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Compositional Analysis of Grain and Forage from MON 87427, an Inducible Male Sterile and Tissue Selective Glyphosate-Tolerant Maize Product for Hybrid Seed Production

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S Supporting Information

ABSTRACT: Conventional maize hybrid seed production has historically relied upon detasseling using either manual methods or semiautomated processes to ensure the purity of the hybrid cross. Monsanto Co. has developed biotechnology-derived MON 87427 maize with tissue-selective glyphosate tolerance to facilitate the production of hybrid maize seed. MON 87427 utilizes a specific promoter and intron combination to drive expression of CP4 EPSPS protein in vegetative and female reproductive tissues, conferring tolerance to glyphosate. This specific combination of regulatory elements also results in limited or no production of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores, which develop into pollen grains, and tapetum cells that supply nutrients to the pollen. Thus, MON 87427 induces a male sterile phenotype after appropriately timed glyphosate applications. To confer additional benefits of herbicide tolerance and/or insect resistance, MON 87427 was combined with MON 89034 and NK603 by conventional breeding to develop MON 87427 × MON 89034 × NK603. The work described here is an assessment of the nutrient, antinutrient, and secondary metabolite levels in grain and forage tissues of MON 87427 and MON 87427 × MON 89034 × NK603. Results demonstrated that MON 87427 is compositionally equivalent to a near-isogenic conventional comparator. Results from this analysis established that the compositional equivalence observed for the single-event product MON 87427 is extendable to the combined-trait product, MON 87427 × MON 89034 × NK603. With increasing global demand for food production, the development of more efficient seed production strategies is important to sustainable agriculture. The study reported here demonstrated that biotechnology can be applied to simplify hybrid maize seed production without affecting crop composition.

KEYWORDS: *maize (Zea mays), inducible male sterility, genetically modified, glyphosate-tolerant, composition, statistical analysis*

INTRODUCTION

Hybrid maize offers numerous agronomic benefits including higher yield, and it comprises >95% of maize acreage in the United States and other major corn-growing regions of the world.[1] Hybrid maize seeds are produced by crossing a female pollen recipient parent with a male pollen donor parent. The majority of hybrid seed production currently relies upon a costly and labor-intensive process involving a combination of mechanical and manual procedures for removal of pollen-producing tassels to prevent self-pollination of the female parent. Steps to reduce the cost and labor associated with this process and still maintain seed quality are therefore important to meet increased demand for hybrid seed production. The ability to produce consistent male sterility of the female parent is a trait that is highly valued in hybrid seed production for maintaining seed purity. Monsanto Co. has developed a biotechnology-derived MON 87427 maize with tissue-selective glyphosate tolerance to facilitate the production of viable hybrid maize seed through induced male sterility. MON 87427 produces the same 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready (Roundup and Roundup Ready are registered trademarks of Monsanto Technology, LLC) crop products, via the incorporation of a *cp4 epsps* coding sequence, which confers tolerance to the herbicide glyphosate. MON 87427 utilizes a specific promoter and intron combination to express CP4 EPSPS protein in vegetative and female reproductive tissues, conferring tolerance to glyphosate. This specific combination of regulatory elements also results in limited or no expression of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores that develop into pollen grains and tapetum cells that supply nutrients to the pollen. Thus, MON 87427 induces a male sterile phenotype after appropriately timed glyphosate applications. This male-sterile phenotype in MON 87427 has the potential to provide major efficiency gains in the hybrid seed production process by allowing specific cross-pollinations to be made without using labor-intensive detasseling methods to control self-pollination.[2] The agronomic utility of MON 87427 for hybrid seed production is further extended by combining the glyphosate-inducible male-sterile system with other herbicide and insect tolerance traits. Monsanto Co. has developed through conventional breeding a MON 87427-containing hybrid that confers additional benefits in weed and pest management. This is MON 87427 × MON 89034 × NK603. MON 89034 produces two insecticidal proteins, Cry1A.105 and Cry2Ab2, and provides protection against multiple lepidopteron insect pests.[3] NK603

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produces the CP4 EPSPS protein,[4] which allows for full vegetative and reproductive tissue glyphosate tolerance in the hybrid seed.

The research reported here compares the composition of grain and forage from MON 87427 and MON 87427 × MON 89034 × NK603 to the composition of near-isogenic conventional hybrid comparators. Compositional analysis is a key component of the comparative assessment process of new crops containing traits derived from modern agricultural biotechnology. It involves comparisons of levels of key nutrients, antinutrients, and secondary metabolites in the new biotech or genetically modified (GM) crop to levels in a near-isogenic conventional counterpart. Assessments of differences between the two comparators typically use statistical significance testing. Harrigan et al.[5] recently reviewed the results of GM crop composition studies conducted over multiple geographies and growing seasons and concluded that significant differences ($\alpha = 0.05$) in mean values between a GM crop and its near-isogenic comparator were typically of very small magnitudes and that these differences were considerably less than the differences resulting from germplasm and environmental factors. This has led to increased emphasis that statistical significance does not necessarily imply biological relevance[6] and that the well-documented sources of compositional variability and its influence on overall crop composition must be considered in comparative assessments. In this investigation, we highlight types of further evaluations to consider in the assessment of compositional outcomes in the context of natural variability.

MATERIALS AND METHODS

Maize Samples for Compositional Analyses. The control for MON 87427 was EXP262, a conventional maize hybrid, with a genetic background similar to that of MON 87427. The control for MON 87427 × MON 89034 × NK603 was MPA636B, a conventional maize hybrid, with a genetic background similar to that of MON 87427 × MON 89034 × NK603. Commercially available conventional reference hybrids were planted concurrently with the experimental (MON 87427 or MON 87427 × MON 89034 × NK603) and control hybrids to provide in-study data on the variability of each compositional component assessed.

Materials for composition analyses were generated in 2010 at eight locations within the primary maize-growing regions. For the MON 87427 study, hybrids were grown in Jackson county, Arkansas; Greene and Jefferson counties, Iowa; Clinton and Stark counties, Illinois; Boone county, Indiana; Pawnee county, Kansas; and York county, Nebraska. For the MON 87427 × MON 89034 × NK603 study, hybrids were grown in Jackson county, Arkansas; Clinton and Stark counties, Illinois; Jefferson county, Iowa; Pawnee county, Kansas; Shelby county, Missouri; York county, Nebraska; and Miami county, Ohio.

At each location, hybrids were planted in a randomized complete block design with four blocks. Within each block, plots (six rows × 6.1 m) were assigned randomly to experimental hybrid (MON 87427 or MON 87427 × MON 89034 × NK603), control (EXP262 or MPA636B), or one of the four of the conventional reference hybrids. For the MON 87427 study, a total of 24 unique reference hybrids were planted across the eight-site multilocation study. For the MON 87427 × MON 89034 × NK603 study, a total of 22 unique reference hybrids were planted across the eight-site multilocation study. These conventional reference hybrids were representative of maize hybrids typically grown at each specific location.

Standard agronomic practices for each region were followed. A single application of Roundup WeatherMAX (Roundup and Roundup WeatherMAX are registered trademarks of Monsanto Technology LLC) was applied, according to the label, at a rate of 1.0 lb ae/acre to the MON 87427 × MON 89034 × NK603 plots at approximately the V2−V4 growth stages for weed control. The genetic purity of MON 87427, MON 87427 × MON 89034 × NK603, control, and commercial reference maize was maintained by bagging the tassels and ear shoots at anthesis and hand-pollinating each plant.

Forage and grain harvest, sampling, shipping, storage and sample preparation protocols for compositional analysis were followed as previously described.[7] Event-specific polymerase chain reaction analysis[8] was conducted to confirm the genetic identity of the harvested grain. From the MON 87427 field production, one experimental sample from one location and a total of 11 samples of nine reference substances from six locations were excluded from compositional analyses due to the adventitious presence of unintended traits. From the MON 87427 × MON 89034 × NK603 field production, 15 samples of nine reference substances from seven locations were also excluded from analysis due to the adventitious presence of unintended traits.

Compositional Analysis Methods. Forage samples were analyzed for proximates (ash, fat, moisture, and protein), acid detergent fiber (ADF), neutral detergent fiber (NDF), and minerals (calcium and phosphorus).[9] Grain samples were analyzed for proximates, ADF, NDF, total dietary fiber (TDF), amino acids, fatty acids (FA), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), secondary metabolites [ferulic acid, 2-furaldehyde (furfural), and *p*-coumaric acid], antinutrients (phytic acid and raffinose), and vitamins [folic acid, vitamin A (β-carotene), niacin, vitamin B_1, vitamin B_2, vitamin B_6, and vitamin E]. Carbohydrate levels in forage and grain were determined by calculation, and moisture levels were measured for the re-expression of fresh weight values on a dry weight basis. The compositional analyses were conducted using industry standard analytical methods (AOAC, AOCS, or other published methods) that are used in routine compositional characterization of foods and feeds[3,10] at Covance Laboratories Inc. (Madison, WI, USA) and EPL Bio-Analytical Services (Niantic, IL, USA). Samples from each study were analyzed in a randomized order to reduce assay bias.

Statistical Analysis of Compositional Data. Statistical analysis of the data involved a comparison of MON 87427 to EXP262 and MON 87427 × MON 89034 × NK603 to MPA636B for every analyte across all sites to determine significant differences ($\alpha = 0.05$) using analysis of variance (ANOVA). To complete a statistical analysis for a component in each separate study, at least 50% of the values for an analyte had to be greater than the assay limit of quantitation (LOQ).[11] The following 13 analytes, which had >50% of observations less than the LOQs, were excluded from statistical analysis from both MON 87427 and MON 87427 × MON 89034 × NK603: capric acid, caprylic acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, heptadecenoic acid, γ-linolenic acid, eicosadienoic acid, eicosatrienoic acid, arachidonic acid, erucic, and furfural. In addition, heptadecanoic acid and sodium in MON 87427 were below the assay LOQ. The fatty acids reported above are present in only low amounts in maize grain, if present at all.[5] This investigation confirmed that this observation extended to MON 87427 and MON 87427 × MON 89034 × NK603.

For analytes with individual measurements less than the LOQ and with >50% of all of the values greater than the LOQ, a value equal to half of the LOQ was assigned prior to statistical analyses.[3] Assigned values for grain in the MON 87427 study included 127 values of palmitoleic acid, four values of behenic acid, and one value of calcium. For the MON 87427 × MON 89034 × NK603 study, assigned grain values included five values of palmitoleic acid, 102 values of heptadecanoic acid, two values of ecosenoic acid, 85 values of behenic acid, three values of copper, 95 values of sodium, 14 values of vitamin A, and 33 values of vitamin B_1.

A studentized PRESS residual test was applied to the adjusted data set to identify outliers. Data points that were outside the ± 6 studentized PRESS residual range were considered for exclusion, as outliers, from the final statistical analysis on the basis of a biological assessment of the data point. For the MON 87427 study, one ash value was identified as an outlier and was removed from the statistical

Table 1. Summary of Maize Grain Fat and Fatty Acids (FA) for MON 87427, MON 87427 × MON 89034 × NK603 and the Respective Conventional Controls

component	MON 87427 mean (SE)[c] range	control EXP62 mean (SE) range	MON 87427 × MON 89034 × NK603 mean (SE) range	control MPA636B mean (SE) range
total fat (% dwt)[a]	3.37 (0.089)	3.33 (0.089)	2.99 (0.14)	2.92 (0.14)
	2.69−3.82	2.74−3.78	1.96−3.98	1.59−3.75
palmitic[b]	11.34 (0.11)[d]	11.67 (0.11)	13.06 (0.39)	12.48 (0.39)
	10.84−12.14	10.87−13.26	11.22−22.74	10.76−18.48
palmitoleic[b]	0.12 (0.0084)	0.10 (0.0083)	0.16 (0.0065)	0.15 (0.0065)
	0.069−0.16	0.067−0.16	0.077−0.26	0.10−0.22
heptadecanoic[b]	<LOQ	<LOQ	0.083 (0.0057)	0.081 (0.0057)
			0.035−0.13	0.034−0.15
stearic[b]	2.01 (0.050)	2.00 (0.049)	2.47 (0.088)	2.29 (0.088)
	1.79−2.29	1.76−2.40	2.00−3.96	1.80−3.27
oleic[b]	22.86 (0.32)[d]	22.50 (0.32)	25.32 (0.44)	24.74 (0.44)
	21.39−25.96	20.22−24.01	22.19−32.00	21.66−29.31
linoleic[b]	61.66 (0.26)	61.69 (0.26)	56.76 (0.88)	58.14 (0.88)
	58.25−63.02	60.58−63.59	38.66−62.42	46.46−63.42
linolenic[b]	1.22 (0.024)	1.25 (0.024)	1.15 (0.043)	1.17 (0.043)
	1.08−1.45	1.15−1.53	0.47−1.46	0.77−1.65
arachidic[b]	0.41 (0.014)	0.41 (0.014)	0.55 (0.021)	0.51 (0.021)
	0.37−0.49	0.36−0.53	0.43−0.89	0.40−0.75
eicosenoic[b]	0.21 (0.0029)	0.21 (0.0029)	0.25 (0.011)	0.26 (0.011)
	0.19−0.23	0.18−0.24	0.037−0.61	0.20−0.34
behenic[b]	0.17 (0.0092)	0.17 (0.0091)	0.19 (0.017)	0.17 (0.017)
	0.14−0.23	0.069−0.25	0.067−0.52	0.069−0.30

[a]dwt = dry weight. [b]Percent total FA. [c]Mean (SE) = least-squares mean (standard error). [d]Statistically significant at 5% level ($\alpha = 0.05$).

analysis. Statistical analysis of the data from samples collected from each separate study was conducted at Certus International, Inc. (Chesterfield, MO, USA). Statistical analyses were conducted on forage and grain using a mixed model analysis of variance from the combination of all sites using the following model:

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

Y_{ijk} is the unique individual observation, U is the overall mean, T_i is the substance effect, L_j is the random location effect, $B(L)_{jk}$ is the random block within location effect, LT_{ij} is the random location by substance interaction effect, and e_{ijk} is the residual error. Within grain and forage, data for each compositional component from the experimental hybrids (MON 87427 or MON 87427 × MON 89034 × NK603) were compared to data from their respective control (EXP262 or MPA636B). Statistically significant differences between the experimental hybrid values and the control values were declared at $\alpha = 0.05$. For both studies, the field designs included other experimental hybrids not related to this study but included in the overall ANOVA. The conventional reference hybrids were used to estimate natural compositional variation that can generally be attributed to differences in the germplasm and environment.

Variance components analysis (VCA) was also conducted for both studies (MON 87427 and MON 87427 × MON 89034 × NK603) separately to estimate the proportion of random effects contributing to the total variance. In this application, the effects of substance, location, location × substance interaction, and replication (block) in the ANOVA model were set as random effects. The SAS procedure PROC MIXED was employed to run the analysis. The output table of covariance parameter estimates from SAS PROC MIXED procedure gives estimates of the variance component parameters for each of model components.[12] The variance component parameters of each model component were divided by the total variance to get the variance proportions for each of the components (see Suppl. Tables 3 and 4 in the Supporting Information). For both studies, the field designs included other experimental substances not related to this work but included in the overall ANOVA and VCA.

RESULTS AND DISCUSSION

Comparisons of MON 87427 and MON 87427 × MON 89034 × NK603 to their respective near-isogenic conventional control were on data combined across all field sites from each separate study (Tables 1−5; Suppl. Table 1 in the Supporting Information) using ANOVA. The analysis from both studies showed few statistically significant differences ($\alpha = 0.05$). For MON 87427, differences were observed for histidine, methionine, proline, palmitic acid, oleic acid, and vitamin B_6 in grain (Tables 1, 2, and 5). No differences were observed for forage (Suppl. Table 1). For MON 87427 × MON 89034 × NK603 differences were observed for calcium, manganese, phosphorus, zinc, niacin, and *p*-coumaric acid in grain (Tables 2−4). No differences were observed for forage (Suppl. Table 1).

These results are consistent with numerous prior studies that have illustrated the lack of impact of transgenesis on composition and highlighted that compositional variability is dominated by germplasm and environmental factors. The purpose of the following section is (i) to further describe the compositional equivalence of the MON 87427 products to their near-isogenic comparators, (ii) to illustrate how well-documented influential sources of compositional variability can be considered in comparative assessments,[5] and (iii) to further explain how statistical significance does not imply biological relevance from a composition or food/feed perspective.[5,6] Evaluation of the differences observed in the combined-site analysis therefore focused on the following logic:

(1) Consideration of the magnitude of the mean difference between experimental hybrid (MON 87427 or MON 87427 × MON 89034 × NK603) and the respective control in the context of the variation observed

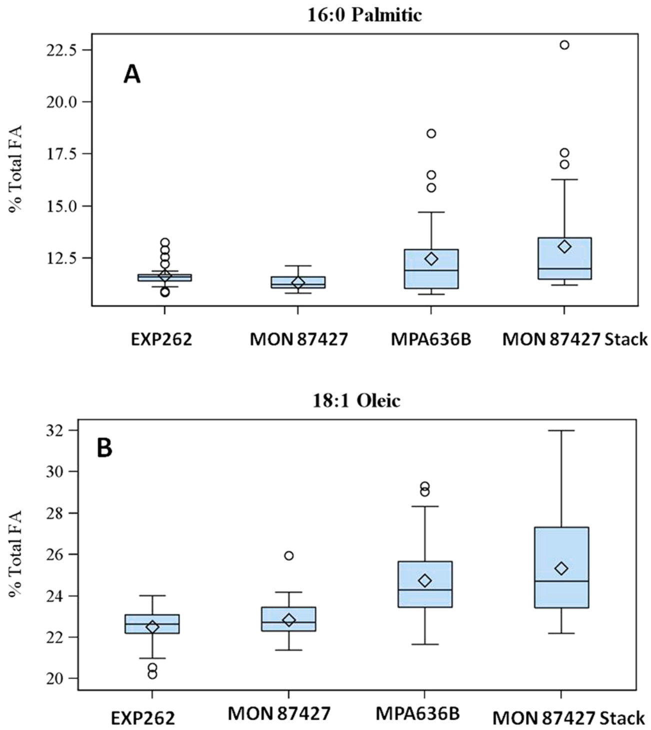

Figure 1. Box plot representing observed variability for palmitic acid and oleic acid values in MON 87427 and MON 87427 × MON 89034 × NK603 (MON 87427 Stack) and their respective conventional control. Each box extends from the lower (25th) to the upper (75th) quartile, and the line in each box represents the median. The whiskers extend to minimum and maximum data points. See Table 1 for details.

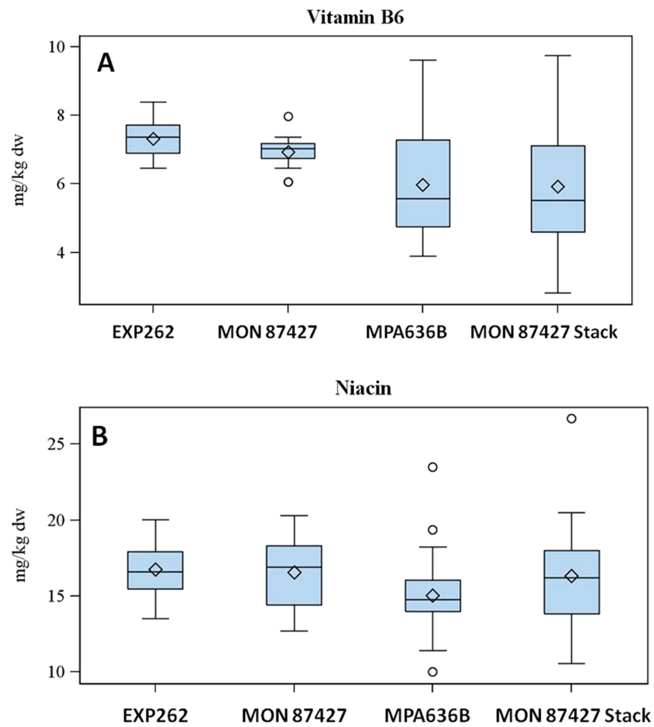

Figure 2. Box plot representing observed variability for niacin and vitamin B_6 values in MON 87427 and MON 87427 × MON 89034 × NK603 (MON 87427 Stack) and their respective conventional control. Legends are the same as described in Figure 1. See Table 2 for details.

(maximum value minus the minimum value) for the control. This allows a meaningful evaluation of the impact of transgene insertion relative to the environmental variability observed in a single genotype (with and without transgene). The individual replicate values of the experimental hybrid and control were also compared to each other to further demonstrate similarity. A significant difference in mean values between an experimental hybrid and the respective control values does not imply a meaningful difference from a compositional perspective, especially if the difference is much less than variation attributable to other sources (e.g., environmental influences).

(2) Comparison of the magnitude of the mean difference between the experimental hybrid (MON 87427 or MON 87427 × MON 89034 × NK603) and the respective control to variation in conventional maize as estimated

Table 2. Summary of Maize Grain Vitamins for MON 87427, MON 87427 × MON 89034 × NK603 and the Respective Conventional Controls

component (mg/kg dwt)[a]	MON 87427 mean (SE)[b] range	control EXP62 mean (SE) range	MON 87427 × MON 89034 × NK603 mean (SE) range	control MPA636B mean (SE) range
folic acid	0.32 (0.020)	0.32 (0.020)	0.99 (0.058)	0.91 (0.058)
	0.25−0.50	0.25−0.47	0.53−2.01	0.43−1.96
niacin	16.61 (0.56)	16.75 (0.56)	16.33 (0.60)[c]	15.06 (0.60)
	12.73−20.29	13.53−20.05	10.56−26.70	10.03−23.52
vitamin A	0.91 (0.036)	0.93 (0.036)	6.54 (0.49)	6.23 (0.49)
	0.66−1.17	0.77−1.12	3.40−9.11	3.03−9.38
vitamin B_1	2.58 (0.099)	2.53 (0.098)	2.48 (0.21)	2.57 (0.21)
	2.01−3.18	1.98−3.22	1.00−3.68	1.00−4.42
vitamin B_2	1.42 (0.043)	1.41 (0.043)	3.38 (0.19)	3.44 (0.19)
	1.05−1.81	1.04−1.83	1.92−5.46	1.72−5.96
vitamin B_6	6.92 (0.11)[c]	7.31 (0.11)	5.92 (0.30)	5.98 (0.30)
	6.05−7.97	6.45−8.39	2.81−9.75	3.90−9.61
vitamin E	18.34 (0.91)	18.62 (0.91)	16.41 (1.08)	15.60 (1.08)
	12.81−27.53	12.82−26.25	11.47−22.96	4.77−21.40

[a]dwt = dry weight. [b]Mean (SE) = least-squares mean (standard error). [c]Statistically significant at 5% level (α = 0.05).

Table 3. Summary of Maize Grain Carbohydrates by Calculation, Fibers, and Minerals for MON 87427, MON 87427 × MON 89034 × NK603 and the Respective Conventional Controls

component [a] (% dwt)	MON 87427 mean (SE)[b] range	control EXP62 mean (SE) range	MON 87427 × MON 89034 × NK603 mean (SE) range	control MPA636B mean (SE) range
carbohydrates by calculation	85.95 (0.23)	86.13 (0.23)	86.16 (0.36)	86.10 (0.36)
	84.75−87.61	84.93−88.12	84.54−88.41	84.36−88.66
acid detergent fiber	3.07 (0.095)	3.25 (0.094)	4.27 (0.073)	4.44 (0.073)
	2.27−4.10	2.18−4.36	3.60−4.79	3.80−4.87
neutral detergent fiber	10.54 (0.38)	10.74 (0.38)	11.51 (0.27)	11.52 (0.27)
	8.79−13.63	8.88−15.11	9.76−14.56	10.46−12.99
total dietary fiber	13.97 (0.45)	14.68 (0.45)	16.27 (0.45)	16.48 (0.45)
	10.39−16.57	11.64−17.68	12.36−19.35	13.70−18.67
ash	1.26 (0.040)	1.33 (0.040)	1.36 (0.025)	1.39 (0.025)
	0.98−1.59	0.96−1.86	1.11−1.50	1.15−1.51
calcium	0.0041 (0.00022)	0.0040 (0.00022)	0.0053 (0.00036)[c]	0.0048 (0.00036)
	0.0032−0.0054	0.0032−0.0053	0.0035−0.0077	0.0036−0.0066
copper (mg/kg dwt)	2.22 (0.059)	2.33 (0.059)	1.46 (0.071)	1.57 (0.071)
	1.79−2.73	1.78−3.34	1.04−2.24	0.98−2.42
iron (mg/kg dwt)	19.92 (0.55)	19.72 (0.55)	22.32 (0.62)	22.31 (0.62)
	16.97−23.51	16.97−24.07	18.60−26.99	17.11−29.39
magnesium	0.11 (0.0024)	0.11 (0.0024)	0.12 (0.0032)	0.12 (0.0032)
	0.099−0.13	0.096−0.13	0.088−0.14	0.098−0.15
manganese (mg/kg dwt)	6.70 (0.28)	6.63 (0.28)	7.53 (0.41)[c]	8.00 (0.41)
	5.01−8.25	5.10−8.45	4.05−9.15	4.61−11.46
phosphorus	0.31 (0.0056)	0.31 (0.0055)	0.31 (0.0069)[c]	0.33 (0.0069)
	0.27−0.33	0.25−0.35	0.24−0.40	0.27−0.39
potassium	0.32 (0.0093)	0.33 (0.0093)	0.36 (0.0079)	0.36 (0.0079)
	0.28−0.37	0.27−0.41	0.30−0.42	0.30−0.45
sodium (mg/kg dwt)	<LOQ	<LOQ	1.46 (0.21)	1.42 (0.21)
			0.35−6.25	0.34−8.60
zinc (mg/kg dwt)	24.91 (0.64)	24.92 (0.63)	25.14 (0.77)[c]	27.67 (0.77)
	20.43−29.36	20.14−29.94	18.67−30.37	20.87−36.63

[a]dwt = dry weight. [b]Mean (SE) = least-squares mean (standard error). [c]Statistically significant at 5% level (α = 0.05).

by in-study reference hybrid values, the scientific literature, and the ILSI Crop Composition Database (ILSI-CCDB). This provides further information that a difference in mean values between the experimental hybrids and respective controls does not imply a meaningful difference from a food and feed safety perspective, especially if the difference is much less than natural variation due to other sources (i.e., environmental and varietal influences).

(3) Evaluations of graphical representations of the compositional data. Proponents of graphical approaches consider that the "location, range, and distribution of numerical values[6a] within a data set should be understood" to provide context to results from significance testing. Box plots (Figures 1−4) are presented to compare the variation observed in the selected experimental hybrid and control components. Results from VCA are also presented to understand and convey sources of variation in the data (Figure 5; Suppl. Tables 3 and 4).

Total Fat and Fatty Acids. No significant differences (α = 0.05) between total fat mean values were observed between the MON 87427 products and their respective near-isogenic controls (Table 1) using ANOVA. Differences were observed for only two of 10 fatty acids assessed (palmitic and oleic acids, Table 1) in MON 87427, and no differences in fatty acids were observed for MON 87427 × MON 89034 × NK603. By evaluating the observed differences for palmitic and oleic acid in MON 87427 using the logic described above, these differences are shown to be irrelevant from a compositional and food/feed perspective. The mean palmitic acid value was 11.34% total FA for MON 87427 and 11.67% total FA for the control, a difference of −0.32% total FA. This difference was less than the range of the control values, 2.39% total FA (Table 1; Figure 1A) calculated from the minimum (10.87% total FA) and maximum (13.26% total FA) palmitic acid values. The mean oleic acid values were 22.86% total FA for MON 87427 and 22.50% total FA for the control, a difference of 0.36% total FA. This was less than the range of the control values, 3.79% total FA (Table 2; Figure 1B), calculated similarly to above from minimum and maximum oleic acid values. The mean differences in palmitic and oleic acid values between MON 87427 and the control were less than the ranges of the control values, indicating that MON 87427 does not affect levels more than natural variation within the control grown at multiple locations. The mean differences in palmitic acid and oleic acid were also less than the variation seen in the reference values (palmitic acid range = 9.27−14.17% total FA and oleic acid range = 21.23−34.93% total FA; Suppl. Table 2), and the MON 87427 mean values were within values observed in the literature and the ILSI-CCDB values[13] (Suppl. Table 5). Maize is known to exhibit extensive variability in total fat and fatty acid levels,[14] and these results are consistent with the literature. Figure 1 illustrates how the concepts of variability discussed

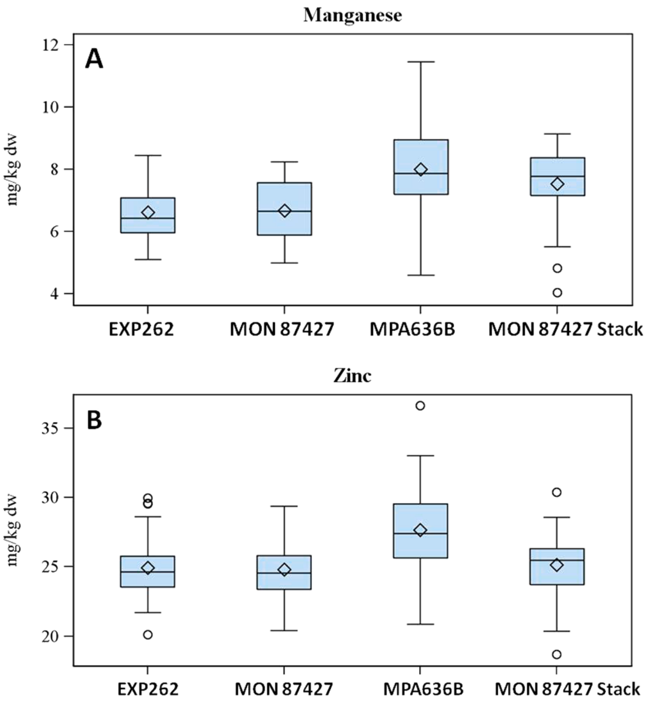

Figure 3. Box plot representing observed variability for manganese and zinc values in MON 87427 and MON 87427 × MON 89034 × NK603 (MON 87427 Stack) and their respective conventional control. Legends are the same as described in Figure 1. See Table 3 for details.

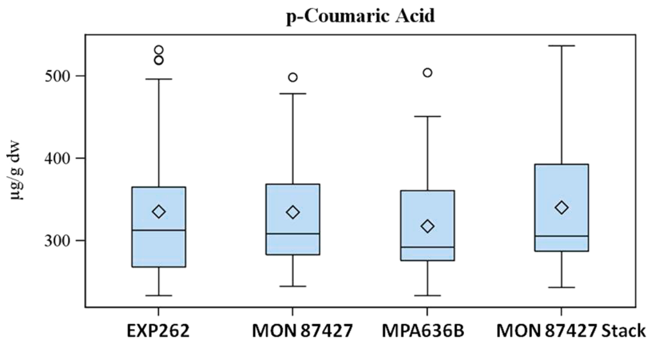

Figure 4. Box plot representing observed variability for *p*-coumaric acid values in MON 87427 and MON 87427 × MON 89034 × NK603 (MON 87427 Stack) and respective conventional control. Legends are the same as described in Figure 1. See Table 4 for details.

above can be presented graphically by comparing the observed variability in the experimental and control substances for palmitic and oleic acid. Figure 1 also demonstrates the similarities observed in values between the experimental and control hybrids. VCA results for all fatty acids are shown in the Supporting Information (Suppl. Tables 3 and 4). Overall, the greatest contributors to variation in both studies were location and residual error. Block and location × substance contributed least to variation in fat and fatty acid levels.

Vitamins. Significant differences (α = 0.05) between vitamin mean values were observed only for vitamin B_6 in MON 87427 compared to the near-isogenic control and for niacin in MON 87427 × MON 89034 × NK603 compared to the near-isogenic control (Table 2).

For vitamin B_6, the mean values were 6.92 mg/kg dwt for MON 87427 and 7.31 mg/kg dwt for the control, a difference of −0.39 mg/kg dwt. This difference was less than the range of the control values, 1.93 mg/kg dwt, indicating that MON 87427 does not affect levels of vitamin B_6 more than natural variation within the control grown at multiple locations. The corresponding range of values for MON 87427 was 1.92 mg/kg dwt (Table 2; Figure 2A).

For niacin, the mean values were 16.33 mg/kg dwt for MON 87427 × MON 89034 × NK603 and 15.06 mg/kg dwt for the control, a difference of 1.27 mg/kg dwt. This difference was less than the range of the control values, 13.48 mg/kg dwt, indicating that MON 87427 × MON 89034 × NK603 does not affect levels of niacin more than natural variation within the control grown at multiple locations. The corresponding range of values for MON 87427 × MON 89034 × NK603 was 16.14 mg/kg dwt (Table 2; Figure 2B). The mean difference in vitamin B_6 and niacin was also less than the variation seen in the respective reference values (vitamin B_6 range in MON 87427 = 5.51−8.22 mg/kg dwt, niacin range in MON 87427 × MON 89034 × NK603 = 9.53−25.00 mg/kg dwt, respectively, Suppl. Table 2) and values observed in the literature and the ILSI-CCDB (Suppl. Table 5). Figure 2 compares the variation observed in experimental and control hybrids in individual values across sites for vitamin B_6 and niacin and emphasizes the observed similarities in values between experimental and control hybrids. VCA results for all vitamins are shown in Suppl. Tables 3 and 4 in the Supporting Information. Overall, the greatest contributions to variation in both studies were location and residual error. Block and location × substance overall contributed least to variation in vitamin levels.

Table 4. Summary of Maize Grain Antinutrients and Secondary Metabolites for MON 87427, MON 87427 × MON 89034 × NK603 and the Respective Conventional Controls

component (% dwt)[a]	MON 87427 (treated) mean (SE)[b] range	control EXP62 mean (SE) range	MON 87427 × MON 89034 × NK603 mean (SE) range	control MPA636B mean (SE) range
phytic acid	0.87 (0.020)	0.90 (0.020)	0.90 (0.021)	0.92 (0.021)
	0.73−0.97	0.66−1.01	0.71−1.10	0.70−1.10
raffinose	0.23 (0.0097)	0.24 (0.0096)	0.26 (0.011)	0.26 (0.011)
	0.18−0.28	0.19−0.28	0.20−0.34	0.22−0.32
ferulic acid (µg/g dwt)	2610.27 (102.62)	2574.27 (102.51)	3166.36 (105.26)	3093.29 (105.26)
	2120.18−3179.78	1943.50−3636.36	2709.97−3710.76	2560.99−3492.06
p-coumaric acid (µg/g dwt)	331.99 (28.49)	335.25 (28.48)	340.37 (27.71)[c]	317.80 (27.71)
	244.09−498.88	232.77−532.51	242.97−537.25	232.58−504.50

[a]dwt = dry weight. [b]Mean (SE) = least-squares mean (standard error). [c]Statistically significant at 5% level (α = 0.05).

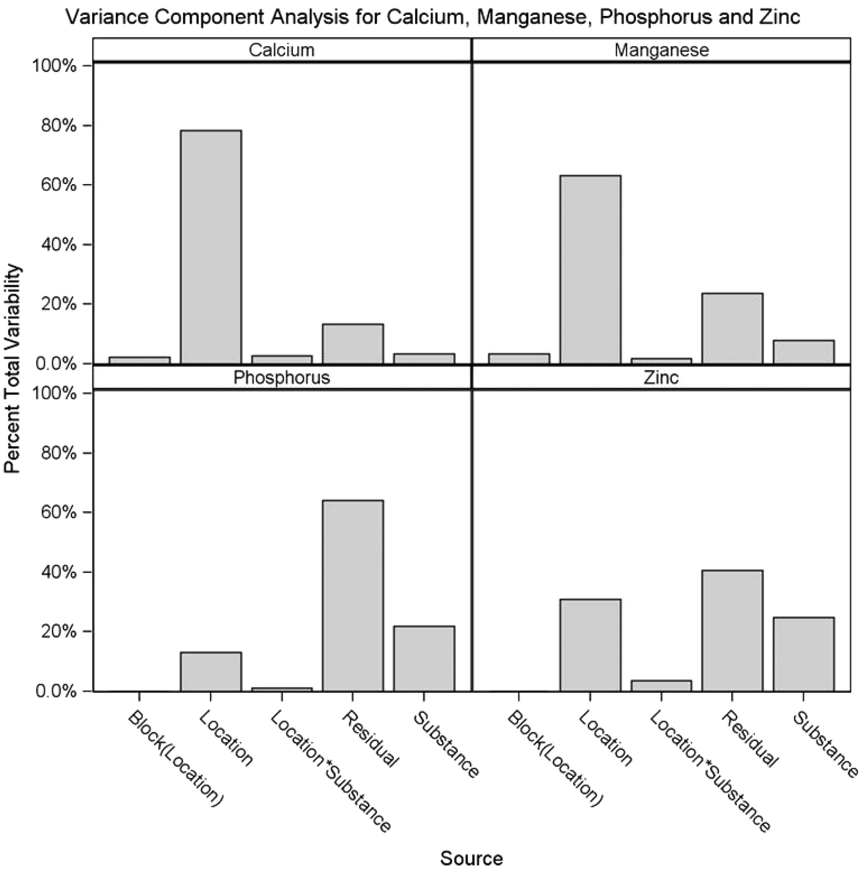

Figure 5. Sources of variation estimates from variance component analysis for calcium, manganese, phosphorus, and zinc values in MON 87427 × MON 89034 × NK603 (MON 87427 Stack).

Ash and Minerals. For MON 87427, no significant difference (α = 0.05) was observed for ash (Table 3) or minerals. For MON 87427 × MON 89034 × NK603, differences were observed for calcium, manganese, phosphorus, and zinc. Similar to the results observed for the fatty acids and vitamins discussed above, the logic applied above illustrated that observed significant mean differences (α = 0.05) for calcium, manganese, phosphorus, and zinc for MON 87427 × MON 89034 × NK603 were small relative to ranges of values observed for the control and reference hybrids as well as in the scientific literature. In other words, these data confirm that MON 87427 by itself or as part of a combined-stack product does not affect levels of minerals more than natural variation within the control grown at multiple locations. Mineral content in maize is known to vary by environment and germplasm.[15] Figure 3 compares the variation observed in experimental and control hybrids for manganese and zinc and highlights observed compositional similarity. VCA results for all minerals are shown in Suppl. Tables 3 and 4. Even though there were clear differences in the contribution of "location", "residual", and "substance" effects for different minerals, the greatest contributor to variation in both studies for most minerals was location. "Block" and "location × substance" overall contributed least to variation in mineral levels. Figure 5 shows results for calcium, manganese, phosphorus, and zinc from the MON 87427 × MON 89034 × NK603 study.

Antinutrient and Secondary Metabolites. Significant differences (α = 0.05) between antinutrient and secondary metabolite mean values were observed only for *p*-coumaric acid between MON 87427 × MON 89034 × NK603 and the near-isogenic control (Table 5). The mean *p*-coumaric acid values for MON 87427 × MON 89034 × NK603 were 340.37 and 317.80 μg/g dwt for the control, a difference of 22.57 μg/g dwt. This difference was much less than the range of the control values, 271.92 μg/g dwt, indicating that MON 87427 × MON 89034 × NK603 does not affect levels of *p*-coumaric acid more than natural variation observed within the control grown at multiple locations. The corresponding range of values from MON 87427 × MON 89034 × NK603 was 294.28 (Table 4; Figure 4). The mean difference was also less than the variation seen in the reference hybrid values (range = 104.18−608.45 μg/g dwt, Table 4) as well as the values observed in the literature and the ILSI-CCDB (Suppl. Table 5). Figure 4 shows the extensive variation associated with *p*-coumaric acid, and this variation can be attributed to location. The estimates of source of variation from variance component analysis are provided in Suppl. Tables 3 and 4 in the Supporting Information and highlight a major contribution (>75%) in both studies due to location.

Protein and Amino Acids. No significant differences (α = 0.05) in protein mean values were observed between the MON 87427 products and their respective near-isogenic controls (Table 5). The actual observed differences were small relative to the control; 2.33% for MON 87427 and −1.01% for MON 87427 × MON 89034 × NK603. To allow an assessment of the difference in amino acids in relation to a difference in protein, relative magnitudes of difference were expressed as a percent of control. Consistent with the changes observed with the protein values, the magnitudes of differences for amino acids were also small with statistical significance observed only for histidine,

Table 5. Summary of Maize Grain Protein and Amino Acids for MON 87427, MON 87427 × MON 89034 × NK603, and the Respective Conventional Controls

component (% dwt)[a]	MON 87427 mean (SE)[b] range	control EXP62 mean (SE) range	MON 87427 × MON 89034 × NK603 mean (SE)[b] range	control MPA636B mean (SE) range
protein	9.43 (0.20)	9.21 (0.20)	9.49 (0.38)	9.58 (0.38)
	8.25−10.74	7.44−10.51	7.47−11.14	6.79−11.90
alanine	0.70 (0.020)	0.68 (0.020)	0.73 (0.034)	0.73 (0.034)
	0.57−0.80	0.54−0.82	0.53−0.93	0.47−0.95
arginine	0.47 (0.0062)	0.47 (0.0062)	0.43 (0.012)	0.44 (0.012)
	0.43−0.53	0.42−0.53	0.35−0.49	0.34−0.52
aspartic acid	0.60 (0.011)	0.59 (0.011)	0.63 (0.025)	0.64 (0.025)
	0.52−0.66	0.52−0.65	0.48−0.79	0.43−0.79
cystine	0.20 (0.0040)	0.20 (0.0040)	0.21 (0.0061)	0.22 (0.0061)
	0.17−0.23	0.16−0.23	0.13−0.27	0.17−0.26
glutamic acid	1.71 (0.049)	1.65 (0.049)	1.88 (0.092)	1.89 (0.092)
	1.36−2.02	1.26−1.95	1.34−2.41	1.17−2.46
glycine	0.37 (0.0052)	0.36 (0.0052)	0.39 (0.0097)	0.40 (0.0097)
	0.34−0.40	0.31−0.41	0.32−0.44	0.32−0.45
histidine	0.27 (0.0048)[c]	0.26 (0.0048)	0.30 (0.0087)	0.31 (0.0087)
	0.23−0.29	0.19−0.30	0.24−0.35	0.23−0.37
isoleucine	0.33 (0.0090)	0.32 (0.0090)	0.35 (0.014)	0.35 (0.014)
	0.25−0.39	0.26−0.37	0.26−0.43	0.24−0.44
leucine	1.11 (0.040)	1.06 (0.040)	1.19 (0.064)	1.20 (0.064)
	0.83−1.31	0.76−1.31	0.83−1.53	0.73−1.60
lysine	0.26 (0.0043)	0.26 (0.0043)	0.31 (0.0079)	0.32 (0.0079)
	0.22−0.30	0.22−0.32	0.25−0.37	0.24−0.37
methionine	0.20 (0.0070)[c]	0.19 (0.0070)	0.23 (0.0086)	0.22 (0.0086)
	0.15−0.25	0.15−0.23	0.14−0.32	0.16−0.30
phenylalanine	0.47 (0.014)	0.45 (0.014)	0.51 (0.025)	0.52 (0.025)
	0.36−0.55	0.35−0.55	0.36−0.63	0.32−0.68
proline	0.82 (0.024)[c]	0.79 (0.024)	0.89 (0.035)	0.89 (0.035)
	0.62−0.95	0.57−0.95	0.67−1.08	0.60−1.11
serine	0.44 (0.0095)	0.43 (0.0094)	0.49 (0.020)	0.50 (0.020)
	0.38−0.53	0.34−0.51	0.37−0.60	0.35−0.63
threonine	0.34 (0.0053)	0.33 (0.0053)	0.37 (0.011)	0.38 (0.011)
	0.31−0.38	0.29−0.37	0.30−0.43	0.28−0.46
tryptophan	0.079 (0.0015)	0.079 (0.0015)	0.065 (0.0018)	0.067 (0.0018)
	0.072−0.087	0.068−0.097	0.057−0.072	0.053−0.077
tyrosine	0.36 (0.0092)	0.34 (0.0091)	0.26 (0.011)	0.27 (0.011)
	0.28−0.43	0.23−0.40	0.19−0.33	0.18−0.36
valine	0.44 (0.0080)	0.43 (0.0079)	0.46 (0.015)	0.47 (0.015)
	0.36−0.50	0.38−0.49	0.36−0.55	0.34−0.57

[a]dwt = dry weight. [b]Mean (SE) = least-squares mean (standard error). [c]Statistically significant at 5% level (α = 0.05).

methionine, and proline in the assessment of MON 87427 and no difference for MON 87427 × MON 89034 × NK603 (Table 5). The rationale applied above for other components reinforced how small these differences were by highlighting that MON 87427 did not affect levels of any amino acids acid more than natural variation within the control grown at multiple locations (Table 5). The mean histidine value was 0.27% dwt for MON 87427 and 0.26% dwt for the control, a difference of 0.0091% dwt. This difference was much less than range of the control values, 0.112% dwt (Table 1). Mean differences in methionine (0.011% dwt) and proline (0.032%) were also much less than the respective control ranges (0.081 and 0.383% dwt, respectively). The mean differences in histidine, methionine, and proline values were also less than the variation seen in the reference values (histidine range = 0.22−0.34% dwt, methionine range = 0.13−0.30% dwt, and proline range = 0.65−1.17% dwt; Suppl. Table 2), and the MON 87427 mean values were within values observed in the literature and the ILSI-CCDB (Suppl. Table 3). Together, these data confirm that the differences in mean values observed for the amino acids histidine, methionine, and proline in MON 87427 were not meaningful from a food/feed safety perspective. It is known that the levels of protein and amino acids in maize can vary widely depending on environmental conditions and agronomic practices.[4,16] The estimates of the source of variation for protein and amino acids from the VCA are provided in Suppl. Tables 3 and 4. The greatest sources of variation were "location" and "residual error".

Fibers and Carbohydrates by Calculation. No significant differences (α = 0.05) between carbohydrates and fiber mean values were observed between the MON 87427 and MON 87427 × MON 89034 × NK603 and their respective near-isogenic controls, respectively (Table 3).

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Concluding Remarks. In summary, comprehensive compositional analysis shows that levels of key nutrients, antinutrients, and secondary metabolites in forage and grain of MON 87427, an inducible male sterile and tissue selective glyphosate-tolerant maize product, are comparable to the near-isogenic conventional control. Multiple sources (germplasm and location) of variation in the study were accounted for to aid in the interpretation of compositional outcomes. This methodology highlighted that differences between MON 87427 and the control were of small magnitude and inconsequential from a food/feed safety perspective. This study also highlights the fact that transgene insertion was a much smaller source of variation in the composition when compared to environmental effects. Results from analysis of MON 87427 × MON 89034 × NK603 confirm that this observation extends to the combined-trait product. With increasing global demand for food production, developments of new strategies for seed production are important to sustainable agriculture. MON 87427 offers a novel biotechnology-derived trait to produce hybrid seeds with decreased costs and labor while maintaining seed purity. The investigation reported here demonstrated that biotechnological approaches can be applied in enhancing hybrid maize seed production without affecting compositional quality.

■ ASSOCIATED CONTENT

Supporting Information

Summary of forage components, analyte ranges, VCA proportions, and literature and ILSI-CCDB ranges. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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