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Glyphosate-Tolerant Alfalfa Is Compositionally Equivalent to Conventional Alfalfa (*Medicago sativa* L.)

MELINDA C. McCANN,*,† GLENNON J. ROGAN,† SHARIE FITZPATRICK,‡
WILLIAM A. TRUJILLO,§ ROY SORBET,‖ GARY F. HARTNELL,†
SUSAN G. RIODAN,† AND MARGARET A. NEMETH†

Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167, Forage Genetics
International, N5292 South Gills Coulee Road, West Salem, Wisconsin 54669, Covance Laboratories
Inc., 3301 Kinsman Boulevard, Madison, Wisconsin 53704, and Certus International, Inc.,
1422 Elbridge Payne Road, Suite 200, Chesterfield, Missouri 63017

Glyphosate-tolerant alfalfa (GTA) was developed to withstand over-the-top applications of glyphosate, the active ingredient in Roundup agricultural herbicides. As a part of the safety assessment, GTA (designated J101 × J163) was grown under controlled field conditions at geographically diverse locations within the United States during the 2001 and 2003 field seasons along with control and other conventional alfalfa varieties for compositional assessment. Field trials were conducted using a randomized complete block design with four replication blocks at each site. Alfalfa forage was harvested at the late bud to early bloom stage from each plot at five field sites in 2001 (establishment year) and from four field sites in 2003 (third year of stand). The concentration of proximate constituents, fibers, amino acids, coumestrol, and minerals in the forage was measured. The results showed that the forage from GTA J101 × J163 is compositionally equivalent to forage from the control and conventional alfalfa varieties.

KEYWORDS: Alfalfa (*Medicago sativa* L.); glyphosate-tolerant; composition; coumestrol

INTRODUCTION

Alfalfa (*Medicago savita* L.) is an important feed crop originating from the Near East and Central Asia (*1*). It was the first forage crop to be domesticated and has adapted to many climates and soils throughout the world. Over 21 million acres of alfalfa hay and two million acres of forage (greenchop) are harvested in the United States annually (*2*). Alfalfa is highly valued as an animal feed because of its high protein content, high intake potential, and digestibility. Given its high nutritive value, alfalfa may serve as the sole plant component in many livestock feeding programs, which demand high-quality alfalfa.

Alfalfa is a perennial crop with multiple cuttings each year possible and a stand life of approximately 5 years (*3*). Weed control during stand establishment is critical because weeds compete with the alfalfa plants for moisture, nutrients, and light, directly reduce the seed yield potential of the stand, and create a loss in the alfalfa forage quality (*4*). For these reasons, weed infestations are the major limiting factor in the production of high-quality alfalfa forage (*5, 6*). The value per ton of alfalfa forage to both commercial hay and livestock producers is based largely on forage quality. Limitations of currently available weed control options often force growers to use alternative approaches, such as (i) seeding with a cover or companion crop, which may suppress weeds but also competes with alfalfa; (ii) delaying the first cutting for a minimum of 60 days, thus sacrificing hay quality to allow surviving alfalfa to get ahead of the competing weeds; or (iii) delaying seeding of new stands until late summer or early fall when there is less weed competition (*4*).

Monsanto Co. and Forage Genetics International have developed glyphosate-tolerant alfalfa [GTA, marketed under the trade name Roundup Ready (Roundup and Roundup Ready are registered trademarks of Monsanto Technology LLC) alfalfa] that is tolerant to glyphosate, the active ingredient in Roundup agricultural herbicides. GTA (designated J101 × J163) has been genetically engineered to produce a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). The CP4 EPSPS protein is functionally similar to native EPSPS enzymes but has a much reduced affinity for glyphosate (*7*). In conventional plants, glyphosate binds to the plant EPSPS enzyme, blocking the biosynthesis of aromatic amino acids thereby depriving plants of these essential components (*8, 9*). In glyphosate-tolerant plants, nutritional requirements for normal growth and development are met by the continued action of the glyphosate-tolerant CP4 EPSPS enzyme in the presence of glyphosate. A comprehensive

* To whom correspondence should be addressed. Tel: 314-694-7556. Fax: 314-694-3080. E-mail: melinda.c.mccann@monsanto.com.
† Monsanto Company.
‡ Forage Genetics International.
§ Covance Laboratories Inc.
‖ Certus International, Inc.

characterization and safety assessment of the CP4 EPSPS protein have been conducted (*10*).

The development of food or feed derived through the techniques of modern biotechnology involves a thorough safety evaluation. One component of the safety evaluation is to compare the biotechnology-derived food/feed to a near-isogenic conventional counterpart (*11*−*13*). A critical part of this comparative safety assessment process is determining whether the common nutrients and antinutrients of the biotechnology-derived food/feed are equivalent to the food/feed derived from a near-isogenic control food/feed. The purpose of this study was to evaluate whether the forage collected from GTA is compositionally equivalent to forage collected from a near-isogenic control and other conventional commercial alfalfa varieties.

MATERIALS AND METHODS

Plant Production. GTA was produced by *Agrobacterium*-mediated transformation of an elite alfalfa line, R2336. J101 and J163 are independent transformation events combined by conventional breeding methods as described by ref *14* to produce GTA J101 × J163. The near-isogenic control alfalfa plants were the null offspring derived from the same ancestor population as the GTA plant starting material. During the process of ensuing natural breeding and directed selection for *cp4 epsps*, a representative transgene-segregating seed source was genotypically and phenotypically identified. From this common source, a subset of control alfalfa plants was genetically identified to be null-segregating siblings (i.e., lacking *cp4 epsps*; used as the control plant starting material ancestors) and a second subset was identified as positive-segregating siblings (i.e., *cp4 epsps* present; used as the GTA plant starting material ancestors). Using Southern blot analysis, it was determined that the null control siblings did not contain the *cp4 epsps* nor any elements of the plasmid used during the plant transformation process. CP4 EPSPS-specific enzyme-linked immunosorbent assay assay of the null control siblings also did not detect CP4 EPSPS whereas the positive-segregating siblings were CP4 EPSPS positive.

Field Trials. Alfalfa plantlets (fall dormancy 4 category) were transplanted into a randomized complete block design with four replication blocks during the spring of 2001 in the states of California, Illinois, New York, Washington, and Wisconsin. These field sites provided a variety of environmental and agronomic conditions representative of regions where alfalfa is grown in the United States. Each plot consisted of seven rows spaced six inches apart with the plants within each row also spaced six inches apart. In addition to plots containing GTA and the control plants, several conventional alfalfa varieties were grown at each of the five field locations. These conventional varieties were Cimmarron VR, Innovator+Z, Macon, Magnum IV, Oneida VR, Ranger, Sommerset, Vernal, Vernema, WL252HQ, 5454, and WL325HQ. USDA-APHIS requirements for the shipment, movement, and environmental release were followed throughout the field trial season. The plots were maintained at each field location from 2001 (initial transplanting) to 2003 (final harvest). Each alfalfa plot had multiple cuttings during the 2001, 2002, and 2003 field seasons, and each GTA plot had multiple applications of a glyphosate herbicide over the course of the growing season coincident with each cutting. All plots were managed to minimize weeds.

Forage Collection and Processing. Forage samples utilized for compositional analyses were harvested from the second field cutting during the year of establishment (2001) and the first field cutting after winter dormancy during the third year of field stand (2003). The forage samples harvested in 2001 for analysis were from all field sites planted. The forage samples harvested in the 2003 field season for analysis were from the same field plots as the 2001 field season for the field locations in California, Illinois, Washington, and Wisconsin. The forage samples from all plots during both field seasons were collected randomly across each plot during the late bud to early bloom stage from all plant tissue more than two inches above ground. Each sample was combined in uniquely labeled bags and placed on dry ice within 10 min of cutting. The plots were harvested in the order of control or conventional variety plots followed by GTA plots. All forage samples were ground to a

fine powder with dry ice and maintained frozen (−20 °C) until compositional analysis. The identity of the forage harvested from each plot was verified by event-specific polymerase chain reaction analysis, and chain-of-custody documentation accompanied all sample shipments.

Compositional Analyses. Ground alfalfa forage samples from the 2001 and 2003 field seasons were analyzed for ash, carbohydrates, moisture, protein, total fat, acid detergent fiber (ADF), lignin, neutral detergent fiber (NDF), amino acids, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. In addition, the 2003 field season samples were analyzed for coumestrol. The analytes measured were considered the most important nutrients and antinutrients in alfalfa food and feed uses. Analytical data were generated by Covance Laboratories Inc. (Madison, WI), and the analytical methods utilized are summarized below. A limit of quantitation (LOQ) was also determined for each method, based on the lowest quantitated standard or control sample. All laboratory activities followed good laboratory practices (*15*).

Proximate Constituent Analysis. Ash content was estimated by igniting the sample with an electric furnace and determining the percent ash gravimetrically (*16*). The moisture content was estimated by loss of weight upon drying the samples in an oven at constant temperature (*17*, *18*). The crude protein concentration was estimated by determining the total nitrogen using the Kjeldahl method, previously described (*19*, *20*). The total fat was estimated by acid hydrolysis with extraction using diethyl ether followed by hexane (*21*, *22*). Ash, moisture, protein, and total fat all had a LOQ of 0.1% fresh weight of sample (fw). The carbohydrate content was calculated using the following equation (*23*):

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

The LOQ for carbohydrate was 1.0% fw.

Fiber Analysis. The ADF content was determined by boiling the sample with sulfuric acid detergent solution, rinsing with acetone, and then determining the percent ADF gravimetrically (*24*). The lignin content was determined by boiling the sample with a detergent solution, rinsing with acetone followed by sulfuric acid, and then determining the percent lignin gravimetrically (*24*). Glass wool was used in place of asbestos. The NDF content was determined by boiling the sample with a neutral detergent solution, adding α-amylase, rinsing with acetone, and then determining the percent NDF gravimetrically (*24*, *25*). NDF, ADF, and lignin all had a LOQ of 0.1% fw.

Amino Acid Analysis. Amino acid composition was determined by three methods (*26*). Tryptophan required a base hydrolysis using sodium hydroxide. Sulfur-containing amino acids required an oxidation using performic acid prior to hydrolysis with hydrochloric acid. Analysis of the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. The individual amino acids were quantitated using an automated amino acid analyzer. For all amino acids, the LOQ was 0.1 mg/g fw.

Mineral Analysis. Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc concentrations were estimated from ashed samples mixed with 5% solution of hydrochloric acid. The amount of each element was determined at appropriate wavelengths using inductively coupled plasma spectroscopy. The emission of the unknown sample was compared with the emission of standard solutions (*27*, *28*). Potassium and sodium each had a LOQ of 100 mg/kg fw. Calcium, magnesium, and phosphorus each had a LOQ of 20 mg/kg fw. The LOQ for copper was 0.5 mg/kg fw, iron was 2 mg/kg fw, manganese was 0.3 mg/kg fw, and zinc was 0.4 mg/kg fw.

Coumestrol Analysis. The coumestrol content was determined by extracting the samples with a methanol:water solution and then saponification with dilute sodium hydroxide (*29*). Coumestrol was measured by a high-performance liquid chromatography system with electrochemical detection. The LOQ for coumestrol was 1.5 mg/kg fw.

Data Reduction and Statistical Analysis. SAS software (*30*) was used by Certus International, Inc. (Chesterfield, MO) to generate all summary statistics and perform all assessments. All sample values were converted to dry weight of sample for comparison, with the exception of moisture and amino acid values (for example, if the method measured the value in mg/kg fresh weight of sample, then the value was converted

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Table 1. Proximate Constituent, Fiber, and Coumestrol Composition of Forage from GTA J101 × J163

	2001 field season			2003 field season			
component[a]	J101 × J163 mean[c] ± SE (range)	control[b] mean[c] ± SE (range)	conventional tolerance interval[e] (range)	J101 × J163 mean[d] ± SE (range)	control[b] mean[d] ± SE (range)	conventional tolerance interval[e] (range)	literature range[a]
ash	14.41[f] ± 2.46 (8.26–32.50)	11.31 ± 2.46 (8.44–15.04)	5.59, 16.40 (8.58–15.25)	9.02 ± 0.60 (6.95–11.16)	9.20 ± 0.59 (7.22–11.69)	5.29, 12.54 (6.86–12.79)	9.5;[g] 5.8–7.5[i]
carbohydrates	63.10[f] ± 3.01 (48.03–74.71)	65.08 ± 3.01 (55.44–73.53)	46.29, 85.59 (58.03–74.38)	67.08 ± 2.00 (59.68–74.85)	66.33 ± 1.99 (58.58–71.80)	53.20, 82.75 (56.63–74.80)	not available
moisture	75.78[f] ± 1.64 (70.70–83.10)	76.77 ± 1.64 (70.70–84.20)	62.91, 88.67 (70.90–82.10)	76.10[f] ± 1.32 (71.90–80.90)	78.68 ± 1.32 (75.10–82.40)	66.89, 88.25 (72.60–83.50)	76–77[g]
protein	20.49 ± 1.24 (15.53–27.11)	21.35 ± 1.24 (16.02–28.20)	7.98, 33.81 (15.29–25.81)	21.42 ± 1.22 (17.99–25.60)	21.07 ± 1.21 (17.02–26.11)	9.20, 31.10 (15.52–28.34)	17–27[g]
total fat	2.12 ± 0.17 (1.50–3.13)	2.26 ± 0.17 (1.45–3.58)	0, 4.61 (1.33–3.15)	2.81[f] ± 0.35 (1.50–4.43)	3.41 ± 0.34 (1.94–4.61)	0.67, 5.27 (1.47–4.49)	not available
ADF	27.01 ± 1.62 (22.09–33.91)	25.79 ± 1.61 (18.81–33.47)	15.76, 40.19 (23.12–33.39)	30.38 ± 2.42 (21.15–39.88)	30.10 ± 2.41 (23.47–36.43)	15.68, 44.63 (21.26–39.25)	13–37[g]
lignin	5.31 ± 0.56 (3.48–8.16)	5.07 ± 0.56 (1.64–8.10)	0, 12.92 (3.86–9.65)	6.81 ± 0.74 (2.81–9.59)	6.35 ± 0.73 (2.00–8.23)	2.10, 10.61 (2.31–13.71)	7;[g] 4.5–7.6[h]
NDF	30.64[f] ± 1.38 (21.87–39.73)	28.09 ± 1.37 (22.25–32.07)	20.01, 41.80 (26.53–35.72)	37.70 ± 3.18 (29.12–49.52)	37.84 ± 3.17 (26.71–51.64)	19.86, 53.29 (26.85–51.09)	40–47;[g] 31–44[h]
coumestrol	not available	not available	not available	47.42 ± 15.10 (3.07–108.00)	37.66 ± 15.05 (3.66–124.50)	0, 145.77 (2.99–104.37)	10–184;[k] 133–278[j]

[a] All data are expressed as percent dry weight of sample except moisture, which is percent fresh weight of sample, and coumestrol, which is mg/kg dry weight of sample. [b] Near-isogenic alfalfa control from null-segregating population. [c] The least-squares mean of 19 values (four replicates from each of four field sites plus three replicates from the New York field site). A replicate from the NY site was not analyzed because we were unable to confirm the identity of the sample. [d] The least-squares mean of 16 values (four replicates from each of four field sites). [e] The tolerance interval is specified to contain 99% of alfalfa commercial variety population with 95% confidence and negative limits set to zero. [f] Value statistically different from the control ($p < 0.05$). The ash content in 2001 contains values from samples considered outliers (see Results and Discussion). [g] Ref 31. [h] Ref 32. [i] Ref 33. [j] Ref 34. [k] Ref 35.

to mg/kg dry weight of sample using the measured moisture value of that sample). Amino acid sample values were converted to percent total amino acid for comparison. Sample values below the LOQ for sodium (41.2% in 2001 and 48.8% of 2003 of the total samples analyzed) and coumestrol (12% in 2003 of the total samples analyzed) were assigned a value equal to half the LOQ prior to statistical analyses. The SAS GLM procedures were applied to all data prior to final analysis to detect potential outliers in the data set by screening studentized PRESS residuals. A PRESS residual is the difference between any value and its predicted value from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distibution when outliers are absent. Extreme data points that are outside of the ±6 studentized PRESS residual range were considered for exclusion, as outliers, from the final analyses. The only data point considered extreme and removed from the statistical evaluation was a single aspartic acid value. Because the amino acid values are totaled for unit conversion, all of the amino acid values for that sample were removed from the comparison.

A total of 35 different components were evaluated in 2001 field season samples, and 36 different components were evaluated in 2003 field season samples. Statistical analyses were conducted using a mixed model analysis of variance for a combination of all five (2001 field season) or four (2003 field season) field sites using the following equation

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

where Y_{ijk} = unique individual observation, U = overall mean, T_i = variety effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by variety interaction effect, and e_{ijk} = residual error. In this analysis, values from GTA samples were compared to the values from control samples to determine statistical differences at $p < 0.05$.

The conventional varieties were not used for statistical comparisons; rather, a range of observed values from the conventional varieties was determined for each analytical component. Additionally, the conventional data were used to develop population tolerance intervals. A tolerance interval is an interval that one can claim, with a specified

degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional varieties. Each tolerance interval estimate was based upon one observation per unique conventional variety. Individual conventional varieties with multiple observations were averaged within a site, then across sites to obtain a single estimate for inclusion in tolerance interval calculations. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

RESULTS AND DISCUSSION

Interpretation of statistical results was reviewed by a tiered process: (i) all test and control values were statistically compared to each other for differences at the 5% level ($p < 0.05$); (ii) if a statistical difference was determined between the test and the control values, then the range of the test values was compared to the 99% tolerance interval (calculated from the conventional variety data); (iii) if the test values fell outside the 99% tolerance interval, then the range of the test values was compared to values published in the literature. Results from the analyses of the combination of all field sites showed that there were no statistical differences observed between GTA J101 × J163 and the control for 24 of the 35 analytes measured in 2001 and 31 of the 36 analytes measured in 2003. For the comparisons observed to be statistically different between GTA and control, all GTA values were within the 99% tolerance interval with the exception of proline, tyrosine, ash, and iron in 2001. A review of the literature for alfalfa forage composition is also presented (**Tables 1−3**). As the majority of the literature values are typically means, they are not true ranges of expected values for any given analyte and must be viewed primarily as providing confirmation that the results reported herein were comparable with those presented in the literature.

Table 2. Amino Acid Composition of Forage from GTA J101 × J163

	2001 field season			2003 field season			
component[a]	J101 × J163 mean[c] ± SE (range)	control[b] mean[c] ± SE (range)	conventional tolerance interval[e] (range)	J101 × J163 mean[d] ± SE (range)	control[b] mean[d] ± SE (range)	conventional tolerance interval[e] (range)	literature range[f]
Ala	6.20 ± 0.097 (6.00–6.79)	6.19 ± 0.097 (6.01–6.56)	5.55, 6.80 (5.93–6.93)	6.07 ± 0.11 (5.65–6.50)	6.07 ± 0.10 (5.71–6.33)	5.48, 6.74 (5.23–6.52)	4.65–6.05
Arg	5.56 ± 0.063 (5.10–5.99)	5.64 ± 0.063 (5.40–6.23)	4.98, 6.21 (5.40–5.90)	5.38 ± 0.18 (4.77–5.84)	5.51 ± 0.18 (4.78–6.01)	4.44, 6.62 (4.37–6.03)	4.05–5.4
Asp	13.16 ± 0.37 (12.05–14.34)	12.86 ± 0.37 (10.95–16.22)	9.75, 16.61 (11.83–15.40)	15.46 ± 1.72 (11.84–23.05)	14.76 ± 1.71 (11.33–20.30)	5.21, 23.47 (10.36–27.93)	9.85–10.75
Cys	1.57[g] ± 0.057 (1.41–1.84)	1.41 ± 0.057 (1.17–1.59)	1.01, 1.96 (1.23–1.76)	1.50 ± 0.10 (1.06–1.79)	1.56 ± 0.10 (1.05–1.93)	0.37, 2.72 (0.79–2.23)	1.7
Glu	11.03 ± 0.077 (10.70–11.33)	11.10 ± 0.077 (10.85–11.79)	10.28, 11.77 (10.75–11.62)	10.78 ± 0.31 (9.57–11.54)	10.81 ± 0.31 (9.57–11.34)	9.03, 12.68 (8.74–11.70)	9.4–12
Gly	5.61 ± 0.044 (5.46–6.23)	5.56 ± 0.044 (5.39–5.97)	5.11, 5.84 (5.35–5.64)	5.24 ± 0.14 (4.68–5.64)	5.28 ± 0.14 (4.87–5.56)	4.33, 6.20 (4.29–5.58)	3.75–5.5
His	2.70 ± 0.045 (2.44–2.88)	2.76 ± 0.044 (2.57–3.01)	2.25, 3.22 (2.43–2.96)	2.64 ± 0.031 (2.49–2.79)	2.61 ± 0.030 (2.41–2.72)	2.45, 2.79 (2.40–2.79)	1.9–3
Ile	4.86[g] ± 0.052 (4.64–5.14)	4.94 ± 0.052 (4.65–5.31)	4.25, 5.58 (4.60–5.20)	4.51 ± 0.14 (3.97–4.97)	4.68 ± 0.14 (4.29–5.06)	4.08, 5.21 (3.76–5.10)	3.35–4.85
Leu	8.55 ± 0.060 (8.24–8.88)	8.66 ± 0.059 (8.32–9.12)	8.08, 9.07 (8.36–8.90)	8.20 ± 0.25 (7.36–8.98)	8.33 ± 0.25 (7.54–8.87)	7.02, 9.78 (6.50–9.11)	5.95–8.1
Lys	6.94 ± 0.098 (6.55–7.39)	7.05 ± 0.098 (6.62–7.34)	6.26, 7.85 (6.27–7.48)	7.46 ± 0.15 (6.74–7.97)	7.32 ± 0.15 (6.79–8.09)	6.62, 8.27 (6.25–7.96)	4.5–5.8
Met	1.90 ± 0.031 (1.71–2.21)	1.89 ± 0.031 (1.57–2.16)	1.56, 2.30 (1.67–2.10)	1.76 ± 0.079 (1.41–2.04)	1.86 ± 0.077 (1.56–2.44)	0.98, 2.66 (1.12–2.36)	1.05–1.85
Phe	5.54[g] ± 0.066 (5.39–6.06)	5.67 ± 0.065 (5.32–6.47)	4.64, 6.61 (5.40–6.16)	5.37 ± 0.15 (4.81–5.99)	5.53 ± 0.15 (5.04–5.85)	4.58, 6.55 (4.34–6.13)	3.9–5.4
Pro	5.49[g] ± 0.11 (5.06–6.16)	5.28 ± 0.11 (4.32–5.97)	4.57, 6.06 (4.86–5.73)	4.86 ± 0.12 (4.30–5.37)	4.83 ± 0.12 (4.02–6.38)	4.24, 5.57 (4.28–6.04)	3.25–6.3
Ser	5.45 ± 0.11 (5.05–5.92)	5.36 ± 0.11 (4.87–5.73)	4.31, 6.57 (4.92–5.91)	5.52[g] ± 0.059 (5.22–5.79)	5.34 ± 0.056 (5.10–5.74)	4.90, 6.08 (5.08–5.98)	3.6–4.75
Thr	4.59 ± 0.067 (4.13–4.88)	4.57 ± 0.067 (4.07–4.79)	3.63, 5.48 (4.10–4.85)	4.77 ± 0.089 (4.41–4.97)	4.75 ± 0.088 (4.38–4.97)	4.31, 5.29 (4.26–5.12)	3.3–5.55
Trp	1.19 ± 0.057 (0.86–1.45)	1.22 ± 0.056 (0.81–1.48)	0.62, 1.84 (0.86–1.38)	1.27 ± 0.051 (1.02–1.43)	1.24 ± 0.050 (1.04–1.48)	0.78, 1.69 (0.76–1.83)	not available
Tyr	3.69[g] ± 0.046 (3.18–3.89)	3.83 ± 0.045 (3.46–4.51)	3.33, 4.07 (3.30–3.94)	3.62 ± 0.23 (2.76–4.19)	3.76 ± 0.23 (3.19–4.38)	2.40, 5.08 (2.57–4.32)	2.65–4.15
Val	6.00 ± 0.052 (5.82–6.27)	6.01 ± 0.051 (5.58–6.41)	5.36, 6.63 (5.69–6.26)	5.58 ± 0.12 (5.02–6.02)	5.75 ± 0.11 (5.48–6.16)	5.16, 6.23 (4.95–6.12)	4.4–5.9

[a] All data are expressed as percent of total amino acids. [b] Near-isogenic alfalfa control from null-segregating population. [c] The least-squares mean of 19 values (four replicates from each of four field sites plus three replicates from the New York field site). A replicate from the NY site was not analyzed because we were unable to confirm the identity of the sample. [d] The least-squares mean of 15 values (four replicates from each of three field sites plus three replicates from the Washington field site). A replicate from the WA site was considered an extreme outlier and was not reported. [e] The tolerance interval is specified to contain 99% of alfalfa commercial variety population with 95% confidence and negative limits set to zero. [f] Literature values reported in percent dry weight (*31, 33*). Converted to percent total protein (assuming 20% protein) for comparison. [g] Value statistically different from the control ($p < 0.05$).

Proximate Constituent, Fiber, and Coumestrol Composition of Alfalfa Forage. Table 1 contains the combined site analyses data for proximate constituents, fiber, and coumestrol for both field seasons. There were no statistical differences ($p \geq 0.05$) between alfalfa forage produced by GTA and control forage in either season, or the GTA values were found to be within the 99% tolerance interval (calculated for each season from the conventional varieties) with the exception of ash in the 2001 field season. During the 2001 field season, the GTA samples at the New York field site were observed to have ash content up to 2-fold higher than the control and up to 3-fold higher content than reported literature ranges. Upon visual inspection and assessment of additional analyses of samples from the same plots (data not shown), it was determined that these samples had soil present on the forage. These ash values of the samples from 2001 are reported in this paper but were considered outliers due to the presence of soil and not representative of the ash content present in alfalfa forage. In the 2003 field season samples, the average ash content of GTA

was similar to the control, confirming that the elevated values observed in the 2001 samples were probably not representative of the ash content in alfalfa and, therefore, not biologically relevant in the determination of equivalence. The proximate constituents, fiber, and coumestrol composition in alfalfa forage as seen in **Table 1** are also similar to values reported in the literature and, therefore, considered to fall within the alfalfa population.

Amino Acid Composition of Alfalfa Forage. The combined site amino acid composition data on alfalfa forage are presented in **Table 2**. There were no statistical differences ($p \geq 0.05$) between the alfalfa forage produced by GTA and the control forage in either season, or the GTA values were found to be within the 99% tolerance interval with the exception of proline and tyrosine in the 2001 field season. At one site, the proline and tyrosine values from one sample were found to fall slightly outside the 99% tolerance interval, while all other samples were within the interval. Notably, the magnitude of the difference between the GTA and the control forage samples for both

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Table 3. Mineral Composition of Forage from GTA J101 × J163

component[a]	2001 field season			2003 field season			
	J101 × J163 mean[c] ± SE (range)	control[b] mean[c] ± SE (range)	conventional tolerance interval[e] (range)	J101 × J163 mean[d] ± SE (range)	control[b] mean[d] ± SE (range)	conventional tolerance interval[e] (range)	literature range[a]
calcium	1.01[f] ± 0.070 (0.81–1.38)	1.12 ± 0.070 (0.88–1.44)	0.48, 1.89 (0.90–1.53)	1.22[f] ± 0.080 (0.90–1.75)	1.39 ± 0.079 (1.15–1.70)	0.62, 2.03 (0.91–1.86)	1.39–2.30[i]
copper	8.24 ± 0.68 (6.42–12.28)	9.41 ± 0.68 (6.76–17.10)	3.12, 12.64 (5.29–10.18)	6.47[f] ± 1.41 (3.36–10.68)	7.51 ± 1.41 (3.83–11.78)	0, 18.56 (3.43–14.72)	10;[g] 3–4;[h] 12–52[i]
iron	730.93[f] ± 230.85 (199.10–2196.43)	410.19 ± 230.60 (184.32–764.23)	0, 892.57 (235.53–1538.46)	250.27 ± 94.44 (79.83–700.00)	195.68 ± 94.13 (82.46–577.46)	0, 583.85 (63.49–709.43)	66–78;[h] 204–489[i]
magnesium	0.24 ± 0.051 (0.10–0.38)	0.26 ± 0.051 (0.11–0.54)	0, 0.68 (0.11–0.45)	0.26 ± 0.041 (0.15–0.45)	0.29 ± 0.041 (0.18–0.49)	0.0099, 0.50 (0.13–0.39)	0.35–0.49;[h] 0.21–0.30[i]
manganese	61.83 ± 8.60 (35.90–112.95)	54.04 ± 8.57 (32.97–81.01)	0, 120.37 (34.60–109.50)	33.17 ± 5.11 (16.09–45.73)	33.36 ± 5.09 (20.57–49.09)	0, 66.81 (15.91–49.60)	48–60;[h] 39–46[i]
phosphorus	0.32 ± 0.027 (0.22–0.42)	0.33 ± 0.027 (0.25–0.45)	0.095, 0.54 (0.22–0.45)	0.34 ± 0.032 (0.23–0.49)	0.35 ± 0.032 (0.28–0.47)	0.17, 0.49 (0.23–0.46)	0.24–0.42[i]
potassium	2.96 ± 0.41 (0.85–4.32)	3.08 ± 0.41 (1.57–4.30)	0.38, 5.75 (1.39–4.31)	2.78 ± 0.26 (1.41–3.66)	2.94 ± 0.26 (1.95–3.33)	1.30, 4.25 (1.92–3.90)	1.34–2.35;[h] 1.48–2.78[i]
sodium	0.10 ± 0.041 (0.017–0.38)	0.079 ± 0.041 (0.018–0.23)	0, 0.31 (0.017–0.21)	0.20 ± 0.099 (0.018–0.75)	0.12 ± 0.099 (0.020–0.45)	0, 0.76 (0.019–0.51)	0.19;[g] 0.0024–0.19[i]
zinc	28.61 ± 2.94 (17.01–37.28)	29.58 ± 2.93 (16.70–46.15)	5.05, 50.21 (18.09–35.98)	29.69 ± 2.24 (20.04–35.81)	29.15 ± 2.23 (20.90–42.96)	6.12, 50.76 (15.20–43.62)	18;[g] 30–65[h]

[a] Calcium, magnesium, phosphorus, potassium, and sodium expressed in percent dry weight of sample; copper, iron, manganese, and zinc expressed in mg/kg dry weight of sample. [b] Near-isogenic alfalfa control from null-segregating population. [c] The least-squares mean of 19 values (four replicates from each of four field sites plus three replicates from the New York field site). A replicate from the NY site was not analyzed due to not being able to confirm the identity of the sample. [d] The least-squares mean of 16 values (four replicates from each of four field sites). [e] The tolerance interval is specified to contain 99% of alfalfa commercial variety population with 95% confidence and negative limits set to zero. [f] Value statistically different from the control ($p < 0.05$). The iron content in 2001 contains values from samples considered outliers (see Results and Discussion). [g] Ref *31*. [h] Ref *33*. [i] Ref *36*.

proline and tyrosine was small (within 4%). In the 2003 field season samples, neither of those analytes was found to be statistically different from the control, indicating that the differences observed earlier were not biologically significant. The similarity in aromatic amino acid concentrations between GTA and control and conventional alfalfa varieties indicates that the presence of the CP4 EPSPS enzyme in GTA and the transformation process had no effect on the distribution of these amino acids.

Mineral Composition of Alfalfa Forage. Table 3 contains the combined site data for the mineral content in alfalfa forage. There were no statistical differences ($p \geq 0.05$) between the alfalfa forage produced by GTA and the control forage in either season, or the GTA values were found to be within the 99% tolerance interval with the exception of iron in 2001 field season. It was determined that the New York GTA samples that had soil present on the forage (as described earlier for ash analyses) were the same samples that showed iron values outside the 99% tolerance interval. These iron values of the samples from 2001 are reported in this paper but were considered outliers due to the presence of soil and not representative of the iron content present in alfalfa forage. In the 2003 field season samples, the average iron content of GTA was similar to the control, confirming that the elevated values observed in the 2001 samples were probably not representative of the iron content in alfalfa and, therefore, not biologically relevant in the determination of equivalence. The mineral content in alfalfa forage as seen in **Table 3** is also similar to reported literature values and, therefore, considered to fall within the alfalfa population.

The results of these compositional analyses show that the components measured in GTA J101 × J163 across two field seasons (35 in 2001 and 36 in 2003) were either not statistically different ($p \geq 0.05$) from the control, were within the ranges observed for the 99% tolerance interval calculated from

conventional alfalfa varieties, or were comparable to those reported in the literature. Therefore, any minor differences are unlikely to be biologically meaningful, and the forage from GTA J101 × J163 is considered compositionally equivalent to that of forage from conventional alfalfa. The results of the compositional analyses, the safety of the CP4 EPSPS protein, and the safe history of use of alfalfa as a common source of animal feed support the conclusion that GTA is compositionally equivalent to, and as safe as, the alfalfa varieties grown commercially today.

LITERATURE CITED

(1) Bolton, J. L.; Goplen, B. P.; Baenziger, H. World distribution and historical developments. In *Alfalfa Science and Technology*; Hanson, C. H., Ed.; American Society of Agronomy: Madison, Wisconsin, 1972; pp 1−34.

(2) NASS-USDA. *Crop Production 2004 Summary*; United States Department of Agriculture: Washington, DC, 2005.

(3) Hower, A. A.; Harper, J. K.; Harvey, R. G. *The Importance of Pesticides and Other Pest Management Practices in U.S. Alfalfa Production*; National Agricultural Pesticide Impact Assessment Program, United States Department of Agriculture: Washington, DC, 1999.

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(4) Peters, E. J.; Linscott, D. L. Weeds and weed control. In *Alfalfa and Alfalfa Improvement*; Hanson, A. A., Barnes, D. K., Hill, R. R., Eds.; American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America: Madison, Wisconsin, 1988; pp 705−735.

(5) Cords, H. P. Weeds and alfalfa hay quality. *Weed Sci.* **1973**, *21*, 400−401.

(6) Temme, D. G.; Harvey, R. G.; Fawcett, R. S.; Young, A. W. Effects of annual weed control on alfalfa forage quality. *Agron. J.* **1979**, *71*, 51−54.

(7) Padgette, S. R.; Kolacz, K.; Delannay, X.; Re, D.; LaVallee, B.; Tinius, C.; Rhodes, W.; Otero, Y.; Barry, G.; Eichholtz, D.; Peshke, V.; Nida, D.; Taylor, N. B. Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci.* **1995**, *35*, 1451−1461.

(8) Haslam, E. *Shikimic Acid: Metabolism and Metabolites*; John Wiley and Sons: Chichester, England, 1993.

(9) Steinrucken, J.; Amrhein, N. The herbicide glyphosate is a potent inhibitor of 5-enolypyryvylshikimic acid-2-phosphate synthase. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1207−1212.

(10) Harrison, L.; Bailey, M.; Naylor, M.; Ream, J.; Hammond, B. G.; Nida, D.; Burnette, B. The expressed protein in glyphosate-tolerant soybean, 5-enolypyryvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. *J. Nutr.* **1996**, *126*, 728−740.

(11) Food and Agriculture Organization. *Biotechnology and Food Safety*; Joint FAO/WHO Consultation: 1996.

(12) Organization for Economic Cooperation and Development. *Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles*; OECD Environment Directorate and Directorate for Science, Technology and Industry: 1993.

(13) World Health Organization. *Application of the Principles of Substantial Equivalence to the Safety Evaluation of Foods and Food Components from Plants Derived by Modern Biotechnology*; WHO: Geneva, Switzerland, 1995.

(14) Samac, D. A.; Temple, S. J. Development and utilization of transformation in *Medicago* species. In *Genetic Transformation in Crops*; Liang, G. H., Skinner, D. Z., Eds.; Haworth Press Inc.: Binghamton, New York, 2004.

(15) U.S. EPA Federal Insecticide, Fungicide and Rodenticide Act: Good laboratory practice standards. *Code of Federal Regulations*; U.S. EPA: Washington, DC, 1999; pp 40, CFR Part 160.

(16) AOAC. AOAC Method 923.03−Ash of flour. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; p 2.

(17) AOAC. AOAC Method 926.08−Moisture in cheese. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; p 70.

(18) AOAC. AOAC Method 925.09−Solids (total) and moisture in flour. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; p 1.

(19) AOAC. AOAC Method 955.04−Nitrogen (total) in fertilizers. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 14−15.

(20) AOAC. AOAC Method 979.09−Protein in grains. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 30−34.

(21) AOAC. AOAC Method 922.06−Fat in flour. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; p 5.

(22) AOAC. AOAC Method 954.02−Fat (crude) or ether extract in pet food. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 33.

(23) U.S. Department of Agriculture. Energy value of foods. In *Agriculture Handbook 74*; U.S. Department of Agriculture: Washington, DC, 1973; pp 2−11.

(24) Goering, H. K.; Van Soest, P. J. Forage and fiber analyses. In *Agriculture Handbook No. 379*; Agricultural Research Service, U.S. Department of Agriculture: Washington, DC, 1970.

(25) AACC. AACC Method 32.20. In *Methods of Analysis*; American Association of Cereal Chemists: Minneapolis, MN, 1998.

(26) AOAC. AOAC Method 982.30−Protein efficiency ratio. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 63−66.

(27) AOAC. AOAC Method 984.27−Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc in infant formula. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 17−18.

(28) AOAC. AOAC Method 985.01−Metals and other elements in plants and pet foods. In *Official Methods of Analysis*; Horwitz, W., Ed.; Association of Official Analytical Chemists International: Gaithersburg, MD, 2000; pp 4−5.

(29) Klump, S. P.; Allred, M. C.; MacDonald, J. L.; Ballam, J. M. Determination of isoflavones in soy and selected foods containing soy by extraction, saponification, and liquid chromatography: Collaborative study. *JAOAC Int.* **2001**, *84*, 1865−1883.

(30) SAS Institute, I. *SAS Software Release 8.1 (TS1M0)*; SAS Institute: Cary, NC, 1999−2000.

(31) National Research Council United States-Canadian Tables of Feed Composition. *Nutritional Data for United States and Canadian Feeds*; National Academy Press: Washington, DC, 1982; pp 8−146.

(32) Julier, B.; Huyghe, C.; Ecalle, C. Wtihin- and among-cultivar genetic variation in alfalfa: Forage quality, morphology, and yield. *Crop Sci.* **2000**, *40*, 365−369.

(33) Smith, D. Influence of temperature on the yield and chemical composition of vernal alfalfa at first flower. *Agron. J.* **1969**, *61*, 470−472.

(34) Moravcová, J.; Kleinová, T.; Loucka, R. The determination of coumestrol in alfalfa (*Medicago sativa*). *Rostl. Vyroba* **2002**, *48*, 224−229.

(35) Lookhart, G. L. Analysis of coumestrol, a plant estrogen, in animal feeds by high-performance liquid chromatography. *J. Agric. Food Chem.* **1980**, *28*, 666−667.

(36) Townsend, M. S.; Henning, J. A.; Smith, D. W.; Ray, I. M.; Currier, C. G. Differential concentration of ten minerals among four ancestral alfalfa germplasms. *Crop Sci.* **1998**, *39*, 574−578.