Composition of forage and grain from genetically modified DP202216 maize is equivalent to non-modified conventional maize (*Zea mays* L.)

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**ABSTRACT.** DP202216 maize was genetically modified to increase and extend the expression of the *zmm28* gene relative to native *zmm28* gene expression, resulting in plants with enhanced grain yield potential. Standard nutritional and compositional parameters for maize grain and forage (e.g., proximates, fiber, minerals, amino acids, fatty acids, vitamins, anti-nutrients, secondary metabolites) from DP202216 maize were compared to grain and forage from non-modified near-isoline maize (control). Three amino acids (glycine, methionine, and serine) and two vitamins (vitamin B1 and vitamin B3) were statistically different between DP202216 and control maize grain but were not statistically different when adjusted using the false discovery rate method. These analyte values also fell within the ranges of natural variation of non-modified commercial maize varieties supporting that statistical differences were not biologically relevant. The composition of grain and forage from DP202216 maize is comparable to grain and forage from non-modified maize with a history of safe use.

**KEYWORDS.** Composition; genetically modified; safety assessment; substantial equivalence; *Zea mays* L.
INTRODUCTION

Genetically modified (GM) crops conferring insect pest protection and tolerance to herbicides have been successfully developed and commercialized for over 20 years. Historically, GM traits protect crop yield (e.g., by reducing damage from insects, competition from weeds, or more recently reducing sensitivity to abiotic stresses) but do not offer a measurable yield advantage under non-stressed conditions. Recognizing the increasing global food, feed, fiber, and fuel demands, new agricultural innovations will be needed to satisfy global productivity and sustainability needs in the future. GM crops are now being developed to improve crop yield. For example, MON 87403 maize (OECD identifier MON-874Ø3-1) has been modified to express a gene from Arabidopsis thaliana for increased ear biomass, and HB4 soybean (OECD identifier IND-ØØ41Ø-5), submitted by Veredca, LLC, has been modified to express a gene from Helianthus annuus for increased yield.

By selecting for desired plant phenotypes, conventional breeding approaches have made incremental improvements in maize grain yield and have altered the expression of endogenous maize genes and genetics over time. For example, MADS-box genes which encode transcription factors related to development have been selected for over time during maize domestication. DP202216 (OECD identifier DP-2Ø2216-6) maize was genetically modified to increase and extend the expression of the zmm28 gene relative to the native zmm28 gene expression. Both the introduced and native zmm28 genes encode the ZMM28 protein, a MADS-box transcription factor. The increased and extended expression of the ZMM28 protein results in plants with enhanced grain yield potential. The zmm28 gene, which encodes the ZMM28 protein, is endogenous to maize, including sweet corn. Using modern biotechnology tools to alter the expression of the endogenous zmm28 gene complements traditional breeding approaches for germplasm improvement. DP202216 maize also contains the phosphinothricin acetyltransferase protein, which confers tolerance to the herbicidal active ingredient glufosinate-ammonium.

Prior to commercialization, food and feed derived from GM crops undergo extensive testing to demonstrate food and feed safety, considering among other things the changes in the composition of the plant as a result of the modification. Substantial equivalence, which uses food and feed with a history of safe use (in this case commercial non-modified maize) as a reference to assess the safety of food and feed from a GM crop, is a key concept used in the compositional assessment. As a screening tool, compositional analytes (proximates, nutrients, antinutrients, etc.) from the GM crop are statistically compared to the same compositional analytes from a near-isoline control (a hybrid that has the same or a very similar genetic background but does not contain the genetic modification) to identify analytes with statistically significant differences. Those analytes are then subject to further evaluation including comparing data from the GM crop to the ranges of natural variation observed in commercial non-modified maize to understand biological relevance of any observed differences. The objective of this paper is to investigate whether the composition of grain and forage from DP202216 maize is substantially equivalent to the grain and forage from non-modified maize.

METHODS

Field study

DP202216 maize, non-modified near-isoline control maize (control maize), and four non-modified commercial maize hybrids (reference maize) were planted during the 2017 growing season at each of eight sites in the United States (Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania, and Texas) and one site in Canada (Ontario), which were selected to represent commercial maize-growing regions of North America. The control maize has the same genetic background as DP202216 maize but does not contain the genetic modification. The four reference maize hybrids grown at each location were selected from...
16 reference maize hybrids (34N84, 35F38, 35P12, P0506, P0589, P0760, P0965, P0987, P0993, XL5140, XL5513, XL5828, XL5840, BK5883, XL5939, and BK6076) based on maturity. All seeds were analyzed by event-specific polymerase chain reaction to confirm the presence of the event in DP202216 and the absence of the event in the control and reference maize. Each field site employed a randomized complete block design, containing four blocks, and each treatment (DP202216 maize, control maize, or one of the four reference maize) was randomly assigned to one six-row plot within each block. Normal pest control and maintenance practices (irrigation, fertilization, herbicide and pesticide applications, etc.), consistent with maize production, were used and were applied uniformly to each entire trial area.

**Forage and grain sample collection and processing**

One forage sample, which consisted of three plants at the R4 growth stage, and one grain (R6) sample, which consisted of grain pooled from five ears, were collected from each of the DP202216 maize plots (N = 32 total DP202216 forage and grain samples), control plots (N = 32 total control forage and grain samples), and from each of the reference maize plots (N = 32 total forage and grain samples for each reference maize hybrid). Forage samples were collected by cutting the aerial portion of the plants 10–15 cm above the soil surface line. Plants were chopped into sections (≤7.6 cm long), plant material was pooled, and approximately one-third of the pooled material was placed into a plastic-lined, cloth bag. Grain samples were collected by husking and shelling five ears, and the grain from the five ears was pooled and placed into a plastic bag which was then placed into a plastic-lined, cloth bag. Forage and grain samples were kept cool using wet ice, artificial ice, or dry ice until placed in a freezer (≤−20°C) at EPL BAS prior to being processed for composition analysis, as described previously.15

**Composition analysis**

Proximates, fiber, and minerals (crude protein, crude fat, crude fiber, acid detergent fiber [ADF], neutral detergent fiber [NDF], ash, carbohydrates, calcium, and phosphorus) were analyzed in forage samples. Grain samples were analyzed for the same proximates, fiber, and minerals as forage, with the addition of total dietary fiber (TDF), copper, iron, magnesium, manganese, potassium, sodium, and zinc. Grain samples were further analyzed for fatty acids (lauric [C12:0], myristic [C14:0], palmitic [C16:0], palmitoleic [C16:1], heptadecanoic [C17:0], heptadecenoic [C17:1], stearic [C18:0], oleic [C18:1], linoleic [C18:2], α-linolenic [C18:3], arachidic [C20:0], eicosenoic [C20:1], eicosadienoic [C20:2], behenic [C22:0], erucic [C22:1], and lignoceric [C24:0] acids), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), vitamins (β-carotene [pro vitamin A], vitamin B1 [thiamine], vitamin B2 [riboflavin], vitamin B3 [niacin], vitamin B5 [pantothenic acid], vitamin B6 [pyridoxine], vitamin B9 [folic acid], α-tocopherol [vitamin E], β-tocopherol, γ-tocopherol, and δ-tocopherol), as well as secondary metabolites and antinutrients (p-coumaric acid, ferulic acid, furfural, inositol, phytic acid, raffinose, and trypsin inhibitor). Composition analysis was performed by EPL BAS using GLP validated methods, as described previously.15,16 Additional modifications for some analytes are briefly described below.

**NDF and TDF**

The NDF detergent solution contained 30.0 g sodium dodecyl sulfate instead of sodium lauryl sulfate. TDF was quantified by an automated method using an ANKOM Dietary Fiber Analyzer. Duplicate samples were gelatinized with heat stable α-amylase then enzymatically
digested with protease and digested with amylo-glucosidase to remove starch and protein. Soluble dietary fiber was precipitated with ethanol. The residue was quantified gravimetrically. Protein analysis was performed on one of the duplicate samples using an automated Kjeldahl technique\textsuperscript{19} employing a Foss Kjeltc\textsuperscript{TM} 8400 Analyzer\textsuperscript{20}, while the other duplicate sample was analyzed for ash.

**Amino Acids**

Amino acids were quantified using reverse phase ultra-performance liquid chromatography (UPLC) with ultraviolet (UV) detection (UPLC/UV). Methionine and cystine analyses employed the sodium metabisulfite alternative with performic acid oxidation.\textsuperscript{21}

**Thiamine (Vitamin B1) and Riboflavin (Vitamin B2)**

Samples were extracted with 10% acetic acid/4.3% trichloroacetic acid solution and then analyzed by reverse phase high performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS).

**Tocopherols (α, β, γ, δ)**

Samples were extracted with hot hexane and extracts analyzed by normal phase UPLC with fluorescence detection.\textsuperscript{22,23}

**Beta-Carotene**

Samples were extracted using a 40:60 acetone:hexane with tert-butylhydroquinone solution and then analyzed by HPLC with UV/visible detection.\textsuperscript{24,25}

**Statistical analysis**

Statistical analysis was conducted to compare the nutrient composition of forage and grain derived from DP202216 maize and the control maize using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). Analytes were analyzed with linear mixed models, with maize line as a fixed effect and site and the interaction between maize line and site as random effects. Means were estimated for each maize line and compared to test whether there was a significant difference (raw \( p \)-value < 0.05) between the means. The approximate degrees of freedom for the statistical test were derived using the Kenward–Roger method.\textsuperscript{26} For each analyte, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or by fitting heterogeneous error variances across sites.

Seven fatty acids (lauric [C12:0], myristic [C14:0], heptadecanoic [C17:0], heptadecenoic [C17:1], eicosadienoic [C20:2], behenic [C22:0], and erucic [C22:1] acids), three vitamins (vitamin B2, β-tocopherol, and δ-tocopherol), and one secondary metabolite (furfural) were not statistically compared because either all samples were below the lower limit of quantification (LLOQ) or more than half of the samples of both maize lines were below the LLOQ (Supporting Information), which violated the assumptions of linear mixed models. Two fatty acids (palmitoleic [C16:1] and lignoceric [C24:0] acids), two minerals (copper and sodium), and one antinutrient (raffinose) had <50% of sample values below the LLOQ (Supporting Information). The false discovery rate (FDR) method\textsuperscript{27,28} was used to control false positive outcomes across all analytes analyzed using linear mixed models, and the adjusted \( p \)-value is reported for analytes with a raw \( p \)-value < 0.05.

**RESULTS AND DISCUSSION**

No statistical differences were observed for proximates, fiber, or minerals between DP202216 and control maize forage (Table 1) or grain (Table 2). No statistical differences were observed for fatty acids (Table 3), or for secondary metabolites or antinutrients between DP202216 maize grain and control grain (Table 4). Only three amino acids (glycine, methionine, and
TABLE 1. Mean and range (minimum and maximum individual values) of proximates, fiber, and mineral composition (% dry weight) in forage from DP202216 maize and non-modified near-isoline control maize (control).

| Component         | DP202216 mean (range) | Control mean (range) | p-Value (adjusted p-value) |
|-------------------|------------------------|----------------------|---------------------------|
| Crude protein     | 8.41 (6.20–10.8)       | NS                   | NS                        |
| Crude fat         | 8.32 (6.30–11.2)       | 8.86 (6.29–5.19)     | 0.14 (0.29–0.27)          |
| Crude fiber       | 4.10 (1.19–6.18)       | 2.98 (1.4–2.97)      | 0.05 (0.03–0.06)          |
| NDF               | 25.8 (18.3–35.3)       | 18.7 (12.5–28.2)     | 0.06 (0.03–0.09)          |
| ADF               | 19.8 (15.4–26.7)       | 22.9 (17.2–36.8)     | 0.10 (0.06–0.15)          |
| Ash               | 4.30 (2.15–6.9)        | 4.31 (2.09–8.6)      | 0.08 (0.05–0.11)          |
| Carbohydrates     | 83.1 (77.9–87.7)       | 81.6 (73.9–89.8)     | 0.09 (0.06–0.12)          |
| Phosphorus        | 0.216 (0.157–0.398)    | 0.253 (0.149–0.347)  | 0.07 (0.04–0.10)          |
| Calcium           | 0.257 (0.125–0.347)    | NS                   | NS                        |

ND: Not detectable, one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified.

Tolerance intervals were derived from DuPont Pioneer’s proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.

N. S: Not significant, where p ≥ 0.05. Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was ≥ 0.05. Adjusted p-value and is not in significant range. Tolerance intervals, literature ranges, and in-study reference ranges provide estimates of the natural variation in analyte values possible for conventional maize varieties grown in diverse environments; however, each of these estimates only partially account for the possible natural variability (because they do not individually consider all the possible growing environments or conventional maize lines that have a history of safe use). If
TABLE 2. Mean and range (minimum and maximum individual values) of proximates, fiber and mineral composition (% dry weight) in grain from DP202216 maize and non-modified near-isoline control maize (control). Tolerance intervals were derived from DuPont Pioneer’s proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.

| Component         | DP202216 mean (range) | Control mean (range) | P-value (adjusted p-value) | Tolerance interval | Literature range b | Reference maize range |
|-------------------|-----------------------|----------------------|----------------------------|-------------------|--------------------|----------------------|
| Total Dietary Fiber | 8.94 (6.96 - 13.2) | 8.88 (6.81 - 12.7) | NS                         | 5.91 - 15.8       | 6.68 - 35.31       | 6.53 - 15.2          |
| Crude Protein     | 8.58 (7.02 - 10.6)   | 8.36 (7.08 - 10.5)   | NS                         | 7.18 - 13.2       | 5.72 - 17.26       | 7.12 - 11.7          |
| Crude Fat         | 4.21 (3.10 - 5.35)   | 4.19 (3.09 - 5.36)   | NS                         | 2.58 - 6.00       | 1.363 - 7.830      | 2.45 - 5.86          |
| Crude Fiber       | 2.39 (1.13 - 3.06)   | 2.36 (1.71 - 3.14)   | NS                         | 1.44 - 3.48       | 0.49 - 5.5         | 1.18 - 4.04          |
| ADF               | 4.55 (2.87 - 6.88)   | 4.24 (3.45 - 5.77)   | NS                         | 2.64 - 6.26       | 1.41 - 11.34       | 2.89 - 7.94          |
| NDF               | 9.48 (6.86 - 11.3)   | 9.74 (6.89 - 11.4)   | NS                         | 7.22 - 20.8       | 4.28 - 22.64       | 5.87 - 12.7          |
| Ash               | 1.30 (0.952 - 1.54)  | 1.27 (0.810 - 1.43)  | NS                         | 0.976 - 1.80      | 0.616 - 6.282      | 0.830 - 1.63         |
| Carbohydrates     | 85.9 (83.9 - 88.5)   | 86.1 (83.6 - 88.0)   | NS                         | 80.2 - 88.0       | 77.4 - 89.7        | 81.5 - 88.1          |
| Calcium           | 0.00340 (0.00271 - 0.00408) | 0.00342 (0.00285 - 0.00435) | NS | 0.00131 - 0.00784 | ND - 0.101 | 0.00212 - 0.00595 |
| Copper            | 0.000125 (<0.0000625c - 0.000212) | 0.000128 (<0.0000625c - 0.000238) | NS | <0.0000625 - 0.000617 | ND - 0.0021 | <0.0000625c - 0.000169 |
| Iron              | 0.00173 (0.00146 - 0.00220) | 0.00168 (0.00151 - 0.00195) | NS | 0.00118 - 0.00261 | 0.0000712 - 0.00191 | 0.00120 - 0.00218 |
| Magnesium         | 0.110 (0.0904 - 0.136) | 0.108 (0.0876 - 0.137) | NS | 0.0787 - 0.163 | 0.0035 - 1.000 | 0.0820 - 0.147 |
| Manganese         | 0.000571 (0.000273 - 0.000850) | 0.000556 (0.000346 - 0.000801) | NS | 0.000328 - 0.00131 | 0.0000312 - 0.0054 | 0.000289 - 0.000992 |
| Phosphorus        | 0.298 (0.205 - 0.351) | 0.296 (0.209 - 0.367) | NS | 0.204 - 0.429 | 0.010 - 0.750 | 0.189 - 0.410 |
| Potassium         | 0.395 (0.316 - 0.451) | 0.399 (0.306 - 0.459) | NS | 0.222 - 0.541 | 0.18 - 0.720 | 0.276 - 0.511 |
| Sodium            | 0.000101 (<0.0000625c - 0.000726) | 0.000158 (<0.0000625c - 0.000961) | NS | 0.00000298 - 0.000366 | ND - 0.150 | <0.0000625c - 0.00207 |
| Zinc              | 0.00226 (0.00166 - 0.00282) | 0.00226 (0.00183 - 0.00277) | NS | 0.00140 - 0.00365 | 0.0000283 - 0.0043 | 0.00150 - 0.00295 |

a NS (not significant), where \( p \geq 0.05 \). Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was significant.

b Literature range.15, 32-36 ND (not detectable): one or more assay values in the published literature references were below the LLOQ and were not quantified.

c Analytes had < 50% of sample values below the LLOQ.
| Component                  | DP202216 mean (range) | Control mean (range) | p-Value (adjusted p-value) | Tolerance interval | Literature (range) | Reference maize (range) |
|----------------------------|-----------------------|----------------------|----------------------------|--------------------|--------------------|------------------------|
| Lauric acid (C12:0)        | <LLOQ (<LLOQ)         | <LLOQ (<LLOQ)        | NA                        | 0–0.209d           | ND–0.698            | <LLOQ                  |
| Myristic acid (C14:0)      | <LLOQ (<LLOQ)         | <LLOQ (<LLOQ)        | NA                        | 0–0.267d           | ND–0.288            | <LLOQ                  |
| Palmitic acid (C16:0)      | 10.6 (10.3–11.3)      | 10.6 (10.3–11.7)     | NS                        | 9.23–26.0          | 6.81–39.0           | 10.0–14.2              |
| Palmitoleic acid (C16:1)   | 0.0787 (0.0385–0.107) | 0.0775               | NS                        | 0–0.463            | ND–0.67             | 0.0349–0.136           |
| Heptadecanoic acid (C17:0) | <LLOQ (<LLOQ)         | <LLOQ (<LLOQ)        | NA                        | 0–0.245            | ND–0.203            | <LLOQ                  |
| Heptadecenoic acid (C17:1) | <LLOQ (<LLOQ)         | <LLOQ (<LLOQ)        | NA                        | 0–0.135d           | ND–0.131            | <LLOQ                  |
| Stearic acid (C18:0)       | 2.09 (1.66–2.42)      | 2.06 (1.77–2.40)     | NS                        | 1.31–3.94          | ND–4.9              | 1.39–2.54              |
| Oleic acid (C18:1)         | 29.9 (27.5–32.5)      | 29.9 (28.3–32.3)     | NS                        | 18.9–39.4          | 16.36–42.81         | 22.4–34.3              |
| Linoleic acid (C18:2)      | 54.9 (51.2–57.3)      | 55.0 (51.3–56.7)     | NS                        | 28.9–64.4          | 13.1–67.68          | 45.5–60.6              |
| c-Linolenic acid (C18:3)   | 1.33 (1.16–1.56)      | 1.33 (1.20–1.53)     | NS                        | 0.0362–2.15        | ND–2.33             | 0.922–2.21             |
| Arachidic acid (C20:0)     | 0.390 (0.344–0.526)   | 0.388 (0.337–0.498)  | NS                        | 0.296–0.916        | 0.267–1.2           | 0.296–0.558            |
| Eicosenoic acid (C20:1)    | 0.258 (0.236–0.304)   | 0.256 (0.234–0.290)  | NS                        | 0.0380–0.693       | ND–1.952            | 0.224–0.521            |
| Eicosadienoic acid (C20:2) | <LLOQ (<LLOQ)         | <LLOQ (<LLOQ)        | NA                        | 0–0.825d           | ND–2.551            | <LLOQ                  |
| Behenic acid (C22:0)       | 0.0871 (0.0710–0.204) | 0.0873               | NA                        | 0–0.453            | ND–0.5              | 0.0691–0.314           |
| Lignoceric acid (C24:0)    | 0.167 (0.0712–0.283)  | 0.165 (0.0708–0.258) | NS                        | 0–0.639            | ND–0.91             | 0.0796–0.391           |

**Notes:**

- **NS:** Not significant, where $p > 0.05$. Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was significant. NA: Not applicable; mixed model analysis was not performed or confidence interval was not determined.
- **Literature range:** ND: Not detectable; one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified.
- **<LLOQ:** all fatty acid sample values were below the assay LLOQ.
- **Historical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.**
- **Tolerance intervals were derived from DuPont Pioneer’s proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.**
- **Palmitoleic Acid (C16:1) and Lignoceric Acid (C24:0) had < 50% of sample values below the LLOQ. Behenic Acid (C22:0) had >50% of sample values below the LLOQ. Erucic acid (C22:1) was not statistically analyzed because all sample values in the study and in historical commercial reference lines were below the lower limit of quantification (LLOQ). This analyte was excluded from the table.**
TABLE 4. Mean and range (minimum and maximum individual values) of secondary metabolites and antinutrients (% dry weight) in grain from DP202216 maize and non-modified near-isoline control maize (control).

| Component                  | DP202216 mean (range) | Control mean (range) | p-Value (adjusted p-value) | Tolerance interval | Literature (range)b | Reference maize (range) |
|-----------------------------|------------------------|----------------------|---------------------------|--------------------|---------------------|------------------------|
| p-Coumaric acid             | 0.0242 (0.0200–0.0297) | 0.0233 (0.0182–0.0296) | NS                        | 0.00715–0.0521     | ND–0.08             | 0.0150–0.0505          |
| Ferulic acid                | 0.213 (0.190–0.254)    | 0.207 (0.170–0.249)   | NS                        | 0.019–0.359        | ND–0.02             | ND–0.135–0.324         |
| Furfural                    | <0.000100 (<0.000100)  | <0.000100 (<0.000100) | NA                        | <0.000100          | ND                  | <0.000100              |
| Inositol                    | 0.0236 (0.0160–0.0362) | 0.0248 (0.0175–0.0351) | NS                        | 0.00684–0.0509     | ND–0.0063–0.48      | 0.0131–0.0344          |
| Phytic acid                 | 0.878 (0.456–1.24)     | 0.895 (0.500–1.27)    | NS                        | 0.516–1.37         | ND–1.940            | <0.355–1.34           |
| Raffinose                   | 0.104 (<0.0800–0.246)  | 0.0995 (<0.0800–0.183) | NS                        | 0–0.440            | ND–0.466            | <0.0800–0.301          |
| Trypsin inhibitor (TIU/mg DW)c | 1.66 (1.05–2.83)      | 1.69 (1.22–3.25)      | NS                        | 1.02–5.68          | ND–8.42             | 1.03–3.01             |

*NS: Not significant, where p ≥ 0.05. Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was significant. NA: Not applicable; mixed model analysis was not performed or confidence interval was not determined.

bLiterature range.15,29–33 ND: Not detectable; one or more assay values in the published literature references were below the LLOQ and were not quantified.

cFurfural had all sample values below the LLOQ. Raffinose had < 50% of sample values below the LLOQ and was subjected to the mixed model analyses. For Phytic acid, one or more reference maize sample values were below the LLOQ.

dTIU/mg DW (trypsin inhibitor units per milligram dry weight).

Tolerance intervals were derived from DuPont Pioneer’s proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.
TABLE 5. Mean and range (minimum and maximum individual values) of amino acids (% dry weight) in grain from DP202216 maize and non-modified near-isoline control maize (control).

| Component   | DP202216 mean (range) | Control mean (range) | p-Value (adjusted p-value) | Tolerance interval | Literature (range) | Reference maize (range) |
|-------------|------------------------|----------------------|----------------------------|--------------------|---------------------|------------------------|
| Alanine     | 0.623 (0.479–0.800)    | 0.609 (0.503–0.803)  | NS                         | 0.492–1.08         | 0.44–1.48           | 0.500–0.937            |
| Arginine    | 0.390 (0.315–0.450)    | 0.380 (0.309–0.429)  | NS                         | 0.317–0.568        | 0.12–0.71           | 0.305–0.502            |
| Aspartic acid| 0.540 (0.412–0.651)    | 0.530 (0.434–0.649)  | NS                         | 0.445–0.916        | 0.33–1.21           | 0.429–0.779            |
| Cystine     | 0.201 (0.126–0.239)    | 0.191 (0.124–0.228)  | NS                         | 0.132–0.303        | 0.12–0.51           | 0.0948–0.272           |
| Glutamic acid| 1.57 (1.20–2.03)       | 1.53 (1.23–2.03)     | NS                         | 1.04–2.70          | 0.97–3.54           | 1.24–2.38              |
| Glycine     | 0.362 (0.303–0.461)    | 0.350 (0.304–0.392)  | 0.00731 (0.215)            | 0.292–0.487        | 0.184–0.685         | 0.291–0.446            |
| Histidine   | 0.256 (0.207–0.297)    | 0.249 (0.206–0.300)  | NS                         | 0.177–0.359        | 0.14–0.46           | 0.200–0.345            |
| Isoleucine  | 0.289 (0.223–0.386)    | 0.282 (0.231–0.389)  | NS                         | 0.229–0.494        | 0.18–0.69           | 0.237–0.421            |
| Leucine     | 1.03 (0.778–1.45)      | 1.01 (0.802–1.46)    | NS                         | 0.763–1.85         | 0.64–2.49           | 0.843–1.62             |
| Lysine      | 0.272 (0.220–0.327)    | 0.263 (0.198–0.319)  | NS                         | 0.186–0.412        | 0.129–0.668         | 0.127–0.391            |
| Methionine  | 0.201 (0.143–0.234)    | 0.187 (0.135–0.231)  | 0.0246 (0.334)             | 0.108–0.342        | 0.10–0.47           | 0.104–0.246            |
| Pheny/lalanine| 0.430 (0.314–0.567)   | 0.418 (0.293–0.570)  | NS                         | 0.342–0.736        | 0.24–0.93           | 0.321–0.626            |
| Proline     | 0.798 (0.616–1.01)     | 0.780 (0.649–1.01)   | NS                         | 0.597–1.25         | 0.46–1.75           | 0.631–1.11             |
| Serine      | 0.446 (0.346–0.609)    | 0.430 (0.342–0.526)  | 0.0197 (0.334)             | 0.296–0.677        | 0.18–0.91           | 0.356–0.595            |
| Threonine   | 0.316 (0.260–0.374)    | 0.310 (0.265–0.371)  | NS                         | 0.179–0.476        | 0.22–0.67           | 0.265–0.413            |
| Tryptophan  | 0.0590 (0.0366–0.0702) | 0.0584 (0.0358–0.0690)| NS                         | 0.0405–0.0913      | 0.027–0.215         | 0.0356–0.0813          |
| Tyrosine    | 0.221 (0.157–0.273)    | 0.216 (0.162–0.283)  | NS                         | 0.164–0.421        | 0.10–0.73           | 0.176–0.315            |
| Valine      | 0.384 (0.316–0.488)    | 0.384 (0.329–0.485)  | NS                         | 0.316–0.626        | 0.21–0.86           | 0.325–0.541            |

*aNS: Not significant, where p ≥ 0.05. Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was significant.

bLiterature range.15,29–33

Tolerance intervals were derived from DuPont Pioneer’s proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.
TABLE 6. Mean and range (minimum and maximum individual values) of vitamins (mg/kg dry weight) in grain from DP202216 maize and non-modified near-isoline control maize (control).

| Component                  | DP202216 mean (range) | Control mean (range) | p-Value (adjusted p-value) | Tolerance interval | Literature (range) | Reference maize (range) |
|---------------------------|-----------------------|----------------------|---------------------------|--------------------|-------------------|------------------------|
| β-Carotene                | 0.962 (0.413–2.30)    | 0.983 (0.429–2.08)   | NS                        | <0.0500–2.06d      | 0.3–5.4           | 0.249–3.51             |
| Vitamin B1 (thiamine)     | 2.54 (1.99–3.23)      | 2.38 (2.08–3.08)     | 0.00466 (0.215)           | 1.71–5.38          | ND–40.00          | 1.97–3.11              |
| Vitamin B2 (riboflavin)   | <0.900c (<0.900c)     | <0.900c (<0.900c)    | NA                       | <0.900–2.27d       | ND–7.35           | <0.900c                |
| Vitamin B3 (niacin)       | 13.5 (9.33–16.2)      | 14.7 (10.9–22.7)     | 0.00947 (0.215)           | 7.86–25.2          | ND–70             | 9.49–66.0              |
| Vitamin B5 (pantothenic acid) | 4.71 (3.16–6.22)  | 5.11 (3.62–7.10)     | NS                       | 3.05–7.66          | 3.0–14            | 3.08–6.51              |
| Vitamin B6 (pyridoxine)   | 4.44 (2.23–8.15)      | 4.54 (2.81–9.48)     | NS                       | 1.37–8.67          | ND–12.14          | 2.51–10.7              |
| Vitamin B9 (folic acid)   | 0.854 (0.235–1.72)    | 0.923 (0.565–2.50)   | NS                       | 0.319–2.41         | ND–3.50           | 0.461–2.70             |
| α-Tocopherol              | 4.44 (1.07–8.92)      | 4.28 (0.969–7.63)    | NS                       | 0–25.1             | ND–68.67          | <0.500c–21.3           |
| β-Tocopherol              | <0.500c (<0.500c)     | <0.500c (<0.500c)    | NA                       | <0.500–1.10d       | ND–19.80          | <0.500c                |
| γ-Tocopherol              | 28.9 (11.4–36.3)      | 25.9 (10.8–35.6)     | NS                       | 0–46.5             | ND–58.61          | 3.06–42.7              |
| δ-Tocopherol              | 0.533 (<0.500c–1.13)  | 0.519 (<0.500c–1.16) | NA                       | <0.500–2.61d       | ND–14.61          | <0.500c–1.14           |
| Total tocopherols         | 32.1 (13.6–42.8)      | 31.0 (12.3–42.2)     | NA                       | 0–61.0             | ND–89.91          | 5.33–52.1              |

*aNS: Not significant, where p ≥ 0.05. Adjusted p-value, which is used to control for false positive outcomes across all analytes analyzed using linear mixed models, is reported if the raw p-value was significant. NA: Not applicable; mixed model analysis was not performed or confidence interval was not determined.

*bLiterature range.15,29–33 ND: Not detectable; one or more assay values in the published literature references were below the LLOQ and were not quantified.

*cVitamin B2 and β-Tocopherol had all sample values below the LLOQ. δ-Tocopherol had >50% of sample values below the LLOQ. For α-Tocopherol, one or more reference maize sample values were below the LLOQ.

*dHistorical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.

Tolerance intervals were derived from DuPont Pioneer's proprietary accumulated data from commercial non-modified maize lines, literature ranges were obtained from published literature, and reference maize ranges were obtained from the four non-modified commercial maize lines grown at each site.
of safe use. In this study, the ranges of the three amino acids and the two vitamins were within the corresponding tolerance intervals, so comparison to the literature ranges (Tables 5 and 6, respectively) was not necessary. Nevertheless, if literature ranges were used, the ranges of the amino acids and vitamins were also within the literature ranges, which again further adds to the weight of evidence that the differences between the DP202216 maize grain and control grain are not biologically relevant.

Finally, the range of analyte observations from DP202216 maize could also be compared to the respective range of the analyte measured in the non-modified reference maize lines grown in this study. However, the in-study reference ranges only partially account for the natural variability of these analytes in maize because they are based on only 16 conventional maize lines and 8 environments, which is a much smaller sample than that of the tolerance intervals or literature ranges. Since the ranges of the three amino acids and two vitamins were determined to fall within the natural variation based on the tolerance intervals or literature ranges, additional consideration of the in-study reference ranges is unnecessary. In some cases, the range of analyte observations for DP202216 maize does not fall within the in-study reference lines (serine, vitamin B1, and vitamin B3). This can be attributed to the limitations of the in-study reference data as an estimate of natural variation.

While compositional analysis studies have been required as part of the regulatory approval process for GM crops for over 20 years, in most cases, compositional assessment does not inform the safety assessment for GM crops. There is extensive literature on GM crop composition that demonstrates that the process used to genetically modify a plant does not result in unintended harmful changes in compositional end points.\textsuperscript{37,38} Compositional assessment should only be conducted if it informs the safety assessment (specifically in cases where a plausible hypothesis can be developed for changes in composition, based on the trait, mode of action, etc.). In the case of DP202216 maize, the expression of the MADS-box transcription factor *zmM28* was extended and increased, resulting in plants with enhanced grain yield potential.\textsuperscript{7} Based on the function of transcription factors (regulating gene expression and endogenous plant pathways), compositional analysis was conducted to investigate whether there were any unintended compositional changes in DP202216 maize grain and forage. All of the composition analytes analyzed for DP202216 maize grain and forage were within the range of normal variation and the few statistical differences detected are not biologically relevant. Therefore, these results support the conclusion that the nutrient composition of forage and grain derived from DP202216 maize is substantially equivalent to that of conventional maize represented by non-modified near-isoline control maize and non-modified commercial maize, which adds to the weight of evidence of safety of DP202216 maize.

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**DISCLOSURE OF INTEREST**

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**SUPPLEMENTARY MATERIAL**

Supplemental data for this article can be accessed on the publisher’s website.
SUPPORTING INFORMATION
DESCRIPTION

Table summarizing the number of samples that were below the lower limit of detection for various analytes in grain.

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