for implementation in routine laboratory activities, and scale-up is expected.

The laws of thermodynamics handle the energy flow in ecosystems. Therefore, energy transfer through the ecosystem is crucial for biological systems (Çatak et al., 2018). Nonetheless, each energy transfer alongside the food chain yields a loss of useable metabolic energy (Özilgen, 2018; Çatak et al., 2022b). Biologicals gain significant amounts of ATP and thermal energy in mitochondria from the energy stored in the chemical bonds of foodstuffs owing to cellular metabolism (Yalçınkaya et al., 2018). Nowadays, scientists have concentrated on the functionality of mitochondria in the regulation of cellular energy production (Yildiz and Özilgen, 2022; Çatak et al., 2021). Recent investigations reveal that the deficiency of certain B vitamins reduces mitochondrial function and disrupts energy metabolism (Mkrtchyan et al., 2018; Udhayabanu et al., 2017). Thus, the deficiency of B vitamins threatens cellular growth, function, and survival.

Fortification of commercial whole wheat bread with vitamins may increase the blood levels of micronutrients. This can be considered an effective public health attitude for controlling and preventing common nutrient deficiencies in Turkey. It has been well known that vitamin deficiencies affect people all over the world. Inadequate vitamin intake can cause many health problems in public nutrition. In Turkey, especially micronutrient deficiency is one of the most critical health problems for many people [Pekcan, 2006]. Food fortification processes helped the nutrition deficiency problem in some of the developed countries with fortifying staple foods with particular nutrients [FAO, 1996]. The critical purpose of food fortification is to fortify commonly consumed foods by deficient nutrients for specific risk groups or the entire population. Bread preferred for food fortification in many countries since bread is a widely consumed food of all ages in society. Besides, the fortification of bread has a practical application compared to other foods in terms of standardization [Czeizel and Merhala, 1998].

Fortifying commercial whole wheat bread with the vitamin may be a satisfactory way to provide nutritional enhancement and provide extra health benefits to consumers. However, nutritionists estimate daily intake levels of vitamins with the values reported in the literature without taking into account the bioaccessible amounts. The daily intake calculation may be overestimated by using the literature amounts. The results of this study may be used as a guideline referring to the bioaccessible amounts.

4. Conclusions

This study revealed that vitamin B content in fortified commercial whole wheat breads is affected by *in vitro* digestion. Vitamins B_1, B_2, B_3, and B_6 have different bioavailability and bioaccessibility. Thus, knowing the content of B vitamins in breads after digestion is required for the healthy nutrition of the population. These results reveal that the vitamins B_2 and B_6 have lower bioaccessibility than vitamins B_1 and B_3. It is believed that the temperature, pH of the gastrointestinal system, dietary fiber, bonding to polysaccharides and polypeptides, and stability considerably affect the bioaccessibility. Our results reveal that *in vivo* bioavailability of studied vitamins may be low.

The results of this study may be used as a source for commercial formulation of vitamin-fortified whole wheat bread because it provides a data that should be applied to reach the most nutritive bakery product; as a reference for governmental bodies to develop National Public Policies for preventing nutritional deficiencies; as a guideline for bread producers with regards to the level of B vitamins in whole wheat breads to achieve the desired digestibility.

Declarations

Author contribution statement

Jale Çatak: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Merve Nur Gizlici: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Additional information

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