

## Composition of Forage and Grain from Second-Generation Insect-Protected Corn MON 89034 Is Equivalent to That of Conventional Corn (*Zea mays* L.)

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Insect-protected corn hybrids containing Cry insecticidal proteins derived from *Bacillus thuringiensis* have protection from target pests and provide effective management of insect resistance. MON 89034 hybrids have been developed that produce both the Cry1A.105 and Cry2Ab2 proteins, which provide two independent modes of insecticidal action against the European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests of corn. The composition of MON 89034 corn was compared to conventional corn by measuring proximates, fiber, and minerals in forage and by measuring proximates, fiber, amino acids, fatty acids, vitamins, minerals, antinutrients, and secondary metabolites in grain collected from 10 replicated field sites across the United States and Argentina during the 2004–2005 growing seasons. Analyses established that the forage and grain from MON 89034 are compositionally comparable to the control corn hybrid and conventional corn reference hybrids. These findings support the conclusion that MON 89034 is compositionally equivalent to conventional corn hybrids.

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**KEYWORDS:** Corn (*Zea mays* L.); corn borer; insect protected corn; composition; substantial equivalence

### INTRODUCTION

Corn (*Zea mays* L.), or maize, is the largest crop grown in the United States in terms of acreage planted and net value. In 2006, 78.3 million acres of corn was planted in the United States. The average yield of corn grain was 149 bushels per acre (60.3 bushels per hectare) with a total production of 10.5 billion bushels valued at U.S. \$33.8 billion. More than 52% of the nation's corn acres were planted with biotechnology-derived (biotech) hybrids. Advances in plant breeding through biotechnology have helped increase corn yields while dramatically reducing the use of chemical pesticides. In 2004, the use of biotech hybrids resulted in a 23.3 million pound (10.57 million kilogram) reduction in the use of chemical pesticides (1).

In 1997, Monsanto commercialized the first-generation Yield-Gard Corn Borer corn, MON 810, which produces the *Bacillus thuringiensis* (Bt) Cry1Ab protein that provides effective protection against damage caused by lepidopteran insect pests

especially the European corn borer (ECB, *Ostrinia nubilalis*) and the corn earworm (CEW, *Helicoverpa zea*). Monsanto Company has developed, through the use of recombinant DNA techniques, MON 89034, a second-generation corn product that produces two Cry proteins, Cry1A.105 and Cry2Ab2, with different modes of action. MON 89034 contains a wider spectrum of activity against lepidopteran pests, strengthens insect resistance management, and facilitates more efficient plant breeding of this insecticidal profile into superior hybrids. Specifically, the Cry1A.105 protein provides increased activity against the fall armyworm (*Spodoptera frugiperda*) and the black cutworm (*Agrotis ipsilon*). The Cry2Ab2 protein provides control from damage caused by the corn earworm. The improved insecticidal profile of MON 89034, with two Cry proteins, potentially reduces the refuge acreage required for insect resistance management purposes and minimizes the potential for mycotoxin accumulation in grain. The safety assessment of foods or feeds derived from biotech crops addresses two sources of potential health consequences: (a) those due to the activity and presence of the introduced trait (most often a protein) and (b) those due to the characteristics of the resulting food or feed crop plant (2–11). This comparative safety assessment process includes quantitative evaluation of crop agronomic/phenotypic

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characteristics and compositional concentration of key nutrients and antinutrients relevant to human or animal health as a means to understand whether the composition of a new biotech crop is consistent with generally accepted definitions or specifications of traditional varieties. The purpose of the present study was to compare the composition of MON 89034 corn with that of a conventional control corn hybrid with similar genetic background and with the composition of commercially available conventional corn hybrids to evaluate any potential changes arising from expression of the *cry1A.105* and *cry2Ab2* genes.

## MATERIALS AND METHODS

**Corn Samples for Compositional Analysis.** MON 89034 was produced by *Agrobacterium*-mediated transformation of corn with the PV-ZMIR245 vector, which is a binary vector containing two separate transfer DNA's (2T-DNA). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* (neomycin phosphotransferase II) expression cassette. During transformation, both T-DNAs were inserted into the genome. The *nptII* selectable marker gene was used for the selection of transformed cells in the presence of neomycin. A significant proportion of the cells selected for resistance to neomycin because of the presence of T-DNA II will also contain T-DNA I. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Traditional breeding was used to produce plants that only contained the *cry1A.105* and *cry2Ab2* expression cassettes (T-DNA I) and that did not contain the *nptII* expression cassette (T-DNA II) thereby producing marker-free MON 89034 corn.

Grain and forage samples were collected from field trials conducted in the United States and in Argentina. In the United States, corn was grown at five replicated field trials (Jefferson County, IA; Jersey County, IL; Warren County, IL; York County, NE; and Fayette County, OH) during the 2004 growing season. In Argentina, corn was grown at five replicated field trials (Pergamino, Buenos Aires; Tacuari, Buenos Aires; Gahan, Buenos Aires; Marcos Juarez, Córdoba; and Uranga, Santa Fe) during the 2004–2005 growing season. The replicated trials were based on a randomized complete block design with three replicates per block of each test, control, and reference substance. Corn plants at the field trials were grown under normal agronomic field conditions for their respective geographic locations. The genetic purity of the MON 89034 corn plants was maintained by bagging the tassels and ear shoots at anthesis and by self-pollinating each plant by hand. The forage was collected at the late dough/early dent stage, and the grain was collected at normal kernel maturity. Forage and grain samples were harvested and shipped to Monsanto. The samples were ground to a fine powder in the presence of dry ice and were maintained frozen until required compositional analysis. The identity of the forage samples was based on sample-handling records. The identity of the grain samples was based on sample-handling records and event-specific polymerase chain reaction analyses of genomic DNA isolated from the grain tissue.

**Compositional Analyses.** Compositional analyses were conducted to measure proximates (protein, fat, ash, carbohydrate by calculation, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids, fatty acids, vitamins (thiamin/*B*<sub>1</sub>, riboflavin/*B*<sub>2</sub>, pyridoxine/*B*<sub>6</sub>, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), secondary metabolites (2-furaldehyde, ferulic acid, and *p*-coumaric acid), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in grain. Proximates, ADF, NDF, and minerals (calcium and phosphorus) were measured in forage. All compositional analyses were performed at Covance Laboratories, Inc. (Madison, WI). Brief descriptions of the methods utilized for the analyses are described below.

**Proximate Analysis.** Protein concentrations were estimated by determining the total nitrogen content using the Kjeldahl method (12). Protein was calculated from total nitrogen using the formula  $N \times 6.25$ . Fat content of the grain was estimated by using the Soxhlet extraction method (13). Fat content of the forage was determined by fat-acid hydrolysis followed by extraction with ether and hexane (14). Ash

content was estimated by ignition of a sample in an electric furnace and by quantitation of the ash by gravimetric analysis (15). Moisture content was determined by loss of weight upon drying in a vacuum oven at 100 °C to a constant weight. (16) Carbohydrate concentrations were estimated by using the fresh weight-derived data and the following equation (17):

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

**Fiber Analysis.** ADF was estimated by treating the sample with an acidic boiling detergent solution to dissolve the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. The lignocellulose fraction was collected and determined gravimetrically (18). The NDF was estimated by treating the sample with a neutral boiling detergent solution to dissolve the protein, enzymes, carbohydrate, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected and determined gravimetrically (18, 19). The TDF was estimated by gelatinizing the samples with alpha-amylase and by digesting with enzymes to break down the starch and protein. Ethanol was used to precipitate the soluble fiber. Samples were then filtered, and the residue was rinsed with ethanol and acetone to remove the starch and protein degradation products and moisture. The protein and ash contents were determined, and the total dietary fiber was calculated using these values (20).

**Minerals.** To estimate the concentrations of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc, inductively coupled plasma emission spectrometry was used as described in the AOAC methods (21) and by Dahlquist and Knoll (22). The sample was dried, precharred, and ashed overnight at approximately 500 °C. The ashed sample was treated with hydrochloric acid, was taken to dryness, and was placed in a solution of 5% (v/v) hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown samples, measured by the inductively coupled plasma, with the emission of a standard solution.

**Amino Acid Composition.** Three procedures described in the literature (23) were used to estimate the values for 18 amino acids in corn grain. The procedure for tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid before hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. The individual amino acids were then quantitated using an automated amino acid analyzer.

**Fatty Acid Composition.** The lipid in the grain samples was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% 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 (GC) using external standards for quantitation (24).

**Vitamin E.** Vitamin E in grain was determined following saponification to break down any fat and to release the vitamin as described by Cort et al. (25). The saponified mixture was extracted with ethyl ether and then was quantitated directly by high-performance liquid chromatography (HPLC) on a silica gel column.

**Riboflavin/Vitamin B<sub>2</sub>.** The amount of riboflavin was measured in grain samples following hydrolysis with dilute acid as described in the literature (26). The quantity of riboflavin in the sample hydrolysates was determined by comparing the growth of *Lactobacillus casei* measured turbidimetrically with the growth response in the presence of varying amounts of riboflavin standard.

**Thiamin/Vitamin B<sub>1</sub>.** Thiamin was extracted by autoclaving the grain samples in the presence of weak acid followed by phosphatase digestion to release any bound thiamin (27). Thiamin was purified from the resulting solution by ion exchange chromatography and then was converted to thiochrome with potassium ferricyanide. The thiochrome was extracted into isobutyl alcohol, and the concentrations were quantitated fluorometrically.

**Pyridoxine/Vitamin B<sub>6</sub>.** The amount of pyridoxine was measured in grain samples following hydrolysis with dilute sulfuric acid as described

Table 1. Fiber, Proximate, and Mineral Composition of Grain from Test MON 89034

component	U.S. trials <sup>a</sup>			2004–2005 Argentina trials <sup>b</sup>			literature range	ILSI <sup>®</sup> database range
	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs <sup>e</sup> tolerance interval <sup>f</sup>	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance inter val <sup>f</sup>		
ADF <sup>g</sup>	5.48 (3.82–7.24)	5.27 (4.17–7.00)	2.77, 7.56	5.66 (4.68–6.84)	5.66 (4.68–6.84)	2.74, 9.13	3.3–4.3 <sup>i</sup>	1.82–11.34
NDF	10.06 (8.59–12.08)	9.75 (8.48–11.75)	5.93, 13.63	10.84 (8.20–14.45)	10.85 (9.22–12.34)	6.21, 16.18	8.3–11.9 <sup>i</sup> ; 7.58–15.91 <sup>i</sup>	5.59–22.64
TDF	15.17 (13.39–17.02)	14.67 (12.82–17.62)	9.20, 20.27	16.14 (11.65–21.87)	15.48 (12.40–18.19)	7.95, 25.13	10.99–11.41 <sup>h</sup>	
ash	1.41 (1.25–1.56)	1.39 (1.28–1.51)	0.74, 1.96	1.43 (1.16–1.70)	1.41 (1.20–1.61)	0.64, 2.18	1.1–3.9 <sup>i</sup> ; 0.89–6.28 <sup>i</sup>	0.616–6.282
carbohydrates	84.85 (83.29–86.52)	84.96 (83.58–86.22)	81.08, 88.80	85.32 (84.17–86.73)	85.42 (84.53–86.22)	81.06, 88.33	77.4–87.2 <sup>i</sup>	77.4–89.5
moisture	9.52 (7.89–12.80)	9.50 (7.86–13.10)	0.45, 19.52	13.44 (12.30–14.50)	13.51 (12.90–14.10)	11.40, 15.35	7–23 <sup>i</sup> ; 8.18–26.2 <sup>i</sup>	6.1–40.5
(% fw)								
protein	10.43 (8.54–11.98)	10.36 (9.22–11.52)	7.54, 13.13	8.01 (7.04–9.05)	7.70 (4.32–8.70)	5.66, 9.31	6–12 <sup>i</sup> ; 9.7–16.1 <sup>i</sup>	6.15–17.26
total fat	3.32 (3.05–3.89)	3.29 (3.05–3.75)	2.20, 4.55	3.29 (2.77–3.66)	3.22 (2.96–3.40)	1.94, 5.07	3.1–5.7 <sup>i</sup> ; 2.48–4.81 <sup>i</sup>	1.742–5.823
calcium	0.0050 (0.0038–0.0066)	0.0049 (0.0040–0.0059)	0.0016, 0.0059	Mineral (mg/kg or % dw)	0.0061 (0.0051–0.0070)	0.0019, 0.0079	0.01–0.1 <sup>i</sup>	0.00127–0.02084
(% dw)								
copper	1.74 (1.33–2.38)	2.07 (1.26–4.54)	0, 4.20	2.37 (1.09–4.82)	4.16 (1.44–18.36)	0, 8.23	0.9–10 <sup>i</sup>	0.73–18.50
(mg/kg dw)								
iron	21.40 (19.23–25.23)	22.20 (19.03–28.26)	8.88, 34.51	18.91 (16.73–21.00)	19.39 (16.63–27.75)	12.48, 29.03	1–100 <sup>i</sup>	10.42–49.07
(mg/kg dw)								
magnesium	0.12 (0.10–0.14)	0.12 (0.11–0.14)	0.075, 0.17	0.13 (0.12–0.14)	0.12 (0.11–0.14)	0.078, 0.18	0.09–1 <sup>i</sup>	0.0594–0.194
(% dw)								
manganese	6.79 (5.43–9.32)	6.51 (5.57–8.00)	3.17, 9.99	6.81 <sup>m</sup> (5.42–7.62)	6.28 (5.33–6.88)	2.33, 11.13	0.7–54 <sup>i</sup>	1.69–14.30
(mg/kg dw)								
phosphorus	0.33 (0.27–0.36)	0.33 (0.29–0.36)	0.18, 0.45	0.34 (0.29–0.39)	0.34 (0.28–0.40)	0.20, 0.48	0.26–0.75 <sup>i</sup>	0.147–0.533
(% dw)								
potassium	0.36 (0.32–0.40)	0.36 (0.34–0.40)	0.26, 0.46	0.38 (0.31–0.43)	0.39 (0.31–0.47)	0.21, 0.58	0.32–0.72 <sup>i</sup>	0.181–0.603
(% dw)								
zinc	22.05 (18.91–26.89)	21.91 (18.81–26.04)	7.16, 38.55	20.74 (17.53–23.60)	20.80 (16.86–23.64)	7.91, 33.26	12–30 <sup>i</sup>	6.5–37.2
(mg/kg dw)								

<sup>a</sup> Data from five replicated U.S. sites. <sup>b</sup> Data from five replicated sites in Argentina. <sup>c</sup> Conventional control. <sup>d</sup> The simple mean of 15 values. <sup>e</sup> Commercial hybrids planted at each trial site. <sup>f</sup> Tolerance interval is specified to contain 99% of the commercial line population; negative limits are set to zero. <sup>g</sup> Reference (44). <sup>h</sup> Reference (44). <sup>i</sup> Reference (48). <sup>j</sup> Reference (49). <sup>m</sup> Statistically different from the control ( $p < 0.05$ ). <sup>n</sup> ADF = acid detergent fiber. NDF = neutral detergent fiber. TDF = total dietary fiber. dw = dry weight.

**Table 2.** Fiber, Proximate, and Mineral Composition of Forage from Test MON 89034

component	2004 U.S. trials <sup>a</sup>			2004–2005 Argentina trials <sup>b</sup>			literature range	ILSI <sup>g</sup> database range
	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>		
ADF <sup>k</sup>	28.95 (22.60–35.85)	27.26 (19.93–35.59)	16.76, 43.76	25.70 (19.22–32.80)	25.72 (20.27–30.52)	17.39, 38.71	18.3–41.0 <sup>i</sup>	16.13–47.39
NDF	39.69 (33.99–46.82)	37.60 (31.44–43.96)	25.94, 55.67	35.50 (29.38–42.68)	36.33 (29.19–46.82)	23.84, 55.56	26.4–54.5 <sup>i</sup>	20.29–63.71
Fiber (% dw)								
ash	3.70 (2.51–4.67)	3.90 (2.59–5.10)	1.93, 6.31	5.22 (4.27–7.22)	4.98 (3.76–5.87)	2.22, 8.69	2–6.6 <sup>i</sup>	1.527–9.638
carbohydrates	86.90 (84.93–89.13)	86.69 (84.36–89.57)	83.05, 90.74	84.44 (82.56–86.11)	84.87 (82.83–88.30)	79.06, 89.42	83.2–91.6 <sup>i</sup>	76.4–92.1
moisture (% fw)	72.20 (68.50–75.40)	71.53 (65.90–76.80)	57.62, 86.45	70.26 (64.20–75.40)	70.13 (65.90–74.10)	56.88, 84.19	55.3–75.3 <sup>i</sup>	49.1–81.3
protein	7.82 (6.34–8.98)	7.70 (6.06–8.87)	4.78, 10.38	8.01 (7.04–9.05)	7.70 (4.32–8.70)	3.90, 12.06	n/a	3.14–11.57
total fat	1.57 (0.63–3.17)	1.71 (0.77–2.91)	0, 4.54	2.33 (1.16–3.49)	2.4 (1.46–3.13)	0, 5.13	0.35–3.62 <sup>i</sup>	0.296–4.570
Mineral (% dw)								
calcium	0.20 (0.16–0.24)	0.19 (0.13–0.28)	0.016, 0.38	0.14 (0.11–0.18)	0.14 (0.11–0.16)	0, 0.32	0.0969–0.3184 <sup>i</sup>	0.0714–0.5768
phosphorus	0.25 <sup>j</sup> (0.22–0.32)	0.21 (0.15–0.25)	0.07, 0.132	0.26 (0.15–0.39)	0.23 (0.14–0.29)	0, 0.56	0.1367–0.2914 <sup>i</sup>	0.0936–0.3704

<sup>a</sup> Data from five replicated U.S. sites. <sup>b</sup> Data from five replicated sites in Argentina. <sup>c</sup> Conventional control. <sup>d</sup> The simple mean of 15 values. <sup>e</sup> Commercial hybrids planted at each trial site. <sup>f</sup> Tolerance interval is specified to contain 99% of the commercial line population; negative limits are set to zero. <sup>g</sup> Reference (44). <sup>h</sup> Reference (49). <sup>i</sup> Statistically different from the control ( $p < 0.05$ ). <sup>k</sup> ADF = acid detergent fiber. NDF = neutral detergent fiber. dw = dry weight.

**Table 3.** Amino Acid Composition of Grain from Test MON 89034

component	2004 U.S. trials <sup>a</sup>			2004–2005 Argentina trials <sup>b</sup>			literature range	ILSI <sup>g</sup> database range
	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>		
Amino Acid (% dw) <sup>h</sup>								
alanine	0.77 (0.64–0.89)	0.78 (0.67–0.89)	0.48, 1.08	0.78 (0.65–0.88)	0.75 (0.69–0.85)	0.54, 1.06	n/a	0.439–1.393
arginine	0.48 (0.38–0.52)	0.47 (0.41–0.51)	0.33, 0.56	0.46 (0.39–0.53)	0.46 (0.39–0.49)	0.33, 0.58	n/a	0.119–0.639
aspartic acid	0.68 (0.56–0.78)	0.67 (0.60–0.76)	0.43, 0.90	0.68 (0.61–0.76)	0.67 (0.61–0.71)	0.50, 0.89	n/a	0.335–1.208
cysteine/cystine	0.23 (0.20–0.26)	0.23 (0.21–0.25)	0.18, 0.27	0.23 (0.20–0.25)	0.23 (0.21–0.25)	0.18, 0.28	n/a	0.125–0.514
glutamic acid	1.97 (1.63–2.29)	1.99 (1.70–2.26)	1.25, 2.75	1.96 (1.64–2.23)	1.89 (1.75–2.14)	1.34, 2.73	n/a	0.965–3.536
glycine	0.38 (0.32–0.41)	0.38 (0.36–0.41)	0.28, 0.46	0.39 (0.36–0.42)	0.38 (0.35–0.40)	0.30, 0.47	n/a	0.184–0.539
histidine	0.31 (0.25–0.35)	0.31 (0.28–0.34)	0.22, 0.38	0.29 (0.27–0.32)	0.2 (0.27–0.30)	0.20, 0.40	n/a	0.137–0.434
isoleucine	0.36 (0.30–0.43)	0.36 (0.30–0.42)	0.23, 0.51	0.35 (0.29–0.39)	0.35 (0.32–0.38)	0.26, 0.47	n/a	0.179–0.692
leucine	1.31 (1.09–1.57)	1.32 (1.08–1.55)	0.77, 1.92	1.31 (1.05–1.49)	1.27 (1.14–1.47)	0.85, 1.89	n/a	0.642–2.492
lysine	0.33 (0.26–0.36)	0.32 (0.29–0.36)	0.20, 0.40	0.31 (0.28–0.36)	0.31 (0.29–0.34)	0.23, 0.37	n/a	0.172–0.668
methionine	0.23 (0.20–0.27)	0.22 (0.20–0.24)	0.14, 0.25	0.22 (0.19–0.24)	0.22 (0.20–0.25)	0.13, 0.28	n/a	0.124–0.468
phenylalanine	0.51 (0.43–0.61)	0.52 (0.43–0.60)	0.32, 0.73	0.53 (0.44–0.60)	0.52 (0.48–0.59)	0.38, 0.73	n/a	0.244–0.930
proline	0.93 (0.79–1.05)	0.93 (0.83–1.01)	0.68, 1.21	0.91 (0.77–1.00)	0.89 (0.82–0.98)	0.66, 1.26	n/a	0.642–1.632
serine	0.52 (0.44–0.61)	0.52 (0.46–0.60)	0.34, 0.71	0.52 (0.47–0.60)	0.51 (0.46–0.56)	0.36, 0.72	n/a	0.235–0.769
threonine	0.33 (0.27–0.37)	0.33 (0.29–0.36)	0.24, 0.41	0.36 (0.31–0.41)	0.36 (0.32–0.39)	0.28, 0.47	n/a	0.224–0.666
tryptophan	0.056 (0.048–0.064)	0.056 (0.045–0.063)	0.032, 0.072	0.065 (0.060–0.071)	0.065 (0.061–0.070)	0.050, 0.075	n/a	0.0271–0.215
tyrosine	0.37 (0.22–0.43)	0.36 (0.24–0.42)	0.17, 0.52	0.34 (0.22–0.40)	0.35 (0.21–0.41)	0.23, 0.48	n/a	0.103–0.642
valine	0.49 (0.40–0.55)	0.49 (0.43–0.55)	0.35, 0.62	0.48 (0.43–0.53)	0.47 (0.45–0.51)	0.37, 0.63	n/a	0.266–0.855

<sup>a</sup> Data from five replicated U.S. sites. <sup>b</sup> Data from five replicated sites in Argentina. <sup>c</sup> Conventional control. <sup>d</sup> The simple mean of 15 values. <sup>e</sup> Commercial hybrids planted at each trial site. <sup>f</sup> Tolerance interval is specified to contain 99% of the commercial line population; negative limits are set to zero. <sup>g</sup> Reference (44). <sup>h</sup> dw = dry weight.

**Table 4.** Fatty Acid Composition of Grain from Test MON 89034

component	2004 U.S. trials <sup>a</sup>			2004–2005 Argentina trials <sup>b</sup>			literature range	ILSI <sup>g</sup> database range
	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>		
Fatty Acid (% Total FA <sup>m</sup> )								
palmitic (16:0)	9.19 (8.98–9.46)	9.12 (8.91–9.34)	6.12, 15.67	8.91 (8.71–9.25)	8.96 (8.80–9.19)	7.54, 13.55	7–19 <sup>k</sup>	7.94–20.71
palmitoleic (16:1)	0.13 (0.11–0.14)	0.12 (0.048–0.14)	0, 0.28	0.11 (0.055–0.14)	0.13 (0.061–0.16)	0.0029, 0.23	1 <sup>k</sup>	0.095–0.447
stearic (18:0)	1.89 <sup>j</sup> (1.79–2.03)	1.82 (1.76–1.87)	0.86, 2.98	1.84 <sup>j</sup> (1.76–1.98)	1.79 (1.73–1.87)	0.63, 3.01	1–3 <sup>k</sup>	1.02–3.40
oleic (18:1)	24.96 (23.38–25.75)	24.84 (23.62–26.66)	7.51, 46.46	24.47 (23.50–25.17)	24.32 (23.22–25.02)	8.77, 43.80	20–46 <sup>k</sup>	17.4–40.2
linoleic (18:2)	61.82 (60.85–63.61)	62.07 (60.51–63.41)	39.41, 76.74	62.66 (61.64–63.86)	62.77 (61.83–64.02)	41.30, 77.09	35–70 <sup>k</sup>	36.2–66.5
linolenic (18:3)	1.19 (1.12–1.23)	1.22 (1.15–1.43)	0.63, 1.77	1.21 (1.11–1.27)	1.21 (1.18–1.27)	0.63, 1.66	0.8–2 <sup>k</sup>	0.57–2.25
arachidic (20:0)	0.39 <sup>j</sup> (0.36–0.42)	0.38 (0.36–0.40)	0.23, 0.54	0.37 (0.35–0.40)	0.37 (0.35–0.47)	0.15, 0.66	0.1–2 <sup>k</sup>	0.279–0.965
eicosenoic (20:1)	0.28 (0.26–0.29)	0.28 (0.25–0.29)	0.15, 0.39	0.29 <sup>j</sup> (0.27–0.31)	0.30 (0.28–0.37)	0.14, 0.48	n/a	0.170–1.917
behenic (22:0)	0.16 (0.13–0.20)	0.15 (0.13–0.18)	0.081, 0.23	0.14 (0.12–0.17)	0.15 (0.13–0.31)	0.059, 0.30	n/a	0.110–0.349

<sup>a</sup> Data from five replicated U.S. sites. <sup>b</sup> Data from five replicated sites in Argentina. <sup>c</sup> Conventional control. <sup>d</sup> The simple mean of 15 values. <sup>e</sup> Commercial hybrids planted at each trial site. <sup>f</sup> Tolerance interval is specified to contain 99% of the commercial line population; negative limits are set to zero. <sup>g</sup> Reference (44). <sup>h</sup> Reference (51). <sup>i</sup> Statistically different from the control ( $p < 0.05$ ). <sup>m</sup> FA = fatty acid.

in the AOAC methods (28). The quantity of pyridoxine was turbidimetrically determined by comparing the growth response of the yeast

*Saccharomyces carlsbergensis* in the sample with the growth response in a pyridoxine standard.

Table 5. Vitamin, Phytic Acid, Raffinose, Ferulic Acid, and *p*-Coumaric Acid Content of Grain from Test MON 89034

component	2004 U.S. trials <sup>a</sup>			2004–2005 Argentina trials <sup>b</sup>			comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>	literature range	ILSI <sup>g</sup> database range
	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance interval <sup>f</sup>	MON 89034 mean <sup>d</sup> (range)	control <sup>c</sup> mean <sup>d</sup> (range)	comm. refs. <sup>e</sup> tolerance inter val <sup>f</sup>			
folic acid	0.35 (0.26–0.48)	0.36 (0.23–0.53)	0.012, 0.69	Vitamin (mg/kg dw) <sup>g</sup> 0.50 (0.27–0.81)	0.56 (0.27–0.92)	0, 1.74	0.3 <sup>i</sup>	0.147–1.464	
niacin	30.08 (25.72–34.84)	29.59 (24.93–35.75)	6.97, 37.83	35.26 (28.39–44.69)	34.18 (27.13–39.47)	0, 56.72	9.3–70 <sup>i</sup>	10.37–46.94	
vitamin B <sub>1</sub>	3.07 (2.39–3.44)	2.94 (2.39–3.36)	0.37, 6.35	3.34 (2.97–3.70)	3.28 (2.76–3.94)	1.92, 5.79	3–8.6 <sup>k</sup>	1.26–40.00	
vitamin B <sub>2</sub>	1.42 (1.24–1.65)	1.42 (1.16–1.61)	0.91, 2.30	1.75 <sup>o</sup> (1.54–1.95)	1.94 (1.65–2.16)	1.19, 3.11	0.25–5.6 <sup>k</sup>	0.50–2.36	
vitamin B <sub>6</sub>	6.22 (5.28–6.99)	6.26 (5.37–6.80)	3.12, 9.30	6.01 (5.23–7.07)	6.55 (5.02–10.83)	2.34, 10.88	5.3 <sup>i</sup> ; 9.6 <sup>k</sup>	3.68–11.32	
vitamin E	6.77 (5.55–8.62)	6.63 (2.72–9.02)	0, 20.49	na	na	na	3–12.1 <sup>k</sup> , 17–47 <sup>i</sup>	1.5–68.7	
phytic acid	0.75 (0.53–0.87)	0.73 (0.56–0.88)	0.21, 1.22	Antinutrient (% dw) 0.81 (0.61–0.98)	0.78 (0.53–1.03)	0.39, 1.12	0.08–0.30 <sup>k</sup>	0.111–1.570	
raffinose	na	na	na	0.060 (0.029–0.081)	0.062 (0.029–0.076)	0, 0.32		0.020–0.320	
ferulic acid	2131.38 (1790.25–2525.31)	2148.05 (1878.66–2669.85)	1136.69, 2806.24	Secondary Metabolites (μg/g dw) 1894.10 <sup>o</sup> (1484.46–2165.31)	1759.10 (1471.61–2034.48)	552.46, 3057.71	113–1194 <sup>m</sup> ; 3000 <sup>o</sup>	291.9–8865.8	
<i>p</i> -coumaric acid	194.25 (166.11–253.04)	183.96 (167.76–210.13)	0, 378.57	155.22 (139.70–185.35)	146.00 (118.66–174.57)	0, 326.22	22–75 <sup>m</sup>	53.4–576.2	

<sup>a</sup> Data from five replicated U.S. sites. <sup>b</sup> Data from five replicated sites in Argentina. <sup>c</sup> Conventional control. <sup>d</sup> The simple mean of 15 values. <sup>e</sup> Commercial hybrids planted at each trial site. <sup>f</sup> Tolerance interval is specified to contain 99% of the commercial line population; negative limits are set to zero. <sup>g</sup> Reference (44). <sup>h</sup> Reference (51). <sup>i</sup> Reference (45). <sup>j</sup> Reference (52). <sup>k</sup> Reference (53). <sup>l</sup> Statistically different from the control ( $p < 0.05$ ). <sup>m</sup>  $dw = dry weight$ .

**Folic Acid.** Folic acid was analyzed using a published procedure (29) in which the grain was hydrolyzed by autoclaving in the presence of ascorbic acid. To release folic acid, the hydrolyzed material was digested by incubation for 18 h with an enzyme preparation from chicken pancreas. The quantity of folic acid in the sample was determined by comparing the growth of *L. casei* measured turbidimetrically with the growth response in the presence of varying amounts of a folic acid standard.

**Raffinose.** The raffinose assay was based on two methods (30, 31) in which the grain samples were extracted with deionized water and the extracts were treated with a solution of hydroxylamine hydrochloride in pyridine containing phenyl- $\alpha$ -D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoroacetic acid and were analyzed by GC with flame ionization detection.

**Phytic Acid.** Phytic acid was quantitated in grain following extraction using ultrasonication as described by Lehrfeld (32, 33). Purification and concentration of the extract was conducted using a silica-based anion exchange (SAX) column followed by quantitation using a polymer HPLC column (PRP-1, 5  $\mu$ m, 150  $\times$  4.1 mm) fitted with a refractive index detector.

**Ferulic and *p*-Coumaric Acids.** Ferulic and *p*-coumaric acids were assayed in grain using the method of Hagerman and Nicholson (34), in which the samples were extracted with methanol, and the extracts were hydrolyzed using 4 N sodium hydroxide, were neutralized, and were filtered. The concentrations of ferulic and *p*-coumaric acids were determined by reversed-phase HPLC with UV detection.

**2-Furaldehyde.** The concentrations of 2-furaldehyde (furfural) were determined using the method of Albala-Hurtado et al. (35) in which the corn grain was extracted with 4% trichloroacetic acid, was centrifuged, was filtered, was concentrated, and was analyzed by reversed-phase HPLC with UV detection.

**Statistical Analysis of Composition Data.** From the 2004 United States study, the following 16 analytes with >50% of the observations at or below the limit of quantitation (LOQ) of the assay were excluded from statistical analysis: sodium, furfural, raffinose, 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, and 20:4 arachidonic acid. With the exception of raffinose and the addition of vitamin E, the same analytes listed above were also excluded from statistical analysis from the 2004–2005 Argentina field trial study. For 16:1 palmitoleic acid, five observations in the United States study and six observations in the Argentinean study were below the LOQ. In addition, two observations for vitamin E and five observations for raffinose in the United States and Argentinean data sets, respectively, were below the LOQ. To include a complete data set for 16:1 palmitoleic acid, vitamin E, and raffinose in the statistical analysis, values equal to half the LOQ were assigned for the missing data points. Three outliers in the United States data set were identified by the studentized PRESS residuals procedure (one test copper, one test iron, and one reference iron), and two of the commercial reference plots in Argentina (copper and zinc) were identified and excluded from the statistical analysis (36). Except for moisture, all component values were converted from a fresh weight to a dry weight basis and into their respective units described in Tables 1–5. A total of 61 different components were therefore evaluated (9 in forage and 52 in grain) in the samples from both the United States and Argentina studies.

Statistical analyses of the composition data were conducted using a mixed model analysis of variance for a combination of all sites for both the United States and the Argentinean studies. The combined trial analysis used the model

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

where  $Y_{ijk}$  = unique individual observation,  $U$  = overall mean,  $T_i$  = substance effect,  $L_j$  = random location effect,  $B(L)_{jk}$  = random block within location effect,  $LT_{ij}$  = random location by substance interaction effect, and  $e_{ijk}$  = residual error. In these analyses, MON 89034 was compared to the conventional control. For each compositional comparison, the  $p$  value for a test of the MON 89034 mean equal to the

control mean, the observed difference of MON 89034 from the control, and the lower and upper 95% confidence intervals for the mean difference of MON 89034 from the control were calculated. Statistical significance was assigned at  $p < 0.05$ . A range of observed values from the reference substances was determined for each analytical component. Additionally, the reference substance data were used to develop population tolerance intervals. A tolerance interval is an interval that one can claim, with a specified degree of confidence,  $100(1 - \alpha)\%$ , which contains at least a specified proportion,  $p$ , of an entire sampled population for the parameter measured. For each compositional analyte, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional references (37, 38). Each tolerance interval estimate was based upon all observations per unique reference substance. As multiple observations existed for a substance, data were first summarized by substance within site and then by substance across sites. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero. SAS software (39) was used by Certus International, Inc. (Chesterfield, MO) to generate all summary statistics and to perform all analyses.

## RESULTS AND DISCUSSION

The safety assessment of biotechnology derived crops has relied on a comparative approach in which the similarities and differences are identified between the food and feed derived from a biotech crop with the food and feed derived from its near isogenic conventional counterpart and with commercially available varieties that have a history of the same consumption (40–43). In this study, the composition of MON 89034 corn was compared with that of a conventional control corn with a similar genetic background which was grown in the same field trials in the United States and Argentina. The evaluation of differences was conducted using a mixed model analysis of variance with statistical significance assigned at the  $p < 0.05$  level. In addition, the compositional profile of MON 89034 was compared with those of traditional corn hybrids by calculating a 99% tolerance interval to describe the compositional variability in the population of conventional corn hybrids in the marketplace. Finally, the compositional values for MON 89034 corn were compared with values obtained from the published literature or from the International Life Sciences Institute (ILSI) Crop Composition Database (44).

**Proximate, Fiber, and Mineral Composition.** Compositional analysis results for corn grain and corn forage are presented in **Tables 1** and **2**, respectively. These results demonstrate that the concentrations of proximate components (fat, protein, ash, and carbohydrate), fiber (ADF, NDF, and TDF), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in the grain as well as proximates and fiber of forage from MON 89034 were comparable to those in the grain and forage of the conventional control. All values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. A significant difference ( $p < 0.05$ ) in the grain between the MON 89034 corn (6.81 mg/kg dw) and the conventional control (6.28 mg/kg dw) was observed in the concentration of manganese for the Argentinean field trial (see **Table 1**). A significant difference ( $p < 0.05$ ) in the forage between the MON 89034 corn (0.25% dw) and the conventional control (0.21% dw) was observed in the concentration of phosphorus for the United States field trial (see **Table 2**). However, the magnitude of differences expressed as a percentage of the control value was small (8.56 and 19.24%, respectively) considering the natural variability. Furthermore, the

means and range of values found for these minerals were both within the calculated 99% tolerance interval for the population of conventional, commercial hybrids grown in their respective field trials. These results demonstrate that, with a confidence level of 95%, the concentrations of proximates, fiber, and minerals for MON 89034 were within the same population as those of conventional, commercially available corn.

**Amino Acid Composition.** The concentrations of the 18 amino acids measured in the grain of MON 89034 corn were comparable to those in the grain of the conventional control (**Table 3**). All values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. No significant differences were observed in the grain between MON 89034 corn and the conventional control (see **Table 3**).

**Fatty Acid Composition.** The concentrations of the fatty acids in the grain of MON 89034 corn were comparable to those observed in the grain of the conventional control (**Table 4**). All values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. Significant differences ( $p < 0.05$ ) in the grain between MON 89034 corn and the conventional control were observed in the concentrations of 18:0 stearic acid and 20:0 arachidic acid for the United States field trials and in the concentrations of 18:0 stearic acid and 20:1 eicosenoic acid for the Argentinean field trials. However, the magnitude of differences expressed as a percentage of the control value was small (2.61–4.35%) considering the natural variability. Furthermore, the means and range of values found for these fatty acids were all within the calculated 99% tolerance interval for the population of conventional, commercial hybrids grown in the respective field trials. These results demonstrate, with a confidence level of 95%, that the concentrations of fatty acids for MON 89034 were within the same population as those of conventional, commercially available corn.

**Vitamin Composition.** Folic acid, niacin, vitamin B<sub>1</sub> (thiamin), vitamin B<sub>2</sub> (riboflavin), vitamin B<sub>6</sub>, and vitamin E were measured in the grain of MON 89034 and were compared to the conventional control (**Table 5**). All values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. A significant difference ( $p < 0.05$ ) in the grain between MON 89034 corn and the conventional control was observed in the concentration of vitamin B<sub>2</sub> for the Argentinean field trial (see **Table 5**). However, the magnitude of difference expressed as a percentage of the control value was relatively small, 9.78%, considering the natural variability. Furthermore, the mean and range of values found for vitamin B<sub>2</sub> were both within the calculated 99% tolerance interval for the population of conventional, commercial hybrids grown in the Argentinean trials. These results demonstrate that, with a confidence level of 95%, the concentrations of vitamins for MON 89034 were within the same population as those of conventional, commercially available corn.

**Antinutrient Composition.** The concentrations of phytic acid and raffinose were measured in the grain of MON 89034 and were compared to the conventional control (**Table 5**). Phytic acid, the hexakis-*o*-phosphate of *myo*-inositol, is widely distributed in plants (45). Seeds accumulate up to 90% of stored organic phosphate as phytic acid, and it has been shown to limit the uptake of minerals such as calcium in higher animals.

Raffinose is a nondigestible oligosaccharide that is considered to be an antinutrient because of gas production and the resulting flatulence caused by its consumption (46). These values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. No significant differences were observed in the grain between MON 89034 and the conventional control (see **Table 5**).

**Secondary Metabolite Composition.** The secondary metabolites, ferulic acid and *p*-coumaric acid, have been shown to be present in corn grain or in processed corn components. Ferulic and *p*-coumaric acids in plants are derived from the aromatic amino acids, phenylalanine and tyrosine (47), and serve as precursors for a large group of phenylpropanoid compounds including flavonoids and coumarins. The ferulic acid and *p*-coumaric acid values in the grain of MON 89034 were comparable with those observed in the grain of the conventional control (**Table 5**). These values were either within the 99% tolerance interval determined for the population of commercial hybrids evaluated in this study, within published literature ranges, or within the range of values obtained from the ILSI database. No significant differences were observed in the grain between MON 89034 and the conventional control (see **Table 5**).

**Conclusions.** The results of compositional analyses generated from corn samples grown in both the United States and Argentina demonstrate that the grain and forage of MON 89034 corn are comparable with those of the conventional control and conventional corn hybrids. The composition of MON 89034 corn grown at each geographical region was shown to fall within the 99% tolerance interval for components in conventional commercial corn hybrids grown concurrently in that geographical region and also within the ranges of values reported for conventional corn in the scientific literature as well as in the ILSI Crop Composition Database. These latter comparisons are important and relevant because it is well recognized that the composition of any crop, including corn, varies as a result of many factors, including variety, and growing conditions. The values for components in MON 89034 corn all fell within the range of natural variability found in conventional corn hybrids.

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