Iron and Folate Contents of Tajik Legumes

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ABSTRACT

Seven varieties of Tajik legumes and two Tajik snack type ready-to-eat (RTE) whole/split chickpeas were analyzed for iron in raw and cooked legumes and for naturally occurring folate content in cooked legumes. Iron was measured according to AACC method 40 - 41B. Folate contents were determined by microbiological (Lactobacillus casei subsp. Rhamnosus ATCC 7469) and high-performance liquid chromatography analysis utilizing a tri-enzyme treatment (protease, α-amylase and conjugase). Folate derivatives of tetrahydrofolate, 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate were identified and quantified. Iron content for Tajik legumes ranged from 5.52 to 13.27 mg/100 g for raw; 2.81 to 4.12 mg/100 g for cooked and 4.37 and 4.76 mg/100 g for RTE chickpeas. The total folate content of cooked legumes ranged from 53 to 81 μg/100 g for beans; 133 to 203 μg/100 g for peas, and from 39 to 22 μg/100 g for small and large lentils, respectively. The predominant form of folate in legumes was tetrahydrofolate, followed by 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate.

Keywords: *Legumes, Ready-to-Eat (RTE) Snack Type Chickpeas, Microbiological Assay, HPLC, Iron, Folate, Tajikistan*

1. Introduction

Vitamin and mineral deficiencies (also known as "hidden hunger") are particularly prevalent in Central Asia with a high prevalence of iron and folate deficiency [1]. Anemia is the most common blood disorder in adults [2]. Both iron and folate deficiencies contribute to the prevalence of anemia [3]. The annual estimate of iron-deficiency anemia is 42% among Tajik women between 15 - 49 years old; approximately 100 maternal deaths per year occur from severe anemia [4]. In the geographic region surrounding Dushanbe (the capital of Tajikistan), but not including the capital, a high rate (73.7%) of folic acid deficiency in women was found [5]. In Tajikistan, over 300 children annually are born with neutral tube defects [4]. Iron intake from animal products was negligible among Tajik women [5]. Folate intake from consumption of green leafy vegetables was 15.6%; 11.6% from melons and pumpkins; and 1.5% from fruits and berries [5].

A food-based approach to reduce micronutrient malnutrition in Tajikistan encourages dietary diversification through the production and consumption of micronutrient-rich foods, including appropriate traditional foods [6]. This strategy is cost-effective, sustainable and income generating; culturally acceptable and feasible to implement; and builds alliances among government, consumer groups, the food industry and other relevant organizations to achieve the shared goal of preventing micronutrient malnutrition [7].

Legumes are grown for direct human consumption, available all year, and important sources of dietary proteins, dietary fiber, iron, folate, zinc, and calcium [8,9]. Legumes can be milled into flour to make bread and bread products and extruded snacks [10]. In Tajikistan, chickpeas are minimally processed into a snack food which is consumed like peanuts in the US. Legumes are an inexpensive source of nutrients and are culturally acceptable both in the diet and as a crop in Tajikistan [11]. The joint FAO/WHO expert consultation on nutrition and mineral requirements states that pulses (legumes), vegetables, including green leafy vegetables and fruits are the preferred way of ensuring optimal nutrition, including micronutrient adequacy [12].

Nutritional assessment by diet analysis of a population includes the evaluation of food consumed and then converting these food items into their nutrients [13]. This process requires a food composition database which lists the nutritional values for a given food portion for foods commonly eaten by the population [13].

Therefore, the objectives of our study were to 1) evaluate the iron content of raw and cooked Tajik legumes by the orthophenanthroline spectrophotometry; and

2) determine the naturally occurring folate contents of cooked Tajik legumes and ready-to-eat snack type chickpeas by microbiological and high-performance liquid chromatography (HPLC) methods.

2. Materials and Methods

2.1. Samples

Legumes (1000 g of each composited variety) obtained from local markets in Tajikistan were: red kidney beans (*Phaseolus vulgaris*); cranberry beans (Romano); black-eye peas (*Vigna unguiculata*), kabuli type chickpeas (*Cicer arietinum*); desi type chickpeas (*Cicer arietinum*); lentils, large (*Lens culinaris*); lentils, small (*Lens culinaris*); and ready-to-eat (RTE) whole or split kabuli type chickpeas (*Cicer arietinum*) (**Table 1**). Legumes were shipped to the US and samples were kept at room temperature until analyzed.

2.2. Cooking Treatments

Fifty g of legumes were soaked in 150 ml distilled water at 4˚C for 16 h. The soaked legumes were held at 4˚C to minimize microbial growth. After 16 h, the soaking water was discarded, 250 ml distilled water was added to the soaked legumes and legumes were then cooked until doneness on an electric hot plate. Doneness was determined visually and subjectively by pressing between the fingers (approximate cooking times for beans, peas and lentils were 30, 20 and 10 min). Cooked legumes were drained before analysis and stored at −80˚C for iron and folate determination. Weights of legumes before and after cooking were recorded. Each variety of raw and cooked legumes was analyzed in triplicate for iron measurements; for folate measurements, cooked legumes were analyzed in triplicate.

2.3. Iron Measurements

The AACC method 40 - 41B [14] was used for the determination of the iron content. Iron reference solution

and 1, 10-phenanthroline monohydrate ($Cl_{12}H_8N_2 \cdot H_2O$) were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) from ACROS Organics (Geel, Belgium). Legumes were homogenized in an Osterizer™ blender. A 5 g sample of raw or cooked legumes were dry ashed and dissolved in 5 ml of concentrated HCl and diluted to 100 ml. A 10 ml of the aliquot was taken for reaction with 1 ml of $NH_2OH \cdot HCl$ solution and 1 ml of orthophenanthroline. The absorbance of each solution was measured spectrophotometrically (Beckman DU-640, Fullerton, CA) at 510 nm and converted to iron concentration (mg/ml) using a calibration curve.

True Retention

True retention of iron content was calculated as described by Murphy *et al.* [15] using the following formula: True retention (%) = [(nutrient content (g/100 g) of cooked legume × g of cooked legume)/(nutrient content (g/100 g) of raw legume × g of raw legume) × 100].

2.4. Folate Measurements

2.4.1. Microbiological Analysis

Microbiological analysis of the total folate contents was based on Rader *et al.* [16] with a modification to the amount of the conjugase source. All procedures were carried out under subdued light conditions. A 25 g sample of cooked legumes was homogenized in 100 ml of 0.1 M phosphate buffer (pH 7.0) and centrifuged for 30 min. A 1 ml (2 mg·ml⁻¹) of protease (*Streptomyces griseus*; Sigma Chemical Co., St. Louis, MO) was added to 25 ml extract and incubated for 3 h at 37˚C. Samples were heated to 100˚C, cooled and brought to pH 7.8 with buffer. A 1 ml (20 mg·ml⁻¹) of α-amylase (*Aspergillus oryzae*; Sigma Chemical Co., St. Louis, MO) was added to each extraction sample and incubated for 2 h at 37˚C. A 4 ml (3 mg·ml⁻¹) aliquot of chicken conjugase (Difco Laboratories, Detroit, MI) was added to each extraction sample and incubated for 16 h at 37˚C. Deconjugated

Table 1. Description of legumes grown and purchased in Tajikistan.

Legumes	Description
Red, kidney beans (*Phaseolus vulgaris*)	- large, kidney-shaped bean; deep reddish-brown; robust, full-bodied flavor
Cranberry beans (Romano)	- large ovals; creamy background with burgundy highlights, pink-colored
Blackeye peas (*Vigna unguiculata*)	- kidney shaped white bean with black eye
Kabuli type chickpeas (*Cicer arietinum*)	-round, beige color; nut-like flavor ad firm texture
Desi type chickpeas (*Cicer arietinum*)	-angular, pigmented seeds, beige color; nut like flavor and firm texture
Lentil, large (*Lens culinaris*)	- seeds are round, about ½ cm in diameter and light green in color
Lentil, small (*Lens culinaris*)	- seeds are round, brown in color mixed with dark brown seeds speckled with black
Chickpeas whole (RTE) (*Cicer arietinum*)	- round, medium size, about ½ cm in diameter and light green in color
Chickpeas split (RTE) (*Cicer arietinum*)	- round, split, yellow color; nut-like flavor and firm texture

extracts were heated to 100˚C and centrifuged. The extracted samples were appropriately diluted and used in the microbiological analysis with *Lactobacillus casei* subsp. *Rhamnosus* (ATCC No. 7469) for total folate. A folic acid (200 µg·ml⁻¹) standard solution was prepared according to AOAC 45.2.03 [17]. *L. casei* subsp. *Rhamnosus* was incubated for 17 h at 37˚C. Turbidimetric readings were carried out by using a spectrophotometer (Beckman DU® 640, Fullerton, CA) at 660 nm. Each variety of cooked legumes was analyzed in triplicate. The growth response of the *L. casei* subsp. *Rhamnosus* (measure as turbidity at 660 nm) of the total folate in the sample was compared quantitatively to standard solutions of folic acid.

2.4.2. HPLC Analysis

Folate derivatives of tetrahydrofolate (THF), 5-formyl-tetrahydrofolate (5-CHO-THF) and 5-methyl tetrahydrofolate (5-CH₃-THF) in cooked legumes were identified and quantified by reversed-phase ion-pair HPLC with fluorometric detection after ion exchange solid phase extraction. Individual folate standards THF, 5-CHO-THF, 5-CH₃-THF, folic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

2.4.2.1. Sample Extraction and Purification

After protease treatment (as described above), the pH was adjusted to 6.8. Rat plasma (Difco Laboratories, Detroit, MI) at a concentration of 0.5 ml was used in 10 ml of extract as a conjugase source. The extracts were purified by solid phase extraction (SPE) on a strong anion exchange (SAX) cartridges [Quaterny amine (N⁺), Baker 7091-3] as described by Osseyi [18]. The cartridges (3-ml SPE column, 500 mg) were conditioned with 3 ml of hexane, followed by 3 ml of methanol, and then equilibrated with 5 ml of 0.1 M K₂HPO₄ buffer (pH 7 - 8) containing 0.1% (w/v) ascorbic acid. A portion (4 ml) of the extract was diluted with 2 ml phosphate buffer before loading into a column, with an elution rate of about 0.6 ml/min; the volume of the portion and the extent of dilution might vary depending on the expected value of folic acid. The column was rinsed with 2 ml diluted (1/5) phosphate buffer. Analytes were eluted with at least 4 ml of 0.1 M sodium acetate (pH 4.5) containing 5% (w/v) Na₂HPO₄ (HPLC grade) and 0.1% (w/v) ascorbic acid. The eluent was injected in 20 µL volumes.

2.4.2.2. Chromatography

The HPLC separation was performed on Microsorb - MV C₁₈ analytical column (100 mm × 4.6 mm I.D., 3 µm particle diameter, Varian Chromatographic Systems, Walnut Creek, CA). A Brownlee (30 mm × 2.1 mm I.D.) guard column with 5 µm ODS packing (Varian Chromatographic Systems, Walnut Creek, CA) which was placed

before a C₁₈ analytical column (Varian Chromatographic Systems, Walnut Creek, CA). The HPLC analysis was performed using a Hitachi L-4000 UV detector operating at 280 nm and Waters 474 scanning fluorescence detector (Waters Corp., Milford, MA) set at an excitation wavelength of 290 nm and emission wavelength of 360 nm. Chromatograms were recorded and peak areas and heights quantified using a Hitachi model D-2500 chromato-integrator. The mobile phase was composed of 26% methanol (v/v) in potassium phosphate buffer (3.5 mM KH₂PO₄ and K₂HPO₄), pH 6.8, containing 5 mM tetrabutylammonium dihydrogen phosphate (Sigma Chemical Co., St. Louis, MO) as an ion-paring agent. Analyses of cooked legumes were conducted in triplicate. Recovery of folates was determined by adding a known amount of each folate form (tetrahydrofolate, 5-formyl-tetrahydrofolate and 5-methyl tetrahydrofolate) into the samples. The concentrations of folate derivatives in the sample were measured by comparing peak area to a standard solution.

2.4.3. Quality Control

Cooked legume samples were spiked with a known amount of folic acid and analyzed by microbiological and HPLC methodologies. Recovery was calculated using the following formula: % Recovery = [(ng folic acid in spiked sample – ng folic acid in unspiked sample)/ng folic acid added in spiked sample. Recovery of added folic acid averaged 93% - 102%.

2.5. Statistical Analysis

For all analysis, the means of three values and standard errors were calculated using statistical analysis software (SAS Institute, Inc., Cary, NC, 2000; Release 8.01). A level of 5% was considered to be significant.

3. Results and Discussion

3.1. Iron Content

Seven varieties of Tajik legumes and two Tajik snack type ready-to-eat (RTE) whole/split chickpeas were analyzed for iron content (**Table 1**). The effect of cooking on the true retention for iron in legumes is presented in **Table 2**. For true retentions of less than 100%, the iron may have been lost in the soaking or cooking water. Murphy *et al*. [15] also reported iron retention greater than 100% in legumes and attributed this increase in foods where moisture is gained during soaking/cooking and the potential of solids are lost during cooking. In our study, the cooking water was not analyzed for iron content.

Results of analyzed iron contents of Tajik legumes were compared with the USDA Nutrient Database [19]. Tajik legumes were 35% lower for raw and 56% higher

for cooked legumes than the USDA Nutrient Database [19] (**Table 2**). These differences may be due to varietal differences, growing conditions, storage conditions and preparation methods. Also, the soaking and cooking process that we used may have affected the higher retentions of iron in the Tajik varieties.

In our study, legumes were soaked overnight for 16 h. Soaking legumes for some period of time is one of the common practices of Tajik households. Soaking improves iron absorption, which degrades the phytate [20]. However, according to Brazaca and Da Silva [21], the effects of tannins and phytic acid were not determining factors in diminishing the amount of dialyzed iron; rather the effect of dietary enhancers (meat and orange juice) increased the amount of iron absorption. Therefore, in addition to soaking, including foods rich in vitamin C (tomatoes, leafy green vegetables, bell peppers, lemon, and green onions) and/or small amount of meat to a cooked legume item may help maximize iron absorption.

3.2. Folate Contents

The same varieties of cooked legumes were analyzed for the folate contents by microbiological analysis and HPLC. In general, the microbiological analysis is based on quantifying the growth response of a specific micro-organism, *L. casei* subsp. *Rhamnosus*, to the total mixture of folate that are present [22,23]. The use of the HPLC analysis allows the identification of the boilogically active forms of folate; tetrahydrofolate (THF), 5--methyl tetrahydrofolate (5-CH$_3$-THF), and 5-formyl tetrahydrofolate (5-CHO-THF).

Legumes are rich in protein and starch; therefore, the complete extraction of folates from the cellular matrix of legume seed is essential. In our study, the tri-enzyme treatment was used, including protease, α-amylase and conjugase [16]. Chicken pancrease was used as a conjugase source for microbiological analysis and rat plasma for HPLC. The total folate contents by microbiological analysis of cooked legumes ranged from 53 to 81 µg/100 g for beans; 133 to 203 µg/100 g for peas, and from 22 to 39 µg/100 g for lentils (**Table 3**). RTE whole or split chickpeas prepared and used as a snack food in Tajikistan had a high folate content (269 and 233 µg/100 g) on a dry basis, respectively (**Table 3**). Our results were simi-

Table 2. Iron contents of raw and cooked legumes (Mean ± SE).

Legumes	Iron content (mg/100g dry matter) of raw legumes		Iron content (mg/100g wet matter) of cooked legumes		TR (%)[1]
	Tajik Data	USDA Data	Tajik Data	USDA Data	Tajik Data
Red, kidney beans (*Phaseolus vulgaris*)	6.98 ± 0.51	6.69 ± 0.24	3.34 ± 0.03	2.94 ± 0.18	92.01
Cranberry beans (Romano)	6.31 ± 0.05	5.00 ± 0.19	3.17 ± 0.01	2.09 ± 0.12	96.19
Blackeye peas (*Vigna unguiculata*)	5.52 ± 0.10	8.27 ± 0.25	2.81 ± 0.11	2.51 ± 0.10	111.00
Kabuli type chickpeas (*Cicer arietinum*)	6.17 ± 0.06	6.24 ± 0.14	3.14 ± 0.43	2.89 ± 0.15	99.75
Desi type chickpeas (*Cicer arietinum*)	6.50 ± 0.01	NA	3.27 ± 0.18	NA	101.57
Lentil, small (*Lens culinaris*)	13.27 ± 0.19	NA	4.08 ± 0.43	NA	60.17
Lentil, large (*Lens culinaris*)	8.45 ± 0.43	9.02 ± 0.37	4.12 ± 0.48	3.33 ± 0.24	100.19
Chickpeas whole (RTE) (*Cicer arietinum*)	*	*	4.37 ± 0.02[2]	NA	NA
Chickpeas split (RTE) (*Cicer arietinum*)	*	*	4.76 ± 0.14[2]	NA	NA

[1]True Retention, reference Murphy *et al*. (1975); NA = Not available; *Analyzed in processed form only.

Table 3. Folate vitamers of cooked legumes (Mean ± SE).

Legumes	THF µg/100 g	5-CHO-THF µg/100 g	5-CH$_3$-THF µg/100 g	Total vitamers (HPLC) µg/100 g	Total folate (MA) µg/100 g	Total folate USDA (MA)
Red, kidney beans (*Phaseolus vulgaris*)	26.2	34.5	13.5	74 ± 14.4	81 ± 5.6	130 ± 3.8
Cranberry beans (Romano)	23.6	22.9	13.3	60 ± 0.6	53 ± 7.5	207 ± 7.8
Blackeye (*Vigna unguiculata*)	51.8	31.0	5.6	88 ± 4.7	133 ± 20.6	208 ± 19.1
Kabuli type chickpeas (*Cicer arietinum*)	51.4	15.3	28.1	95 ± 11.6	189 ± 1.9	172 ± 9.7
Desi type chickpeas (*Cicer arietinum*)	34.4	23.0	26.7	84 ± 4.7	203 ± 7.7	NA
Lentil, small (*Lens culinaris*)	48.3	23.7	16.0	88 ± 2.3	39 ± 1.2	NA
Lentil, large (*Lens culinaris*)	19.0	ND	2.1	21 ± 5.8	22 ± 1.0	181 ± 7.0
Chickpeas whole (RTE) (*Cicer arietinum*)	84.2	ND	4.7	89 ± 1.1	269 ± 20.8	NA
Chickpeas split (RTE) (*Cicer arietinum*)	74.4	ND	5.1	80 ± 17.3	233 ± 29.3	NA

MA = Microbiological Analysis; ND = Not detectable; NA = Not available.

lar to folate contents of cooked legumes, soaked overnight for 16 h, reported by Hoppner and Lampi [24]. However, the total folate contents were lower than those reported in the USDA Nutrient Database (**Table 3**). Measurement of folate contents by different extraction methodologies, as well as the nature of the growing conditions, weather, location and cultivar has an impact on food components [25-28] storage conditions, legumes may become dry and cracked, which may influence the loss of vitamins from outer layers during soaking, as most B vitamins are located in the outer layers.

Seven varieties of cooked legumes analyzed by HPLC contained 49.9% of folates in the form of THF; 29.5% in the form of 5-CHO-THF; and 20.6% in the form of 5-CH_3-THF (**Table 3**). RTE chickpeas contained mainly 95% of folates in the THF form (**Table 3**). The distributions of folates derivatives in kabuli type of chickpeas (**Table 3**) were higher (28.1 µg/100 g for 5-CH_3-THF, 15.3 µg/100 g for 5-CHO-THF, 51.4 µg/100 g for THF) than the results of Ruggeri *et al*. [27]; 9.1 µg/100 g for 5-CH_3-THF, 6.1 µg/100 g for 5-CHO-THF and not detectable for THF. Another researcher, Rychlik *et al*. [29] determined the folate content of legumes using a stable isotope dilution assays and found that 5-CH_3-THF was the predominate form in frozen peas, dried lentils, dried blackeye peas and mung beans; THF was the most abundant in fresh beans and soybeans; and 5-CHO-THF was predominately found in peanuts.

Typically THF, the most unstable form, was detected in meat and meat products [27,30-32]. The 5-CH_3-THF form was found to be the main folate in vegetables and legumes [22,25,29,30,32-34]. In vegetables, fruit, bread, milk products, potatoes, and meat products, 62% of all folate forms (THF, 5-CH_3-THF, 5-CHO-THF, 105-HCO-folic acid and 10-HCO-H_2folate) were 5-CH_3-THF [30]. Our results for cooked varieties of Tajik legumes differed from these published data. These differences may be due to growing conditions, varietal and/or the methodological differences.

The mean differences of folate as measured by the HPLC analysis were approximately 29% lower than the total folate amounts analyzed by microbiological analysis. Differences may be due to the identification of only three folate forms (THF, 5-CH_3-THF, and 5-CHO-THF) by HPLC analysis that are found in food; therefore, not including other folate compounds which may be found in minute quantities and not measured. The higher total folate values that we found as measured by the microbiological analysis may be due to non-folate compounds that stimulate bacterial growth [30].

In our study, we did not analyze folate in raw legumes. Folate losses may occur through leaching (soaking for 16 h) and cooking. Leaching of folates also depends on the type and variety of legumes [35], size and the surface area to volume ratio during soaking [24]. In our study, for example, small lentils were higher in total folate values (39 µg/100 g) compared to large lentils (22 µg/100 g); and desi type chickpeas (small) had higher total folate content (203 µg/100 g) compared to kabuli type chickpeas (large) which contained 189 µg folate/ 100 g.

Differences of folate forms from our study compared to other research may be attributed to discarding the soaking and cooking water rather than the destruction of folate forms during cooking [24,36]. However, legumes soaked overnight for 16 h have been shown to have higher folate retentions compared to no presoak or quick soak treatments [24]. Folate content of the cooking water was not determined in our study. It has been suggested to use the cooking water from legumes for further consumption [36]. In Tajikistan, the most common way of preparing legumes is cooking the legume and using the water as broth for stew, soup or other food items.

4. Conclusions

Iron and folate contents of Tajik varieties of cooked legumes could be used in dietary research and to make recommendations to Tajiks (especially women of childbearing age) to improve their iron and folate status. Using our data and calculations, the dietary iron intake for Tajik women (18 or above) consuming 100 mg of cooked legumes would have an iron intake of 18% from cranberry and red kidney beans, 17% peas, 28% lentils, and 25% from RTE chickpeas, based on 18 mg of iron per day as advised by Dietary Reference Intakes. The dietary folate intake for Tajik women (18 or above) consuming 100 g of cooked legumes would have a folate intake of 17% from cranberry and red kidney beans, 44% peas, 8% lentils, and 63% from RTE chickpeas, based on 400 µg of folate.

5. Acknowledgements

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