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Determination of Folate Concentrations in Pulses by a Microbiological Method Employing Trienzyme Extraction

JEE-YUP HAN* AND ROBERT T. TYLER

Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A8, Canada

Over the past two decades, the role of folate in human nutrition has been of much interest because of its relationship to diseases such as neural tube defects and heart disease. Since 1998, the U.S. Food and Drug Administration has mandated that cereal products be fortified with 140 μg of folic acid/100 g. It is important, therefore, to be able to determine accurately the folate concentrations in cereals and other grains to ensure proper dietary intake of folate. In this study, a microbiological method employing a trienzyme extraction procedure was applied to the analysis of folate in several starchy grain legumes (pulses). Differences in the folate content of dry bean were observed among some market classes but not between cultivars in the same market class. Location had a significant effect on the folate content of lentil and dry pea; cultivar did not. The significant effect of market class, cultivar, and growth environment on the levels of folate in pulses is of particular importance to pulse processors and pulse breeders.

KEYWORDS: Folate; folic acid; microbiological analysis; dry bean; lentil; pea

INTRODUCTION

Folate was originally found in yeast by Willis (*1*) in 1931 and was initially called vitamin M or vitamin Bc. Today, folate is a generic term that refers to all derivatives of folic acid, a B-vitamin. Among the many naturally occurring folates, the predominant derivatives in plants, particularly cereal grains and legume seeds, are polyglutamyl forms of tetrahydrofolic acid (THF), 5-methyl-THF and 10-formyl-THF (*2*). Folate is an essential dietary component and is necessary for the formation of red and white blood cells and epithelial cells of the digestive tract (*3*). Folate is important in human health because a deficiency can result in megaloblastic anemia, neural tube defects (*4*, *5*), chronic disease (*6*), coronary heart disease (*7*−*9*), and malignant transformation (*10*).

Over the past four decades, a variety of techniques have been employed in the analysis of folate, including biological, microbiological, radio-immunoassay, and chemical (colorimetric and fluorometric) methods, gel filtration and ion exchange chromatography, high-performance liquid chromatography, high-voltage electrophoresis, and an enzymatic folate binding protein method. These methods have been reviewed by several researchers (*10*−*17*). The microbiological assay has been the most widely used method for folate determination in foods. Although the microbiological assay has been modified and improved over the years, the basic concept of the assay has not changed. *Lactobacillus casei* L. has been used most widely for the determination of folate in foods because it responds similarly to a wide variety of folate derivatives (*16*, *18*). For sample preparation, Martin et al. (*19*) reported that a series of three enzyme treatments (α-amylase, protease, and chicken pancreas conjugase) in the extraction procedure for folate was superior to a single enzyme treatment (conjugase). The triple-enzyme treatment increased the measurable folate content of several foods, including cereal grains and pulses. The roles of α-amylase and protease in releasing folate bound to starch and protein, respectively, by physical adsorption were discovered by several researchers (*20*, *21*). In light of recent developments in folate analysis methodology, such as thermal treatment, enzymatic deconjugation, and trienzymatic digestion, and adoption of an approved method by the American Association of Cereal Chemists (AACC) in 2001 (*22*), it is necessary to examine the folate content of various foods using the latest methodology. The availability of more precise folate content data for various grains and seeds would be beneficial to both food processors and consumers because of the U.S. Food and Drug Administration (FDA) regulations requiring the addition of folic acid to a wide range of enriched cereal products.

In this study, a microbiological assay employing trienzyme extraction was applied to the determination of folate contents in samples of several dry bean, lentil, and dry pea (green and yellow) cultivars. The effect of cultivar and growth environment on the level of folate in pulses was assessed by analyzing samples from several cultivars, locations, and crop years.

MATERIALS AND METHODS

Two cultivars each of three dry bean classes were analyzed in duplicate for their folic acid contents: pinto bean (*Phaseolus vulgaris* L. cv. Pinnacle and cv. Camino) grown at Melfort, SK; navy bean (*P.*

* Author to whom correspondence should be addressed [telephone (306) 966-2402; fax (306) 966-8898; e-mail hanjy@duke.usask.ca].

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vulgaris L. cv. Envoy and cv. Laser) grown at Hensall, ON; and Great Northern bean (*P. vulgaris* L. cv. Crocus and cv. US1140) grown at Grassy Lake, AB. Two lentil cultivars (*Lens culinaris* L. cv. Laird and cv. Richlea) grown in three locations in Saskatchewan (Elrose, Rosthern, and Saskatoon) were analyzed. All dry bean and lentil samples were from the 1999 crop year. Two cultivars of green pea (*Pisum sativum* L. cv. Nitouche and cv. CDC Verdi) grown in three locations in Saskatchewan (Saskatoon, Nipawin, and Rosthern) in the 1999 and 2000 crop years and two cultivars of yellow pea (*Pisum sativum* L. cv. CDC Mozart and cv. Alfetta) grown at six locations in Saskatchewan (Assiniboia, Jedbergh, Kernen, Luseland, Nipawin, and Rosthern) in the 2000 crop year were analyzed. Folic acid (pteroylglutamic acid) and 5-methyltetrahydrofolic acid (5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, barium salt) were used as standards and were obtained from Sigma Chemical Co. (St. Louis, MO).

To verify the accuracy and reproducibility of the folate analytical method used in this study, two commercially available folate reference samples were analyzed along with the pulse samples. A freeze-dried whole blood control (Lypocheck Level 2) was obtained from Bio-Rad Laboratories (Hercules, CA). The stated folate content of the freeze-dried human blood standard was 400 ± 123 ng/mL of reconstituted human blood. The other reference standard, wheat wholemeal flour (CRM 121), was obtained from the Institute for Reference Materials and Measurements, European Union—Joint Research Centre (Geel, Belgium). The concentration of folate in CRM 121 was stated as 50 ± 7 μg/100 g (*23*).

To further verify the efficiency of the method, several pulse samples were intentionally spiked with known amounts of commercial folic acid (Sigma). The results were reported as percent of folic acid recovered.

The folate analytical method used in this study was very similar to the method (AACC Method 86-47) recently approved by the AACC (*22*), as it employed trienzyme extraction, the same microorganism, a 96-well microtiter plate, etc. The methods were developed independently and were based on studies by Vahteristo et al. (*24*), Wilson and Horne (*25*), and Rader et al. (*26*). Only significant differences between the methodology used in this study and the approved AACC method are described here.

Homogenization. The main difficulty with folate analysis lies in its extraction and purification from food samples, especially plant materials, due to their complex chemical composition (high protein and starch contents). Two grams of finely ground sample was dispersed in 20 mL of extraction buffer [75 mM potassium phosphate buffer (pH 6.0) containing 52 mM sodium ascorbate and 0.1% (v/v) 2-mercaptoethanol] with a few drops of 1-octanol as an antifoaming agent. The mixture was then homogenized for 30 s using an Ultra-Turrax T25-S1 homogenizer (IKA-Laboratechnik, Berlin, Germany). The homogenized sample was placed in a boiling water bath for 10 min and then cooled on ice.

Trienzyme Treatments. The homogenized sample was treated with three enzymes as described in the AACC method (*22*), with the following modifications. The sample was incubated at 37 °C with 1 mL of α-amylase from *Aspergillus oryzae* (20 mg/mL, Sigma A-0273) for 4 h and then 2 mL of protease from *Streptococcus griseus* (2 mg/mL, Sigma P-5147) for 1 h, followed by incubation with 0.2 mL of conjugase isolated from chicken pancrease (1 mg/mL, Difco Laboratories, Detroit, MI) for 4 h. The enzymes were deactivated by placing the tubes in a boiling water bath, and the sample was filtered through a 0.2 μm pore size nylon membrane filter (Acrodisc; Gelman Science, Ann Arbor, MI) into sterile vials, flushed with N_2, and stored at −70 °C until assayed.

Microbiological Assay. Extracts of pulse samples were diluted to appropriate concentrations and assayed microbiologically using *Lactobacillus rhamnosus* L. strain ATCC 7469 (ATCC, Manassas, VA). Folic Acid Casei Medium was obtained from Difco.

A vial of lyophilized *L. rhamnosus* was prepared as described in the AACC method (*22*). All procedures were performed using aseptic techniques. All procedures involving standard solutions or extracts were carried out under yellow (550−600 nm) fluorescent light to protect labile folates and ascorbic acid from photochemical degradation (*24*). All solutions were prepared using deionized water.

Table 1. Folate Concentrations and Recoveries from Folate-Spiked Samples of Commercially Available Reference Samples As Determined by a Microbiological Assay[a]

	folate content[b]	recovery[c] (%)
wheat wholemeal flour[d]	52.7 ± 3.8 μg/100 g	93.4 ± 4.2
human blood[e]	381.8 ± 34.4 ng/mL	95.1 ± 3.7

[a] Values are means of quadruplicate determinations. [b] Folate content determined using a 5-methylfolate standard curve. [c] Recovery of folate from the folate-spiked reference samples. Reference samples were spiked by adding 30 μg/100 g and 200 ng/mL of commercial folic acid to wheat wholemeal flour and human blood, respectively. [d] Folate content provided by the supplier was 50 ± 7 μg/100 g wheat wholemeal flour. [e] Folate content provided by the supplier was 400 ± 123 ng/mL human blood standard.

Standard Curves. Standard curves were prepared for folic acid and 5-methyltetrahydrofolic acid. Stock standard solutions were prepared by dissolving 10 mg of standard in 2 mL of 0.1 M NaOH and further diluting this solution to 500 mL with 0.5% (w/v) sodium ascorbate. The stock solutions were stored in the dark in the refrigerator for up to 2 months. Working standard solutions were prepared by diluting 2.5 mL of stock standard solution to 100 mL with 0.5% (w/v) sodium ascorbate. Aliquots (2 mL) were portioned into vials and stored in the dark at −70 °C until needed.

Sample Analysis. Appropriate dilutions were made of the sample extract through trial and error such that the growth response of the *L. rhamnosus* was within the linear portion of the standard curve. The standard curve was constructed by dispensing appropriate amounts of working standard solution into the wells of the microtiter plate (in quadruplicate) and following the procedure below.

The sample extract (50 μL) was diluted to 5 mL with 0.1 M potassium phosphate buffer containing 0.1% (w/v) ascorbic acid. The samples were plated (in quadruplicate) by adding 0, 10, 20, 40, 60, 80, 100, and 150 μL of sample extract. An appropriate amount of 0.1 M potassium phosphate buffer (pH 6.1) containing 0.1% (w/v) sodium ascorbate was added to each well to achieve a final volume of 150 μL. Preinoculated *L. rhamnosus* medium (150 μL) was added to each well. The wells in the first row of the microtiter plate were filled with 300 μL of deionized water as blanks. The loaded plate was incubated at 37 °C for 24 h in the dark. The plate was then read at 530 nm using a microplate reader (model 550, Bio-Rad Laboratories), and the results were expressed as micrograms of folic acid per 100 g of sample (dry weight basis).

RESULTS AND DISCUSSION

Table 1 presents the folate concentrations in two commercially available reference samples as determined by the microbiological assay. Values were derived using a 5-methylfolate standard curve; 5-methylfolate is one of the folate monoglutamates present in plant materials, including pulses and other grains (*2*). *L. rhamnosus* cells (3.8×10^5, determined using a Klett−Summerson photoelectric colorimeter, Klett Manufacturing Co., New York) were added to each sample well. The folate contents measured in the reference samples were in agreement with the values provided by the suppliers, which indicated that the microbiological assay used in this study was valid and accurate. The reference samples were also intentionally spiked by adding 30 μg/100 g and 200 ng/mL of commercial folic acid to the wheat wholemeal flour sample and the human blood sample, respectively, to further verify the accuracy of the method. The folate recoveries (naturally occurring plus spiked) obtained were 93.4 ± 4.2 and 95.1 ± 3.7% for wheat wholemeal flour and human blood, respectively, indicating a high efficiency of sample extraction, preparation, and assay. These tests verified that the microbiological method used in this study was reproducible and accurate.

Table 2. Folate Contents of Dry Bean Samples As Determined by a Microbiological Assay[a]

market class	cultivar	folate content (μg/100 g)	standard deviation
pinto	Pinnacle	143.1 a	10.3
	Camino	145.2 a	15.1
navy	Envoy	156.8 b	11.4
	Laser	160.4 b	13.6
Great Northern	Crocus	152.5 ab	18.0
	US1140	144.5 a	8.0

[a] Values are means of quadruplicate determinations. Means with the same letter are not significantly different ($P > 0.05$).

Table 3. Folate Contents of Lentil Samples As Determined by a Microbiological Assay[a]

cultivar	location	folate content (μg/100 g)	standard deviation
Laird	Saskatoon	165.8 a	7.5
	Rosthern	150.6 b	6.9
	Elrose	194.0 c	8.8
Richlea	Saskatoon	167.3 a	9.1
	Rosthern	150.9 b	5.8
	Elrose	201.2 c	8.5

[a] Values are means of quadruplicate determinations. Means with the same letter are not significantly different ($P > 0.05$).

Table 4. Folate Contents of Green Pea Samples As Determined by a Microbiological Assay[a]

cultivar	year	location	folate content (μg/100 g)	standard deviation
Nitouche	1999	Kernen	27.4 a	1.8
		Nipawin	35.4 ab	5.9
		Rosthern	24.9 a	4.7
	2000	Kernen	36.1 b	5.5
		Nipawin	30.0 ab	2.7
		Rosthern	25.0 a	3.5
CDC Verdi	1999	Kernen	25.4 ab	2.7
		Nipawin	64.8 c	5.5
		Rosthern	37.8 b	1.9
	2000	Kernen	24.9 ab	2.4
		Nipawin	62.2 c	3.9
		Rosthern	42.2 b	3.3

[a] Values are means of quadruplicate determinations. Means with the same letter are not significantly different ($P > 0.05$).

The folate contents of dry bean, lentil, and green and yellow pea were determined by the microbiological assay. Results for dry bean (**Table 2**), lentil (**Table 3**), and pea (**Tables 4** and **5**) fell within the range of published findings, namely, 117−506, 68−433, and 21−274 μg/100 g of sample, respectively (*27*−*30*). Due to variations in the methodology employed in previous studies, reported folate contents of pulse samples vary widely, making it difficult to compare specifically folate values from this study with those reported by others.

Table 2 presents the folate contents of dry bean samples as determined by the microbiological method. No significant differences in folate content were observed between cultivars in the same market class. However, differences in folate content were observed among market classes. The average folate contents of pinto bean (144.5 μg/100 g) and navy bean (158.6 μg/100 g) were higher than the previously reported value of 100 μg/100 g for both bean classes (*28*). This was probably due to the superior sample extraction methodology used in this study. The average folate content of Great Northern bean (148.5 μg/100 g) was within the range of previously reported values (*27*−*30*). No value for the folate content of bean samples determined using the trienzyme extraction method has been reported previously.

Table 5. Folate Contents of Yellow Pea Samples As Determined by a Microbiological Assay[a]

cultivar	location	folate content (μg/100 g)	standard deviation
CDC Mozart	Assiniboia	46.4 bc	4.3
	Jedbergh	39.5 b	3.8
	Kernen	23.7 a	3.5
	Luseland	55.6 c	3.2
	Nipawin	44.0 b	1.5
	Rosthern	37.9 b	5.6
Alfetta	Assiniboia	46.8 bc	6.1
	Jedbergh	30.2 ab	1.2
	Kernen	26.5 a	2.3
	Luseland	48.7 bc	4.5
	Nipawin	44.3 b	4.8
	Rosthern	33.1 ab	3.0

[a] Values are means of quadruplicate determinations. Means with the same letter are not significantly different ($P > 0.05$).

Table 3 presents the folate contents of lentil samples as determined by the microbiological method. Differences in folate content were observed among lentil samples grown in different locations but not between cultivars grown in the same location. Lentil from Elrose contained the highest concentration of folate (194.0 μg/100 g in Laird and 201.2 μg/100 g in Richlea). Samples of the respective cultivars from Saskatoon contained 165.8 and 167.3 μg of folate/100 g, whereas samples from Rosthern contained 150.6 and 150.9 μg of folate/100 g, respectively. The results clearly indicate that the folate content of lentil is influenced by environmental factors such as weather and soil type.

Table 4 presents the folate contents determined for green pea. No effect of crop year on folate content was observed for either green pea cultivar with the exception of Nitouche grown at Kernen (**Table 4**). Location, however, significantly affected the folate content of each cultivar, CDC Verdi in particular. For example, CDC Verdi grown at Nipawin was significantly higher in folate (average = 63.5 μg/100 g) compared to the same cultivar grown at Kernen or Rosthern (averages of 25.2 and 40.0 μg/100 g, respectively).

Table 5 presents the folate contents of yellow pea grown at six different locations in the 2000 crop year. No differences in folate content were observed between CDC Mozart and Alfetta when grown at the same location. The folate content of each cultivar was significantly affected by location, however. Samples from Assiniboia and Luseland were significantly higher in folate (average contents of 46.6 and 52.2 μg/100 g, respectively) than those grown at other locations, whereas samples from Kernen were significantly lower in folate (average contents of 25.1 μg/100 g) than those grown at other locations.

Conclusions. The total folate analytical method employed in this study, which was similar to the approved AACC microbiological assay using trienzyme extraction and 96-well microtiter plates (*22*), was useful for determining the folate content of pulses. The accuracy and reproducibility of the method were verified by analyzing two commercially available reference samples (human blood and wheat wholemeal flour) and by intentionally spiking the reference samples with folate.

Differences in folate content were observed among market classes of dry bean but not between cultivars of dry bean of the

same market class. The average folate content determined for each dry bean class was generally higher than comparable values reported previously. Location (i.e., growth environment) had a significant effect on the folate content of lentil; cultivar did not. A significant effect of location on folate content was also seen for both green and yellow pea; the effect was marked in a green pea cultivar. The significant effect of cultivar and environment on the level of folate in pulses is of particular importance to pulse processors and pulse breeders.

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