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Evaluation of Compositional Equivalence for Multitrait Biotechnology Crops

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Supporting Information

ABSTRACT: Compositional analysis is an important tool in the evaluation of the safety and nutritional status of biotechnology-derived crops. As part of the comparative assessment of a biotechnology-derived crop, its composition is evaluated by quantitative measurement of the levels of key nutrients, antinutrients, and secondary metabolites and compared to that of conventional crops. To evaluate the effect of combining multiple biotech traits through conventional breeding, the forage and grain compositions of the double combinations MON 810 × NK603, MON 863 × MON 810, and MON 863 × NK603 and the triple combination MON 863 × NK603 × MON 810 were compared to their respective near-isogenic, conventional control hybrids. Overall, a total of 241 statistical comparisons between the multitrait biotechnology crop and its corresponding conventional controls were conducted. Of these comparisons 192 (79.7%) were not statistically significantly different ($p > 0.05$), and all 49 of the differences were within the 99% tolerance interval for commercial hybrids grown in the same field or related field trials. These data on combined trait biotechnology-derived products demonstrated that the forage and grain were compositionally equivalent to their conventional comparators, indicating the absence of any influence of combining insect protection and herbicide tolerance traits by conventional breeding on compositional variation.

KEYWORDS: Biotechnology, multitrait, stacks, composition, maize (*Zea mays* L.), safety assessment

INTRODUCTION

The development of herbicide-tolerant and insect-protected maize, cotton, and soybean crops using the tools of modern agricultural biotechnology has increased productivity and decreased the environmental impact of agricultural practices worldwide.[1] Originally, these crops were developed to contain only a single biotechnology-derived trait. Combined trait products (also known as "stacks") have been developed through conventional breeding in which more than one biotech trait is present in the same seed. In 2009, 85% of the national maize crop in the United States was biotech, and 75% of it was hybrids with either double- or triple-stacked traits. Biotech cotton occupied approximately 90% or more of the national area of cotton in the United States, Australia, and South Africa in 2009, with double-stacked traits occupying 75−88% of all biotech cotton in those countries.[1]

Comparisons of the levels of key nutrients and antinutrients in crops containing biotechnology-derived traits with those of conventional varieties represent one of several important considerations in nutritional and safety assessments.[2−8] In consultation with government agencies, the Organization for Economic Cooperation and Development (OECD) has promoted a list of well-defined constituents for assessment in compositional studies of novel crops, including those for maize.[9,10] Crop tissues are collected from replicated field trials conducted at multiple locations in various world areas and are analyzed using internationally accepted validated methods.

Previous studies for two insect-protected products in maize, MON 810[11] and MON 863,[12] and a herbicide-tolerant maize product, NK603,[13] have demonstrated compositional equivalence between these single-trait biotechnology-derived products and their conventional comparators. To evaluate the effect of combining multiple biotech traits through conventional breeding, the compositions of MON 810 × NK603, MON 863 × MON 810, and MON 863 × NK603 and the triple combination MON 863 × NK603 × MON 810 were compared to their respective near-isogenic, conventional comparators. The forage and grain samples for MON 810 × NK603 were obtained from field trials conducted in Europe in 2000, and the forage and grain samples for MON 863 × MON 810 were grown in Argentina in 1999. MON 863 × NK603 and the triple combination MON 863 × NK603× MON 810 were grown concurrently in Argentina during 2002−2003. The availability of both double- and triple-stack combinations provided the opportunity to examine the effect of increasing trait complexity on compositional equivalence.

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Table 1. Proximate and Fiber Composition of Forage from MON 810 × NK603 and MON 863 × MON 810

	MON 810 × NK603 (IP1 × HT)			MON 863 × MON 810 (IP2 × IP1)				
component[a]	IP1 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	IP2 × IP1 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] [99% tolerance interval][e]	lit. range[d]	ILSI[h] range[d]
ash	4.03[j] (2.91−5.12)	3.52 (2.99−4.19)	(2.11−7.09) [1.30, 7.03]	6.41 (5.36−9.44)	6.32 (4.88−8.23)	[2.33, 7.70]	2.43−9.64[f] 2−6.6[g]	1.527−9.638
carbohydrates	85.94 (82.15−88.42)	86.44 (83.87−89.50)	(79.47−89.73) [79.16, 92.40]	83.23 (81.27−84.90)	82.61 (81.09−84.68)	[78.37, 91.73]	76.5−87.3[f] 83.2−91.6[g]	76.4−92.1
moisture	69.02 (64.00−75.20)	67.87 (62.20−73.10)	(56.40−76.00) [50.06, 83.63]	74.58 (72.00−78.40)	74.13 (70.20−77.70)	[56.69, 87.10]	56.5−80.4[f] 55.3−75.3[g]	49.1−81.3
protein	7.45 (4.43−10.82)	7.30 (4.52−9.49)	(3.14−11.06) [0.18, 14.77]	8.48[j] (7.59−9.77)	9.52 (8.35−10.60)	[0.22, 15.79]	4.98−11.56[f]	3.14−11.57
fat	2.58 (1.78−3.35)	2.74 (2.35−3.28)	(1.31−4.12) [0.66, 4.49]	1.88 (0.82−2.82)	1.56 (0.71−2.37)	[0, 4.49]	1.42−4.57[f] 0.35−3.62[g]	0.296−4.570
ADF[i]	22.93 (19.15−27.34)	22.40 (20.15−25.29)	(16.13−29.69) [13.77, 30.79]	26.28 (21.70−37.31)	27.22 (22.83−30.32)	[15.09, 34.96]	17.5−38.3[f] 18.3−41.0[g]	16.13−47.39
NDF[i]	38.67 (33.86−42.42)	36.14 (22.84−44.19)	(20.29−52.02) [25.68, 47.27]	40.31[j] (34.48−53.24)	43.20 (39.15−47.21)	[24.59, 55.98]	27.9−54.8[f] 26.4−54.5[g]	20.29−63.71

[a] Percent dry weight of sample, except moisture. [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Ridley et al.[13] [g] Sidhu et al.[54] [h] International Life Sciences crop composition database, Ridley et al.[55] [i] ADF, acid detergent fiber; NDF, neutral detergent fiber. [j] Statistically significantly different ($p < 0.05$) from control.

Components examined in forage samples included proximates (protein, fat, ash, and moisture), carbohydrates by calculation, acid detergent fiber (ADF), and neutral detergent fiber (NDF). Components examined in grain samples included proximates carbohydrates by calculation, ADF, NDF, total dietary fiber (TDF), amino acid composition, fatty acid composition, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), vitamins [vitamin B_1 (thiamin), vitamin B_2 (riboflavin), vitamin B_6 (pyridoxine), vitamin E, niacin, and folic acid), furfural, trypsin inhibitor, raffinose, inositol, phytic acid, *p*-coumaric acid, and ferulic acid. A range of commercially available conventional corn hybrids were also included as reference substances for each field trial to provide data for the development of a 99% tolerance interval for each component analyzed. Both univariate and multivariate statistical analyses were utilized.

MATERIALS AND METHODS

Maize Samples for Compositional Analysis. The maize hybrids discussed in this paper, MON 810 × NK603, MON 863 × MON 810, MON 863 × NK603, and MON 863 × NK603 × MON 810, were produced by conventional breeding of individual inbreds containing the single events MON 810 (IP1), NK603 (HT), and MON 863 (IP2). MON 810 contains a gene that produces a variant of the insecticidal protein Cry1Ab derived from *Bacillus thuringiensis* and confers resistance to the European corn borer (ECB) and other lepidopteran insect pests. NK603 contains two genes that express 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) proteins from *Agrobacterium* sp. strain CP4 that confer tolerance to glyphosate, the active ingredient in the Roundup family of herbicides.[14] The MON 863 event contains the gene that expresses a variant of the wild-type Cry3Bb1 insecticidal protein from *B. thuringiensis*, which protects maize plants from feeding damage caused by corn rootworm (CRW, *Diabrotica*).[15] For each biotechnology-derived hybrid a conventional (nontransgenic) maize hybrid with comparable background genetics was used as the control. Commercial, conventional hybrids were included as references and grown concurrently with the trait containing hybrids and their respective comparators.

MON 810 × NK603. In 2000, three replicated trials at L'Isle Jordain, Samatan, and Labrihe, France, were planted with MON 810 × NK603, the near-isogenic control, and five reference maize hybrids randomly assigned within each of four replication blocks in a randomized complete block design. Within each replicate, events were grouped according to herbicide tolerance. Herbicide-tolerant plots received a single application of Roundup herbicide containing 360 mg/L glyphosate acid equivalent at a rate of 3 L/ha.

MON 863 × MON 810. Four replicated trials were grown in 1999 at two sites in Fontezuela and at one site each in Salto and Rojas, Argentina. At each site, MON 863 × MON 810, the near-isogenic control, and four commercial references were planted as four replicates in a randomized complete block design.

MON 863 × NK603 and MON 863 × NK603 × MON 810. In 2002−2003 replicated trials were planted at four sites in Argentina, two in Buenos Aires (Pergamino and Tacuari-Salto) and two in Cordoba (Marfredi and Marcos Juarez). MON 863 × NK603 and MON 863 × NK603 × MON 810, the near-isogenic control, and four commercial,

Table 2. Proximate and Fiber Composition of Grain from MON 810 × NK603 and MON 863 × MON 810

	MON 810 × NK603 (IP1 × HT)			MON 863 × MON 810 (IP2 × IP1)				
component[a]	IP1 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	IP2 × IP1 mean (range)	control[b] mean (range)[d]	commercial references[c] [99% tolerance interval][e]	lit. range[d]	ILSI[k] range[d]
ash	1.50 (1.26−1.74)	1.50 (1.23−2.24)	(1.07−2.25) [1.06, 1.69]	1.49 (1.31−1.64)	1.51 (1.32−1.80)	[0.97, 1.76]	0.89−6.28[f] 1.1−3.9[g]	0.616−6.282
carbohydrates	84.23 (83.24−87.01)	84.73 (82.73−86.68)	(82.85−89.50) [79.23, 92.35]	84.41 (81.62−86.06)	84.49 (83.84−85.92)	[77.60, 92.24]	77.4−87.2[f] 82.2−88.1[h]	77.4−89.5
moisture	14.28 (12.50−16.70)	13.72 (12.30−15.10)	(7.02−13.70) [0.34, 18.55]	12.88 (11.40−16.30)	12.73 (11.60−15.30)	[0, 20.94]	7−23[g] 8.18−26.2[f]	6.1−40.5
protein	11.04[m] (8.23−12.05)	10.37 (8.33−12.10)	(6.15−11.75) [3.27, 15.87]	10.32 (8.70−12.66)	10.40 (9.30−10.92)	[3.37, 16.57]	6−12[g] 9.7−16.1[i]	6.15−17.26
fat	3.23 (2.73−3.72)	3.40 (3.17−3.93)	(2.35−4.12) [1.65, 4.90]	3.77 (3.27−4.42)	3.60 (2.83−3.94)	[1.26, 6.25]	2.48−4.81[f] 3.1−5.7[g]	1.742−5.823
ADF[l]	3.59 (2.60−4.84)	3.35 (2.51−4.00)	(2.51−5.59) [2.24, 5.25]	3.08 (2.19−4.08)	3.25 (2.58−4.44)	[1.35, 5.75]	2.46−11.34[f,h] 3.3−4.3[g]	1.82−11.34
NDF[l]	14.07[m] (9.44−18.91)	12.28 (10.37−15.79)	(8.72−17.75) [4.02, 19.77]	10.52 (8.48−13.14)	11.60 (8.49−18.12)	[4.35, 17.20]	7.58−15.91[f] 8.3−11.9[g]	5.59 −22.64

[a] Percent dry weight of sample, except moisture. [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Sidhu et al.[54] [g] Watson.[56] [h] Ridley et al.[13] [i] Jugenheimer.[57,58] [k] International Life Sciences crop composition database, Ridley et al.[55] [l] ADF, acid detergent fiber; NDF, neutral detergent fiber;. [m] Statistically significantly different ($p < 0.05$) from control.

conventional maize references were planted in a randomized complete block design with three replicates per block. A total of 13 unique commercial references were included because three of the same references were grown at two sites. All plots containing herbicide-tolerant traits received a single application of Roundup UltraMAX herbicide at a product rate of approximately 1.9 L/ha.

For both the European and Argentine field trials, the genetic purity of the maize plants was maintained by bagging the tassels and ear shoots at anthesis and self-pollinating each plant by hand. The forage was collected at the late dough/early dent stage and frozen on dry ice, and the ears were collected at normal kernel maturity. Maize ears were dried to a moisture level of approximately 12−14%, and kernels were shelled from the ears. Forage and grain samples were shipped to Monsanto, St. Louis, MO, where they were ground to a fine powder in the presence of dry ice and maintained frozen until required for compositional analysis.

The identity of the forage and grain samples was confirmed by chain of custody records and molecular analysis of the grain for the presence or absence of the events included in the individual field productions.

Compositional Analyses. Components assessed are described in the main text and listed in Tables 1−6 and in the Supporting Information. 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 levels were estimated by determining the total nitrogen content using the Kjeldahl method.[16,17] The protein was calculated from the total nitrogen (N) using the formula N × 6.25. The fat content of the grain was determined by Soxhlet extraction.[18] The fat content of the forage was determined by acid hydrolysis, followed by extraction with ether and hexane.[19,20]

The ash content was determined by ignition in an electric furnace and quantitation of the ash by gravimetric analysis.[21] The moisture content was determined by the loss of weight upon drying in a vacuum oven at 100 °C to a constant weight.[22,23] Carbohydrate levels were estimated using the fresh weight-derived data and the following equation:[24]

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

Fiber Analysis. The ADF was determined by treating the samples 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.[25] The NDF was determined by treating the samples 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.[25,26] For TDF, duplicate samples were treated with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values.[27]

Minerals. To determine the levels of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc, inductively coupled plasma emission spectrometry was used as described in the AOAC methods[28,29] and Dahlquist and Knoll.[30] The sample was dried, precharred, and ignited in an electric furnace overnight at approximately

Table 3. Total Amino Acid Composition of Grain from MON 863 × NK603 and MON 863 × NK603 × MON 810

	MON 863 × NK603 (IP2 × HT)			MON 863 × NK603 × MON 810 (IP2 × HT × IP1)			
component[a]	IP2 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	IP2 × HT × IP1 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	literature[f] range[d]
alanine	7.60[g] (7.42−7.78)	7.69 (7.55−7.82)	(7.46−8.13) [7.14, 8.33]	7.66 (7.35−7.91)	7.69 (7.55−7.82)	(7.46−8.13) [7.14, 8.33]	6.4−9.9
arginine	4.72 (4.48−5.17)	4.53 (4.18−4.93)	(3.91−5.35) [3.29, 5.97]	4.60 (4,25−5.21)	4.53 (4.18−4.93)	(3.91−5.35) [3.29, 5.97]	2.9−5.9
aspartic acid	6.71[g] (6.40−7.10)	6.57 (6.13−6.73)	(6.09−7.18) [5.49, 7.75]	6.56 (6.21−6.91)	6.57 (6.13−6.73)	(6.09−7.18) [5.49, 7.75]	5.8−7.2
cystine	2.05 (1.91−2.30)	2.12 (2.00−2.23)	(1.74- 2.35) [1.50, 2.56]	2.03 (1.85−2.21)	2.12 (2.00−2.23)	(1.74−2.35) [1.50, 2.56]	1.2−1.6
glutamic acid	19.16 (18.75−19.50)	19.36 (18.91−19.67)	(18.08−20.44) [17.23, 21.57]	19.31 (18.79−19.89)	19.36 (18.91−19.67)	(18.08−20.44) [17.23, 21.57]	12.4−19.6
glycine	3.90 (3.65−4.14)	3.81 (3.73−4.05)	(3.20−4.38) [2.69, 4.89]	3.82 (3.59−4.17)	3.81 (3.73−4.05)	(3.20−4.38) [2.69, 4.89]	2.6−4.7
histidine	2.99 (2.85−3.09)	2.97 (2.89−3.11)	(2.67−3.22) [2.42, 3.43]	2.95 (2.81−3.09)	2.97 (2.89−3.11)	(2.67−3.22) [2.42, 3.43]	2.0−2.8
isoleucine	3.42 (3.24−3.58)	3.42 (3.18−3.62)	(3.13−3.61) [3.15, 3.70]	3.45 (3.27−3.62)	3.42 (3.18−3.62)	(3.13−3.61) [3.15, 3.70]	2.6−4.0
leucine	12.45[g] (11.57−13.09)	12.76 (12.16−13.19)	(11.42−14.08) [9.87, 15.67]	12.71 (11.57−13.34)	12.76 (12.16−13.19)	(11.42−14.08) [9.87, 15.67]	7.8−15.2
lysine	3.34 (3.06−3.74)	3.24 (3.11−3.48)	(2.70−3.98) [1.99, 4.56]	3.27 (2.98−3.65)	3.24 (3.11−3.48)	(2.70−3.98) [1.99, 4.56]	2.0−3.8
methionine	2.13 (1.73−2.37)	2.20 (1.90−2.35)	(1.77−2.50) [1.52, 2.71]	2.07[g] (1.81−2.35)	2.20 (1.90−2.35)	(1.77−2.50) [1.52, 2.71]	1.0−2.1
phenylalanine	5.08 (4.87−5.22)	5.11 (4.95−5.25)	(4.82−5.52) [4.45, 5.79]	5.12 (4.84−5.24)	5.11 (4.95−5.25)	(4.82−5.52) [4.45, 5.79]	2.9−5.7
proline	9.04 (8.60−9.36)	9.07 (8.87−9.23)	(8.13−9.42) [7.77, 9.92]	9.25 (9.01−9.80)	9.07 (8.87−9.23)	(8.13−9.42) [7.77, 9.92]	6.6−10.3
serine	5.14 (4.86−5.50)	5.14 (4.81−5.44)	(4.75−5.42) [4.71, 5.49]	5.10 (4.86−5.38)	5.14 (4.81−5.44)	(4.75−5.42) [4.71, 5.49]	4.2−5.5
threonine	3.49[g] (3.34−3.70)	3.39 (3.09−3.61)	(3.12−3.75) [2.81, 3.97]	3.44 (3.24−3.72)	3.39 (3.09−3.61)	(3.12−3.75) [2.81, 3.97]	2.9−3.9
tryptophan	0.60 (0.56−0.65)	0.60 (0.51−0.70)	(0.51−0.76) [0.44, 0.78]	0.61 (0.53−0.72)	0.60 (0.51−0.70)	(0.51−0.76) [0.44, 0.78]	0.5−1.2
tyrosine	3.50 (2.78−3.72)	3.36 (2.29−3.70)	(2.42−3.86) [2.49, 4.55]	3.36 (2.47−3.73)	3.36 (2.29−3.70)	(2.42−3.86) [2.49, 4.55]	2.9−4.7

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Table 3. Continued

component[a]	MON 863 × NK603 (IP2 × HT) IP2 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	MON 863 × NK603 × MON 810 (IP2 × HT × IP1) IP2 × HT × IP1 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	literature[f] range[d]
valine	4.70 (4.48−4.89)	4.65 (4.36−4.86)	(4.32−5.08) [4.21, 5.20]	4.69 (4.55−4.85)	4.65 (4.36−4.86)	(4.32−5.08) [4.21, 5.20]	2.1−5.2

[a] Percent of total amino acids. [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Most literature reports on amino acid composition express values as percent of total amino acids (Watson[56]). [g] Statistically significantly different ($p < 0.05$) from control.

Table 4. Fatty Acid Content Composition of Grain from MON 863 × NK603 and MON 863 × NK603 × MON 810

component[a]	MON 863 × NK603 (IP2 × HT) IP2 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	MON 863 × NK603 × MON 810 (IP2 × HT × IP1) IP2 × HT × IP1 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit. range[d]	ILSI[g] range[d]
16:0 palmitic	13.93 (13.56−14.50)	13.52 (8.15−14.52)	(8.03−13.16) [4.45, 16.08]	13.88 (13.32−14.51)	13.52 (8.15−14.52)	(8.03−13.16) [4.45, 16.08]	7−19[f]	7.94−20.71
16:1 palmitoleic	0.12 (0.11−0.13)	0.12 (0.11−0.13)	(0.056- 0.16) [0, 0.23]	0.13 (0.11−0.13)	0.12 (0.11−0.13)	(0.056−0.16) [0, 0.23]	1[f]	0.095−0.447
18:0 stearic	1.62 (1.52−1.70)	1.68 (1.51−2.20)	(1.65−2.27) [1.37, 2.53]	1.60[h] (1.53−1.68)	1.68 (1.51−2.20)	(1.65−2.27) [1.37, 2.53]	1−3[f]	1.02−3.40
18:1 oleic	30.30[h] (28.97−31.30)	29.48 (26.10−30.90)	(22.02−35.24) [15.95, 40.11]	31.73[h] (30.58−32.48)	29.48 (26.10−30.90)	(22.02−35.24) [15.95, 40.11]	20−46[f]	17.4−40.2
18:2 linoleic	51.88[h] (50.28−53.44)	53.06 (50.62−61.62)	(48.77−62.71) [42.62, 72.43]	50.63[h] (49.17−52.07)	53.06 (50.62−61.62)	(48.77−62.71) [42.62, 72.43]	35−70[f]	36.2−66.5
18:3 linolenic	1.14 (1.07−1.24)	1.16 (0.98−1.25)	(0.86−1.44) [0.58, 1.73]	1.05[h] (0.97−1.12)	1.16 (0.98−1.25)	(0.86−1.44) [0.58, 1.73]	0.8−2[f]	0.57−2.25
20:0 arachidic	0.45[h] (0.42−0.49)	0.42 (0.38−0.46)	(0.38−0.54) [0.31, 0.59]	0.44[h] (0.42−0.49)	0.42 (0.38−0.46)	(0.38−0.54) [0.31, 0.59]	0.1−2[f]	0.279−0.965
20:1 eicosenoic	0.37 (0.33−0.41)	0.37 (0.31−0.40)	(0.27−0.38) [0.23, 0.44]	0.37 (0.32−0.41)	0.37 (0.31−0.40)	(0.27−0.38) [0.23, 0.44]		0.170−1.917
22:0 behenic	0.19 (0.18−0.21)	0.18 (0.14−0.20)	(0.14−0.23) [0.095, 0.27]	0.17[h] (0.16−0.19)	0.18 (0.14−0.20)	(0.14−0.23) [0.095, 0.27]		0.110−0.349

[a] Expressed as % of total fatty acid. The method included the analysis of the following fatty acids, which were not detected in the majority of samples analyzed: caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1), pentadecanoic acid (15.0), pentadecenoic acid (15:1), heptadecanoic acid (17:0), heptadecenoic acid (17:1), γ-linolenic acid(18:3), eicosadienoic acid (20:2), eicosatrienoic acid (20:3), and arachidonic acid (20:4). [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Watson.[59] [g] International Life Sciences crop composition database, Ridley et al.[55] [h] Statistically significantly different ($p < 0.05$) from control.

500 °C. The resulting ash was treated with hydrochloric acid, evaporated to dryness, and 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 inductively coupled plasma emission spectrometry, with the emission of standard solutions.

Amino Acid Composition. Three procedures described in the literature[31] were used to determine the concentration of 18 amino acids in maize grain. The procedure for tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to 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 utilizing postcolumn ninhydrin derivatization.

Table 5. Mineral Composition of Grain from MON 810 × NK603 and MON 810 ×MON 863

	MON 810 × NK603 (IP1 × HT)			MON 863 × MON 810 (IP2 × IP1)				
component[a]	IP1 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	IP2 × IP1 mean (range)	control[b] mean (range)[d]	commercial references[c] [99% tolerance interval][e]	lit. range[d]	ILSI[h] range[d]
calcium	0.0052 (0.0040−0.0072)	0.0052 (0.0044−0.0070)	(0.0030−0.0083) [0.0018, 0.0093]	0.0041 (0.0027−0.0049)	0.0044 (0.0033−0.0055)	[0.0016, 0.0090]	0.01−0.1[f,g]	0.00127−0.02084
copper	1.82 (1.48−2.15)	1.68 (1.53−1.91)	(0.85−3.54) [0, 3.69]	1.98[i] (1.70−2.26)	2.82 (2.32−3.22)	[0, 3.91]	0.9−10[f,g]	0.73−18.50
iron	25.06 (19.70−30.97)	24.27 (20.28−30.65)	(10.58−28.04) [4.13, 36.90]	22.61[i] (18.35−27.15)	25.33 (22.84−27.19)	[2.49, 37.25]	1−100[f,g]	10.42−49.07
magnesium	0.12 (0.11−0.14)	0.11 (0.11−0.12)	(0.085−0.15) [0.074, 0.16]	0.13 (0.11−0.16)	0.13 (0.12−0.14)	[0.074, 0.17]	0.09 −1[f,g]	0.0594 −0.194
manganese	5.38 (3.92−6.07)	4.98 (3.72−6.10)	(3.67−9.39) [0.82, 11.04]	7.69 (5.55−10.38)	7.58 (6.04−9.05)	[0.90, 11.97]	0.7−54[f,g]	1.69−14.30
phosphorus	0.34 (0.31−0.38)	0.32 (0.31−0.34)	(0.25−0.38) [0.27, 0.37]	0.34 (0.25−0.41)	0.36 (0.31−0.39)	[0.25, 0.39]	0.26−0.75[f,g]	0.147−0.533
potassium	0.39 (0.36−0.43)	0.38 (0.36−0.40)	(0.29−0.47) [0.23, 0.50]	0.41[i] (0.33−0.46)	0.43 (0.41−0.46)	[0.23, 0.52]	0.32−0.72[f,g]	0.181−0.603
zinc	24.88 (19.12−29.26)	24.98 (20.79−28.85)	(16.67−31.38) [7.52, 38.15]	25.75[i] (22.07−31.31)	28.13 (24.38−31.63)	[6.10, 40.05]	12−30[f,g]	6.5−37.2

[a] Percent dry weight of sample, except copper, iron, manganese and zinc which are expressed as mg/kg dry weight. [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Watson.[56] [g] Watson.[59] [h] International Life Sciences crop composition database, Ridley et al.[55] [i] Statistically significantly different ($p < 0.05$) from control.

Fatty Acid Composition. The lipid in the grain samples was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponified 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 using external standards for quantitation.[32]

Vitamin E. The amount of vitamin E in the grain was determined following saponification to break down any fat and release the vitamin as described by Cort et al.[33]

The saponified mixture was extracted with ethyl ether and the amount of vitamin E determined by normal phase HPLC with fluorescence detection using external standard calibration.

Riboflavin. The amount of riboflavin was measured in grain samples following hydrolysis with dilute acid as described in the literature.[34] 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 various amounts of a riboflavin standard.

Thiamin. Thiamin was extracted by autoclaving the grain samples in the presence of weak acid followed by phosphatase digestion to release any bound thiamin.[35−37] Thiamin was purified from the resulting solution by ion exchange chromatography and then converted to thiochrome with potassium ferricyanide. The thiochrome was extracted into isobutyl alcohol, and the levels were quantitated fluorometrically.

Folic Acid. Folic acid was analyzed using a published procedure[38,39] 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 various amounts of a folic acid standard.

Pyridoxine. The sample was hydrolyzed with dilute sulfuric acid in the autoclave, and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces carlsbergensis*, with the growth response of a pyridoxine standard. The response was measured turbidimetrically.[40] Results were reported as pyridoxine hydrochloride.

Phytic Acid. Phytic acid was quantitated in grain following extraction using ultrasonication as described by Lehrfeld.[41,42] Purification and concentration of the extract were conducted using a silica-based anion exchange column followed by quantitation using a polymer HPLC column (PRP-1, 5 mm, 150 mm × 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[43] in which the samples were extracted with methanol, and the extracts were hydrolyzed using 4 N sodium hydroxide, neutralized, and filtered. The levels of ferulic and *p*-coumaric acids were determined by reversed-phase HPLC with UV detection.

Furfural (2-Furaldehyde). The levels of furfural were determined using the method of Albala-Hurtado et al.[44] in which the maize grain was extracted with 4% trichloroacetic acid, centrifuged, filtered, and analyzed by reversed-phase HPLC with UV detection. The limit of quantitation (LOQ) for furfural was 0.5 ppm based on fresh weight.

Raffinose. The analysis of raffinose was based on two methods[45,46] 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-α-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoroacetic acid and analyzed by gas chromatography with flame ionization detection.

Table 6. Vitamin, Antinutrient, and Secondary Metabolite Composition of Grain from MON 863 × NK603 and MON 863 × NK603 × MON 810

	MON 863 × NK603 (IP2 × HT)			MON 863 × NK603 × MON 810 (IP2 × HT × IP1)				
component[a]	IP2 × HT mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	IP2 × HT × IP1 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit. range[d]	ILSI[h] range[d]
folic acid	0.63 (0.40−1.07)	0.64 (0.39−0.81)	(0.32−1.12) [0.20, 1.15]	0.74 (0.34−1.28)	0.64 (0.39−0.81)	(0.32−1.12) [0.20, 1.15]	0.3[f]	0.147−1.464
niacin	20.69 (19.27−22.50)	20.81 (19.42−22.05)	(18.23−31.02) [13.51, 34.47]	18.13[i] (15.81−19.89)	20.81 (19.42−22.05)	(18.23−31.02) [13.51, 34.47]	9.3−70[f,g]	10.37−46.94
riboflavin/vitamin B_2	1.31 (1.08−1.67)	1.36 (1.08−1.90)	(1.13−1.95) [0.63, 2.17]	1.27 (1.09−1.64)	1.36 (1.08−1.90)	(1.13−1.95) [0.63, 2.17]	3−8.6[f,g]	0.50−2.36
thiamin HCl/ vitamin B_1	3.98 (3.64−4.19)	3.92 (3.64−4.16)	(3.04−5.14) [2.42, 5.64]	4.01 (3.58−4.31)	3.92 (3.64−4.16)	(3.04−5.14) [2.42, 5.64]	0.25−5.6[f,g]	1.26−40.00
pyridoxine HCl/ vitamin B_6	5.50 (4.91−6.01)	5.40 (4.75−6.28)	(5.39−8.20) [3.75, 9.49]	5.40 (4.83−6.17)	5.40 (4.75−6.28)	(5.39−8.20) [3.75, 9.49]	5.3;[f] 9.6[g]	3.68−11.32
vitamin E	7.34 (6.93−8.18)	7.16 (6.70−8.02)	(7.11−13.72) [2.89, 17.33]	6.89 (5.85−8.44)	7.16 (6.70−8.02)	(7.11−13.72) [2.89, 17.33]	3−12.1[g] 17−47[f]	1.5−68.7
phytic acid (% DW)	0.63 (0.50−0.72)	0.60 (0.057−0.86)	(0.34−1.19) [0.49, 1.02]	0.61 (0.44−0.86)	0.60 (0.057−0.86)	(0.34−1.19) [0.49, 1.02]	0.48−1.12[j]	0.111−1.570
raffinose (% DW)	0.13 (0.090−0.17)	0.13 (0.085−0.17)	(0.057−0.20) [0.016, 0.19]	0.13 (0.11−0.16)	0.13 (0.085−0.17)	(0.057−0.20) [0.016, 0.19]	0.08−0.30[f]	0.020−0.320
ferulic acid	2012[i] (1667−2528)	2187 (1333−2694)	(1478−2669) [948, 3316]	2007[i] (1458−2452)	2188 (1333−2694)	(1478−2669) [948. 3316]	113−1194[k] 3000[l]	291.9−3885.8
p-coumaric acid	167.2 (137.8−196.6)	177.4 (116.4−226.7)	(138.4−289.4) [13.5, 374.2]	169.7 (128.8−210.4)	177.4 (116.4−226.7)	(138.4−289.4) [13.5, 374.2]	22−75[k]	53.4−576.2

[a] Expressed as mg/kg dry weight except as indicated . [b] Nontransgenic control. [c] Commercial references planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial maize population where negative limits are set to zero. [f] Watson.[56] [g] Watson.[59] [h] International Life Sciences crop composition database, Ridley et al.[55] [i] Statistically significantly different ($p < 0.05$) from control. [j] Ridley et al.[13] [k] Classen et al.[60] [l] Dowd and Vega.[61]

Inositol. Grain was assayed for inositol by extraction with dilute hydrochloride at high temperature. Inositol in the resulting extract was determined by comparing the growth response of the sample measured turbidiometrically using *S. carlsbergenensis* with the growth response of an inositol standard.[47,48]

Trypsin Inhibitor. The trypsin inhibitor activity in the samples was based on AOCS method BA 12-75[49] and was determined by suspending the ground, defatted sample in 0.1 N sodium hydroxide. An appropriate dilution of the suspension was made, and an increasing series of aliquots of the diluted suspension was mixed with trypsin and benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA). The reaction was incubated for 10 min and stopped by the addition of acetic acid, and the filtered or centrifuged suspension was measured at 410 nm. One trypsin inhibitor unit was defined as an increase of 0.01 absorbance unit at 410 nm per 10 mL of the reaction mixture under the condition of the assay.

Niacin. The sample 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 measured turbidimetrically, using the bacteria *Lactobacillus plantarum*, with the growth response of a niacin standard (USP, niacin, 100%).[50]

Statistical Analysis of Composition Data. In all, 59 analytical components (7 in forage, 52 in grain) were statistically analyzed for MON 810 × NK603, and 58 components (7 in forage, 51 in grain) were analyzed for MON 863 × MON 810. For these two combined trait hybrids, calcium and phosphorus were not measured in forage, and vitamin B_6 and niacin were not measured in grain. For MON 863 × NK603 and MON 863 × NK603 × MON 810 a total of 62 analytical components were statistically analyzed (9 in forage and 53 in grain). For these two combined trait hybrids, trypsin inhibitor and inositol were not analyzed. The following 15 analytes with >50% of the observations at or below the LOQ of the assay were excluded from statistical analysis: sodium, furfural, 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 γ-linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid. In some cases when there were fewer than 50% of the analyses for a particular analyte below the LOQ, a value of half the LOQ was assigned to include a complete data set in the analysis. Except for moisture, fatty acids, and amino acids, all component values were converted from a fresh weight to a dry weight basis and into their respective units.

PRESS residuals were used to identify outliers. A PRESS residual is the difference between any value and its predicted value from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be with ± 3. Extreme data points that were outside the ± 6 studentized PRESS residual ranges were considered for exclusion, as outliers, from the final analyses.

Components were statistically analyzed using a mixed-model analysis of variance. The replicated sites were analyzed both individually (individual site data not presented) and in combined-site analyses.

Combined site analyses for MON 810 × NK603 used the model

$$Y_{ijkl} = U + S_i + L_j + B(L)_{jk} + S^*L_{ij} + H(B)_{kl} + e_{ijkl}$$

where Y_{ijkl} = unique individual observations, U = overall mean, S_i = substance effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, S^*L_{ij} = random location by substance interaction effect, $H(B)_{kl}$ = herbicide spray group within block effect, and e_{ijkl} = residual error.

Combined-site analyses for MON 863 × MON 810 used the model

$$Y_{ijkl} = U + S_i + L_j + B(L)_{jk} + H^*L_{jl} + H(S)_{il} + e_{ijkl}$$

where Y_{ijkl} = unique individual observations, U = overall mean, S_i = substance effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, H^*L_{jl} = random location by hybrid interaction effect, $H(S)_{il}$ = hybrid within substance effect, and e_{ijkl} = residual error.

The combined-site analyses for the MON 863 × NK603 and MON 863 × NK603 × MON 810 used the model

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

where Y_{ijk} = unique individual observation, U = overall mean, S_i = substance effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LS_{ij} = random location by substance interaction effect, and e_{ijk} = residual error. For each compositional component, the forage and harvested seed from the test substance were compared to the conventional control.

The reference substance data were used to develop population tolerance intervals for each field production. A tolerance interval is an interval that one can claim, with a specified degree of confidence, 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. For MON 810 × NK603 and MON 863 × MON 810, five and four references, respectively, per site were grown, and for these field trials the same reference hybrids were used at each site. The data sets from these hybrids were supplemented with additional data from six commercial hybrids grown in Europe in 1999 and analyzed concurrently for the construction of a 99% tolerance interval describing compositional variability in a population of commercial maize hybrids. For MON 863 × NK603 and MON 863 × NK603 × MON 810 13 unique references derived from four hybrids grown per site. Tolerance interval estimation was based upon one observation per unique reference substance. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS (SAS is a registered trademark of SAS Institute Inc.) software was used to generate all summary statistics and perform all analyses (SAS Institute, 2002−2003). All statistical analyses were conducted by Certus International, Inc. (Chesterfield, MO).

Multivariate Analysis of Composition. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) of compositional data were performed using JMP 8.1 software. The multivariate analyses were conducted using mean values for compositional components analyzed for MON 863 × NK603, MON 863 × NK603 × MON 810, and the single traits (MON 863, MON 810, and NK603, data not shown).

RESULTS AND DISCUSSION

Although the composition of crops containing a single biotechnology trait has been reported extensively in the literature,[11−13,51] to date, few composition data have been reviewed in the literature for crops containing biotech traits combined by conventional breeding. Therefore, the compositions of three "double combinations" (MON 810 × NK603, MON 863 × MON 810, and MON 863 × NK603) and one "triple combination" (MON 863 × NK603 × MON 810) were compared to their respective near-isogenic, conventional control hybrids. The multitrait biotechnology crops covered in this paper were evaluated for proximates (protein, fat, ash, and moisture), carbohydrate by calculation, fiber, amino acids, fatty acids, vitamins, minerals, antinutrients, and secondary metabolites. Data are contained in Tables 1−6 in the text and Tables S1−S7 in the Supporting Information.

Proximate and Fiber Composition of Forage and Grain. Compositional analysis results for the combined site analysis of the proximate and fiber for forage and grain from MON 810 × NK603 and MON 863 × MON 810 are presented in Tables 1 and 2. These results demonstrated that the levels of proximate components and fiber for MON 810 × NK603 and MON 863 × MON 810 were comparable to those in the conventional control. Only one statistically significant difference (ash) was observed for MON 810 × NK603, as were two differences (protein and NDF) for MON 863 × MON 810 in the forage (Table 1). In all cases the relative magnitude of the difference was <15%, and the values of the test fell within the 99% tolerance interval for commercial hybrids in the trials, literature values, and the range of values in the International Life Sciences Institute (ILSI) crop composition database. Evaluation of the proximates in grain (Table 2) showed two statistically significant differences (protein and NDF) for MON 810 × NK603 and no differences for MON 863 × MON 810. Similarly, the relative magnitudes of the differences were relatively small (<15%), and the values for the combined trait hybrids were within the natural variability of commercial hybrids represented by the 99% tolerance interval, literature values, and ILSI database ranges.

Similar results were obtained for proximate and fiber values from forage and grain for MON 863 × NK603 and MON 863 × NK603 × MON 810 (Supporting Information, Tables S1 and S2). Comparisons with the conventional control were either not statistically significantly different or were within the 99% tolerance interval determined for commercial varieties evaluated for each respective field trial and within published ranges.

Amino Acid Composition of Grain. The levels of the 18 amino acids expressed as percent of total amino acids measured in the grain of MON 863 × NK603 and MON 863 × NK603 × MON 810 were comparable to those in the grain of their

Table 7. Summary of Results for Multitrait Maize Products

product	trait	location	no. of combined-site comparisons	no. of stat diff, $p < 0.05$	no. of diff with test values in 99% tolerance interval
MON 810 × NK603	IP1 × HT	Europe	59[a]	15 (25.4%)	15 (100%)
MON 863 × MON 810	IP2 × IP1	Argentina	58[a]	12 (20.7%)	12 (100%)
MON 863 × NK603	IP2 × HT	Argentina	62[b]	9 (14.5%)	9 (100%)
MON 863 × NK603 × MON 810	IP2 × HT × IP1	Argentina	62[b]	13 (21.0%)	13(100%)
all			241	49 (20.3%)	49 (100%)

[a] Results for MON 810 × NK603 and MON 863 × MON 810 are contained in Tables 1, 2, and 5 and Tables S3, S4, S6, and S7 in the Supporting Information. [b] Results for MON 863 × NK603 and MON 863 × NK603 × MON 810 are contained in Tables 3, 4, and 6 and Tables S1, S2 and S5 in the Supporting Information.

respective conventional control (Table 3). For the four amino acids (alanine, aspartic acid, leucine, and threonine) found to be statistically significantly different when MON 863 × NK603 was compared to its near-isogenic control, the relative magnitude of the differences was small (<5%). Only one amino acid, methionine, for MON 863 × NK603 × MON 810 was found to be significantly different from the near-isogenic control. In all cases for both MON 863 × NK603 and MON 863 × NK603 × MON 810 the amino acid values fell within the 99% tolerance interval for commercial hybrids and within literature values. Analyses of amino acids for the two other multitrait maize hybrids, MON 810 × NK603 and MON 863 × MON 810 (see the Supporting Information, Table S3) were similar, and when statistically significant differences were found, the values from the combined trait hybrids were within the 99% tolerance interval for commercial hybrids and values published in the literature.

Fatty Acid Composition of Grain. The levels of nine fatty acids in the grain of MON 863× NK603 and MON 863 × NK603 × MON 810 were compared to those observed in the grain of the conventional control (Table 4). Statistically significant differences were seen for three fatty acids in MON 863× NK603 and for six fatty acids in MON 863 × NK603 × MON 810. The relative magnitude of the differences were all <10%, and in all cases the mean values for the event containing hybrids fell within the 99% tolerance interval for the commercial hybrids, the range of values in the published literature, and the ILSI crop composition database. Analyses of fatty acids for the two other multitrait maize hybrids, MON 810 × NK603 and MON 863 × MON 810 (see the Supporting Information, Table S4), were similar, and when statistically significant differences were found, the values from the combined trait hybrids were within the 99% tolerance interval for commercial hybrids and values published in the literature.

Mineral Composition of Grain. Minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc) in the grain of MON 810 × NK603 and MON 863 × MON 810 were analyzed and compared with their conventional controls as shown in Table 5. No statistically significant differences in the minerals for MON 810 × NK603 compared to its control were observed, whereas four differences (copper, iron, potassium, and zinc) were seen for MON 863 × MON 810. With the exception of copper, the magnitude of the differences between MON 863 × MON 810 and its control was <11%. For copper the difference between MON 863 × MON 810 and the control was approximately 30%, which likely reflects the influence of variable soil mineral content. In all cases the mineral levels were within the 99% tolerance interval for conventional hybrids and the range of values for maize published in the literature. Similar results were obtained for MON 863× NK603 and MON 863 × NK603 × MON 810 as shown in Table S5 of the Supporting Information.

Vitamin, Antinutrient, and Secondary Metabolite Composition of Grain. Compositional analysis showed that the levels of folic acid, niacin, riboflavin (vitamin B_2), thiamin (vitamin B_1), pyridoxine (vitamin B_6), and vitamin E in the grain of MON 863 × NK603, MON 863 × NK603 × MON 810 were comparable to those of the conventional controls (Table 6). The only statistically significant difference was for niacin in MON 863 × NK603 × MON 810, and in this case the mean value for niacin was within the tolerance interval determined for commercial varieties evaluated at each respective field trial and within literature ranges. These results demonstrated that the levels of niacin and the other vitamins in the grain of MON 863 × NK603, MON 863 × NK603 × MON 810 were within the same population as those of the conventional, commercially available maize references.

Phytic acid, the hexakis-*o*-phosphate of *myo*-inositol, has been suggested as an antinutrient because it can limit the uptake of minerals such as calcium in higher animals. Raffinose is a nondigestible oligosaccharide that is considered to be an antinutirent due to gas production and resulting flatulence caused by its consumption.[9] Ferulic and *p*-courmaric acids are secondary metabolites derived from the aromatic amino acids phenylalanine and tyrosine in plants and serve as precursors for a large group of phenylpropanoid compounds. No statistically significant differences between MON 863 × NK603 and MON 863 × NK603 × MON 810 and their respective controls were found for phytic acid, raffinose, and *p*-coumaric acid (see Table 6). Ferulic acid was shown to be statistically different for both MON 863 × NK603 and MON 863 × NK603 × MON 810, but the magnitude of the difference was small (<10%) and the values for both multitrait biotech products were within the 99% tolerance interval for conventional hybrids and the range of values for maize published in the literature. Similar results were observed for vitamins, antinutrients, and secondary metabolites for MON 810 × NK603 and MON 863 × MON 810 as shown in the Supporting Information, Tables S6 and S7.

Summary of Results. The summary of compositional analyses for grain of MON 810 × NK603, MON 863 × MON 810, MON 863 × NK603, and MON 863 × NK603 × MON 810 is shown in Table 7. Overall, a total of 241 statistical comparisons between the multitrait biotechnology crop and its corresponding conventional controls were conducted. Of these comparisons 192 (79.7%) were not statistically significantly different ($p > 0.05$), and all 49 of the differences were within the 99% tolerance

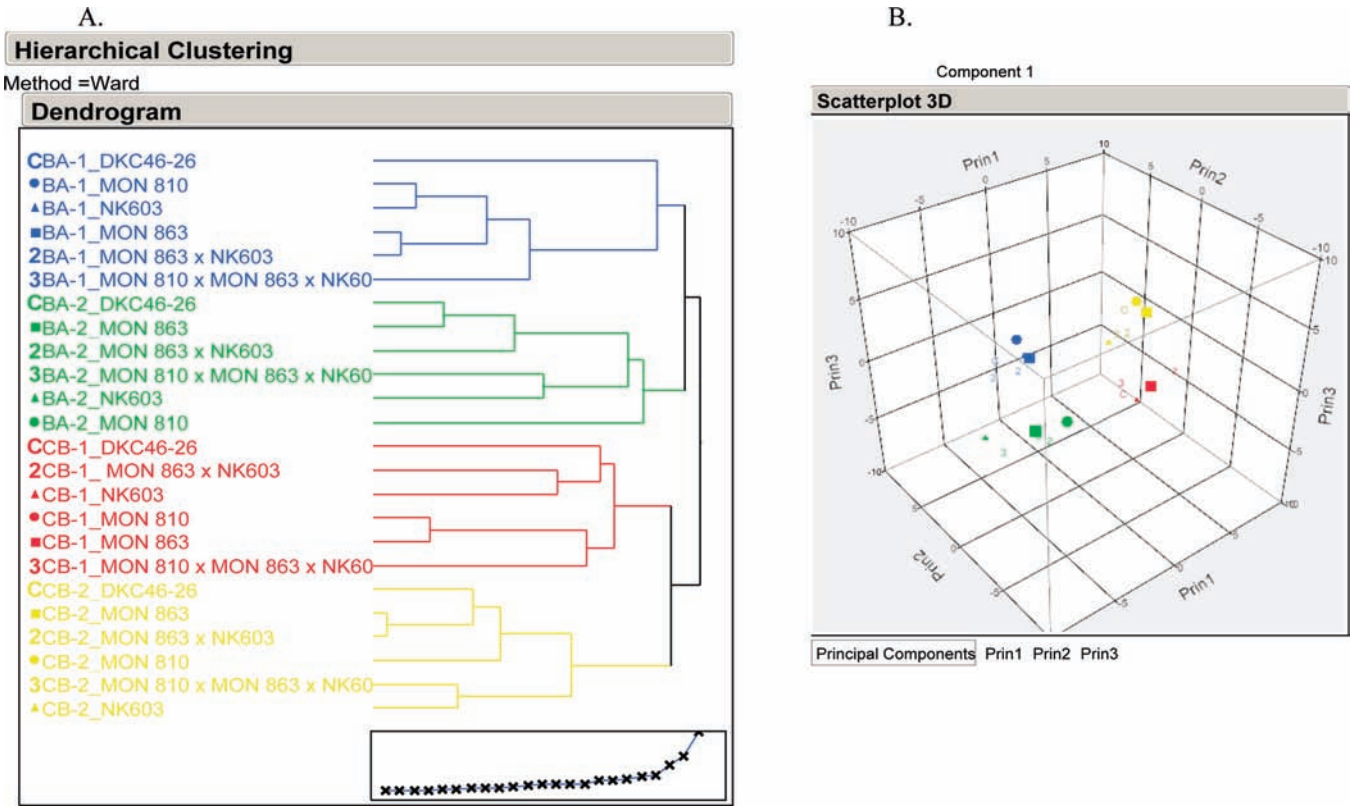

Figure 1. Multivariate analysis of composition data for grain of MON 863 × NK603 and MON 863 × NK603 × MON 810 grown at four field sites in Argentina: (A) hierarchical clustering analysis (HCA); (B) principal component analysis (PCA). Field sites are BA-1 (blue), BA-2 (green), CB-1 (red), and CB-2 (yellow). Specific materials are identified by letter, symbol, or number as indicated: C, control (DKC46-26); single trait, MON 810 (●); NK603 (▲), MON 863 (■); MON 863 × NK603 (2); MON 863 × NK603 × MON 810 (3).

interval for commercial hybrids grown the same field or related field trials. Five percent of the statistical comparisons are expected to be different on the basis of chance alone.

Multivariate Analyses. Results from the univariate analysis confirmed the compositional equivalence of the combined-trait products to the conventional control. Multivariate analysis was conducted on a representative data set to present a graphical overview of compositional variation within that data set. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were conducted for MON 863× NK603 and MON 863 × NK603 × MON 810 data sets from four replicated sites, and the results are shown in Figure 1. The purpose of the HCA conducted on the maize forage and grain samples was to group these samples into subsets or "clusters", such that the composition of those within each cluster was more closely related to one another than to the composition of maize samples assigned to different clusters. The four individual sites in Argentina (BA-1, BA-2, CD-1, and CD-2) are represented with different colors in Figure 1. It is clear from the dendrogram in panel A that the individual test and control substances from an individual site tend to group together. In other words, MON 863 × NK603 and MON 863 × NK603 × MON 810 and their control comparator (DKC46-26) are more similar at the BA-1 site than the combined trait hybrids and controls are to each other at different sites.

PCA is a data reduction technique and was used here in an exploratory and qualitative evaluation to determine how growing regions affected differences in compositional space. The three-dimensional score plot shown in panel B of Figure 1 is consistent with the HCA because compositional data from an individual site (represented by a different color) clustered together when the three principal components that account for a majority of the variability are plotted. The multivariate analysis demonstrated that variability in the compositional analysis for MON 863 × NK603, MON 863 × NK603 × MON 810, and their control comparators was primarily due to the growing region rather than differences between the multitrait biotechnology crop and its control. These results are consistent with demonstrations that single-trait biotechnology-derived products contribute minimally to compositional variation[51] and imply that this observation extends to multitrait products.

Conclusions. It has been shown that combining biotechnology-derived traits through conventional breeding of maize to produce multitrait products did not cause significant changes in any of the key forage or grain nutritional or antinutritional components analyzed in this study. Overall, results of compositional analyses derived from three separate studies demonstrated that the levels of key nutrients, antinutrients, and secondary metabolites in the grain and forage of MON 810 × NK603, MON 863 × MON 810, MON 863 × NK603, and MON 863 × NK603 × MON 810 were comparable to those of the conventional controls and commercially available maize. Variability in the compositional analysis for MON 863 × NK603, MON 863 × NK603 × MON 810, and their control comparators was shown to be primarily due to the growing region rather than differences between the multitrait biotechnology crop and its control. In addition, other studies with maize from these stacked products using broiler chickens have demonstrated nutritional equivalence of the single-trait products and the combined-trait products,

MON 810 × NK603 and MON 863 × MON 810.[52,53] In summary, these studies are consistent with the hypothesis that the use of conventional breeding to combine agricultural biotechnology traits into a single crop does not affect the composition of the resulting multitrait crop. For the studies described in this paper, compositional equivalence was comparable whether two agronomic traits, in various combinations, or three traits were combined by traditional breeding. A corollary to the hypothesis stated above is that the compositional evaluation of single-trait biotechnology-derived crops is sufficient to assess compositional equivalence for that trait, whether alone or in combination through traditional breeding with other agronomic traits that have been shown to be compositionally equivalent to conventional counterparts. If the composition of a multitrait crop is assessed, the largest combination is likely to be the most informative because it contains all of the traits of interest.

■ ASSOCIATED CONTENT

Supporting Information. Additional composition data tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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