Journal of Food Composition and Analysis 42 (2015) 134–140

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Genetic diversity of folate profiles in seeds of common bean, lentil, chickpea and pea

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ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form 15 March 2015
Accepted 17 March 2015
Available online 4 April 2015

Keywords:
Cultivars
Folates
Pulses
Liquid chromatography
Mass spectrometry
Quantification
Food analysis
Food composition

ABSTRACT

Folates are water-soluble B vitamins and act as cofactors in many metabolic functions in the human body. Pulses have traditionally been considered as a good dietary source of folates. The objectives of this study were (1) to determine the concentration of folates in four cultivars each of common bean, lentil, chickpea and pea, and (2) to determine the effect of growing location on folate concentration. Six folate monoglutamates were quantified by ultra-performance liquid chromatography coupled with mass spectrometry (UPLC–MS/MS). Total folate concentration ranged from 351 to 589 μg/100 g in chickpea, 165 to 232 μg/100 g in common bean, 136 to 182 μg/100 g in lentil, and 23 to 30 μg/100 g in pea. The 5-methyltetrahydrofolate (5-MTHF) and 5-formyltetrahydrofolate (5-FTHF) folates were most abundant in common bean, lentil and chickpea, whereas 5-MTHF and tetrahydrofolate (THF) were the predominant forms in pea. Significant differences were detected among cultivars for all folates across the pulses, except for 5,10-methenyltetrahydrofolate (5,10-MTHF) in lentil, 5-MTHF in chickpea, and 5,10-MTHF and folic acid (FA) in pea. Significant effects for location and cultivar by location were also observed for the majority of the folates.

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1. Introduction

Pulse crops play a significant role in traditional diets of people in many parts of the world as they are among the richest source of proteins and amino acids (Messina, 1999; Duranti, 2006). Globally, the consumption of pulses is increasing due to their nutritional value and many health benefits (Curran, 2012). Common bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) are among the most important pulse crops grown worldwide (Duranti, 2006). Pulses and other legumes, as well as beef liver, spinach, asparagus, lettuce, and Brussels sprouts, are rich in folates (USDA-ARS, 2012).

Tetrahydrofolate (THF) and its derivatives, collectively called folates, are involved in one carbon transfer reactions in many organisms including humans (Jabrin et al., 2003; Hayashi et al., 2007). Folates are water-soluble B vitamins and act as cofactors in many metabolic functions including the biosynthesis of nucleic acids, metabolism of amino acids, methylation of hormones, lipids, proteins, and DNA (Bailey and Gregory, 1999; Scott et al., 2000; Forges et al., 2007). In plants, folates are vital for biosynthesis of lignin, alkaloids, betaines and chlorophyll, and are indispensable in photorespiration (Hanson and Roje, 2001).

Humans cannot synthesize folates and thus depend upon plant and animal sources (Scott et al., 2000; Basset et al., 2005). The majority of people in developing countries depend on staple crops such as rice, maize, plantain and potato to fulfil their basic food requirements, however these crops are low in folates (USDA-ARS, 2012). Folate deficiency poses serious problems in both developed and developing nations and can cause serious health issues including neural tube defects (NTDs), impaired cognitive function, and cardiovascular diseases (Geisel, 2003; Ramos et al., 2005; McCully, 2007). It is also associated with numerous neurodegenerative disorders, including Alzheimer's disease (Seshadri et al., 2002), and various cancers (Choi and Friso, 2005). The Recommended Dietary Allowance (RDA) of folates is 400 μg for adults and 600 μg for pregnant women (Institute of Medicine. Food and

Abbreviations: FA, folic acid; 10-FFA, 10-formylfolic acid; 5-FTHF, 5-formyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; UPLC–MS, ultra-performance liquid chromatography–mass spectrometry.

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http://dx.doi.org/10.1016/j.jfca.2015.03.006
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Nutrition Board, 1998). Folate-rich diets are recommended to pregnant women as they play an important role in various metabolic processes including nucleotide biosynthesis during cell division (Geisel, 2003). Insufficient folate consumption increases the risks of preterm delivery, low birth weight, and foetal growth retardation (Scholl and Johnson, 2000). Besides their role in megaloblastic anaemia prevention in pregnancy, folates are also essential for human reproductive health (Tamura and Picciano, 2006). Wallock et al. (2001) reported that seminal plasma folate was correlated with blood plasma folate and hence important in male reproduction.

Intake of folates can be increased by the consumption of folate-rich foods, fortification of food with folic acid, and folic acid supplements (Hefni et al., 2010). Among various approaches, biofortification, enriching the nutritional value of staple crops, is a balanced and economic way to improve the health status of low-income consumers (Bouis, 2002; Bouis et al., 2011; Blancquaert et al., 2014). In developing countries, biofortification of staple food crops including rice and pulses is a new approach to control deficiencies of folate, β-carotene, iron, and zinc (HarvestPlus, 2007).

Several methods have been used to determine the levels of folates in food samples, including legumes. Microbiological assays have been around for many years (early literature summarized in Toepfer et al. (1951)). Sample preparation in these early methods typically used a conjugase digestion, but modern methods now use a trienzyme (protease, α-amylase, and rat conjugase) treatment (Tamura et al., 1997). The trienzyme method showed significant increases in measured folates indicating that the use of conjugase alone underestimated folate levels (Tamura et al., 1997). Microbiological assays are still used to estimate total folates (Shrestha et al., 2000; Chew et al., 2012); however, more recently, methods using liquid chromatography (LC) have been employed as LC allows for the detection of specific folate forms. LC with fluorescence detection (LC-FD) (Hefni et al., 2010; Sen Gupta et al., 2013), and LC with mass spectrometry (MS) detection (Rychlik et al., 2007; De Brouwer et al., 2008, 2010; Vishnumohan et al., 2011; Camara et al., 2013) are commonly used. Tandem MS methods (MS/MS) are extremely selective as both the parent ion and a specific fragment ion are required for detection, thereby greatly reducing chemical interference. In addition, the use of isotopically labelled internal standards enables very accurate quantification, as they will account for losses in sample preparation or degradation. Recently, De Brouwer et al. (2010) used the higher resolution and faster separation capabilities of ultra-performance LC (UPLC) to develop a UPLC–MS/MS method for analysis of folates in rice. This method has a short run time that allows for a more efficient analysis of the highly labile folates before they decompose in the autosampler.

Although studies have been conducted to measure folate concentrations in various legume crops (Rychlik et al., 2007; Sen Gupta et al., 2013), knowledge of the diversity in folate profiles of pulse crop cultivars grown in different locations is not available. Biofortification of pulse crops is one of the goals of the pulse crop-breeding program at the Crop Development Centre (CDC), University of Saskatchewan. The objectives of this research were: (1) to determine the concentration of folates in four cultivars of each of common bean, lentil, chickpea and pea, and (2) to determine the effect of growing location on folate concentration. This information can be used to determine the scope for future biofortification of folates in pulse crops through conventional or molecular breeding approaches.

2. Materials and methods

2.1. Materials

Four cultivars each of common bean, lentil, chickpea and pea developed at the CDC, University of Saskatchewan, were used in the analysis (Table 1). Seeds from field trials conducted at Saskatoon (common bean, lentil and pea), Limerick (lentil, chickpea), Rosthern (common bean), Elrose (chickpea), and Meath Park (pea), Saskatchewan in 2012 were used for analyses. Trials were conducted in a randomized complete block design with three replicates per location. Saskatchewan is in the Chernozemic soil order with four soil zones. Each soil has different colour of the surface horizon based on the amount of soil organic matter (SOM) stored in the soil (www.soilsofsask.ca). Meath Park and Rosthern are located in the Black soil zone with 4.5–5.5% SOM, Saskatoon is in the Dark Brown soil zone with 3.5–4.5% SOM, Elrose and Limerick are located in the Brown soil zone with 2.5–3.5% SOM. Temperature was similar at five locations in 2012, however total precipitation differed. Meath Park experienced highest rainfall during the growing season (May 1–September 30) followed by

Table 1
Phenotypic characters of common bean, lentil, chickpea, and pea cultivars used in assessment of folates. Samples were derived from 2012 regional variety trials grown at indicated sites in Saskatchewan, Canada.

Crop	Cultivar	Species	Seed coat colour	Cotyledon colour	Grain yield (kg/ha)		Seed weight (g/100 seeds)	
					Saskatoon	Rosthern	Saskatoon	Rosthern
Common bean	CDC Blackcomb	*Phaseolus vulgaris*	Black	White	3083	1337	184	138
	CDC Pintium	*Phaseolus vulgaris*	Cream with brown flecks	White	2635	1031	370	288
	CDC Sol	*Phaseolus vulgaris*	Yellow	White	2751	1242	400	351
	CDC WM-2	*Phaseolus vulgaris*	Cream with brown flecks	White	3086	1260	377	308
					Limerick	Saskatoon	Limerick	Saskatoon
Lentil	CDC Maxim	*Lens culinaris*	Gray	Red	1847	1771	41	39
	CDC QG-1	*Lens culinaris*	Green	Green	1784	1743	47	46
	CDC SB-2	*Lens culinaris*	Gray dotted	Yellow	1964	1738	38	35
	CDC Greenstar	*Lens culinaris*	Green	Yellow	2484	1482	71	61
					Elrose	Limerick	Elrose	Limerick
Chickpea	CDC Leader	*Cicer arietinum*	Non-pigmented	Yellow	3723	3310	383	362
	CDC Consul	*Cicer arietinum*	Light tan	Yellow	3668	2849	324	311
	CDC Cory	*Cicer arietinum*	Tan	Yellow	3899	2559	279	285
	CDC Frontier	*Cicer arietinum*	Non-pigmented	Yellow	3771	2789	350	347
					Meath Park	Saskatoon	Meath Park	Saskatoon
Pea	CDC Amarillo	*Pisum sativum*	Non-pigmented	Yellow	2584	2715	NA	209
	CDC Dakota	*Pisum sativum*	Tan	Yellow	2679	2855	NA	193
	CDC Meadow	*Pisum sativum*	Non-pigmented	Yellow	2138	2099	NA	186
	CDC Striker	*Pisum sativum*	Non-pigmented	Green	2101	2377	NA	236

NA, not available.

Saskatoon and Rosthern, Elrose and Limerick (www.climate.weather.gc.ca, www.theweathernetwork.com).

Approximately 5 g of harvested seeds of common bean, lentil, chickpea, and pea were air-dried to 14% moisture and stored at −20 °C until milled, whereas finely ground seed samples were stored at −80 °C until analysis. With the exception of folic acid (PteGlu), all non-labelled standards, 10-CHO–PteGlu (10-FFA), (6S)-5-CH_3–H_4PteGlu (5-MTHF), (6S)-5-CHO–H_4PteGlu (5-FTHF), (6R,S)-5,10-CH^+–H_4PteGlu (5,10-MTHF), and (6S)-H_4PteGlu (THF) were purchased from Schircks Laboratories (Jona, Switzerland). $^{13}C_5$-labelled folates (i.e. $^{13}C_5$-FA, $^{13}C_5$-MTHF, $^{13}C_5$-FTHF) were purchased from Merck Eprova (Schaffhausen, Switzerland). Folic acid, α-amylase (from porcine pancreas), protease (from *Streptomyces griseus*), potassium phosphate monobasic and dibasic, ascorbic acid, and dithiothreitol (DTT) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). LC/MS grade acetonitrile and formic acid were purchased from Thermo Fisher Scientific (Nepean, ON, Canada). Unfiltered rat serum was purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Endogenous folates were removed from both protease and rat serum (separately) by mixing with one tenth volume of activated charcoal, vortexing for 15 s, and incubating for 1 h on ice with occasional mixing. After centrifugation at 14,000 rpm for 15 min, aliquots of the supernatant were extracted and stored at −80 °C until required.

2.2. Stock solutions

Similar to De Brouwer et al. (2008), all manipulations were carried out under subdued light conditions. Except for 5,10-MTHF, all folate stock solutions were prepared at ~2 mM in 50 mM of a phosphate buffer consisting of potassium phosphate monobasic and dibasic added to give a pH of 7.2. For 5,10-MTHF, the pH was adjusted to 6. To the stock solutions, 1% ascorbic acid and 0.5% dithiothreitol (DTT) were also added to help with stability (De Brouwer et al., 2008) and the solutions were stored at −80 °C until used. Working solutions were prepared daily when required by appropriate dilution with the phosphate buffer. Solutions of α-amylase and protease were prepared and stored as described previously (De Brouwer et al., 2008).

2.3. Folate extraction using the tri-enzyme method

A tri-enzyme extraction method similar to that of De Brouwer et al. (2010) was used with some modifications. As recommended by Hyun and Tamura (2005) when adapting the tri-enzyme method, the optimal conditions were verified to avoid underestimating the folate content. For our experiments, both the amount of enzyme added as well as the length of incubation at each step were optimized resulting in the following procedure. Seed samples were individually ground in a Udy grinder (Fort Collins, CO, USA) using a vacuum to clean equipment between each sample. Approximately 150 mg of finely ground seed sample was weighed in a 2 mL Eppendorf tube. To each sample, 1.2 mL of a solution containing the three labelled internal standards in a 20 mM potassium phosphate buffer (pH 7) containing 1% ascorbic acid and 0.5% DTT was added. Each sample tube was vortexed (30 s) followed by shaking (230 rpm) for 1 h (VWR signature™ bench top shaking incubator). The solution was boiled for 10 min then cooled on ice. Next, 10 μL of α-amylase was added and vortexed (20 s) and the sample was kept at room temperature for 10 min. 150 μL of protease was added and incubated with shaking (230 rpm) for 1 h at 37 °C. The capped tube was kept in boiling water in a 2 L beaker for 10 min at 100 °C to stop the enzymatic reaction. The tube was then cooled on ice for 20 min. Hundred microliters of rat serum was added to the solution, followed by incubation with shaking (230 rpm) for 2 h at 37 °C. Again the enzyme was inactivated by boiling for 10 min at 100 °C, followed by cooling on ice for 20 min, and then centrifugation at 14,000 rpm for 30 min. The supernatant from each sample was transferred to a new 2 mL Eppendorf tube and 250 μL of supernatant was ultrafiltered through a 10 kDa filter (EMD Millipore, Merck KGaA, Darmstadt, Germany) at 12,000 rpm for 10 min. Samples were stored at −20 °C until analysis.

2.4. UPLC–MS/MS analysis

Mass spectrometry (MS) detection was carried out using a Thermo TSQ Quantum operated in positive electrospray ionization mode (Thermo Fisher, San Jose, CA, USA). Optimal mass spectrometry conditions for multiple reaction monitoring (MRM) were determined for each of the folates individually using infusion (Table 2). The chromatographic method was adapted from De Brouwer et al. (2010). Chromatography was performed using a Waters Acquity ultra high performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, USA). A Waters HSS T3 column (150 mm × 2.1 mm, 1.8 μm particle size) with a Waters VanGuard pre-column (50 mm × 2.1 mm, 1.8 μm particle size) was used. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate was 0.3 mL/min and the gradient was set to 97% solvent A to start and held at 97% for 1 min. Over the next 10 min solvent A was ramped to 72%. From 11 to 13 min, solvent A was ramped from 72% to 5% and then held at 5% for an additional 2 min. The composition was then changed back to 97% solvent A over the next minute and remained there until the completion of the 20 min run. To maximize the dwell time, the data acquisition was split into two segments. The first segment (first 6.5 min) acquired data only for THF, 5-MTHF, $^{13}C_5$-MTHF, and 5,10-MTHF. The second segment (last 13.5 min) acquired data only for 10-FFA, FA, $^{13}C_5$-FA, FTHF, and $^{13}C_5$-FTHF. The column oven was maintained at 30 °C, the autosampler at 4 °C, and the autosampler door was shielded to prevent light from entering.

2.5. Calibration and concentration determination

Preliminary analyses of subsets of six samples from each crop (24 total) were used to determine approximate levels and from

Table 2
Summary of the calibration data for the individual folates measured in common bean, lentil, chickpea, and pea.

Folate	Transition (eV)	LOQ (μg/100 g)	Slope (×10^{-3})	Intercept (×10^{-3})	R^2	Linear range (μg/100 g)
FA	442 → 295 (21)	0.25	15.50 ± 0.10	4.30 ± 1.8	0.999	0.2–62.8
10-FFA	470 → 295 (23)	0.26	2.35 ± 0.20	1.40 ± 5.0	0.997	0.3–66.8
THF	446 → 299 (18)	2.00	2.10 ± 0.01	−1.10 ± 8.9	0.993	2.0–570.0
5-MTHF	460 → 313 (16)	4.00	2.91 ± 0.01	4.70 ± 5.8	0.999	4.0–1046.0
5,10-MTHF	456 → 412 (29)	0.41	1.04 ± 0.02	−0.84 ± 0.5	0.992	0.4–107.0
5-FTHF	474 → 327 (16)	1.30	2.85 ± 0.02	29.60 ± 5.5	0.998	1.3–354.0

FA, folic acid; 10-FFA, 10-formyl folic acid; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahyrofolate; 5-FTHF, 5-formyltetrahydrofolate.

those results, levels for the internal standards and the ranges of the calibration curve were determined. For the values reported herein, all samples for one pulse crop were prepared and analyzed on the same day; different pulse crops were analyzed on different days. Solutions for a six-point calibration curve, plus a blank containing the labelled internal standards, were also prepared the same day of the analysis using sequential dilutions starting from a concentrated working solution of a mixture of the six folate monoglutamates, a concentrated working solution of the labelled internal standards, and phosphate buffer (pH 7.2). The standards for the calibration curve were injected at the beginning and the end of the sample list and the area ratios of the standards to their internal standards were found to have good reproducibility. In addition, chickpea cultivar CDC Jade was included with each pulse crop analysis to ensure good reproducibility among the analyses. Table 2 summarizes the calibration data for the individual folates measured in the pulse crops. Note that the calibration data is an average of all calibration curves for each of the pulse crops (i.e. eight total). Folate concentrations were expressed as micrograms per 100 g dry seed weight.

2.6. Statistical analysis

Analysis of variance (ANOVA) was calculated using PROC MIXED implemented in SAS®9.3 (SAS Institute Inc. Cary, NC, USA). Cultivar and location were treated as fixed and replication as random. Homogeneity of variance test (HOVTEST) was used to assess the homogeneity of variance among replications.

3. Results and discussion

3.1. Effect of cultivar and location on folate concentrations

The method we developed to measure folates in pulse crops was based on the UPLC–MS/MS method reported by De Brouwer et al. (2008, 2010) with some modifications. UPLC–MS/MS was chosen because of its specificity and ability to distinguish the six folate monoglutamates as well as its high accuracy in quantification of the results from the use of isotopically labelled internal standards. In this study, FA, 10-FFA, THF, 5-MTHF, 5,10-MTHF, and 5-FTHF were quantified using UPLC–MS/MS (Table 3). Analysis of variance (ANOVA) was carried out (Table 3) and in several cases, significant differences ($P < 0.05$) are observed. Most notably, significant differences in all folate monoglutamates and total folate were observed among the cultivars in each pulse crop with the exceptions being 5,10-MTHF in lentil, 5-MTHF in chickpea, and FA and 5,10-MTHF in pea. Significant differences due to location were observed for about half of the folate monoglutamates in lentil, chickpea, and common bean, but only in 5-MTHF for pea. Some significant differences due to cultivar X location were observed especially in common bean. The results indicate that in western Canada where each location had different soil texture and rainfall, both cultivars and locations can influence total folate concentrations in each of the four crops grown. Khanal et al. (2013) also observed significant variation for 5-MTHF and total folate among dry beans grown under field conditions near Elora, ON, Canada. Sen Gupta et al. (2013) detected a significant genotype X location X year interaction for total folate concentration in lentils grown in North Dakota, USA.

3.2. Variation in concentration of folates within cultivars of each crop and among crops

Table 4 shows the concentration for each of the six folate monoglutamates as well as the total folate concentration, which represents the sum of these six species, in the seeds of four pulse crops. The total folate concentration was the highest in chickpea (351–589 μg/100 g), followed by common bean (165–232 μg/100 g), lentil (136–182 μg/100 g), and pea, which was much lower than the other three crops (23–30 μg/100 g). Thus, a normal serving of 35 g of chickpea, common bean, lentil, or pea would provide 41%, 17%, 13%, or 2% of the RDA (400 μg/100 g) requirement for adults, respectively. This trend that we observed is in agreement with the results reported using an LC–MS method by Rychlik et al. (2007) that showed levels of 275 μg/100 g (chickpea), 106–164 μg/100 g (white bean), 110–154 μg/100 g (green lentil), and 10–20 μg/100 g (pea). Although our results are higher for all crops, the differences in the values could be due to the specific varieties used and/or the locations at which they were grown. Contrary to our results and those of Rychlik et al. (2007), Sen Gupta et al. (2013) using LC-FD reported higher total folate concentration in field pea (41–202 μg/100 g) grown in the United States compared to chickpea (42–125 μg/100 g). They also reported greater folate concentration in cultivars SGDP (202 μg/100 g) and Arcadia (156 μg/100 g) than the other pea cultivars. The folate concentration of 59 and 52 μg/100 g was observed using LC–MS in green peas consumed in Finland (Vahteristo et al., 1997) and Egypt (Hefni et al., 2010), respectively. Han and Tyler (2003) reported 24.9–64.8 μg/100 g folates in green peas grown in 1999 and 2000 at two locations and 23.7–55.6 μg/100 g in yellow peas grown in 2000 at six different locations in Saskatchewan, Canada using a microbiological assay.

In the present study, seed coat and cotyledon colour had no effect on folate concentrations. To the best of our knowledge, there

Table 3
F values from the analysis of variance (ANOVA) for folate concentration measured in common bean, lentil, chickpea and pea cultivars grown in Saskatchewan, Canada.

Crop	Effect	FA	10-FFA	THF	5-MTHF	5,10-MTHF	5-FTHF	Total folate
Common bean	Cultivar	92.44***	8.05**	75.8***	18.52***	26.95***	78.84***	35.36***
	Location	0.07 ns	0.01 ns	15.1**	0.10 ns	29.48***	37.41***	19.31***
	Cultivar × location	2.89 ns	0.44 ns	6.58**	16.32***	3.56*	9.81**	13.26***
Lentil	Cultivar	24.16***	23.23***	20.53***	9.56**	3.16 ns	7.19**	3.44*
	Location	0.17 ns	8.81*	7.76*	18.27***	0.25 ns	3.06 ns	21.91***
	Cultivar × location	1.231 ns	4.41*	8.03**	3.85*	0.70 ns	3.02 ns	4.25*
Chickpea	Cultivar	9.21***	7.66**	28.8***	1.75 ns	47.18***	38.76***	37.78***
	Location	260.24***	38.14***	1.62 ns	0.41 ns	96.82***	473.78***	458.45***
	Cultivar × location	1.12 ns	0.58 ns	0.95 ns	7.89**	2.26 ns	3.85*	1.91 ns
Pea	Cultivar	0.23 ns	4.83*	17.28***	13.05***	0.94 ns	42.33***	8.3**
	Location	1.88 ns	2.42 ns	0.59 ns	5.84*	0.17 ns	3.63 ns	0.28 ns
	Cultivar × location	0.58 ns	0.74 ns	6.47**	3.96*	1.46 ns	4.07*	6.19**

ns, not significant; FA, folic acid; 10-FFA, 10-formyl folic acid; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahyrofolate; 5-FTHF, 5-formyltetrahydrofolate.
* $P < 0.05$
** $P < 0.01$.
*** $P < 0.001$.

Table 4
Least square means (LSM), mean and least significant difference (LSD) for folate concentration (μg/100 g dry seed weight) for common bean, lentil, chickpea and pea cultivars obtained from two locations with three biological replicates per location in Saskatchewan, Canada in 2012.

Crop	Cultivar	FA		10-FFA		THF		5-MTHF		5,10-MTHF		5-FTHF		Total folate	
		Sask	Ros	Sask	Ros	Sask	Ros	Sask	Ros	Sask	Ros	Sask	Ros	Sask	Ros
Common bean	CDC Blackcomb	12.6	13.7	3.5	3.7	39.0	36.1	103.5	75.0	8.8	6.9	64.9	57.4	232.4	192.8
	CDC Pintium	9.0	9.4	3.7	3.4	21.5	22.6	67.7	81.8	11.0	10.2	51.9	54.4	164.6	181.9
	CDC Sol	13.2	12.3	3.1	3.0	35.1	25.3	61.9	68.3	16.9	12.5	86.6	70.0	216.8	191.4
	CDC WM-2	8.5	8.2	2.3	2.5	22.2	20.1	70.2	75.2	14.7	9.7	63.9	56.2	181.8	171.9
	Mean	10.8	10.9	3.2	3.2	29.4	26.0	75.8	75.1	12.8	9.8	66.8	59.5	198.9	184.5
	$LSD_{0.05}$	1.4	1.0	1.1	0.7	4.7	3.7	11.6	10.4	3.9	1.0	5.3	8.6	11.6	18.6
		Lim	Sask	Lim	Sask	Lim	Sask	Lim	Sask	Lim	Sask	Lim	Sask	Lim	Sask
Lentil	CDC Maxim	7.9	8.7	4.4	5.4	17.4	13.7	58.1	58.5	11.8	12.7	56.4	59.8	156.0	158.8
	CDC QG-1	6.9	6.7	2.6	3.3	19.5	20.0	72.1	51.9	10.1	9.9	56.5	53.8	176.6	145.6
	CDC SB-2	7.0	6.8	4.4	3.9	24.5	19.3	46.0	40.3	11.4	10.8	59.4	55.4	152.7	136.5
	CDC Greenstar	9.3	9.2	4.2	5.3	17.3	19.1	60.7	39.3	12.0	10.9	62.3	59.7	182.4	143.4
	Mean	7.8	7.9	3.9	4.4	19.7	18.0	59.2	47.5	11.3	11.1	58.7	57.2	166.9	146.1
	$LSD_{0.05}$	0.8	1.3	1.1	1.2	1.5	3.5	10.7	15.6	2.7	2.1	5.4	4.0	27.4	17.0
		Elr	Lim	Elr	Lim	Elr	Lim	Elr	Lim	Elr	Lim	Elr	Lim	Elr	Lim
Chickpea	CDC Leader	5.5	9.9	5.7	8.8	4.2	5.6	164.0	202.7	18.3	25.3	153.9	238.8	351.5	491.0
	CDC Consul	7.4	11.5	9.1	13.1	9.1	9.1	165.9	172.7	33.0	47.1	214.3	335.4	438.7	588.8
	CDC Cory	5.7	10.8	3.9	9.5	5.5	5.4	191.8	146.7	23.2	37.6	187.5	317.9	417.6	527.8
	CDC Frontier	5.8	9.6	5.1	10.1	6.1	6.5	192.6	175.8	20.0	30.3	181.0	307.3	410.6	539.6
	Mean	6.1	10.4	6.0	10.4	6.2	6.6	178.6	174.5	23.6	35.1	184.2	299.8	404.6	536.8
	$LSD_{0.05}$	1.3	1.1	2.7	4.5	2.4	3.1	18.0	37.1	4.3	8.6	27.4	21.2	36.2	23.5
		MP	Sask	MP	Sask	MP	Sask	MP	Sask	MP	Sask	MP	Sask	MP	Sask
Pea	CDC Amarillo	0.7	0.8	0.8	0.8	3.8	5.1	14.7	14.2	0.8	1.0	5.5	6.6	26.3	28.4
	CDC Dakota	0.7	0.7	1.1	1.0	6.3	6.9	15.6	16.6	0.9	0.8	2.2	3.6	26.9	29.6
	CDC Meadow	0.7	0.8	0.8	0.8	6.3	5.1	17.0	14.0	0.8	0.7	2.8	1.9	28.5	23.3
	CDC Striker	0.7	0.7	0.8	0.6	6.0	5.9	13.4	11.8	0.6	0.8	2.5	3.0	24.0	22.8
	Mean	0.7	0.8	0.9	0.8	5.6	5.8	15.2	14.1	0.8	0.8	3.3	3.8	26.4	26.0
	$LSD_{0.05}$	0.2	0.2	0.5	0.2	1.1	1.0	2.7	1.1	0.5	0.5	2.0	0.7	5.2	2.3

Sask, Saskatoon; Ros, Rosthern; Lim, Limerick; Elr, Elrose; MP, Meath Park; FA, folic acid; 10-FFA, 10-formyl folic acid; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahyrofolate; 5-FTHF, 5-formyltetrahydrofolate.

are no reports of association of seed coat and/or cotyledon colour with folates in the available literature. In addition, folate concentration was not significantly correlated with either grain yield or seed weight in the four pulse crops at any locations.

3.3. Major forms of folate

Fig. 1 shows a graphical representation of the composition of the six folate monoglutamates in the four pulse crops. The figure shows relative similarities among the crops as well as some significant differences. 5-MTHF and 5-FTHF were the two major folates, representing 35–39% and 33–51% of total folate in common bean, lentil, and chickpea, respectively (Fig. 1). In pea, 5-MTHF and THF were the two most abundant folates, representing 56% and 22% of the total folate, respectively. Similar to our findings, 5-MTHF was identified as the major folate in common bean (Hefni et al., 2010; Khanal et al., 2013), lentil (Rychlik et al., 2007; Hefni et al., 2010), and chickpea (Hefni et al., 2010). Besides legume grains, 5-MTHF was the major folate in cereals, vegetables, fruit, bread, milk, and meat products (Konings et al., 2001; Hefni et al., 2010).

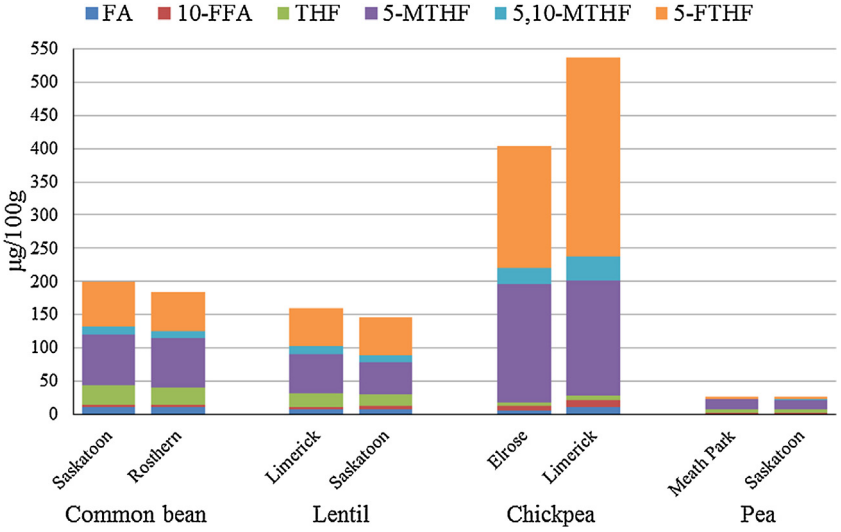

Fig. 1. Graphical representation of the mean composition of six folates in four cultivars each of common bean, lentil, chickpea, and pea grown at two locations in Saskatchewan, Canada in 2012. FA, folic acid; 10-FFA, 10-formyl folic acid; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahyrofolate; 5-FTHF, 5-formyltetrahydrofolate.

5-MTHF is the most abundant form during germination and plant growth (Roos et al., 1968) and plays an important role in methionine biosynthesis (Dodd and Cossins, 1970) and methylation via S-adenosylmethionine (SAM) (Dodd and Cossins, 1969). Methyltransferase enzymes use SAM in the methyl cycle and transfer methyl groups to a wide range of substrates, including proteins, lipids, DNA, and hormones (Scott et al., 2000). 5-MTHF is also the major form in human beings and plays an important role in human health. 5-MTHF was the main form of folate in plasma (Henderson et al., 1995) and it represented 76% of total folate in seminal plasma (Wallock et al., 2001). In plasma and red cells, 5-MTHF, the major folate, is a strong determinant of homocysteine concentrations (SobczyNska-Malefora et al., 2014). An increase in homocysteine (hyperhomocysteinaemia) due to lower 5-MTHF concentration was associated with various diseases including cardiovascular disease (McCully, 2007). 5-MTHF, the major folate observed in the current study, is important for plants as well as humans, and could be a target for improvement by breeders.

3.4. Strategies for improvement of folates

Folate levels in diets can be improved by consuming naturally folate-rich foods, fortification of food with folic acid, use of folic acid supplements (Hefni et al., 2010; Blancquaert et al., 2013), and by consuming sprouted seed (Shohag et al., 2012). The use of naturally occurring 5-MTHF has important advantages over synthetic folic acid including the prevention of potential negative effects of unconverted folic acid in the peripheral circulation (Scaglione and Panzavolta, 2014).

Among various strategies, biofortification of foods through biotechnology or plant breeding to enhance folate concentration is the most sustainable and economical approach and could be a strategy to reduce folate deficiencies globally (Bouis, 2002; Bouis et al., 2011; Blancquaert et al., 2014). Increases in folate by means of metabolic engineering was successfully achieved in tomato and rice (Blancquaert et al., 2013, 2014) raising the baseline folate concentration in rice more than 100-fold by overexpression of *Arabidopsis thaliana* pterin and para-aminobenzoate genes, precursors of the folate biosynthesis pathway (Storozhenko et al., 2007). A two- to fourfold increase was achieved in *Arabidopsis* by overexpressing a gene involved in pterin biosynthesis (Hossain et al., 2004). Biofortification through plant breeding provides an alternative approach. Political and regulatory restrictions are in place in many countries on genetically modified crops, which encouraged HarvestPlus to take a lead in global efforts to address vitamin and mineral deficiencies through conventional breeding (Nestel et al., 2006; Saltzman et al., 2013). Breeding of staple foods rich in vitamins and minerals is not only a cost-effective, sustainable, and long-term strategy, but has the potential to benefit populations residing in relatively remote rural areas (Bouis, 2002; Bouis et al., 2011; Saltzman et al., 2013). In addition, recurrent costs are low and germplasm can be shared internationally without having adverse effect on agronomic productivity (Nestel et al., 2006; Bouis et al., 2011).

4. Conclusions

Significant differences were observed among four pulse crops, and to a lesser extent among cultivars within crops, in overall amounts and relative proportions of the six folates quantified using UPLC–MS/MS. For the four crops in this study, the new understanding of the variation in folate profiles and concentration represents the first step towards biofortifying these crops. They are staple foods in many regions, and can contribute substantial amounts of the RDA for folates. Sen Gupta et al. (2013) indicated higher total folate concentration in two pea cultivars and these could be useful sources of variation for breeding for improved folate concentration in pea. Genetic variation was detected among the four cultivars evaluated in each crop, setting the stage for wider surveys to increase the folate concentration through identification of useful genetic variation. We hope to identify accessions with greater folate concentration after evaluation of a wider set of germplasm, especially for peas, and this trait will be introgressed into adapted cultivars using conventional and molecular breeding approaches.

Acknowledgments

The NSERC Industrial Research Chair Program, the Saskatchewan Pulse Growers, and the Saskatchewan Ministry of Agriculture are gratefully acknowledged for financial support.

We thank Thermo Fisher for the use of the TSQ Quantum as part of a collaboration between the National Research Council and Thermo Fisher. We are also thankful to the Pulse Field Crew members for technical assistance. Thanks to Marie Caudill and her group at Cornell University for sharing their knowledge in folate analysis and providing insightful guidance in setting up this research.

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