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The Composition of Grain and Forage from Glyphosate Tolerant Wheat MON 71800 Is Equivalent to That of Conventional Wheat (*Triticum aestivum L.*)

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Glyphosate tolerant wheat MON 71800, simply referred to as MON 71800, contains a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) that has a reduced affinity for glyphosate as compared to the endogenous plant EPSPS enzyme. The purpose of this work was to evaluate the compositional equivalence of MON 71800 to its nontransgenic parent as well as to conventional wheat varieties. The compositional assessment evaluated the levels of proximates, amino acids, fatty acids, minerals, vitamins, secondary metabolites, and antinutrients in wheat forage and grain grown during two field seasons across a total of eight sites in the United States and Canada. These data demonstrated that with respect to these important nutritional components, the forage and grain from MON 71800 were equivalent to those of its nontransgenic parent and commercial wheat varieties. These data, together with the previously established safety of the CP4 EPSPS protein, support the conclusion that glyphosate tolerant wheat MON 71800 is as safe and nutritious as commercial wheat varieties.

KEYWORDS: Wheat (*Triticum aestivum*); glyphosate tolerant; genetically modified; composition

INTRODUCTION

Since its first cultivation in antiquity, wheat (*Triticum aestivum* L.) production has grown to over 550 million tons of grain annually worldwide (*1*). Wheat is grown throughout the world, represents a staple food group for many cultures, and is recognized as an important source of dietary fiber, B vitamins, and trace minerals (*2*). In addition to its extensive use in a wide variety of human foods, wheat is a component of animal feed and has nonfood, industrial applications. Between 1997 and 1999, United States utilization of wheat was divided primarily between human consumption (69%) and animal feed (24%) with the remainder for seed or industrial uses (*1*). Within the United States, spring wheat, excluding Durum, was grown on 13.8 million acres in 2000 (*3*). That year, weed control for spring wheat amounted to over 9.6 million pounds of herbicides, primarily 2,4-D and MCPA, applied onto a total of 13.1 million acres (*3*).

Glyphosate is used globally for nonselective weed control. In plants, glyphosate inhibits the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), thereby blocking the production of essential aromatic amino acids and the secondary metabolites for which they are precursors (*4*, 5). The EPSPS enzyme from *Agrobacterium* species CP4 is a 45.7 kDa polypeptide, which is functionally similar to the plant EPSPS but has a reduced affinity for glyphosate (*6*). Expression of the *cp4 epsps* gene in plants has been demonstrated to confer tolerance to glyphosate through the production of the CP4 EPSPS protein (*6*). Through the techniques of biotechnology, the *cp4 epsps* gene was inserted into the Bobwhite cultivar of wheat to generate a hard red spring wheat, designated MON 71800, with the commercial name of Roundup Ready Wheat, that is tolerant to glyphosate-based agricultural herbicides.

The safety of a food derived through the techniques of biotechnology typically is evaluated by a combination of approaches that result in a determination of substantial equivalence, a concept that has been adopted by leading international food and regulatory bodies including the World Health Organization (*7*, 8), the United Nations Food and Agricultural Organization (*9*), the Organization for Economic Cooperation and Development (*10*−*12*), and the International Life Sciences Institute (*13*). For a biotechnology-derived food to be considered substantially equivalent, it must be shown that except for the introduced trait, it does not differ in a meaningful way from its

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Table 1. Fiber, Mineral, and Proximate Composition of Forage from Glyphosate Tolerant Wheat MON 71800

	1999 field trials			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component [e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range) [c]	range[d] (99% TI)[f]	literature (range)
ADF	23.40 (18.42–27.45)	24.27 (20.07–28.11)	18.48–29.81 (4.98, 43.91)	23.54 (19.89–29.49)	23.70 (21.12–28.27)	20.40–29.56 (17.11, 32.66)	25.1–40.3[g]
NDF	29.51 (21.72–34.03)	29.96 (20.56–36.22)	21.57–38.43 (1.13, 57.34)	34.58 (24.13–44.07)	34.49 (24.78–44.21)	28.36–42.16 (20.01, 52.83)	46.1–63.8[g,h]
calcium	4366 (3119–5909)	4523 (3306–6795)	2909–5713 (1269, 6838)	4222 (3005–5986)	4249 (2906–6174)	2392–5388 (1041, 6246)	2400[g]
phosphorus	4626 (2964–7633)	4692 (3373–7248)	2605–6573 (0, 11171)	3541 (2650–5071)	3610 (2827–5015)	2324–4791 (931, 5686)	3500[g]
ash	11.50 (7.51–15.07)	12.01 (9.05–16.18)	10.00–13.40 (7.03, 17.40)	10.99 (8.18–14.13)	11.83 (7.95–18.70)	7.74–16.41 (2.88, 21.16)	not available
carbohydrates	57.45 (45.27–68.88)	56.10 (47.62–63.73)	46.81–64.65 (26.80, 83.56)	61.09 (50.52–74.34)	60.08 (46.88–74.82)	45.64–71.37 (28.41, 90.46)	not available
moisture	83.34[i] (81.80–85.00)	84.74 (83.90–85.60)	81.80–86.40 (77.88, 89.78)	83.16 (78.90–87.00)	83.51 (79.10–86.40)	77.90–86.60 (74.81, 92.98)	not available
protein	26.23 (19.72–33.92)	27.60 (22.97–33.61)	21.11–35.79 (4.64, 51.39)	23.93 (12.48–30.79)	24.21 (12.31–30.81)	14.93–34.19 (1.92, 46.85)	22.45–30.90[g]
total fat	4.82 (3.54–6.51)	4.29 (2.59–5.46)	3.40–7.02 (0, 9.31)	3.99 (2.34–5.67)	3.88 (2.62–5.05)	2.63–5.46 (0.96, 7.35)	not available

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 20 values (four replicates from each of five field sites). ADF was the mean of 19 samples for the control. [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 2000. [e] ADF, NDF, ash, carbohydrates, protein, and total fat in % dry weight; calcium and phosphorus in mg/kg dry weight; and moisture in % fresh weight. [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *71*. [h] Ref *72*. [i] Value statistically different than the control; $p < 0.05$.

conventional counterpart, which is generally regarded as safe based on its historical consumption as a human food or animal feed. The overall substantial equivalence assessment includes demonstration of the safety of the transgene and its derived protein and of the equivalence to conventional counterparts with respect to phenotypic, agronomic, and compositional parameters. Additionally, toxicological evaluation in rodents and animal feed performance assessments of the derived feed in appropriate models can be conducted to evaluate any unintended effects that may not have been detected by the other methods to support a conclusion of substantial equivalence (*14*).

The safety of the CP4 EPSPS protein has been established on the basis of rapid in vitro digestibility, the lack of similarity to known toxins and allergens, lack of acute oral toxicity to mice (*15*), the ubiquitous presence of EPSPS activity in foods of plant origin, and the known biochemical function of the EPSPS protein (*6*). Compositional equivalence assessments have been reported for other glyphosate tolerant crops including soybean (*16*, 17), corn (*18*, 19), and cotton (*20*). Field evaluations of glyphosate tolerant wheat MON 71800 have demonstrated agronomic equivalency to nontransgenic wheat with respect to yield, morphology, and performance (*21*). This paper describes the compositional analyses and the comparison of glyphosate tolerant wheat MON 71800 to its conventional counterparts at eight locations within the U.S. and Canada over two growing seasons.

MATERIALS AND METHODS

Wheat Samples for Compositional Analyses. Glyphosate tolerant wheat MON 71800 was produced by the insertion the *cp4 epsps* gene into the Bobwhite cultivar of spring wheat (*T. aestivum* L). The R_0 generation was backcrossed with the Bobwhite cultivar, and the progeny selected for glyphosate tolerance until homozygosity was achieved (*22*). The control wheat for this compositional assessment was the parental, nontransformed Bobwhite cultivar. Commercially available spring wheat varieties were grown alongside MON 71800 and the Bobwhite control or were purchased separately to collectively provide reference materials from a broad range of spring wheat cultivars. These commercial reference varieties were chosen by local growers as being representative of wheat grown in their regions and included AC Barrie, AC Crystal, AC Cora, AC Domain, AC Morse, Amidon, Cavalier, CDC Teal, Earnst, Express, Forge, Grandin, Hank, Ingot, Katepwa, Majestic, McNeal, Oxen, Penewawa, Russ, Vanna, Westbred 936, Yecora Roja, Zeke, and 2375 and were distributed between the various field sites and 1999−2000 field seasons.

The field sites consisted of four replicate blocks, with each block containing MON 71800 and the control variety, Bobwhite, in randomized plots of approximately 500 ft^2 each. Additionally, at each field site, four commercial reference varieties were planted as nonreplicated plots in border strips surrounding each of the four sides of the blocked test area. In 1999, one site was located in the state of Washington and two sites were in the Canadian province of Manitoba. In 2000, there was one site each in the states of Washington, North Dakota, and Minnesota and the provinces of Manitoba and Alberta. These geographies represent regions in North America where spring wheat is grown commercially as a significant crop in terms of acreage and agricultural production.

At least 500 g of forage material, including all plant parts more than one inch above ground, was collected nonsystematically from each plot at the wheat jointing stage of development (Feekes stage 6−8) and frozen on dry ice within 10 min to maintain sample integrity. Grain material, defined as the kernel, was mechanically harvested when the moisture level was expected to be 12−14%. Forage and grain were shipped to Monsanto Company, and samples were homogenized by grinding with dry ice into a fine powder and frozen until compositional analysis.

The genetic identity of the grain was confirmed by sample handling records, event specific Southern blot analyses (1999), polymerase chain reaction of genomic DNA isolated from grain tissue (2000), or by determining the presence of the CP4 EPSPS protein by enzyme-linked immunosorbant assay (1999).

Compositional Analysis Methods. Forage samples were analyzed for proximates (fat, protein, ash, and moisture), acid detergent fiber

Table 2. Amino Acid Composition of Grain from Glyphosate Tolerant Wheat MON 71800

	1999 field trial			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component[e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range)[c]	range[d] (99% TI)[f]	literature (range)
alanine	3.58 (3.41–3.75)	3.53 (3.41–3.66)	3.29–4.05 (2.84, 4.16)	3.65 (3.40–3.80)	3.63 (3.23–3.85)	3.26–3.73 (3.15, 3.96)	3.1[g]–3.6[h]
arginine	4.78 (4.45–5.77)	4.65 (4.29–4.90)	3.96–5.27 (3.56, 5.62)	4.59 (4.18–4.95)	4.69 (4.40–4.98)	3.98–4.79 (3.82, 5.24)	4[g]–6[i]
aspartic acid	5.26 (4.90–5.70)	5.16 (4.89–5.55)	4.78–5.78 (4.09, 5.98)	5.24 (4.71–5.67)	5.24 (4.56–5.63)	4.72–5.65 (4.33, 6.02)	4.7[g]–5[g]
cystine	2.10 (1.54–2.53)	2.08 (1.76–2.23)	1.84–2.39 (1.35, 2.84)	2.28 (2.06–2.53)	2.26 (2.05–2.67)	1.91–2.50 (1.75, 2.70)	2.2[i]–2.8[g]
glutamic acid	31.56 (30.54–32.49)	31.78 (30.97–32.63)	29.84–33.74 (29.10, 35.52)	31.39 (30.22–33.21)	31.37 (30.37–33.48)	30.72–34.22 (29.24, 34.61)	29.9[h]–32[g]
glycine	4.39 (4.26–4.55)	4.36 (4.26–4.45)	3.92–4.49 (3.52, 4.79)	4.37 (4.09–4.63)	4.33 (4.11–4.54)	3.58–4.42 (3.52, 4.72)	3.8[g]–4.1[h]
histidine	2.33 (2.27–2.39)	2.33 (2.27–2.39)	2.38–2.57 (2.27, 2.62)	2.36 (2.31–2.42)	2.36 (2.27–2.44)	2.36–2.52 (2.29, 2.55)	1.95–2.45[i]
isoleucine	3.72[k] (3.47–3.88)	3.64 (3.51–3.77)	3.53–3.85 (3.39, 4.00)	3.60 (3.50–3.70)	3.58 (3.47–3.92)	3.44–3.75 (3.25, 3.83)	3[g]–4.7[i]
leucine	6.87 (6.73–7.03)	6.82 (6.75–6.93)	6.67–7.16 (6.44, 7.46)	6.81 (6.73–6.88)	6.79 (6.64–6.91)	6.72–7.13 (6.61, 7.13)	6.3[g]–6.79[j]
lysine	2.81 (2.63–3.02)	2.72 (2.61–2.87)	2.42–3.04 (2.07, 3.26)	2.84 (2.61–3.01)	2.82 (2.49–3.01)	2.51–2.99 (2.36, 3.13)	2.3[g]–3.4[i]
methionine	1.56 (1.15–1.82)	1.54 (1.31–1.63)	1.42–1.90 (1.05, 2.17)	1.68 (1.48–1.85)	1.67 (1.42–1.99)	1.46–1.97 (1.24, 2.20)	1.2[g]–2.1[j]
phenylalanine	4.69 (4.55–4.80)	4.69 (4.59–4.80)	4.64–5.14 (4.39, 5.44)	4.74 (4.60–4.86)	4.75 (4.64–4.90)	4.73–5.18 (4.60, 5.30)	4.5[h]–4.96[j]
proline	11.17 (10.46–11.87)	11.31 (10.85–11.78)	10.20–11.59 (9.71, 12.18)	11.19 (9.81–11.97)	11.25 (10.41–12.56)	10.45–11.69 (9.63, 12.35)	9.9[h]–10.4[j]
serine	4.53 (4.38–4.72)	4.59 (4.37–4.76)	4.44–4.76 (4.26, 4.93)	5.04 (4.96–5.15)	5.02 (4.88–5.21)	4.93–5.28 (4.76, 5.44)	4.2[g]–4.6[h]
threonine	2.77 (2.67–2.92)	2.79 (2.62–3.02)	2.36–2.96 (2.09, 3.24)	2.67 (2.50–2.91)	2.69 (2.45–2.95)	2.11–2.81 (2.05, 3.19)	2.4[g]–2.93[l]
tryptophan	0.96 (0.71–1.18)	0.99 (0.91–1.09)	0.80–1.07 (0.64, 1.23)	0.93 (0.82–1.08)	0.93 (0.81–1.11)	0.83–1.07 (0.66, 1.23)	1.28[j] –1.5[g]
tyrosine	2.41 (1.92–2.65)	2.61 (2.56–2.66)	1.57–3.02 (1.37, 3.37)	2.32 (1.68–2.82)	2.33 (1.78–2.76)	1.80–2.66 (1.46, 3.13)	2.7[g]–3.72[j]
valine	4.50 (4.22–4.66)	4.42 (4.30–4.57)	4.32–4.84 (4.02, 5.04)	4.29 (4.14–4.40)	4.27 (3.99–4.52)	4.08–4.43 (3.93, 4.62)	3.6[g]–4.5[j]

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 19 values (four replicates from each of four field sites and three replicates from one field site). [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 2000. [e] Percent of total amino acids. [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *73* (g/16 gN or % protein). [h] Ref *74* (g/16 gN). [i] Ref *75* (g/16 gN). [j] Ref *76* (% protein). [k] Value statistically different than the control at $p < 0.05$. [l] Ref *77* (g/16 gN).

(ADF), neutral detergent fiber (NDF), calcium, and phosphorus. Grain samples were analyzed for proximates, total dietary fiber (TDF), amino acids, fatty acids, sugars (arabinose, fructose, galactose, glucose, mannose, maltose, raffinose, stachyose, sucrose, and xylose), starch, vitamin E, niacin, riboflavin (vitamin B_2), thiamin (vitamin B_1), vitamin B_6, minerals (cadmium, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium, and zinc), and phytic acid. In addition, the 2000 field samples were also analyzed for folic acid, ferulic acid, oxalic acid, *p*-coumaric acid, and malonic acid. Total carbohydrate levels in forage and grain were determined by calculation. Compositional analyses were conducted at Covance Laboratories Inc. in Madison, Wisconsin. The order of sample analysis was randomized for each tissue by site to minimize assay bias. A nondescriptive laboratory information systems number identified the samples at the laboratory.

Proximate Analysis. Protein levels were calculated from total nitrogen using the Kjeldahl method (*23*, 24) and the equation $N \times 6.25$. Fat content of the grain was determined by the Soxhlet extraction method (*25*). Fat content of the forage was determined by fat acid hydrolysis, followed by extraction with ether and hexane (*26*, 27). Ash content was determined by ignition in an electric furnace and gravimetric quantitation of the remaining ash (*28*). Moisture content was determined by loss of weight upon drying in a vacuum oven at 100 °C to a constant weight (*29*, 30). Carbohydrate levels were calculated using the fresh weight derived data and the following equation (*31*):

$$\%\ \text{carbohydrate} = 100\% - (\%\ \text{protein} + \%\ \text{fat} + \%\ \text{ash} + \%\ \text{moisture})$$

Fiber Analysis. ADF was determined by treating the sample with an acidic, boiling, detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed fats and pigments. The remaining lignocellulose fraction was determined gravimetrically (*32*). NDF was determined by treating the samples with a neutral, boiling, detergent solution to dissolve the protein, carbohydrate, and ash. Fats and pigments were removed using an acetone wash. The hemicellulose, cellulose, and lignin fractions were determined gravimetrically (*33*, 34). For TDF, duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch, protein degradation products, and moisture. Protein content was determined for one of the duplicates; ash content was determined for

Table 3. Fatty Acid Composition of Grain from Glyphosate Tolerant Wheat MON 71800

	1999 field trials			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component[e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range)[c]	range[d] (99% TI)[f]	literature (range)
16:0 palmitic	19.03 (18.12–20.02)	18.92 (17.97–19.82)	15.82–19.29 (14.10, 21.39)	18.49 (17.59–19.23)	18.66 (17.63–19.64)	16.44–19.97 (14.80, 21.60)	11–32[g]
16:1 palmitoleic	0.22 (0.098–0.37)	0.18 (0.097–0.28)	0.090–0.30 (0, 0.48)	0.29 (0.14–0.41)	0.30 (0.14–0.46)	0.10–0.82 (0, 0.75)	3.44[h]
18:0 stearic	1.29 (1.15–1.47)	1.23 (1.08–1.52)	0.74–1.38 (0.38, 1.69)	1.47 (1.20–1.89)	1.38 (1.10–1.80)	0.81–2.45 (0.032, 2.38)	0–4.6[g]
18:1 oleic	18.21 (17.27–20.15)	18.25 (17.17–19.69)	14.59–21.36 (10.41, 24.17)	20.07 (18.82–22.06)	19.38 (17.08–21.00)	14.41–21.45 (10.62, 24.54)	11–29[g]
18:2 linoleic	55.29 (53.37–56.23)	55.50 (53.82–56.53)	55.10–59.82 (53.10, 62.89)	54.21 (52.19–55.86)	54.82 (52.43–56.76)	51.30–62.04 (49.24, 65.62)	37.9[h]–74[g]
18:3 linolenic	4.27 (4.12–4.83)	4.30 (3.90–4.99)	3.58–5.53 (2.15, 6.52)	3.96 (3.74–4.37)	4.00 (3.54–4.96)	3.35–5.04 (2.66, 5.70)	0.71–4.84[g]
20:0 arachidic	0.25 (0.12–0.35)	0.24 (0.11–0.31)	0.068–0.28 (0, 0.48)	0.28 (0.14–0.39)	0.24 (0.12–0.35)	0.090–0.30 (0, 0.35)	not available
20:1 eicosenoic	1.18 (1.13–1.25)	1.17 (1.10–1.24)	0.80–1.37 (0.46, 1.54)	1.24 (1.09–1.36)	1.22 (1.03–1.47)	0.68–1.42 (0.40, 1.56)	not available
22:0 behenic	0.25[i] (0.23–0.33)	0.22 (0.11–0.27)	0.086–0.36 (0, 0.44)	<LOQ[j]	<LOQ[j]	<LOQ[j]	not available

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 20 values (four replicates from each of five field sites). [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 2000. [e] Percent total fatty acids. [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *78*. [h] Ref *79* (% lipid). [i] Value statistically different than the control; $p < 0.05$. [j] More than 50% of the observations for this analyte were below the LOQ of 0.004% fresh weight.

the other. The TDF in the sample was calculated using the protein and ash values (*35*).

Amino Acid Composition. The sample was assayed by three methods to obtain the full profile of 18 amino acids. The procedure for tryptophan required a base hydrolysis using sodium hydroxide. The sulfur-containing amino acids required an oxidation using performic acid prior to hydrolysis with hydrochloric acid. Analysis of the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. Glutamine and asparagine are converted to glutamate and aspartate, respectively. The individual amino acids were quantitated using an automated amino acid analyzer (*36*).

Fatty Acid Profile. Lipids in samples were extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation (*37*).

Ferulic and p-Coumaric Acids. Extracted samples were base hydrolyzed and then acidified and filtered. Ferulic and *p*-coumaric acids were quantitated by high-performance liquid chromatography (HPLC) with UV detection (*38*).

Folic Acid. Grain samples were suspended in buffer and autoclaved in the presence of ascorbic acid. Free folic acid was enzymatically released and quantitatively determined microbiologically by its effect on the growth of *Lactobacillus casei* (*39*−*41*).

Malonic Acid and Oxalic Acid. Grain samples were extracted with a weak sulfuric acid solution, and the acid levels were quantitated by ion exchange HPLC with UV detection (*42*).

Minerals. This method was used to estimate the levels of nine minerals in the sample: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. Samples were dried, pre-charred, and ashed overnight in a muffle furnace. Ashed samples were treated with hydrochloric acid, taken to dryness, and dissolved in 5% (v/v) hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, using inductively coupled plasma, with the emission of standard solutions (*43*−*45*). To determine the levels of cadmium, the grain was precharred and ashed in a muffle furnace for 5−16 h. The sample was cooled, treated with nitric acid, reashed, and dissolved in hydrochloric acid solution. The amount of cadmium in the unknown samples was determined by atomic absorption spectrophotometry with an external standard curve (*46*−*48*). For selenium, the grain was digested in a nitric−perchloric−hydrochloric acid mixture, in which any selenium present formed selenous acid. The selenous acid was reacted with 2,3-diaminonaphthalene to form 2,3-4,5-benzopiazselenol that was then extracted into an organic solvent. The amount of selenium was then determined by comparing the absorbance of the unknown sample, measured by fluorescence spectroscopy, with the absorbance of standard solutions (*49*−*53*).

Niacin. The grain was hydrolyzed with sulfuric acid, and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum*, with the growth response of a niacin standard (*54*).

Phytic Acid. Samples were extracted in 0.5 M HCl with ultrasonication. Purification and concentration were performed using a silica-based anion exchange column. Sample analysis was conducted using a macroporous polymer HPLC column connected to a refractive index detector (*55*, 56).

Riboflavin (Vitamin B_2). The sample was hydrolyzed with dilute hydrochloric acid, and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *L. casei*, with the growth response of riboflavin standard. This growth response was measured turbidimetrically (*57*).

Sugars. After extraction from the sample with deionized water, the sugars (fructose, glucose, sucrose, maltose, raffinose, and stachyose) were treated with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucoside as the internal standard. The resulting oximes were converted to silyl derivatives with hexamethyldisilazane and trifluoroacetic acid treatment and analyzed by gas chromatography using a flame ionization detector (*58*, 59).

Additional Sugars. Samples were refluxed with dilute sulfuric acid. After the sample was neutralized, an aliquot was taken to dryness and the sugars (arabinose, xylose, mannose, and galactose) were converted first to oximes and then to aldonitrile peracetates and analyzed by gas chromatography using a flame ionization detector (*60*).

Table 4. Proximate and Mineral Composition of Grain from Glyphosate Tolerant Wheat MON 71800

	1999 field trials			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component[e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range)[c]	range[d] (99% TI)[f]	literature (range)
protein	16.71 (14.70–19.11)	16.95 (15.15–19.14)	15.04–21.60 (13.05, 24.55)	16.66 (15.14–19.68)	16.90 (14.80–20.27)	15.13–21.31 (12.41, 23.88)	8.3–19.3[g]
total fat	1.33 (1.13–1.49)	1.36 (1.18–1.66)	1.21–1.95 (0.81, 2.15)	1.25 (0.99–1.56)	1.24 (0.96–1.86)	1.04–1.69 (0.80, 1.85)	1.9[h]–2.86[i]
ash	1.84 (1.45–2.34)	1.87 (1.45–2.28)	1.50–2.29 (1.10, 2.86)	1.99 (1.60–2.48)	1.91 (1.59–2.24)	1.53–2.29 (1.27, 2.55)	1.17–2.96[g]
carbohydrates	80.12 (77.68–82.46)	79.82 (77.34–81.78)	74.92–81.37 (71.82, 83.67)	80.10 (76.83–81.74)	79.89 (75.54–81.85)	75.31–81.61 (72.79, 84.45)	65.4–78.9[g]
TDF	14.93 (13.42–18.10)	14.67 (13.47–17.31)	12.37–18.54 (9.59, 20.92)	16.82 (14.34–20.92)	17.22 (13.97–23.55)	13.98–22.38 (8.34, 25.85)	12.2[j]
moisture	11.31 (9.17–14.30)	11.83 (9.52–14.20)	8.33–18.70 (1.01, 21.20)	11.78 (7.79–14.80)	11.96 (8.91–15.60)	9.16–14.30 (5.90, 17.08)	7.8–14.8[g]
cadmium	<LOQ[k]	<LOQ[k]	<LOQ[k]	0.050 (0.023–0.12)	0.053 (0.022–0.10)	0.022–0.11 (0, 0.14)	not available
calcium	609 (380–762)	648 (505–733)	312–803 (0, 945)	572 (438–704)	553 (416–688)	277–725 (72.7, 861)	250[j]–538[g]
copper	4.19 (2.55–5.21)	3.74 (2.64–4.77)	2.19–6.33 (0.99, 8.72)	4.51 (3.27–6.40)	4.58 (3.55–5.81)	3.09–6.44 (1.86, 7.73)	4.25–5.84[g]
iron	41.81 (39.36–44.04)	39.54 (35.42–43.71)	36.20–51.56 (28.03, 60.61)	45.00 (37.17–57.75)	44.24 (33.89–59.16)	34.79–62.27 (21.55, 70.57)	33[l]–79[g]
magnesium	1760[m] (1618–1905)	1677 (1560–1879)	1532–1808 (1376, 1939)	1763 (1564–1991)	1751 (1424–2270)	1502–2060 (1188, 2297)	1240[j]–1802[i]
manganese	30.58 (15.10–43.81)	30.53 (20.09–42.45)	19.07–60.90 (0, 82.50)	36.15 (22.17–57.66)	38.43 (20.13–57.68)	17.13–62.88 (0, 82.52)	38–63.2[g]
phosphorus	4355 (3652–5307)	4289 (3566–5201)	3590–5216 (2920, 6180)	4211 (3134–5068)	4258 (2932–5786)	3406–5764 (2226, 6510)	3320[j]–5160[g]
potassium	4779 (3810–6716)	4661 (3937–6161)	3700–6046 (1824, 7330)	4739 (3721–5542)	4586 (3738–5654)	3788–6041 (2348, 6747)	3400[j]–5180[g]
selenium	0.26 (0.10–0.60)	0.26 (0.090–0.47)	0.1–0.69 (0, 1.02)	0.45 (0.029–1.53)	0.47 (0.029–1.50)	0.028–0.98 (0, 1.25)	0.04–0.71[l]
zinc	40.45 (25.98–59.54)	39.75 (31.31–58.69)	28.57–65.39 (4.48, 81.99)	44.58 (31.05–66.97)	43.25 (28.43–70.90)	25.59–73.84 (4.59, 77.70)	24–47[g]

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 20 values (four replicates from each of five field sites), ash, and iron for control are 19 values each. [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 2000. [e] Minerals (ppm dry weight), proximates (% dry weight), and moisture (% fresh weight). [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *78*. [h] Ref *76*. [i] Ref *80*. [j] Ref *79* (fresh weight). [k] More than 50% of observations were below the LOQ of 0.04 ppm fresh weight. [l] Ref *81*. [m] Value statistically different than the control; $p < 0.05$.

Starch. The sample was extracted with alcohol to remove carbohydrates other than starch and hydrolyzed to glucose with α-amylase and amyloglucosidase. Glucose was oxidized with glucose oxidase to form peroxide, which reacted with a dye in the presence of peroxidase to give a stable colored product proportional to glucose concentration. The glucose concentration was quantitated by a spectrophotometer at 540 nm. Percent starch was then calculated from the glucose concentration (*61*).

Thiamin (Vitamin B_1). The sample was autoclaved under weak acid conditions to extract the thiamin. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamin, after which an ion exchange cleanup column was used. An aliquot was reacted with potassium ferricyanide to convert thiamin to thiochrome that was extracted into isobutyl alcohol and read on a fluorometer against a known standard (*62*−*64*).

Vitamin E. Samples were saponified to break down fat and release vitamin E. The saponified mixture was extracted with ethyl ether, and α-tocopherol was quantitated directly by HPLC on a silica column (*65*−*67*).

Statistical Analysis of Compositional Data. The following 16 analytes with >50% of observations below the limit of quantitation (LOQ) for their respective assays were excluded from statistical analysis of results from both field trial years: sodium, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, mannose, and stachyose. Additionally, cadmium (1999), malonic acid (2000), and 22:0 behenic acid (2000) with >50% of observations below the LOQ for their respective assays were excluded from the statistical analyses for the noted years. Otherwise, for results below the quantitation limit, values equal to half the quantitation limit were assigned prior to statistical analyses. In the 2000 field season data, five outliers were identified by studentized PRESS residuals. Outliers were restricted to one replicate sample each at the following field sites: ADF for the control wheat at Minnesota, histidine and lysine for MON 71800 at North Dakota, and iron and ash for the control wheat at Manitoba. These outliers were excluded from the statistical analysis. With the removal of two amino acids in one replicate sample from North Dakota, the calculation of percent total amino acids was not possible and all amino acid data from this replicate were excluded.

The data from each year were statistically analyzed independently. There were a total of 65 components (nine in forage and 56 in grain) and 70 components (nine in forage and 61 in grain) for the 1999 and 2000 field seasons, respectively. Statistical analyses were conducted using a mixed model analysis of variance for compositional data from the combination of all sites for each field season in forage and grain. The combined trial analysis used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

Table 5. Sugar Composition of Grain from Glyphosate Tolerant Wheat MON 71800

	1999 field trials			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component[e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range)[c]	range[d] (99% TI)[f]	literature (range)
starch	61.49 (57.75–65.93)	61.06 (58.11–64.88)	54.00–63.76 (50.99, 67.11)	53.63 (47.05–61.98)	54.36 (44.24–61.10)	45.41–65.05 (36.25, 72.28)	59.9[g]–71.9[h]
arabinose	2.78 (1.96–4.29)	2.74 (1.87–3.12)	1.84–3.10 (1.28, 3.88)	2.73 (2.38–3.01)	2.71 (2.46–3.09)	2.32–3.14 (1.97, 3.53)	not available
fructose	0.29 (0.076–0.54)	0.27 (0.070–0.41)	0.071–0.51 (0, 0.73)	0.19 (0.073–0.43)	0.18 (0.086–0.36)	0.081–0.29 (0, 0.34)	0.06–0.08[i]
galactose	0.50 (0.41–0.58)	0.49 (0.42–0.55)	0.41–0.71 (0.20, 0.87)	0.49 (0.40–0.59)	0.49 (0.40–0.58)	0.40–0.59 (0.31, 0.71)	0.02[i]
glucose	0.33 (0.064–0.66)	0.28 (0.061–0.49)	0.064–0.51 (0, 0.80)	0.23 (0.094–0.51)	0.21 (0.095–0.41)	0.10–0.36 (0, 0.42)	0.03–0.09[i]
maltose	0.091 (0.028–0.13)	0.086 (0.028–0.15)	0.027–0.13 (0, 0.22)	0.070 (0.027–0.15)	0.063 (0.028–0.13)	0.028–0.14 (0, 0.19)	0–0.18 [i]
raffinose	0.27 (0.15–0.47)	0.27 (0.14–0.50)	0.15–0.50 (0, 0.70)	0.30 (0.18–0.45)	0.32 (0.20–0.49)	0.20–0.48 (0.022, 0.66)	0.19–0.68[i]
sucrose	0.51 (0.19–0.94)	0.50 (0.25–1.00)	0.31–0.85 (0.044, 1.23)	0.51 (0.25–0.69)	0.56 (0.35–0.79)	0.51–0.85 (0.33, 1.07)	0.54–1.55[i]
xylose	3.60 (3.08–4.07)	3.48 (2.98–4.05)	2.70–4.36 (1.90, 5.37)	4.24 (3.66–4.55)	4.14 (3.97–4.34)	3.30–4.45 (2.83, 4.95)	not available

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 20 values (four replicates from each of five field sites). [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canada field sites in 2000. [e] Percent dry weight. [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *82*. [h] Ref *76*. [i] Ref *83*.

where Y_{ijk} = unique individual observation, U = overall mean, T_i = line effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by line interaction effect, and e_{ijk} = residual error. MON 71800 was compared to the nontransgenic control line, Bobwhite, to determine statistically significant differences at $p < 0.05$.

The commercial reference varieties' data from each year were not included in the statistical analyses but rather were used to develop population tolerance intervals expected to contain, with 95% confidence, 99% of the values expressed in the population of commercial wheat varieties. Because negative quantities are not possible, calculated lower tolerance bounds that were negative were set to zero. SAS software (*68*) was used to generate all summary statistics and perform all analyses.

The compositional analysis data and statistical evaluation are summarized in **Tables 1**−**6**. For each component and year of field trials, least-squares means and the range of observed values are presented for the test event and control line. The calculated 99% tolerance interval is also presented for each field season.

RESULTS AND DISCUSSION

Proximates, Fibers, and Minerals in Forage. As presented in **Table 1**, the results indicate that with the exception of moisture in 1999, there were no statistically significant differences between forage produced by MON 71800 and its parental control in either year. The range of values for moisture in MON 71800 in 1999 fell within the 99% tolerance interval and therefore was considered to be within the population of commercial wheat. The levels of all proximates, fibers, and minerals in forage fell within the tolerance interval of commercial varieties.

Amino Acid Composition in Grain. The amino acid composition data are presented in **Table 2**. The only statistically significant difference between MON 71800 and its parental control was for isoleucine in 1999. However, the range of values for isoleucine in MON 71800 in 1999 fell within the 99% tolerance interval and therefore was considered to be within the population of commercial wheat. The close agreement in levels of aromatic amino acids indicates that the presence of the CP4 EPSPS enzyme has no effect on the distribution of these amino acids.

Fatty Acid Composition in Grain. Table 3 contains the data for the fatty acid composition. There were no statistically significant differences in fatty acid composition between MON 71800 and its control with the exception of 22:0 behenic acid in 1999. The magnitude of the difference for this very low abundance fatty acid was 13.6%, and the range of observations for this fatty acid in MON 71800 fell within the 99% tolerance interval of commercial wheat varieties.

Proximates, Minerals, and Fiber Composition of Grain. Table 4 contains the data for proximates, fiber, and minerals. For these analytes, there were no statistically significant differences between MON 71800 and its control. The sole exception to this was magnesium in 1999 with a 4.9% difference between MON 71800 and the control. The range of observations for magnesium in MON 71800 in 1999 fell well within the tolerance interval generated for the commercial wheat from 1999.

Starch and Sugar Composition of Grain. Starch and 10 sugars were evaluated in wheat grain. Mannose and stachyose fell below the LOQ and were excluded from the statistical analyses. The data for the remaining eight sugars and starch are presented in **Table 5**. There were no statistically significant differences in the starch or sugar content of MON 71800 as compared to its parental control for either field season.

Vitamin, Secondary Metabolite, and Phytic Acid Composition of Grain. The data for the statistical analysis of niacin, riboflavin, thiamin, vitamin B6, vitamin E, folic acid, ferulic acid, oxalic acid, *p*-coumaric acid, phytic acid, and starch in wheat grain are presented in **Table 6**. Ferulic acid and *p*-coumaric acid, which serve in plant defense and structural roles, are downstream metabolites of the aromatic amino acids tyrosine and phenylalanine (*69*). Oxalic acid and phytic acid can both interfere with the absorption of dietary calcium (*70*).

Table 6. Vitamin, Phytic Acid, and Secondary Metabolite Composition of Grain from Glyphosate Tolerant Wheat MON 71800

	1999 field trials			2000 field trials			
	MON 71800	control	commercial	MON 71800	control	commercial	
component[e]	mean[a] (range)[a]	mean[a] (range)[a]	range[b] (99% TI)[f]	mean[c] (range)[c]	mean[c] (range)[c]	range[d] (99% TI)[f]	literature (range)
niacin	49.57 (38.12–65.47)	51.01 (35.31–69.20)	25.72–82.85 (2.96, 106.12)	59.42 (44.84–79.93)	58.59 (47.99–77.35)	57.19–89.19 (36.13, 105.88)	38–93[g]
riboflavin	1.32 (0.99–1.49)	1.26 (1.07–1.52)	0.97–1.68 (0.36, 2.05)	1.31 (1.00–1.53)	1.25 (0.96–1.55)	0.90–1.48 (0.60, 1.78)	1–1.7[g]
thiamin	4.93 (4.00–7.04)	5.02 (4.31–5.86)	4.22–8.18 (2.03, 9.43)	4.28 (3.61–5.06)	4.62 (3.96–5.68)	3.95–6.88 (2.36, 7.80)	3.3–6.5[g]
vitamin B_6	NA[h]	NA[h]	NA[h]	1.92 (1.61–2.22)	1.86 (1.42–2.19)	1.70–2.43 (1.30, 2.70)	0.7–3.7[i,j]
vitamin E	48.71 (6.70–111.12)	62.06 (6.71–112.73)	7.03–119.74 (0, 165.08)	9.35 (7.17–11.25)	9.99 (7.89–12.63)	7.97–15.40 (4.52, 17.89)	4.9–58[g]
folic acid	NA[h]	NA[h]	NA[h]	0.72 (0.46–1.03)	0.77 (0.49–1.15)	0.52–1.24 (0.13, 1.53)	0.43% fw[k]
phytic acid	1.03 (0.78–1.23)	0.99 (0.74–1.42)	0.78–1.27 (0.55, 1.52)	0.96 (0.68–1.27)	0.90 (0.60–1.18)	0.62–1.30 (0.28, 1.54)	0.49–0.93[l]
ferulic acid	NA[h]	NA[h]	NA[h]	982 (767–1251)	997 (834–1285)	670–1023 (527, 1180)	780–1980[m]
p-coumaric acid	NA[h]	NA[h]	NA[h]	29.2 (20.4–45.1)	37.1 (22.7–79.4)	12.4–84.0 (0, 104.0)	not available
oxalic acid	NA[h]	NA[h]	NA[h]	0.055 (0.035–0.068)	0.054 (0.035–0.073)	0.045–0.087 (0.024, 0.094)	0.040–0.073[n]

[a] The mean and range of 12 values (four replicates from each of three field sites). [b] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 1999. [c] The mean and range of 20 values (four replicates from each of five field sites). [d] The range of sample values for commercial varieties purchased separately or grown at the same U.S. or Canadian field sites in 2000. [e] Niacin, riboflavin, thiamin, vitamin B_6, vitamin E, folic acid, *p*-coumaric acid, and ferulic acid in mg/kg dry weight; phytic acid and oxalic acid in % dry weight. [f] TI = tolerance interval, specified to contain 99% of the commercial variety population with 95% confidence; negative limits set to zero. [g] Ref *78*. [h] NA indicates that these analytes were not evaluated in the 1999 field year samples. [i] Ref *84*. [j] Ref *85*. [k] Ref *79* (fresh weight). [l] Ref *86*. [m] Ref *87* (durum wheat, fresh weight). [n] Ref *88*.

For vitamins, secondary metabolites, and phytic acid, there were no statistically significant differences in the content of MON 71800 as compared to its parental control for either field season. The absence of significant differences in ferulic and *p*-coumaric acids between MON 71800 and the nontransgenic wheat indicates that there is no effect on the flux of aromatic amino acids due to the presence of the *cp4 epsps* gene.

CONCLUSIONS

The results of these compositional analyses show that the 88 components measured in glyphosate tolerant wheat MON 71800 across two field seasons were not statistically different ($p < 0.05$) from the nontransgenic control or were within the 99% tolerance interval calculated from commercial wheat lines analyzed concurrently with MON 71800 and its control. The compositional data were also consistent to those reported in the literature. Depending on the source, the literature data may be derived from a single set of data or may represent a compilation of many studies. For this reason, literature values are presented, but not directly compared, to the data generated in the current work. These data demonstrate, with a confidence level of 95%, that the levels of all key nutrients and other evaluated components for MON 71800 were not statistically different from the nontransgenic control or were within the same population established from commercially available wheat varieties. Therefore, any minor differences noted from the statistical comparisons are unlikely to be biologically meaningful, and the forage and grain from MON 71800 are considered compositionally equivalent to those of conventional wheat.

These data demonstrate that the tolerance interval is a useful statistical tool that can account for natural variability in any measured parameter; in this case, the parameters are food and feed nutritional profiles as measured by biochemical composition. The large number of nutritional and antinutritional components analyzed as part of this assessment provides a thorough compositional evaluation of MON 71800. The limited number of significant differences between MON 71800 and its control, combined with the agreement between the data from MON 71800 and the commercial varieties and data reported in the literature, demonstrate that no unintended effects were observed on the composition of MON 71800. Considering the principle of substantial equivalence as articulated by the World Health Organization, the Organization for Economic Cooperation and Development, and the United Nations Food and Agriculture Organization, these data, along with the safety of the CP4 EPSPS protein and the safe history of use of wheat as a common source of animal feed and human food, demonstrate that glyphosate tolerant wheat MON 71800 is as safe and nutritious as conventional varieties of wheat currently on the market.

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