Comparison of the Nutritional Profile of Glyphosate-Tolerant Corn Event NK603 with That of Conventional Corn (*Zea mays* L.)

William P. Ridley,*,† Ravinder S. Sidhu,† Paul D. Pyla,†
Margaret A. Nemeth,† Matthew L. Breeze,‡ and James D. Astwood†

Product Safety Center, Monsanto Company, 800 North Lindbergh Boulevard,
St. Louis, Missouri 63167, and Covance Laboratories, Inc., 3301 Kinsman Boulevard,
Madison, Wisconsin 53704

The composition of glyphosate-tolerant (Roundup Ready) corn event NK603 was compared with that of conventional corn grown in the United States in 1998 and in the European Union in 1999 to assess compositional equivalence. Grain and forage samples were collected from both replicated and nonreplicated field trials, and compositional analyses were performed to measure proximates, fiber, amino acids, fatty acids, vitamin E, nine minerals, phytic acid, trypsin inhibitor, and secondary metabolites in grain as well as proximates and fiber in forage. Statistical analysis of the data was conducted to assess statistical significance at the $p < 0.05$ level. The values for all of the biochemical components assessed for corn event NK603 were similar to those of the nontransgenic control or were within the published range observed for nontransgenic commercial corn hybrids. In addition, the compositional profile of Roundup Ready corn event NK603 was compared with that of traditional corn hybrids grown in Europe by calculating a 99% tolerance interval to describe compositional variability in the population of traditional corn varieties in the marketplace. These comparisons, together with the history of the safe use of corn as a common component of animal feed and human food, support the conclusion that Roundup Ready corn event NK603 is compositionally equivalent to, and as safe and nutritious as, conventional corn hybrids grown commercially today.

KEYWORDS: Corn (*Zea mays* L.); glyphosate tolerant; composition; nutritional profile

INTRODUCTION

Herbicide tolerance has been introduced, through genetic modification, into a number of crops including corn. Glyphosate, the active ingredient in the Roundup family (Roundup, Roundup Ultra and Roundup Ready are registered trademarks of Monsanto Technology LLC) of agricultural herbicides, is one of the most widely used herbicides in the world. Since 1996, glyphosate-tolerant or Roundup Ready crops have been developed and commercialized for soybean (*Glycine max*) (*1, 2*), canola (*Brassica napus*), cotton (*Gossypium hirsutum*) (*3*), and corn (*Zea mays* L.) (*4*). Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds and has superior environmental and toxicological characteristics, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds, and fish (*5*).

Roundup Ready corn event NK603 (corn event NK603) was produced by the stable insertion of two gene cassettes that express 5-enolpyruvylshikimate-3-phosphate synthases from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Corn event NK603 differs from Roundup Ready corn event GA21 that expresses a modified corn EPSPS (mEPSPS) (*4*). The *cp4 epsps* genes from *Agrobacterium* sp. strain CP4 have been completely sequenced and encode ∼47.6 kDa proteins consisting of a single polypeptide of 455 amino acids (*6*). The CP4 EPSPS proteins are functionally similar to plant EPSPS enzymes but have a much reduced affinity for glyphosate. Glyphosate acts by inhibition of EPSPS, an enzyme involved in the shikimic acid pathway for aromatic acid biosynthesis in plants and microorganisms (*7*). EPSPS is present in plants, bacteria, and fungi but not in animals (*8*). In plants, EPSPS is localized in the chloroplasts or plastids (*9*). Expression of CP4 EPSPS fused to a chloroplast transit peptide enables targeting of this protein to the chloroplast, thereby conferring glyphosate tolerance to the corn plant while meeting the plant's needs for the production of aromatic amino acids. A comprehensive safety assessment of CP4 EPSPS protein has been described in the literature (*10*).

The safety assessment of foods or feeds derived from genetically enhanced crops addresses two major sources of potential health consequences: (1) those due to the activity and

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presence of the introduced trait (most often a protein) and (2) those due to the characteristics of the resulting food or feed crop plant (*11*). It was necessary for food and feed safety evaluation of corn event NK603 to determine if any significant changes in the nutritional profile of the crop resulted from the insertion of the *cp4 epsps* genes into the corn genome or from the presence of the CP4 EPSPS proteins. Because safety assessment of crops has the advantage that comparisons can be made to traditional crops, in a process referred to as substantial equivalence [World Health Organization (WHO; *12*, *13*), United Nations Food and Agriculture Organization (FAO; *14*), and Organization for Economic Cooperation and Development (OECD; *15*−*17*)], the purpose of this study was to evaluate the nutritional profile of corn event NK603 compared with that of a nontransgenic control of similar genetic background as well as with those of traditional corn hybrids available in the United States and the European Union.

MATERIALS AND METHODS

Corn Samples for Compositional Analysis. Grain and forage samples were collected from field trials conducted in 1998 and 1999. In 1998 corn was grown at six nonreplicated trials in the United States (Richland, IA; Webster City, IA; Bagley, IA; Carlyle, IL; Indianapolis, IN; and Andale, KS) and three replicated trials (Jerseyville, IL; New Holland, OH; and Claude, TX). Corn event NK603 (containing the *cp4 epsps* genes) in an LH82 inbred background was crossed with the nontransgenic inbred, B73, to form the test hybrid. A hybrid formed from the cross of two related nontrangenic inbreds, LH82 and B73, both of which lacked the *cp4 epsps* gene, was used as the control. For the nonreplicated sites, corn event NK603 was planted in one plot at each site and the control was planted in a second plot. For the replicated sites, corn event NK603 and its control were planted in a randomized complete block design with four blocks or replications. The NK603 plots were treated with three applications of Roundup Ultra at pre-emergence, at early postemergence (V4−V6 stage), and at late postemergence (V8 to 30 in. tall, whichever came first). The genetic purity of the Roundup Ready corn plants was maintained by bagging the tassels and ear shoots at anthesis and self-pollinating each plant by hand. Forage was collected at the late dough/early dent stage, and grain was collected at normal kernel maturity. The forage and grain from the Texas site were not representative of the test and control hybrids due to above normal temperatures, below normal rainfall, and a disease infestation with *Ustilago maydis*. Grain from the Kansas site was also of poor quality and poor yield caused by weather and *Ustilago* infestation. Consequently, these samples from both the Texas and Kansas sites were not used for compositional analysis. Forage and grain samples were ground to a fine powder in the presence of dry ice and maintained frozen until required for compositional analysis. The identity of forage samples was based on sample-handling records and CP4 EPSPS enzyme-linked immunosorbed assay (ELISA) analyses. The identity of the grain samples was based on sample-handling records, CP4 EPSPS ELISA, and Southern blot analyses of genomic DNA isolated from the grain.

In 1999, grain and forage samples were collected from four replicated field sites in the European Union (EU) at Germignonville, Janville, and L'Isle Jourdain, France, and at Banarola, Italy. Corn event NK603 was the test hybrid, and the related nontransgenic hybrid was the control in these trials. In addition to the test and control corn hybrids, a total of 19 different conventional, commercial hybrids (five per site with one hybrid planted at two sites) were planted as references. The conventional, commercial hybrids with the supplier noted in parentheses were Chantal, Oural, Rival, Liberal, Radial, Total, and Tevere (Asgrow); Alvina, Cecilia, Kelada, and Balka (Pioneer); Aramis and Santos (Dekalb); DK312 and DK300 (Ragt); Anjou 285 (Angevin); Banguy (Nickerson); Cherif (Verneuil semences); and Capitol (Maisadour). The EU replicated trials contained four replications of the test and control plots and were based on a randomized complete block design at the L'Isle Jourdain, France, and Banarola, Italy, sites. Due to space limitations at the Germingnonville and Janville sites in France, the test

and control lines were not planted in the same block, and therefore an incomplete block design was used for these two sites. A single postemergent application of Roundup herbicide (MON 52276) at the V4−V6 stage was made to plots containing Roundup Ready corn plants. The genetic purity of plants was maintained, and forage and grain samples collected as described for the 1998 U.S. field trials.

Compositional Analyses. Compositional analyses were conducted to measure proximates (protein, fat, ash, carbohydrate, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, fatty acids, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), vitamin E, phytic acid, and trypsin inhibitor contents of grain. Proximates, ADF, and NDF contents were measured in forage. The secondary metabolites, ferulic acid, *p*-coumaric acid, 2-furaldehyde, and raffinose, were measured in grain. All compositional analyses were performed at Covance Laboratories, Inc. (Madison, WI). Brief descriptions of the procedures used are given below.

Proximate Analysis. Protein levels were estimated by determining the total nitrogen content using the Kjeldahl method, as previously described (*18*, *19*). Protein was calculated from total nitrogen using the formula N × 6.25. Fat content of the grain was estimated by using the Soxhlet extraction method (*20*). Fat content of forage was determined by fat−acid hydrolysis, followed by extraction with ether and hexane (*21*, *22*).

Ash content was estimated by ignition of a sample in an electric furnace and quantitation of the ash by gravimetric analysis (*23*). Moisture content was determined by loss of weight upon drying in a vacuum oven at 100 °C to a constant weight (*24*, *25*). Carbohydrate levels were estimated by using the fresh weight-derived data and the following equation (*26*):

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

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 (*27*). 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 (*27*, *28*).

Minerals. To estimate the levels of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc, inductively coupled plasma emission spectrometry was used as described in AOAC methods (*29*, *30*) and the literature method of Dahlquist et al. (*31*). The sample was dried, precharred, and ashed overnight at ∼500 °C. The ashed sample was treated with hydrochloric acid, taken 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 sample, measured by the inductively coupled plasma, with the emission of a standard solution.

Amino Acid Composition. Three procedures described in the literature (*32*) 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 using external standards for quantitation (*33*).

Vitamin E. Vitamin E in grain was determined following saponification to break down any fat and release the vitamin as described by

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Table 1. Fiber, Mineral, and Proximate Composition of Grain from Corn Event NK603

	1998[a]		1999[b]				
	NK603 mean (range)[h]	control[d] mean (range)[h]	NK603 mean (range)[h]	control[d] mean (range)[h]	commercial hybrids[e] tolerance interval[f] (range)[h]	lit. (range)	historical[g] (range)[h]
component[c]							
protein	12.20 (10.30–14.77)	12.60 (11.02–14.84)	12.07 (10.23–13.92)	11.34 (10.13–13.05)	6.84, 14.57 (7.77–12.99)	(6.0–12.0)[i] (9.7–16.1)[j]	(9.0–13.6)
total fat	3.61 (2.92–3.94)	3.67 (2.88–4.13)	4.16[k] (3.87–4.48)	3.60 (3.24–3.84)	1.55, 5.75 (2.57–4.95)	(3.1–5.7)[i] (2.9–6.1)[j]	(2.4–4.2)
ash	1.45 (1.28–1.62)	1.49 (1.32–1.75)	1.38 (1.23–1.65)	1.34 (1.25–1.50)	0.77, 2.22 (1.02–1.94)	(1.1–3.9)[i]	(1.2–1.8)
ADF[l]	3.72 (3.14–5.17)	3.60 (2.79–4.28)	3.21 (2.63–3.87)	3.03 (2.30–3.68)	1.96, 4.71 (2.46–6.33)	(3.3–4.3)[i]	(3.1–5.3)
NDF[l]	10.06 (7.89–12.53)	10.00 (8.25–15.42)	10.08 (8.50–12.00)	10.57 (9.35–11.63)	7.26, 14.64 (8.45–14.75)	(8.3–11.9)[i]	(9.6–15.3)
carbohydrates	82.76 (80.71–84.33)	82.29 (80.23–83.70)	82.39 (80.49–84.57)	83.73 (81.93–84.92)	79.38, 88.91 (82.18–88.14)	not reported in this form	(81.7–86.3)
moisture	11.13 (9.01–13.30)	11.78 (8.56–14.80)	7.62 (7.34–7.82)	7.81 (7.55–8.28)	7.06, 9.53 (7.43–9.94)	(7–23)[i]	(9.4–15.8)
calcium	0.0047 (0.0037–0.0056)	0.0046 (0.0033–0.0058)	0.0053 (0.0050–0.0058)	0.0053 (0.0050–0.0058)	0.0028, 0.0082 (0.0039–0.0076)	(0.01–0.1)[i]	(0.003–0.006)
copper	1.79 (1.19–2.37)	1.90 (1.50–2.33)	1.89 (1.77–1.99)	1.83 (1.69–1.97)	0.45, 3.16 (1.16–2.78)	(0.9–10)[i]	na[m]
iron	22.71 (19.08–25.94)	22.95 (18.77–26.62)	22.73 (17.43–26.91)	21.81 (18.52–25.87)	10.60, 33.63 (15.42–29.34)	(1–100)[i]	na
magnesium	0.12 (0.11–0.13)	0.12 (0.11–0.13)	0.12 (0.096–0.13)	0.11 (0.10–0.12)	0.079, 0.16 (0.089–0.15)	(0.09–1.0)[i]	na
manganese	6.47 (4.64–9.63)	6.55 (4.96–8.83)	6.73 (5.18–7.90)	6.42 (5.63–7.32)	2.50, 12.03 (3.86–10.47)	(0.7–54)[i]	na
phosphorus	0.36 (0.32–0.39)	0.36 (0.32–0.39)	0.36 (0.31–0.39)	0.35 (0.32–0.37)	0.27, 0.42 (0.27–0.39)	(0.26–0.75)[i]	(0.288–0.363)
potassium	0.36 (0.35–0.39)	0.36 (0.34–0.41)	0.36[k] (0.34–0.38)	0.38 (0.36–0.39)	0.31, 0.45 (0.32–0.45)	(0.32–0.72)[i]	na
zinc	28.35 (20.23–33.17)	28.72 (23.47–33.26)	23.78 (15.95–31.45)	23.21 (17.87–29.88)	9.89, 31.52 (13.51–27.98)	(12–30)[i]	na

[a] Data from five nonreplicated U.S. sites and two replicated U.S. sites; NK603 grain harvested from plants treated with Roundup Ultra herbicide. [b] Data from two replicated EU sites; NK603 grain harvested from plants treated with Roundup (MON 52276) herbicide. [c] Percent dry weight of sample, except moisture as percent fresh weight and copper, iron, manganese, and zinc as mg/kg of dry weight. [d] Nontransgenic control. [e] Commercial hybrids; local hybrids planted at each EU site. [f] Tolerance interval is specified to contain 99% of the commercial line population, negative limits set to zero. [g] Range for nontransgenic control lines planted in Monsanto Co. field trials conducted in 1993 and 1995. [h] Range denotes the lowest and highest individual values across sites. [i] Watson (*55*). [j] Jugenheimer (*56*). [k] Statistically significantly different from the control at the 5% level ($p < 0.05$). [l] ADF, acid detergent fiber; NDF, neutral detergent fiber. [m] na = not available.

Cort et al. (*34*). The saponified mixture was extracted with ethyl ether and then quantitated directly by high-performance liquid chromatography (HPLC) on a silica gel column.

Phytic Acid. Phytic acid was quantitated in grain following extraction using ultrasonication as described by Lehrfeld (*35, 36*). 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 μm, 150 × 4.1 mm) fitted with a refractive index detector.

Trypsin Inhibitor. Trypsin inhibitor activity in grain was determined using AOCS method Ba 12-75 (*37*). The ground, defatted sample was suspended in dilute sodium hydroxide, an appropriate dilution of the suspension was made, and a series of aliquots resulting in increased levels of the diluted suspension was mixed with trypsin and the synthetic substrate, benzoyl-DL-arginine-*p*-nitroanilide. After 10 min, the action of trypsin was stopped by the addition of acetic acid, the mixture was centrifuged or filtered, and the absorbance of the supernatant or filtrate was measured at 410 nm. Trypsin inhibitor activity was calculated from the change in absorbance versus aliquot volume and expressed in trypsin inhibitor units (TIU) per milligram of fresh weight of sample.

Ferulic and p-Coumaric Acids. Ferulic and *p*-coumaric acids were assayed in grain using the method of Hagerman and Nicholson (*38*), 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 acid were determined by reversed-phase HPLC with UV detection. The limit of quantitation based on fresh weight was 5.0 ppm for both analytes.

2-Furaldehyde. The levels of 2-furaldehyde were determined using the method of Albala-Hurtado et al. (*39*), in which the corn grain was

extracted with 4% trichloroacetic acid, centrifuged, filtered, concentrated, and analyzed by reversed-phase HPLC with UV detection. The limit of quantitation for 2-furaldehyde was 0.5 ppm based on fresh weight.

Raffinose. The raffinose assay was based on two methods (*40, 41*) 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.

Statistical Analysis of Composition Data. The following 15 analytes with >50% of the observations at or below the limit of quantitation of the assay were excluded from statistical analysis: sodium, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:1 palmitoleic 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. Except for moisture, all component values were converted from a fresh weight to a dry weight basis (**Tables 1–5**). A total of 51 components were evaluated (7 in forage and 44 in grain) in both 1998 and 1999. The 44 components in grain resulted from the difference between the initial 59 components minus the 15 components having levels below the limit of quantitation. In addition to the nutritional components, the secondary metabolites ferulic acid, *p*-coumaric acid, and raffinose were analyzed in grain. The levels of 2-furaldehyde were below the limit of quantitation of the method (<0.5 ppm) in all samples, and therefore this secondary metabolite was not statistically analyzed.

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Table 2. Fiber and Proximate Composition of Forage from Corn Event NK603

	1998[a]		1999[b]			
	NK603 mean (range)[h]	control[d] mean (range)[h]	NK603 mean (range)[h]	control[d] mean (range)[h]	commercial hybrids[e] tolerance interval[f] (range)[h]	historical[g] (range)
component[c]						
protein	7.14 (5.57–8.98)	6.80 (5.49–8.69)	8.71 (6.37–10.79)	8.86 (7.03–10.96)	4.02, 12.46 (4.98–11.56)	(4.8–8.4)
ash	3.81 (2.36–6.80)	4.02 (2.46–6.28)	4.38 (2.82–6.44)	4.44 (3.35–5.80)	0, 12.47 (2.43–9.64)	(2.9–5.1)
ADF[i]	25.72 (17.01–33.52)	24.84 (19.53–31.83)	23.53 (19.27–26.13)	22.07 (19.39–26.90)	9.80, 44.43 (17.54–38.31)	(21.4–29.2)
NDF[i]	42.09 (36.39–49.03)	42.45 (35.44–53.24)	37.34 (31.77–44.35)	37.75 (34.85–41.86)	20.77, 61.87 (27.93–54.75)	(39.9–46.6)
total fat	2.36 (0.69–3.64)	2.17 (0.61–3.42)	3.24 (2.06–4.49)	3.05 (2.09–4.02)	0.84, 4.80 (1.42–4.57)	(1.4–2.1)
carbohydrates	86.71 (82.68–90.32)	87.11 (83.71–90.03)	83.67 (80.43–87.53)	83.65 (80.64–85.52)	75.55, 91.37 (76.50–87.29)	(84.6–89.1)
moisture	67.02 (60.30–75.00)	66.24 (61.00–73.70)	67.53 (61.60–75.20)	66.30 (60.40–72.60)	45.40, 96.42 (56.50–80.40)	(68.7–73.5)

[a] Data from five nonreplicated U.S. sites and two replicated U.S. sites; NK603 forage harvested from plants treated with Roundup Ultra herbicide. [b] Data from two replicated EU sites; NK603 forage harvested from plants treated with Roundup (MON 52276) herbicide. [c] Percent dry weight of sample, except for moisture. [d] Nontransgenic control. [e] Commercial hybrids; local hybrids planted at each site. [f] Tolerance interval is specified to contain 99% of the commercial line population, negative limits set to zero. [g] Range for nontransgenic control lines planted in Monsanto Co. field trials conducted in 1994 and 1995. [h] Range denotes the lowest and highest individual values across sites. [i] ADF, acid detergent fiber; NDF, neutral detergent fiber.

Statistical analyses of the composition data were conducted using a mixed model analysis of variance (randomized complete block design) for a combination of all sites for 1998 data and a combination of the two sites (L'Isle Jourdain, France, and Banarola, Italy) for the 1999 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 = line effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by line interaction, and e_{ijk} = residual error. In these analyses, corn event NK603 was compared to the nontransgenic control. For each compositional measure, the *p* value for a test of corn event NK603 mean equal to the control mean, the observed difference of NK603 from the control, and lower and upper 95% confidence intervals for the mean difference of NK603 from the control were calculated. Statistical significance was assigned at *p* < 0.05.

Compositional data from the commercial reference hybrids in the 1999 study were not included in the statistical analysis. However, a range of the reference values was determined for each component. Additionally, commercial reference data were used to develop population tolerance intervals. A tolerance interval is an interval 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 analysis component, tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the values expressed in the population of commercial lines. Because negative quantities are impossible, calculated lower tolerance bounds were limited to zero. SAS software (*42−44*) was used by Certus International, Inc., Chesterfield, MO, to generate all summary statistics and perform all analyses. Additional analyses of the individual replicated sites in 1998 with a randomized complete block design (Jerseyville, IL, and New Holland, OH) and 1999 (L'Isle Jourdain, France, and Banarola, Italy) were conducted, and the results (data not shown) of these additional analyses were consistent with the conclusions reached in this paper.

RESULTS AND DISCUSSION

The safety assessment of genetically enhanced crops has relied on a comparative approach focusing on similarities and differences between the food and feed derived from genetically enhanced crop and its conventional counterpart. In this paper the nutritional composition of corn event NK603 was compared with that of a nontransgenic control with a similar genetic background that was grown in the same field trials in the United States and Europe. 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 corn event NK603 was compared with those of traditional corn hybrids grown in Europe by calculating a 99% tolerance interval to describe the compositional variability in the population of conventional corn hybrids in the marketplace. Finally, the composition values for corn event NK603 were compared with values obtained from the published literature or historical conventional control values determined in previous studies.

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 levels of proximate components (protein, ash, carbohydrate), fiber (ADF and NDF), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, and zinc) in the grain and forage of corn event NK603 were comparable to those in the grain and forage of the nontransgenic control. In addition, these values were either within published literature ranges, within the tolerance interval determined for commercial varieties evaluated in the 1999 field trials, or within the range of historical conventional control values determined from previous studies. No measurable differences were observed for the content of fat or potassium in forage data from either 1998 or 1999 field trials and the grain data from the 1998 field trials. Although the contents of fat and potassium in the grain of corn event NK603 were significantly different statistically from those in the nontransgenic control in data from 1999 field trials, the range of values for both analytes of corn event NK603 fell within the 99% tolerance interval for the commercial varieties grown at the same field trials. These results demonstrate, with a confidence level of 95%, that the levels of fat and potassium for corn event NK603 were within the same population as those of nontransgenic, commercially available corn hybrids.

Amino Acid Composition. The content of the 18 amino acids measured in the grain of corn event NK603 was comparable to that in the grain of the nontransgenic control (**Table 3**). In

Table 3. Amino Acid Composition of Grain from Corn Event NK603

| amino acid[a] | 1998[b] | | 1999[c] | | | | |
	NK603 mean (range)[i]	control[d] mean (range)[i]	NK603 mean (range)[i]	control[d] mean (range)[i]	commercial hybrids[e] tolerance interval[f] (range)[i]	lit.[g] (range)	historical[h] (range)[i]
alanine	7.93 (7.78–8.22)	7.89 (7.65–8.17)	8.04[j] (7.87–8.18)	7.95 (7.88–8.05)	7.20, 8.35 (7.38–8.13)	(6.4–9.9)	(7.2–8.8)
arginine	4.16 (3.79–4.49)	4.24 (3.90–4.63)	4.00[j] (3.74–4.27)	4.27 (4.09–4.36)	3.45, 5.03 (3.77–4.98)	(2.9–5.9)	(3.5–5.0)
aspartic acid	6.45 (6.29–6.62)	6.40 (6.18–6.56)	6.45 (6.27–6.96)	6.28 (6.18–6.37)	5.53, 7.61 (6.02–7.51)	(5.8–7.2)	(6.3–7.5)
cysteine/cystine	2.00 (1.69–2.27)	2.00 (1.63–2.22)	1.82 (1.66–1.98)	1.92 (1.61–2.09)	1.56, 2.43 (1.68–2.51)	(1.2–1.6)	(1.8–2.7)
glutamic acid	19.84 (19.16–20.47)	19.81 (19.19–20.41)	19.93[j] (18.98–20.62)	19.40 (18.69–19.92)	18.03, 20.76 (18.38–20.08)	(12.4–19.6)	(18.6–22.8)
glycine	3.49 (3.22–3.74)	3.51 (3.22–3.86)	3.44 (3.23–3.64)	3.60 (3.44–3.77)	3.06, 4.15 (3.27–4.01)	(2.6–4.7)	(3.2–4.2)
histidine	2.72 (2.45–2.81)	2.74 (2.56–2.88)	2.65[j] (2.56–2.74)	2.77 (2.69–2.85)	2.34, 3.36 (2.58–3.15)	(2.0–2.8)	(2.8–3.4)
isoleucine	3.87 (3.59–4.06)	3.80 (3.65–3.93)	3.77 (3.54–3.97)	3.76 (3.61–3.85)	3.35, 3.97 (3.34–3.85)	(2.6–4.0)	(3.2–4.3)
leucine	14.20 (13.63–14.79)	14.07 (13.59–14.60)	14.02 (13.38–14.71)	13.69 (13.27–13.96)	11.73, 14.76 (12.18–14.34)	(7.8–15.2)	(12.0–15.8)
lysine	2.69 (2.42–2.96)	2.67 (2.35–3.00)	2.71[j] (2.37–3.03)	2.83 (2.56–3.20)	2.22, 3.68 (2.58–3.67)	(2.0–3.8)	(2.6–3.5)
methionine	1.94 (1.76–2.16)	2.03 (1.74–2.21)	1.77[j] (1.66–1.85)	1.89 (1.67–2.06)	1.39, 2.49 (1.49–2.32)	(1.0–2.1)	(1.3–2.6)
phenylalanine	5.32 (5.18–5.52)	5.24 (5.09–5.36)	5.28 (5.13–5.46)	5.25 (5.20–5.29)	4.59, 5.61 (4.85–5.54)	(2.9–5.7)	(4.9–6.1)
proline	8.88 (8.44–9.10)	8.96 (8.59–9.26)	9.33 (8.89–9.71)	9.16 (8.83–9.31)	8.61, 10.09 (8.74–9.91)	(6.6–10.3)	(8.7–10.1)
serine	4.87 (4.72–5.09)	4.86 (4.68–4.99)	4.84 (4.47–5.17)	4.90 (4.82–5.09)	4.36, 5.19 (4.41–5.22)	(4.2–5.5)	(4.9–6.0)
threonine	3.37 (3.26–3.46)	3.33 (3.19–3.50)	3.31 (3.14–3.57)	3.29 (3.15–3.50)	3.14, 3.69 (3.24–3.66)	(2.9–3.9)	(3.3–4.2)
tryptophan	0.53 (0.44–0.58)	0.54 (0.48–0.60)	0.58 (0.49–0.64)	0.62 (0.57–0.69)	0.45, 0.76 (0.49–0.79)	(0.5–1.2)	(0.4–1.0)
tyrosine	3.02 (2.36–3.73)	3.25 (2.43–3.64)	3.24 (2.11–3.65)	3.52 (2.69–3.69)	3.00, 4.03 (2.32–3.90)	(2.9–4.7)	(3.7–4.3)
valine	4.74 (4.59–4.85)	4.71 (4.62–4.94)	4.81 (4.55–5.00)	4.90 (4.74–5.04)	4.64, 5.38 (4.65–5.29)	(2.1–5.2)	(4.2–5.3)

[a] Values expressed as percent of total amino acids for statistical comparisons. [b] Data from five nonreplicated U.S. sites and two replicated U.S. sites; NK603 grain harvested from plants treated with Roundup Ultra herbicide. [c] Data from two replicated EU sites; NK603 grain harvested from plants treated with Roundup (MON 52276) herbicide. [d] Nontransgenic control. [e] Commercial hybrids; local hybrids planted at each EU site. [f] Tolerance interval is specified to contain 99% of the commercial line population, negative limits set to zero. [g] Watson (*57*). Values are percent of total protein. [h] Range for nontransgenic control lines planted in Monsanto Co. field trials conducted between 1993 and 1995; values are percent of total protein. [i] Range denotes the lowest and highest individual values across sites. [j] Value statistically significantly different than the control at the 5% level ($p < 0.05$).

addition, these values were either within published literature ranges, within the 99% tolerance interval for commercial varieties evaluated in 1999 field trials, or within the range of historical conventional control values determined from previous studies. Because EPSPS catalyzes a step in the aromatic amino acid biosynthetic pathway, it was important to assess whether expression of CP4 EPSPS influenced the levels of the aromatic amino acids in corn event NK603. EPSPS is not the rate-limiting step in aromatic amino acid biosynthesis (*45*, *46*) and, therefore, greater EPSPS activity is unlikely to increase the levels of aromatic compounds in plants. No statistically significant differences were observed in the content of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, between corn event NK603 and the nontransgenic control in either 1998 or 1999 field trials (**Table 3**).

A majority of the amino acids in corn event NK603 were comparable to the control in the 1999 field trials. However, small statistically significant differences (1.1−6.4%) were observed for alanine, arginine, glutamic acid, histidine, lysine, and methionine ($p < 0.05$). No differences were found for these amino acids in the 1998 field trials, and in all cases the range

of values found for corn event NK603 fell within the 99% tolerance interval for conventional commercial varieties grown in the same field trials (**Table 3**). These results demonstrate, with a confidence level of 95%, that the levels of these amino acids were within the same population as those of nontransgenic, commercially available corn hybrids.

Fatty Acid Composition. The content of the fatty acids in grain of corn event NK603 was comparable to that observed in the grain of the nontransgenic control (**Table 4**). In addition, these values were either within published literature ranges, within the 99% tolerance interval determined for commercial hybrids evaluated in 1999 field trials, or within the range of historical conventional control values determined from previous studies. Statistically significant differences between corn event NK603 and the nontransgenic control were observed in the levels of 18:1 oleic acid, 16:0 palmitic acid, and 18:0 stearic acid for the 1998 field trials and 20:0 arachidic acid in the 1999 trials. In general, the magnitude of the differences was small (2.6−4.8%), and in no case was a fatty acid level found to be statistically different in corn event NK603 when compared to the control for more than one year. Furthermore, the ranges of

Table 4. Fatty Acid Composition of Grain from Corn Event NK603

fatty acid[a]	1998[b] NK603 mean (range)[i]	1998[b] control[d] mean (range)[i]	1999[c] NK603 mean (range)[i]	1999[c] control[d] mean (range)[i]	commercial hybrids[e] tolerance interval[f] (range)[i]	lit.[g] (range)	historical[g] (range)[h]
arachidic (20:0)	0.36 (0.34–0.39)	0.37 (0.33–0.40)	0.36[j] (0.34–0.39)	0.35 (0.33–0.37)	0.17, 0.64 (0.31–0.74)	(0.1–2)	(0.3–0.5)
behenic (22:0)	0.16 (0.14–0.19)	0.16 (0.14–0.19)	0.16 (0.12–0.20)	0.18 (0.15–0.19)	0.093, 0.24 (0.073–0.22)	(not reported)	(0.1–0.3)
eicosenoic (20:1)	0.29 (0.28–0.32)	0.30 (0.27–0.34)	0.30 (0.28–0.34)	0.29 (0.28–0.31)	0.21, 0.42 (0.26–0.40)	(not reported)	(0.2–0.3)
linoleic (18:2)	64.62 (63.79–65.80)	64.26 (63.07–65.65)	63.73 (61.94–65.25)	63.15 (61.63–64.04)	44.59, 73.50 (49.72–65.98)	(35–70)	(55.9–66.1)
linolenic (18:3)	1.11 (1.07–1.17)	1.11 (1.07–1.20)	1.02 (0.97–1.05)	1.09 (1.05–1.12)	0.54, 1.72 (0.71–1.50)	(0.8–2)	(0.8–1.1)
oleic (18:1)	22.40[j] (21.37–23.12)	23.08 (22.15–24.14)	23.80 (22.82–24.95)	24.20 (23.52–25.56)	12.65, 39.86 (20.21–34.64)	(20–46)	(20.6–27.5)
palmitic (16:0)	9.13[j] (8.67–9.57)	8.89 (8.41–9.44)	8.90 (8.47–9.36)	9.00 (8.89–9.13)	7.35, 14.72 (9.12–12.62)	(7–19)	(9.9–12.0)
stearic (18:0)	1.92[j] (1.80–2.06)	1.83 (1.67–1.98)	1.73 (1.59–1.88)	1.74 (1.67–1.81)	1.02, 2.27 (1.19–2.02)	(1–3)	(1.4–2.2)

[a] Value of fatty acids 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), palmitoleic acid (16:1), heptadecanoic acid (17:0), heptadecenoic acid (17:1), gamma linolenic (18:3), eicosadienoic acid (20:2), eicosatrienoic acid (20:3), and arachidonic acid (20:4). [b] Data from five nonreplicated U.S. sites and two replicated U.S. sites; NK603 grain harvested from plants treated with Roundup Ultra herbicide. [c] Data from two replicated EU sites; NK603 grain harvested from plants treated with Roundup (MON 52276) herbicide. [d] Nontransgenic control. [e] Commercial hybrids; local hybrids planted at each EU site. [f] Tolerance interval is specified to contain 99% of the commercial line population, negative limits set to zero. [g] Watson (57). Values expressed as % of total fat except for palmitic acid (16:1), which is expressed as % of triglyceride fatty acids. [h] Range for nontransgenic control lines planted in Monsanto Co. field trials conducted between 1993 and 1995; values are expressed as % of total fatty acids. [i] Range denotes the lowest and highest individual values across sites. [j] Statistically significantly different from the control at the 5% level ($p < 0.05$).

Table 5. Phytic Acid, Trypsin Inhibitor, Vitamin E, and Secondary Metabolite Content of Grain from Corn Event NK603

component	1998[a] NK603 mean (range)[h]	1998[a] control[c] mean (range)[h]	1999[b] NK603 mean (range)[h]	1999[b] control[c] mean (range)[h]	commercial hybrids[d] tolerance interval[e] (range)[h]	lit.[f] (range)	historical[g] (range)
phytic acid (% dw)	0.97 (0.70–1.06)	1.00 (0.81–1.21)	0.79 (0.51–0.89)	0.70 (0.55–0.77)	0.32, 1.18 (0.48–1.12)	to 0.9%	na[i]
trypsin inhibitor (TIU/mg dw)	3.16 (2.34–5.08)	2.67 (1.39–5.14)	1.56 (0.54–2.57)	1.15 (0.54–2.38)	0, 3.63 (0.54–4.13)	na	na
vitamin E (mg/g of dw)	0.0088 (0.0070–0.010)	0.0090 (0.0064–0.011)	0.0062 (0.0046–0.0080)	0.0070 (0.0050–0.014)	0, 0.021 (0.0027–0.015)	(0.017–0.047)	(0.008–0.015)
ferulic acid (% dw)	0.20 (0.15–0.25)	0.20 (0.17–0.23)	na	na	na	na	(0.17–0.27)[j]
p-coumaric acid (% dw)	0.016 (0.012–0.022)	0.015 (0.012–0.020)	na	na	na	na	(0.011–0.030)[j]
raffinose (% dw)	0.13 (0.098–0.20)	0.13 (0.082–0.21)	na	na	na	na	(0.053–0.16)[j]

[a] Data from five nonreplicated U.S. sites and two replicated U.S. sites; NK603 grain harvested from plants treated with Roundup Ultra herbicide. [b] Data from two replicated EU sites; NK603 grain harvested from plants treated with Roundup (MON 52276) herbicide. [c] Nontransgenic control. [d] Commercial hybrids; local hybrids planted at each EU site. [e] Tolerance interval is specified to contain 99% of the commercial line population, negative limits set to zero. [f] Watson (50). [g] Range for nontransgenic control hybrids planted in Monsanto Co. field trials conducted between 1993 and 1995. [h] Range denotes the lowest and highest individual values across sites for each line. [i] na, not available. [j] Range for 13 commercial varieties planted in Monsanto Co. field trials or purchased from growers in 1998.

values found for these fatty acids were in all cases within the 99% tolerance interval for the commercial varieties grown in the 1999 field trials, demonstrating that corn event NK603 was within the same population as conventional, commercially available corn hybrids.

Phytic Acid, Trypsin Inhibitor, and Vitamin E Composition. Phytic acid, the hexakis-*o*-phosphate of *myo*-inositol, is widely distributed in plants (47). Seeds accumulate up to 90% of stored organic phosphate as phytic acid, and it limits uptake of minerals such as calcium in higher animals. The trypsin inhibitors in several hybrids of corn have been compared and found to be similar in physicochemical and immunological properties (48). The trypsin inhibitors of soybeans have been well studied and affect the nutritive value of raw soybeans (49); however, the soybean levels of these materials are significantly higher than those measured in corn. Corn is also considered to be a good source of vitamin E (50).

The content of phytic acid, trypsin inhibitor, and vitamin E in the grain of corn event NK603 was comparable with that observed in the grain of the nontransgenic control (**Table 5**). In addition, these values were either within published literature ranges, within the 99% tolerance interval for the commercial varieties in the 1999 field trials, or within the range of historical conventional control values determined from previous studies.

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No measurable differences in the levels of these analytes between corn event NK603 and the nontransgenic control were observed in the data from both 1998 and 1999 field trials.

Secondary Metabolite Composition. The secondary metabolites, 2-furaldehyde, ferulic acid, *p*-coumaric acid, and raffinose, are present in corn grain or processed corn components. Acid hydrolysis of the pentosans contained in corncobs, oat hulls, and other crop residues are a major source of 2-furaldehyde (furfural) (*51*). Ferulic and *p*-coumaric acids are derived from aromatic amino acids, phenylalanine, and tyrosine, in plants (*52*) and serve as precursors for a large group of phenylpropanoid compounds including flavonoids and coumarins. Raffinose is a nondigestible oligosaccharide that is considered to be an antinutrient due to gas production and resulting flatulence caused by its consumption (*53*).

The levels of 2-furaldehyde were below the limit of quantitation (<0.5 ppm of fresh weight) for all corn grain samples analyzed from the 1998 field trials. The levels of ferulic acid, *p*-coumaric acid, and raffinose in the grain of corn event NK603 were comparable with the levels found in the grain from the nontransgenic control (**Table 5**). No statistically significant differences were observed in the comparisons conducted for the 1998 field trials. These secondary metabolites were not analyzed in the grain samples from the 1999 trials.

Conclusions. The results of compositional analyses generated from nine field sites over a period of two years demonstrate that the grain and forage of corn event NK603 are comparable in their composition with those of the nontransgenic control and conventional corn varieties. The use of multiyear data and incorporation of reference corn hybrids into field trials suggests that the few statistically significant differences observed are unlikely to be of biological relevance. Moreover, the composition of corn event NK603 was shown to fall within the 99% tolerance interval for components in 19 nontransgenic commercial corn hybrids grown as part of the 1999 field trials in Europe and within the ranges of values reported for nontransgenic corn in the literature as well as in historical data. 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, growing conditions, and methods of analysis. The values for components in corn event NK603 all fell within the range of natural variability found in nontransgenic corn hybrids.

The analysis of the data reported herein illustrates that the tolerance interval is a useful statistical tool that can account for extant natural variability in any measured parameter, especially food and feed nutritional profiles as measured by biochemical composition. From the perspective of safety assessment, the biochemical sampling described in this paper provides a robust measure of unexpected effects due to the insertion of the *cp4 epsps* genes into the corn genome. It has been shown, by targeted nutritional analysis, that the genetic enhancement of conventional corn to produce corn event NK603 did not produce significant changes in 51 biologically and nutritionally important components. Also, feeding performance studies in broiler chickens (*54*) have demonstrated that corn event NK603 is equivalent in nutritive value to conventional corn. On the basis of the principle of substantial equivalence as articulated by the World Health Organization, the Organization for Economic Cooperation and Development, and the United Nations Food and Agriculture Organization, these data support the conclusion that Roundup Ready corn event NK603 is as safe and nutritious as conventional varieties of corn on the market today.

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