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Determination of riboflavin bioaccessibility in legumes by *in vitro* digestion using different cooking methods

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ABSTRACT

This research aimed to investigate the riboflavin cooking losses and bioaccessibilities according to the cooking methods in legumes. Considerable nutrient losses occur during the cooking/preparation of foods. Updating old data is important today to support and complement a healthy diet. The riboflavin contents of legumes were measured in raw, pressure-cooked, pan-cooked, and digested cooked legumes. For the bioaccessibility determination, *in vitro* method was used to simulate the human gastrointestinal tract. Average riboflavin cooking losses of pressure- and pan-cooked legumes were 13.5% and 38.6% and the average riboflavin bioaccessibility was 58.1% and 57.6%, respectively. As a result of High Performance Liquid Chromatography analysis, it has been determined that there were fewer cooking losses and higher bioaccessibility in the pressure cooking method. However, there was no statistical difference between the two cooking methods. The pressure cooking method may be recommended due to fewer riboflavin cooking losses.

Keywords: Bioaccessibility, cooking loss, legumes, riboflavin

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INTRODUCTION

Legumes are a good source of protein and nutrients for developing countries in the world. They provide fiber, B group vitamins, and other micro-nutrients such as magnesium, zinc, phosphorus, iron, and copper[1]. The consumption of legumes worldwide is 21 grams/person/day but the consumption of meat as a source of protein is more than legumes[2].

Legumes contain 0.062 mg/100 g of riboflavin as a good source[3]. The main source of riboflavin in the diet comes from eggs, organ meats, lean meats, and milk, but some vegetables are also rich in riboflavin[4]. Riboflavin, also known as lactoflavin composed of ribitol and lumichrome, and its chemical formula is $C_{17}H_2ON_4O_6$. It is heat-stable and water-soluble like other group B vitamins[5] Riboflavin can be converted to FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) in tissues, and these coenzyme forms release energy from carbohydrates, fats, and protein through the Krebs cycle, mitochondrial electron transport, and other electron transfer mechanisms. These compounds play essential roles in accepting electrons in biochemical reactions. In addition, it plays essential roles in protein stabilization and apo-protein synthesis[6]. Recommended Dietary Allowance (RDA) of riboflavin for women and men respectively: 1.1 mg/day and 1.3 mg/day[7].

Riboflavin has low stability in alkaline conditions and maximum stability at pH 2.0 –5.0 against heat degradation[8]. In addition, riboflavin, FMN, and FAD can easily degrade by visible light[9]. For these reasons, exposure to light as sun-drying processes causes riboflavin losses. Another factor that causes riboflavin loss is spilling the cooking water. The levels of B vitamins in foods are affected by cooking. During cooking, the losses of B vitamins occur due to their high ability to be dissolved and thermal uncertainty[10,11]All the listed factors affect the bioaccessibility of riboflavin and the amount of losses that occur during cooking[12].

The energy circulating in the ecosystem is essential for all possessions. The laws of thermodynamics handle the energy flow in ecological systems[13,14]. However, each energy transfer along the food chain causes the loss of usable metabolic energy[15,16]. Biologicals gain ATP and thermal energy in mitochondria from the energy stored in the food's chemical bonds due to cellular metabolism[17,18]. Mitochondria are named as a "the cell powerhouses" due to the its functions that are regulating ATP production and cellular metabolism[19]. Lately, researchers have given their attention to the role of mitochondria in the regulation of cellular energy production[20] Recent investigations reveal that the deficiency of

some B-group vitamins decreases mitochondrial function and disrupts energy metabolism[21,22]. The B-group vitamins deficiency is vital for energy metabolism. If it occurs, these situations threaten ,cellular growth, function, and survival.

Bioaccessibility is the quantity of digested nutrients that potentially become available for absorption in the gastrointestinal tract[23-25]. Bioavailability studies are important for making nutrition plans and providing the closest requirements. However, bioavailability studies are disadvantageous in terms of both procedures and time and cost[26]. Therefore, this study aimed to update the limited and outdated data and determine the cooking losses and bioaccessibility of riboflavin with an *in vitro* gastrointestinal model.

2. METHODOLOGY

2.1. Reagents and Materials

The riboflavin standard and acid phosphatase from potatoes, taka diastase from *Aspergillus oryzae*, alpha-amylase from *Aspergillus oryzae*, beta-glucosidase from almonds, pepsin from porcine gastric mucosa, pancreatin, lipase, hydrochloric acid (HCl, 37%), NaCl, $CaCl_2.2H_2O$, KCl, $NaHCO_3$, mucin, bovine serum albumin, urea, uric acid, bile salts mixture, methanol (MeOH), acetonitrile (ACN), trichloroacetic acid (TCA), 1-heptane sulfonic acid and a sodium salt were supplied from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Samples and Cooking Methods

In this study, four different legumes (dry beans, chickpeas, red lentils, and green lentils) were obtained from local markets in Istanbul, Turkey. Legume samples were divided into 3 groups with 3 legumes each. The first group (raw) was uncooked, while the other two groups were cooked by pressure and pan cooking methods.

After 130 grams of chickpeas and beans were weighed, soaked in 500 mL of water for 12 hours. Then, 150 grams of each sample and 600 mL of water were added to the pan and pressure cooker, and the cooking process was carried out. No pre-cooking preparation was made for the red lentil sample, but 300 grams of green lentils were boiled by adding 800 mL of water for 10 minutes, and the boiling water was spilled. For both samples, 150 grams were weighed, and two different cooking methods were applied. While 800 mL of water was used in both methods during cooking for red lentils, 500 mL of water was used for green lentils.

The legumes were put on the pressure cooker and cooked at 125 °C for 1 hour (chickpeas), 45 minutes (beans), 10 minutes (red lentils), and 20 minutes (green lentils). Pan cooking of samples was performed in a pan at 100 °C for 1 hour 50 min for chickpeas, 1 hour 45 min for beans, 20 minutes for red lentils, and 30 minutes for green lentils. Finally, the samples were homogenized with the blender together with the cooking water. Each sample was analyzed 3 times in each cooking method. The cooked legume samples were cooled to room temperature.

2.3. Extraction of Riboflavin from Legumes and HPLC Analysis of Riboflavin

The extraction and HPLC determination method for riboflavin described by Çatal *et al.*

was used with some modifications[27]. First, a 5 g homogenized sample was added to a 100 mL Erlenmeyer flask. Next, 60 mL 0.1 N HCl solution was added and autoclaved at 121 °C for 30 min. An enzymatic procedure was accomplished to release the phosphorylated forms of riboflavin (FAD and FMN). The solution was cooled to room temperature and the pH was adjusted to 4.5 using a sodium acetate (2.5 mM) solution. In the enzymatic extraction stage, 100 mg taka diastase and 10 mg acid phosphatase were added to the sample and incubated at 37 °C for 3 h in a shaking water bath. Then, after cooling to room temperature, the volume was completed to 100 mL with 0.1 HCl solution, filtered, and injected into HPLC for riboflavin analysis.

2.4. HPLC Determination of Riboflavin

A Shimadzu Nexera-i HPLC with a Shimadzu RF-20A fluorescence detector (Shimadzu Corporation, Kyoto, Japan) was used for the separation of riboflavin. The mobile phase consisted of 75% deionized water and 25% methanol. The separation was with an Eclipse X08-C18, 5 **µm**, 4.6×150mm column (Agilent, USA) with a flow rate of 1 mL/min. The fluorescence detector excitation and emission wavelengths were 290 and 395 nm, respectively. The column oven temperature was 25 °C.

2.5. *In-Vitro* Gastrointestinal Model and Riboflavin Bioaccessibility

In vitro analysis was achieved via the methodology described by Lee *et al.* with some modifications[28]. The preparation of the *in vitro* digestion condition and the digestion process are shown in Fig. 1. Saliva solution, gastric, duodenal, and bile juices were prepared using organic and inorganic chemicals and enzymes (Fig. 1). In this *in vitro* human digestion model, organic and inorganic constituents were prepared with 500 mL of distilled water for each digestive enzyme. Next, each enzyme was mixed into that solution. Then, using 1M HCl or 0.2M NaOH, the pH was adjusted to the proper value for each solution (given in Fig. 1).

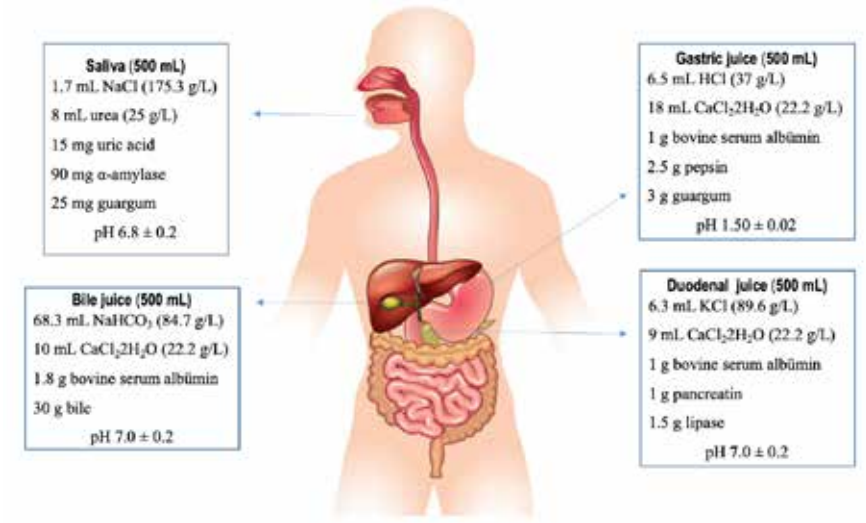

Fig 1. *In vitro* digestion model and digestion process

In the mouth step, a 5 g test sample was mixed with 5 mL saliva solution in a 50 mL falcon tube for 20 s with a vortex. This mixture was incubated for 5 min at 37 °C in a shaking water bath. After this stage, in the gastric step, 12 mL of gastric juice was added to the test sample provided from the mouth stage, and this mixture was allowed to incubate once more in a shaking water bath at 37 °C for two hours. Next, 10 mL of duodenal juice and 5 mL of bile juice were added to the test sample provided from the gastric stage. This mixture was incubated for two hours at 37 °C in a shaking water bath. Using trichloroacetic acid, the pH of the solution was adjusted to 4.5 after the digestion procedure was accomplished, finishing volume was diluted to 50 mL by deionized water and centrifuged for 10 minutes at 8000 rpm. The obtained solution was used in riboflavin determination analysis.

2.6. Statistical Analysis

Analyses with samples were performed three times and the mean value was used. Significant differences within groups were statistically identified by ANOVA ($p < 0.05$, Tukey's test).

3. RESULTS AND DISCUSSION

Scope of the study, 4 different legume species were cooked with two different methods (pan, and pressure cooker) and digested by *in vitro* methods. At each step, riboflavin levels were measured, and cooking losses and bioaccessibility values were calculated due to the cooking methods. The HPLC chromatogram of riboflavin in the chickpea sample is given in Fig. 2.

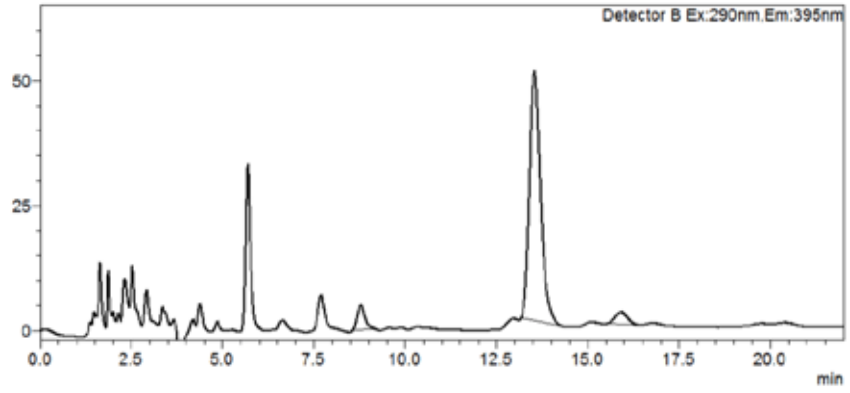

Fig 2. HPLC chromatogram of riboflavin in chickpea

3.1. Content of Riboflavin in Samples

Pre-cooking, and post-cooking riboflavin levels and cooking loss rates in legumes are given in Table 1. The amount of riboflavin in raw legumes varied between 130.33 and 219.67 μg/100 g. The chickpea had the highest riboflavin level in two different cooking methods performed on legumes (pressure cooker: 176.33 μg/100 g; pan: 115.33 μg/100 g). The riboflavin cooking losses were between 4.6% and 21.4% when cooked with a pressure cooker, and between 23% - 47.7% in a pan. The riboflavin contents and riboflavin cooking losses in legumes are listed in Table 1.

Table 1. Content of riboflavin in legumes and riboflavin cooking losses.

Samples	Raw (µg/100 g)	Cooked with a pressure cooker (µg/100 g)	Cooking loss in a pressure cooker (%)	Cooked with a pan (µg/100 g)	Cooking loss in pan (%)
Bean	196.33±7.23[a]	154.0±5.0[b]	21.4	111.0±5.5[c]	43.3
Chickpea	219.67±8.79[a]	176.33±7.51[b]	20.0	115.33±4.51[c]	47.7
Red lentil	130.33±5.34[a]	124.33±4.79[a]	4.6	100.0±3.87[b]	23.0
Green lentil	154.67±6.51[a]	142.33±5.51[b]	8.3	92.33±3.51[c]	40.6

Different letters within the same row indicate statistical differences between the applications ($p < 0.05$).

3.2. *In-Vitro* Digestion Results

The riboflavin amount of digested legume samples and bioaccessibility values are given in Table 2. After digestion, the amount of riboflavin in cooked legumes varied between 57.67 and 128.0 µg/100 g in a pressure cooker, 40.33 and 95.67 µg/100 g in a pan. As seen in the table, the riboflavin bioaccessibility in cooked legumes varied between 41.1 – 73.2% in a pressure cooker and 40.4 – 83% in a pan (Table 2).

Table 2. Bioaccessibility of riboflavin in legumes.

Samples	Cooked with a pressure cooker (µg/100 g)	Cooked with pressure cooker+digested (µg/100 g)	Bioaccessibility by a pressure cooker (%)	Cooked with a pan (µg/100 g)	Cooked with pan+digested (µg/100 g)	Bioaccessibility by pan (%)
Bean	154.0±5.0[b]	80.67 ±0.31[a]	52.2	111.0±5.5[c]	56.0±0.5[b]	50.4
Chickpea	176.33±7.5[b]	128.0±3.03[a]	73.2	115.33±4.51[c]	95.67±4.51[b]	83.1
Red lentil	124.33±4.7[a]	80.33±0.31[a]	65.7	100.0±3.87[b]	40.33±0.51[b]	40.4
Green lentil	142.33±5.5[b]	57.67±0.35[a]	41.1	92.33±3.51[c]	52.33±0.55[a]	56.5

Different letters within the same row indicate statistical differences between the applications ($p < 0.05$).

Considering the results of the analysis, the riboflavin losses in legumes were higher by pan cooking than by pressure cooking. Since riboflavin undergoes photochemical degradation (like sunlight exposure, and cooking in an open pan), all processes, such as cooking should be done in the dark or under subdued red light. Studies suggested that exposing milk to sunlight in glass bottles can destroy more than half of the riboflavin in one day[29]. On the contrary, riboflavin is stable in the heat process. So, the riboflavin content of foods is not affected by sterilization, canning, or cooking[30]. In line with this information, since the lid is never opened in pressure cookers, the product is not exposed to light. However, since the pans we used in the study had glass lids, and the lids were frequently opened to check whether they were cooked, there was constant exposure to light.

Riboflavin in cereal grains is mainly found in seeds and bran, and grinding or hulling, which removes these tissues, causes significant losses in vitamin content. About half of the riboflavin in whole-grain rice and more than one-third of the riboflavin in whole wheat is lost when these grains are milled[23,31-33]. During the processes carried out within the scope of the study, husking was not applied to red and green lentils. Therefore, the loss of riboflavin in red and green lentils was considerably lower than in chickpeas and beans.

The environment's pH is another factor that affects riboflavin stability and absorption. The maximum stability for riboflavin is between pH 2 and 4 [31]. Since the pH of the small intestine environment *in vitro* is 7, it is thought that vitamins are affected by this pH, and their bioaccessibility decreases. In a study, it was shown that 70% of the riboflavin content of chickpeas was lost by soaking in a sodium bicarbonate solution and cooking. It was seen that the soaking of chickpeas/beans in carbonated water, which is common among people, also causes high vitamin losses[32] In our study, soaking processes were carried out in plain drinking water for all samples.

Dietary fiber content affects riboflavin bioaccessibility. In the study on how dietary fiber addition in breads affects riboflavin bioaccessibility, it was found to be 40.9 – 91.2% for riboflavin[33]. Similarly, bioaccessibility was found to be low in our study. In addition, forms of riboflavin can be found bound to proteins by non-covalent bonds, and denaturation of proteins may be less in an alkaline environment in the gastrointestinal tract *in vitro*[31,34,42]. This causes the forms of these vitamins to become less free, which can therefore lead to low bioaccessibility.

Today, the consumption of vegetable protein sources and their effects on health are the subject of more studies[35,36]. For this reason, it is essential to study and update the nutritional and bioaccessibility values of legumes. In the Turkey Nutrition Health Survey 2017, the rate of cooking legumes in a pressure cooker was determined 45% [37]. Processes such as long cooking times in a pan and changing the boiling water increase the loss of vitamins. For this reason, the pressure cooker does not pose any danger when used correctly and is a good cooking method to reduce losses.

A group of population studies available about the vitamin status report on riboflavin deficiency in children, young adults, and especially in young women[38]. These studies achieved on riboflavin status are old. When we look at the situations that are getting riboflavin deficiency worse, the current changes in lifestyle, especially in well-nourished countries, with diets based on a lack of dairy and species of meat combination with more exercise, could potentially increase the risk of riboflavin deficiency. In line with these studies, studies on the daily riboflavin intake of people who adhere to a vegan diet lifestyle have shown that these individuals meet less than 48% of the daily intake level recommendation, which increases the risk for riboflavin deficiency[39-41].

This research was conducted *in vitro* by simulating the gastrointestinal system and aimed to evaluate bioaccessibility. From this point of view, the interaction of foods consumed together during digestion and whether their bioaccessibility changes as a result of the interaction have not been tested. However, since *in vivo* studies are expensive and require a lot of equipment, an *in vitro* model, which is a more advantageous and faster method, has been developed. In addition, the strongest aspect of this study is that it determines the most realistic vitamin content and bioaccessibility by taking into account the losses due to the cooking method in the vitamin content declaration.

As a result of our study, it has been seen that using the vitamin contents of raw foods in food composition charts can lead to misleading/incomplete guidance and results. At the end of this study, we concluded that bioaccessibility is important in determining how much of the daily nutrient requirement can be met by diet. In line with this knowledge, we think that it is realistic and necessary to include the nutritional content values after cooking and digestion in the food composition tables. When the bioaccessibility data obtained as a result of the study is evaluated, another noteworthy issue is that the evaluation of bioaccessibility only on the baked product may be misleading. When we look at the literature; the term bioaccessibility compares the state of the food when it first enters the digestive system with the state when it comes out. The point that is

overlooked here is that it seems to affect bioaccessibility positively due to the high cooking losses in the food. Vitamin losses are so high that there is no more vitamin to be lost. In conclusion; although the amount of vitamins absorbed and used in the digestive system is much higher in products with low cooking loss; it was found that the bioaccessibility of the products with high cooking loss was high, and the bioaccessibility of the products with low cooking loss was found to be low.

CONFLICT OF INTEREST STATEMENT

The authors affirm that they have no competing interests in relation to the work reported in this paper.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

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