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Impact of Genetics and Environment on Nutritional and Metabolite Components of Maize Grain

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The Organization for Economic Co-operation and Development (OECD) recommends the measurement of specific plant components for compositional assessments of new biotechnology-derived crops. These components include proximates, nutrients, antinutrients, and certain crop-specific secondary metabolites. A considerable literature on the natural variability of these components in conventional and biotechnology-derived crops now exists. Yet the OECD consensus also suggests measurements of any metabolites that may be directly associated with a newly introduced trait. Therefore, steps have been initiated to assess natural variation in metabolites not typically included in the OECD consensus but which might reasonably be expected to be affected by new traits addressing, for example, nutritional enhancement or improved stress tolerance. The compositional study reported here extended across a diverse genetic range of maize hybrids derived from 48 inbreds crossed against two different testers. These were grown at three different, but geographically similar, locations in the United States. In addition to OECD analytes such as proximates, total amino acids and free fatty acids, the levels of free amino acids, sugars, organic acids, and selected stress metabolites in harvested grain were assessed. The major free amino acids identified were asparagine, aspartate, glutamate, and proline. The major sugars were sucrose, glucose, and fructose. The most predominant organic acid was citric acid, with only minor amounts of other organic acids detected. The impact of genetic background and location was assessed for all components. Overall, natural variation in free amino acids, sugars, and organic acids appeared to be markedly higher than that observed for the OECD analytes.

KEYWORDS: Maize; *Zea mays* L.; compositional variation; metabolic profiling; GM crops; comparative safety assessment process; substantial equivalence

INTRODUCTION

The major worldwide crops, such as maize and rice, have been subject to extensive selective breeding to improve nutrition, agronomics, and yield. The key nutritional and antinutritional components of grain from major crops are now well characterized. There is increasing evidence of extensive natural variation in the levels of these components. Contributing factors to this variation can include (i) differences between ecotypes adapted for growth and productivity in different geographical regions and environments; (ii) differences arising from changes in climatic conditions and from different levels of abiotic stress; (iii) differences in responses to biotic stresses including herbivore attack and pathogen infestation; (iv) differences in exposure to levels and types of biotic stresses; and (v) breeding for different endpoints such as protein composition, yield, or morphology. Many recent compositional studies that address natural variation in maize composition have focused on a list of well-defined metabolites promoted by the Organization for Economic Co-operation and Development (OECD) for assessment in compositional studies of biotechnology-derived crops (*1*−*8*). The OECD produces internationally accepted recommendations to facilitate multilateral agreement in many spheres of economic development. Its consensus documents on ap-

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proaches to compositional equivalence form the basis of requirements for regulatory submissions of biotechnology-derived crops (see www.oecd.org). The OECD approach emphasizes measurements of essential nutrients, and known antinutrients and toxicants, and is predicated on the premise that such targeted analyses will most effectively identify potential safety and antinutritional concerns. Yet the OECD consensus also suggests measurements of any metabolites that may be directly associated with a newly introduced trait. This study therefore sought to further define the metabolite composition of grain harvested from maize by surveying a wide genetic range of maize hybrids. We expanded metabolite coverage beyond the OECD components (*9*) typically used in maize compositional analyses (proximates, total amino acids, free fatty acids) to include free amino acids, organic acids, sugars, total glycerol, abscisic acid, and glycine betaine, metabolites that might reasonably be expected to be affected by new traits addressing, for example, nutritional enhancement or improved stress tolerance. The study was also intended to provide additional insights into the effect of genetic by environmental interactions on nutrient and metabolite composition in grain, and hybrids were therefore grown at three different, but geographically similar, sites in the United States.

MATERIALS AND METHODS

Biological Material. Seeds of the various maize (*Zea mays* L.) hybrids were planted at three different locations in Iowa. These were Cambridge, Huxley, and South Amana. Planting at Cambridge was on April 29, 2005, and planting at Huxley and South Amana was on May 5. Ninety-six plots representing 48 inbred lines crossed with the two different testers (C103 and Iodent) were planted per location. Mature grain was collected from five plants for each line selected at random and bulked for compositional analyses.

Analytical Methods. All chemicals are of standard reagent grade unless otherwise stated.

Oven-Extractable Moisture. Ground samples (0.5−2 g) were dried in an oven, and the weight difference before and after drying was determined. Oven temperatures were set to 130 ± 10 °C, and drying was for a period of 2 h ± 5 min.

Total Protein Content. Total protein content was determined indirectly on a LECO FP-Nitrogen analyzer. Ground sample (∼250 mg) was combusted at 900 °C. Released N_2 was measured by a thermal conductivity detector, and levels were calculated on the basis of a calibration curve (using atropine as a standard sample) prepared prior to analysis. All calculations related to percent N_2 and percent protein were automatically calculated by the LECO software based on nitrogen peak areas of standards. Results were reported on a dry matter basis.

Total Oil Content. Ground sample (∼2 g) was placed in an extraction thimble containing 80 ± 3 mL of hexane. The extraction thimble was then placed in a Foss (Eden Prairie, MN) Tecator 2050 Soxtec Avanti Automatic System. Sample was washed with refluxing solvent for 40 min to remove hexane-soluble extractable oil. Extracted oil was determined gravimetrically after distilling off hexane and subsequent drying in an oven set to 105 ± 5 °C for 30 min. Results were reported on a dry matter basis.

Fatty Acid Profiles of Extracted Oil. Aliquots of oil (20−60 mg) derived from hexane extraction as described above were used for fatty acid analysis. Fatty acid methyl esters were formed by trans-esterification of the extracted oil with sodium methoxide at 37 °C for 20 min. A sodium chloride solution was added to the vial to quench the reaction and form an aqueous solution. Hexane was added to the vial to extract the fatty acid methyl esters (FAME). The upper liquid phase was analyzed by capillary gas chromatography with a flame ionization detector (HP 6890 series, Agilent). A 7 min elevated temperature run on a Supelco Omegawax 320 fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness) was used to separate the FAMEs in order of increasing carbon chain length (from C10 to C24). The gas chromatograph was calibrated to identify a minimum of nine FAMEs: methyl palmitate (16:0), methyl stearate (18:0), methyl oleate (18:1, n-9), methyl linoleate (18:2, n-6), methyl linolenate (18:3, n-3), methyl arachidate (20:0), methyl eicosenoate (20:1, n-9) methyl eicosodienoate (20:2, n-6), and methyl behenate (22:0). The instrument detection limits were 2.5 μg/mL. The GC integration program was unable to consistently separate the methyl oleate (18:1, n-9) and methyl vaccenate (18:1, n-7) isomers from each other; therefore, the peak areas for these two FAMEs are summed together for reporting purposes. Data were recorded as area percent of fatty acid composition. Fatty acid methyl ester standards were purchased from Matreya (Pleasant Gap, PA).

Total Amino Acid Determination. Ground sample (∼60 mg) was subjected to acid hydrolysis (6 M HCl, 10 mL) at 110 ± 2 °C for 24 h under argon. Sample tubes also included 10 μL of melted phenol. After hydrolysis, 100 μL aliquots of each sample were dried under vacuum at 45 ± 2 °C until completely dried followed by reconstitution in 500 μL of 0.1 M HCl. The sample was filtered through a 0.45 μm filter plate. The amino acids were then separated by reversed-phased high-pressure liquid chromatography on a Zorbax Eclipse XDB-C_{18} column (4.6 × 75 mm, 3.5 μm, Agilent, Palo Alto, CA) fitted with a Kromasil (Xpertek,) C_{18} guard column (3 × 15 mm, 5 μm). The column compartment was set to maintain 40 ± 0.8 °C. The binary mobile phase system included 40 mM NaH_2PO_4 (pH 7.8) with 0.001% sodium azide as solvent A and acetonitrile/methanol/H_2O (45:45:10; v/v/v) as solvent B.

The 12 min HPLC run used a mobile phase flow rate of 2 mL/min with an initial solvent A/solvent B ratio of 95:5 held for 1 min followed by a linear increase in solvent B to 35% at 9.80 min, then 100% at 12 min. Precolumn derivatization of amino acids to *o*-phthalaldehyde (OPA, Agilent) derivatives was performed by an injection program that exposes sample to OPA just prior to column loading. The resulting amino acid adducts were detected by a fluorescence detector (excitation, 340 nm; emission, 450 nm). Cysteine, proline, asparagine, glutamine, and tryptophan are not included in this amino acid screen.

Total Tryptophan Determination. Ground sample (∼60 g) was subjected to base hydrolysis (4.2 M NaOH, 10 mL) at 110 ± 2 °C for 20 h under argon. After cooling, 0.1 M NaOAc (pH 4.5, 3 mL) was added and mixed welll; 6 M HCl (7 mL) was then added followed by mixing. An aliquot of the resultant solution (1 mL) was filtered through a 0.45 μm syringe filter, and this was used for the HPLC (OPA) analyses. The HPLC (OPA) derivatization methodology was as described above.

Free Amino Acid Analysis. Ground sample (∼30 mg) was suspended in a 5% trichloroacetic acid solution (1 mL) and vortexed for 30 ± 5 min. Samples were allowed to sit overnight in a refrigerator set to maintain 4 ± 2 °C. The following day samples were vortexed for 30 ± 5 min at room temperature and then centrifuged for 20 ± 5 min at 3000*g* in an Eppendorf centrifuge model 5804. The sample was filtered through a 0.45 μm filter plate, and this was used for HPLC analysis. The HPLC (OPA) derivatization methodology was as described above. The limit of quantitation was 10 ppm. Cysteine and proline are not included in this amino acid screen.

Free Proline Analysis. Ground sample (∼30 mg) was treated as for free amino acid analysis prior to reversed-phase HPLC analysis on an Asentis C_{18} (4.6 × 100 mm, 3 μm) column. The binary mobile phase system included 60 mM NaOAc (pH 5.7) as solvent A and methanol as solvent B. All solvents were of HPLC grade. The 7 min HPLC run used a mobile phase flow rate of 1 mL/min with an initial solvent A/solvent B ratio of 35:65 (v/v) followed by a linear increase in solvent B to 72.5% at 5.0 min and then to 90% at 5.5 min, a 0.5 min hold, and then a return to 65% at 6.5 min. Precolumn derivatization of amino acids to their 9-fluorenylmethylchloroformate (FMOC) derivative was performed by an injection program that exposes sample to FMOC just prior to column loading. The resulting amino acid adducts were detected by a fluorescence detector (excitation, 266 nm; emission, 313 nm). The limit of quantitation was 10 ppm.

LC-MS/MS of Sugars. Ground sample (∼50 mg) was suspended in 1 mL of 80:20 ethanol/H_2O (v/v) and vortexed for about 15 min. Samples were then allowed to sit overnight in a referigerator set to maintain 4 °C. The following day water (0.65 mL) was added to the samples, which were then vortexed for a further 15 min at room temperature and then centrifuged for 5 min at 3000*g* in an Eppendorf

centrifuge model 5804. Supernatant (600−700 μL) was aliquoted and then recentrifuged for 5 min at 2000*g* to allow further filtration. A supernatant aliquot (20 μL) was then subjected to dilutions with water as appropriate to ensure analytes were within the range of the method. The dilution step also incorporated spiking of deuterated internal standards. This sample is used for injection after spiking with an internal standard solution. HPLC separations were performed on an Asahipak NH_2P-50 4B column (4.6 mm × 50 mm, PN:00C-4375-E0, Shodex). The binary mobile phase system comprised acetonitrile/H_2O/NH_4OH 90:10:0.1 (v/v/v) as solvent A and 0.1% NH_4OH as solvent B. The initial solvent composition was 100% solvent A, which linearly progressed to 90:10 solvent A/solvent B at 1.7 min, then to 75:25 solvent A/solvent B at 4 min, and then to 20:80 solvent A/solvent B at 4.1 min, at which it was held for 1 min further. The flow rate was 1.5 mL/min. The flow entering the MS/MS instrument (Micromass Quattro Ultima System, Waters, Billerica, MA) was split ∼1:5 so that the amount entering the MS was ∼300 μL/min. Electrospray ionization was used, and the MS was run in negative mode. The source block and desolvation temperatures were 120 and 300 °C, respectively. The MS/MS parameters varied for each analyte and are presented here in the following format: analyte (dwell time, collision energy, cone voltage, *m*/*z* parent ion/ daughter ion); $^{13}C_6$-fructose internal standard (0.10 s, 10 eV, 30 V, *m*/*z* 185.12/ 92.13); $^{13}C_6$, d_7-glucose internal standard (0.10 s, 10 eV, 30 V, *m*/*z* 192.19/ 128.17); fructose (0.07 s, 10 eV, 30 V, *m*/*z* 179.24/ 89.31); glucose (0.10 s, 10 eV, 30 V, *m*/*z* 179.24/ 89.31); inositol (0.10 s, 16 eV, 30 V, *m*/*z* 179.24/ 87.17); mannitol (0.10 s, 15 eV, 35 V, *m*/*z* 181.12/ 89.25); $^{13}C_{12}$-sucrose internal standard (0.10 s, 20 eV, 35 V, *m*/*z* 353.20/ 92.27); sucrose (0.10 s, 20 eV, 35 V, *m*/*z* 341.20/ 89.27); trehalose (0.10 s, 20 eV, 35 V, *m*/*z* 341.20/ 89.27); raffinose (0.10 s, 30 eV, 35 V, *m*/*z* 503.12/ 221.11); stachyose (0.10 s, 35 eV, 50 V, *m*/*z* 665.21/ 383.09).

The method profiled sugar by comparing the peak areas of each sugar against an internal standard. The measurement used is the *response unit*, which is the peak area of the analyte divided by the peak area of the internal standard. The method is not recommended for absolute quantitation, but the typical analytical range is 0.05−28 μg/mL in standards, which corresponds to approximately 125−62500 ppm in tissue.

LC-MS/MS Analysis of Organic Acids. Ground sample (∼20 mg) was suspended in 1 mL of methanol/H_2O/formic acid 50:50:1 (v/v/v) and vortexed for about 10 min. Samples were then allowed to sit overnight in a freezer set to maintain −20 °C. The following day samples were vortexed for 30 ± 5 min at room temperature and then centrifuged for 5 min at 3000*g* in an Eppendorf centrifuge model 5804. Supernatant (∼700 μL) was aliquoted and then recentrifuged for 5 min at 2000 rpm to allow further filtration. A supernatant aliquot (50 μL) was then subjected to dilutions with 0.1% formic acid as appropriate to ensure analytes were within the range of the method. The dilution step also incorporated spiking of deuterated internal standards. This sample was used for injection after spiking with an internal standard solution. HPLC separations were performed in reversed-phase mode on an Alltima C_{18} column (4.6 mm × 150 mm, 5.0 μm, Alltech). The binary mobile phase system comprised 0.1% formic acid as solvent A and 0.05% formic acid in methanol as solvent B. The initial solvent composition (100% solvent A) was maintained for 1 min and linearly progressed to 20% solvent B at 3.7 min then to 100% solvent B at 8 min. The solvent composition was then held at 100% solvent B for a further 2 min, at which time the run time was complete. The flow rate was 1 mL/min. The flow entering the MS/MS instrument (Micromass Quattro Ultima System) was split ∼1:4 so that the amount entering the MS was ∼250 μL/min. Electrospray ionization was used, and the MS was run in negative mode. The source block and desolvation temperatures were 120 and 300 °C, respectively. The MS/MS parameters varied for each analyte and are presented here in the following format: analyte (dwell time, collision energy, cone voltage, *m*/*z* parent ion/ daughter ion); pyruvic acid (0.08 s, 8 eV, 10 V, *m*/*z* 87.00/ 43.00), fumaric acid (0.03 s, 7 eV, 30 V, *m*/*z* 115.01/ 71.09); succinic acid (0.03 s, 12 eV, 30 V, *m*/*z* 117.01/ 73.17); d_4-succinic acid internal standard (0.10 s, 12 eV, 30 V, *m*/*z* 121.30/ 77.30); oxaloacetic acid (0.08 s, 7 eV, 5 V, *m*/*z* 133.00/ 87.13); malic acid (0.08 s, 11 eV, 50 V, *m*/*z* 133.15/ 71.22); d_3-malic acid internal standard (0.10 s, 11 eV, 50 V, *m*/*z* 136.20/ 73.30); anthranilic acid acid (0.10 s, 16 eV, 30 V, *m*/*z* 136.04/ 92.14); salicylic acid (0.10 s, 16 eV, 30 V, *m*/*z* 136.80/ 93.10); α-ketoglutaric acid (0.08 s, 8 eV, 30 V, *m*/*z* 144.94/ 101.10); glutaric acid (0.03 s, 8 eV, 30 V, *m*/*z* 130.80/ 87.00); β-phenyl pyruvic acid (0.10 s, 10 eV, 10 V, *m*/*z* 163.00/ 91.10); shikimic acid (0.08 s, 12 eV, 30 V, *m*/*z* 173.05/ 93.30); *p*-hydroxyphenyl pyruvic acid (0.10 s, 8 eV, 30 V, *m*/*z* 179.06/ 107.10); citric acid (0.03 s, 11 eV, 30 V, *m*/*z* 191.01/ 111.02); d_4-citric acid internal standard (0.10 s, 11 eV, 30 V, *m*/*z* 195.20/ 113.00); isocitric acid (0.08 s, 11 eV, 30 V, *m*/*z* 191.01/ 111.02); chorismic acid (0.10 s, 6 eV, 30 V, *m*/*z* 206.80/ 178.90); prephenic acid (0.10 s, 5 eV, 25 V, *m*/*z* 208.90/ 163.20); saccharopine (0.30 s, 15 eV, 25 V, *m*/*z* 274.90/ 256.90); homogentisic acid (0.10 s, 12 eV, 30 V, *m*/*z* 167.04/ 123.02).

The method profiled organic acids by comparing the peak areas of each organic acid against an internal standard. The measurement used was the *response unit*, which is the peak area of the analyte divided by the peak area of the corresponding deuterated internal standard. The method is not recommended for absolute quantitation, but the typical analytical range is 0.04−38 μg/mL in standards, which corresponds to approximately 19−19000 ppm in tissue.

LC-MS/MS of Glycine Betaine. Ground sample (∼30 mg) was suspended in 1 mL of methanol/H_2O/formic acid 50:50:1 (v/v/v) containing internal standard (2.5 μg/mL d_9-glycine betaine) and vortexed for 20 ± 5 min. Samples were then allowed to sit for 15−24 h in a refrigerator set to maintain 4 °C. With the expectation of low glycine betaine concentrations, a sample aliquot (500 μL) was then transferred to an analytical 96 deep well plate for analysis. This sample is used for injection. HPLC separations were performed in reversed-phase mode on an Alltima C_{18} column (4.6 mm × 150 mm, 3.0 μm, Alltech). The ternary mobile phase system comprised H_2O as solvent A, methanol as solventB,B and 1% formic acid as solvent C. Solvent C was maintained at 10% throughout the 2 min run. The percent volumes of solvents A and B were 5:85 at 0.00 min, 85:5 at 0.02 min, 85:5 at 1.30 min, and 5:85 at 2.00 min. The flow rate was 620 μL/min. The flow entering the MS/MS instrument (API2000, Applied Biosystems) was split ∼1:3 so that the amount entering the MS was ∼200 μL/min. Electrospray ionization was used, and the MS was run in positive mode. For MS/MS the collision energy was 40 eV. The parent/daughter ions analyzed for glycine betaine were *m*/*z* 118 and 58 and those for the d_9-glycine betaine standard, *m*/*z* 127.0 and 66.0. The limit of quantitation was 0.0391 μg/mL.

LC-MS/MS of Abscisic Acid. Ground sample (∼25 mg) was suspended in acetone (1.5 mL) containing 1% formic acid and internal standard (d_6-abscisic acid) and vortexed for 10 min. Samples were then allowed to sit for 2 h at room temperature followed by re-vortexing for 10 min. A sample aliquot (1 mL) was decanted and evaporated to dryness on a Zymark 96 Turbovap without heating. The dried extract was suspended in chloroform/methanol [1:1 (v/v), 400 μL] for analysis. HPLC separations were performed in reversed-phase mode on a Zorbax SB-C_8 column (4.6 mm × 150 mm, 3.5 μm, Agilent). The binary mobile phase system comprised 0.1% formic acid as solvent A and 0.05% formic acid in methanol as solvent B. The initial solvent composition (30% solvent A) was maintained for 1 min, linearly progressed to 100% solvent B at 3 min. The solvent composition was then held at 100% solvent B for a further 0.5 min at which time the run time was complete. The flow rate was 1 mL/min. The flow entering the MS/MS instrument (Micromass Quattro Ultima System) was split ∼1:5 so that the amount entering the MS was ∼200 μL/min. The MS was run in negative mode. The source block and desolvation temperatures were 120 and 300 °C, respectively. The capillary and cone voltages were 4.0 and 40 V, respectively. For MS/MS the collision energy was 10 eV. The parent/ daughter ions analyzed for abscisic acid were *m*/*z* 263.0 and 153.0 and those for the d_6-abscisic acid standard, *m*/*z* 269.0 and 159.0. The limit of quantitation was 0.01 μg/mL.

Statistical Model and Analysis. Analytes with >50% of observations below the assays limit of quantitation were excluded from summaries and analysis. These included 4 fatty acids and 11 organic acids as described in the text. Otherwise, results below the quantitation limit have a value equal to zero. The following analytes had values below the assay limit of quantitation: free methionine (12 measure-

Table 1. Proximate Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana						
component[a]	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction[b]	lit.[c] (range)	ILSI[d] mean (range)
moisture	7.85 (6.09–8.50)	7.98 (6.10–8.59)	7.89 (5.71–8.93)	7.94 (6.39–8.76)	7.79 (5.89–8.38)	7.71 (5.95–8.58)	0.6229	0.0510	0.3836	(7.0–23.0)	12.55 (10.4–16.2)
oil	3.88 (3.24–4.52)	4.26 (3.49–4.93)	3.98 (3.22–4.75)	4.13 (3.19–4.72)	3.64 (2.79–4.58)	4.17 (3.57–4.88)			0.0004	(3.1–5.8)	3.56 (1.74–5.82)
protein	8.30 (7.13–10.14)	8.31 (7.00–9.68)	8.91 (7.11–11.41)	8.47 (6.54–9.76)	9.01 (7.64–11.61)	9.25 (8.09–11.32)			0.0161	(6.0–12.7)	10.30 (6.15–17.26)

[a] Percent dry weight, except for moisture. [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [c] OECD, 2002 (*9*). [d] ILSI (*10*).

ments), free tryptophan (3), fumaric acid (2), glutaric acid (3), isocitric acid (2), malic acid (1), succinic acid (1), inositol (1), and stachyose (12).

The data were analyzed across locations with a separate analysis performed for each analyte. A model of the following form was fit for each analyte

$$y_{ijk} = \mu + s_i + t_j + (st)_{ij} + \epsilon_{ijk} \quad (1)$$

where y_{ijk} is the kth response for the jth tester at the ith location, μ is the overall mean, s_i is the effect of the ith location, t_j is the effect of the jth tester, $(st)_{ij}$ is the effect of the interaction between the jth tester and the ith location, and ϵ_{ijk} is the random error.

To satisfy the model assumptions for abscisic acid, free alanine, free lysine, and glycine betaine, the analysis was performed on the log(e) transformed data for these analytes. The model assumptions were satisfied for the remaining analytes.

Model 1 was fit for each analyte using SAS PROC GLM to conduct an outlier analysis. Observations with studentized residuals that are ≤ -6 or ≥ 6 were removed from the analysis dataset. This resulted in the removal of 16 measurements: free arginine (1), free glutamine (1), free leucine (1), free phenylalanine (1), fumaric acid (1), glutaric acid (1), succinic acid (1), malic acid (1), inositol (1), raffinose (3), sorbitol/mannitol (1), and sucrose (3).

On the dataset with the excluded analytes and outliers removed as described above, SAS PROC MEANS (*10*) was used to calculate the sample mean and range for each analyte, location, and tester.

The final analysis dataset consists of the transformations described above and the removal of the observations deemed to be outliers. Using the final analysis dataset, model 1 was fit for each analyte using SAS PROC MIXED. If the interaction between locations and testers is significant, then the location and tester main effects should not be interpreted. If the interaction between locations and testers is not significant, then the location and tester main affects may be assessed. All statistical comparisons are made at the 5% level of significance (i.e., $p < 0.05$).

RESULTS AND DISCUSSION

Proximate Composition. Compositional analysis results for proximates are presented in **Table 1**. The means and ranges of values recorded for moisture and oil contents from all locations were consistent with those reported in the literature (*9*) and in the ILSI Crop Composition Database (*11*); protein levels were slightly lower than reported previously. There was a statistically significant interaction effect between tester and location for oil and protein levels. This would imply that levels of oil and protein level in maize grain are particularly susceptible to changes due to natural variation. In other words, differences in genetic backgrounds and location act synergistically, resulting in enhanced variability in the level of these proximates.

Fatty Acid Composition. A total of nine analytes were measured. Only 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, and 18:3 linolenic acid were consistently detected at levels above the assay limit of quantitation. Measured levels in harvested grain from all three locations were comparable to those recorded in the literature (*9*) and in the ILSI Crop Composition Database (**Table 2**) (*11*). The other measured fatty acids, which were not detected at levels above the assay limit of quantitation, included 20:0 arachidic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, and 22:0 behenic acid. These analytes are known to be present at only very low levels in grain (*9*, *11*, *12*). Mean values for oleic acid and palmitic acid were higher in inbreds crossed with Iodent than for inbreds crossed with C103 (and stearic acid was correspondingly lower). Linoleic, oleic, and stearic acids showed a significant tester and location interaction effect. As described in the above paragraph on proximates, this would imply differences in genetic backgrounds and location combine synergistically to enhance natural variation in the levels of these fatty acids.

Total Amino Acid Composition. The range of values recorded for total amino acids was consistent with those recorded in the literature (*9*) and in the ILSI Crop Composition Database (*11*), although mean values were slightly lower (**Table 3**) commensurate with the lower protein content noted above. For most amino acids magnitude differences in the mean values recorded for the different tester lines at the different locations were small but statistically significant. A significant location effect was detected for 13 of the 18 total amino acids, and a significant tester effect was detected for four amino acids (**Table 3**). **Table 3** also shows that statistically significant ($p < 0.05$) interaction effects were observed for four (proline, serine, cysteine, methionine) of the 18 total amino acids. Amino acids that showed both a (non-interacting or nonsynergistic) tester and a location effect included arginine, histidine, and glycine. The fact that the 18 total amino acids are differentially susceptible to tester, location, and/or interaction effects may suggest that variation in genetic background and growing location, even within a geographically circumscribed area, differentially affects different protein components in maize grain.

Free Amino Acid Composition. As expected, recorded values of free amino acids (**Table 4**) were considerably lower than that recorded for total amino acids (**Table 3**) (note that values for free amino acids are recorded in parts per million dry weight, whereas values for total amino acids are recorded in percent dry weight). Indeed, for several free amino acids, especially leucine, methionine, phenylalanine, and tryptophan, recorded values were very close to the assay limits of quantitation. The most abundant free amino acids were asparagine, aspartic acid, glutamic acid, and proline. This is consistent with values reported for both wild-type maize and maize with reduced

Table 2. Fatty Acid Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana						
component[a]	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction[b]	lit.[c] (range)	ILSI[d] mean (range)
C18:2 linoleic acid	57.88 (52.90–61.20)	55.29 (49.20–58.20)	56.95 (49.30–60.70)	55.38 (50.00–60.50)	58.41 (53.70–61.40)	55.23 (50.50–58.00)			0.0136	(0.67–2.31)	56.5 (43.1–64.4)
C18:3 linolenic acid	1.18 (1.00–1.70)	1.14 (0.90–1.50)	1.14 (1.00–1.70)	1.14 (1.00–1.70)	1.18 (1.00–1.70)	1.16 (1.00–1.50)	0.1863	0.4512	0.7741	(0.03–0.10)	1.10 (0.71–1.42)
C18:1 oleic acid	27.58 (24.10–31.70)	30.38 (26.50–36.60)	28.52 (24.90–35.80)	30.11 (25.30–35.80)	27.20 (23.90–32.50)	30.32 (27.10–35.70)			0.0149	(0.70–1.39)	26.7 (19.4–40.2)
C16:0 palmitic acid	11.35 (9.90–13.30)	11.52 (10.50–12.70)	11.24 (10.20–12.40)	11.51 (10.70–12.80)	11.07 (9.60–12.80)	11.53 (10.20–13.00)	0.0001	0.3790	0.2830	(0.29–0.79)	11.37 (7.94–14.32)
C18:0 stearic acid	1.91 (1.50–2.20)	1.58 (1.40–2.00)	1.87 (1.50–2.20)	1.65 (1.40–1.90)	2.06 (1.60–2.30)	1.67 (1.40–1.90)			0.0003	(0.04–0.17)	1.87 (1.33–2.66)

[a] Values of fatty acids expressed as percent of total fatty acid. The method included the analysis of the following fatty acids, which were not detected in the majority of samples analyzed: C20:0 arachidic acid, C20:1 eicosenoic acid, C20:2 eicosadienoic acid, and C22:0 behenic acid. [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [c] OECD, 2002 (*9*). [d] ILSI (*10*).

Table 3. Total Amino Acid Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana						
component[a]	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction	lit.[b] (range)	ILSI[c] mean (range)
alanine	6.27 (5.27–7.73)	6.42 (5.21–7.58)	6.65 (4.93–8.54)	6.39 (4.55–7.69)	6.86 (5.58–9.27)	7.02 (5.94–8.57)	0.9043	<0.0001	0.0767	(5.6–10.4)	8.22 (5.83–11.03)
arginine	4.68 (3.90–5.77)	4.62 (3.80–5.23)	4.78 (3.89–5.71)	4.57 (3.60–5.28)	4.88 (4.14–5.94)	4.81 (4.24–5.78)	0.0115	0.0007	0.2871	(2.2–6.4)	4.59 (3.11–5.69)
aspartate	5.71 (4.55–7.31)	5.82 (4.39–7.27)	5.85 (4.54–7.07)	5.72 (4.25–6.84)	6.12 (4.89–8.21)	6.24 (5.23–8.01)	0.6753	<0.0001	0.3042	(4.8–8.5)	6.95 (5.28–8.58)
cysteine	1.95 (1.65–2.48)	1.88 (1.55–2.31)	2.19 (1.70–2.71)	1.98 (1.62–2.50)	2.17 (1.78–2.62)	2.05 (1.77–2.69)			0.0429	(0.8–3.2)	2.22 (1.67–3.09)
glutamate	16.74 (13.53–21.45)	17.04 (13.49–20.73)	17.97 (13.14–23.66)	17.04 (11.84–20.94)	18.60 (14.75–25.69)	18.86 (15.65–23.53)	0.6281	<0.0001	0.0853	(12.5–25.8)	20.77 (14.69–28.08)
glycine	3.42 (2.97–4.05)	3.40 (2.91–3.86)	3.55 (2.94–4.14)	3.40 (2.75–3.77)	3.63 (3.09–4.59)	3.58 (3.21–4.25)	0.0180	<0.0001	0.1363	(2.6–4.9)	3.88 (3.06–4.58)
histidine	2.51 (2.03–3.30)	2.40 (1.94–2.81)	2.60 (2.00–3.21)	2.40 (1.87–2.79)	2.67 (2.20–3.44)	2.55 (2.06–3.20)	<0.0001	<0.0001	0.4435	(1.5–3.8)	3.14 (2.41–4.18)
isoleucine	3.08 (2.46–3.97)	3.10 (2.41–3.96)	3.15 (2.22–4.33)	3.07 (2.13–3.76)	3.26 (2.56–4.64)	3.36 (2.77–4.48)	0.7295	0.0002	0.3588	(2.2–7.1)	3.78 (2.65–5.17)
leucine	10.37 (8.28–13.26)	10.62 (8.29–12.98)	11.05 (7.67–14.96)	10.53 (7.14–13.08)	11.40 (8.96–16.23)	11.73 (9.48–14.90)	0.8953	<0.0001	0.0851	(7.9–24.1)	13.95 (9.23–19.53)
lysine	2.57 (2.22–3.29)	2.51 (2.15–2.80)	2.59 (2.20–3.08)	2.52 (2.12–2.88)	2.59 (2.27–2.87)	2.57 (2.26–3.09)	0.0257	0.3688	0.6784	(0.5–5.5)	3.12 (2.38–5.57)
methionine	1.58 (1.20–2.12)	1.56 (1.23–1.95)	1.90 (1.35–2.47)	1.74 (1.30–2.04)	1.94 (1.57–2.26)	1.89 (1.58–2.30)			0.0336	(1.0–4.6)	2.15 (1.30–3.44)
phenylalanine	3.49 (2.88–4.36)	3.62 (2.83–4.51)	3.65 (2.59–4.76)	3.58 (2.46–4.37)	3.79 (3.00–5.31)	3.93 (3.26–4.91)	0.2156	<0.0001	0.1759	(2.9–6.4)	5.43 (3.87–7.43)
proline	7.88 (6.57–10.09)	7.85 (6.25–9.29)	8.79 (6.99–11.96)	8.20 (6.19–10.23)	8.60 (6.66–10.97)	8.72 (6.89–10.48)			0.0208	(6.3–13.6)	9.77 (6.61–13.29)
serine	4.16 (3.42–5.15)	4.30 (3.60–5.34)	4.40 (3.32–5.66)	4.22 (3.26–5.29)	4.55 (3.81–6.00)	4.63 (3.95–5.51)			0.0402	(3.5–9.1)	5.42 (3.65–7.35)
threonine	3.18 (2.60–3.95)	3.22 (2.50–3.66)	3.35 (2.62–4.12)	3.20 (2.52–3.74)	3.44 (2.91–4.17)	3.45 (3.01–4.14)	0.3224	<0.0001	0.0609	(2.7–5.8)	3.51 (2.73–4.58)
tryptophan	0.68 (0.52–1.04)	0.68 (0.54–1.01)	0.75 (0.61–1.05)	0.71 (0.56–0.88)	0.76 (0.61–1.15)	0.74 (0.60–1.20)	0.1350	<0.0001	0.4044	(0.4–1.3)	0.59 (0.36–0.88)
tyrosine	3.57 (2.86–4.71)	3.64 (2.81–4.45)	3.75 (2.76–4.76)	3.61 (2.59–4.37)	3.94 (3.12–5.18)	3.99 (3.34–5.06)	0.9258	<0.0001	0.1695	(1.2–7.9)	3.57 (1.89–5.07)
valine	4.35 (3.45–5.63)	4.34 (3.27–5.58)	4.47 (3.23–5.90)	4.31 (3.01–5.28)	4.68 (3.73–6.52)	4.68 (3.90–6.16)	0.3588	<0.0001	0.5312	(2.1–8.5)	5.09 (3.81–6.59)

[a] Percent dry weight. [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [c] OECD, 2002 (*9*). [d] ILSI (*10*).

zein storage protein levels (*13*−*15*). Whereas the range of values for total amino acids was rarely >2-fold for any given amino acid, the range was typically much larger for free amino acids, generally around 5-fold (**Table 4**) if not greater. For example, the values for free lysine ranged from 21 to 250 ppm, a nearly 12-fold difference combined across all locations. Only a 1.5-fold range (from 2.15 to 3.29% dry weight) was observed for total lysine.

Table 4. Free Amino Acid Composition of Grain Harvested from All Three Sites

component[a]	Cambridge C103 mean (range)	Cambridge Iodent mean (range)	Huxley C103 mean (range)	Huxley Iodent mean (range)	South Amana C103 mean (range)	South Amana Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction	lit.[b,c] (range)
alanine[d]	57.26 (34.00–86.00)	84.07 (50.00–128.00)	85.66 (40.00–153.00)	91.36 (52.00–155.00)	78.31 (45.00–139.00)	111.67 (63.00–206.00)			0.0002	(56–134)
arginine	77.67 (48.00–151.00)	88.58 (62.00–164.00)	82.36 (38.00–160.00)	83.00 (41.00–146.00)	76.06 (54.00–128.00)	86.70 (53.00–172.00)	0.0074	0.8625	0.2214	(26–74)
asparagine	264.41 (129.00–409.00)	366.79 (233.00–588.00)	265.85 (141.00–411.00)	295.32 (176.00–440.00)	250.54 (144.00–396.00)	340.79 (216.00–617.00)			0.0008	(185–272)
aspartate	231.61 (138.00–379.00)	235.91 (159.00–348.00)	251.45 (160.00–462.00)	238.86 (144.00–360.00)	236.90 (161.00–382.00)	252.92 (178.00–448.00)	0.6827	0.2485	0.1741	(113–173)
glutamate	378.30 (291.00–572.00)	374.00 (247.00–524.00)	357.13 (234.00–516.00)	354.11 (206.00–463.00)	302.98 (202.00–443.00)	330.60 (232.00–538.00)	0.3391	<0.0001	0.1099	(216–289)
glutamine	42.15 (20.00–153.00)	62.09 (34.00–121.00)	52.45 (24.00–150.00)	59.34 (23.00–139.00)	38.98 (21.00–70.00)	64.83 (33.00–161.00)			0.0108	(29–141)
glycine	18.37 (12.00–45.00)	22.37 (15.00–46.00)	22.17 (13.00–44.00)	21.75 (13.00–50.00)	17.81 (12.00–33.00)	21.63 (15.00–48.00)			0.0402	(12–56)
histidine	34.22 (16.00–57.00)	38.56 (18.00–59.00)	34.43 (14.00–55.00)	37.09 (15.00–53.00)	34.81 (16.00–51.00)	37.60 (16.00–69.00)	0.0031	0.8911	0.7883	(20–30)
isoleucine	12.74 (8.00–24.00)	16.81 (11.00–25.00)	15.68 (10.00–26.00)	18.16 (10.00–34.00)	14.40 (10.00–23.00)	18.96 (13.00–28.00)	<0.0001	0.0001	0.1247	(8–18)
leucine	10.87 (7.00–29.00)	17.21 (11.00–43.00)	12.28 (7.00–26.00)	15.86 (9.00–28.00)	10.46 (6.00–24.00)	17.15 (11.00–41.00)			0.0433	(7–20)
lysine[d]	69.48 (30.00–250.00)	76.91 (33.00–220.00)	74.02 (27.00–208.00)	70.16 (21.00–219.00)	44.67 (24.00–94.00)	53.81 (25.00–113.00)	0.3213	<0.0001	0.1790	(13–47)
methionine	5.63 (0–11.00)	9.00 (4.00–15.00)	8.17 (0–18.00)	9.86 (0–15.00)	6.06 (0–11.00)	9.56 (0–19.00)	<0.0001	0.0004	0.0688	(11–13)
phenylalanine	10.65 (5.00–18.00)	16.63 (10.00–27.00)	12.28 (8.00–26.00)	15.98 (9.00–26.00)	11.67 (6.00–17.00)	17.06 (11.00–35.00)	<0.0001	0.4323	0.1225	(7–37)
proline	626.98 (278.47–971.78)	668.02 (222.68–1045.15)	640.43 (340.95–1121.28)	669.08 (412.37–1127.00)	404.78 (136.67–651.37)	643.72 (370.09–873.55)			<0.0001	
serine	36.50 (24.00–72.00)	49.14 (27.00–85.00)	48.87 (29.00–100.00)	54.41 (31.00–78.00)	34.48 (24.00–63.00)	50.27 (32.00–77.00)			0.0054	(29–90)
threonine	15.96 (9.00–33.00)	22.98 (15.00–35.00)	20.60 (12.00–42.00)	24.16 (12.00–35.00)	16.15 (10.00–31.00)	24.04 (15.00–37.00)			0.0159	(10–22)
tryptophan	12.09 (7.00–21.00)	15.44 (8.00–22.00)	12.62 (0–19.00)	15.07 (11.00–21.00)	13.23 (0–24.00)	15.02 (9.00–24.00)	<0.0001	0.7301	0.2672	(7–15)
tyrosine	44.35 (30.00–68.00)	52.77 (32.00–81.00)	47.57 (32.00–80.00)	54.91 (34.00–91.00)	46.92 (33.00–75.00)	54.98 (39.00–90.00)	<0.0001	0.1948	0.9435	(28–40)
valine	26.28 (16.00–53.00)	33.35 (22.00–53.00)	29.87 (20.00–59.00)	35.27 (22.00–56.00)	29.88 (19.00–57.00)	36.29 (26.00–54.00)	<0.0001	0.0120	0.7754	(23–43)

[a] Parts per million dry weight. [b] Huang et al., 2005 (*13*). [c] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] Huang et al. (*14*). [d] To fit the assumptions of the statistical models used, analyte was treated as a log-transformed variable (see Materials and Methods).

Furthermore, although differences in growing location appeared to be the major contributor to variation in total amino acid content (at least in terms of the number of amino acids significantly affected), the picture appears to be more complex when free amino acid levels are assessed. Only five of the free amino acids showed a non-interacting location effect (i.e., no synergistic combination of location with genetic background) (**Table 4**). Of these, three amino acids (methionine, isoleucine, and lysine) are biosynthetically linked (via aspartate-4-semialdehyde). Eight free amino acids showed a significant tester effect. In general, there appeared to be higher mean values for most free amino acids in hybrids derived from the Iodent group, and this was most pronounced for asparagine and glutamine. Statistical significance for a tester effect was also observed from phenylalanine, tryptophan, and tyrosine, amino acids that share the same biosynthetic precursors through the shikimic acid pathway. Analogously, histidine and arginine, which share glutamate as a biosynthetic precursor, also showed a tester effect. Significant interaction effects were detected for eight free amino acids. Notably, this included the osmoprotectant, proline. Increased free proline accumulation in maize seedlings (*16*) and roots (*17*) subject to osmotic stress has been reported, although there is little information on free proline levels in maize grain prior to this study.

The fact that, at least in this study, total amino acids are, in general, more susceptible to location effects, whereas free amino acids levels are, in general, more susceptible to a tester effect, may imply that these two sets of compositional components are under different genetic or regulatory control. It is also known, however, that a reduction in levels of the major zein storage proteins (a reduction that effectively results in elevated levels of total lysine) in maize grain is known to elevate levels of certain free amino acids, especially asparagine, aspartic acid, and glutamic acid, the major free amino acids in maize (*13*−*15*). It is worthwhile recognizing that the interaction of free amino acid metabolic networks with protein composition, an area one would expect to be thoroughly well researched in the pursuit of nutritionally enhanced crops, remains only partially understood and an active area of research (e.g., ref *18*).

Sugar Composition. Simple sugars, with the exception of the antinutrient raffinose, are rarely measured in mature grain harvested from maize. In this study, a comparative screening approach was adopted to facilitate rapid measurement of the large sample set and because semiquantitative data were deemed to be adequate for the statistical analyses. Of the sugars measured here (**Table 5**) glucose, fructose, sucrose, and raffinose were observed in most samples, with responses falling in the general range of the calibration standards; inositol, sorbitol/

Table 5. Sugar Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana					
component[a]	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction[b]	ILSI[c] mean (range)
fructose	1.38 (0.56–3.46)	1.51 (0.64–3.77)	1.45 (0.70–4.13)	1.51 (0.74–3.81)	0.85 (0.40–1.56)	1.28 (0.51–3.82)	0.0032	<0.0001	0.0718	
glucose	2.45 (0.66–6.92)	2.85 (1.00–7.91)	2.50 (0.92–9.00)	2.74 (0.80–7.18)	1.45 (0.30–2.62)	2.33 (0.52–8.19)	0.0009	<0.0001	0.1920	
inositol	0.021 (0.011–0.098)	0.026 (0.011–0.098)	0.021 (0.011–0.087)	0.021 (0.011–0.043)	0.019 (0–0.044)	0.022 (0.011–0.098)	0.1076	0.2835	0.6031	1469[d] (1236.0–2009.5)[d]
raffinose	0.93 (0.50–1.47)	0.93 (0.53–3.19)	0.88 (0.39–3.25)	0.88 (0.21–3.08)	1.04 (0.27–2.06)	0.88 (0.30–1.38)	0.2522	0.2990	0.2079	0.142[e] (0.056–0.290)[e]
sorbitol/ mannitol	1.87 (0.53–5.67)	2.81 (0.53–8.04)	1.86 (0.61–7.67)	2.66 (0.84–9.25)	1.13 (0.22–3.62)	2.44 (0.80–8.16)	<0.0001	0.0050	0.3370	
stachyose	0.015 (0.011–0.054)	0.017 (0.011–0.044)	0.011 (0–0.044)	0.012 (0–0.044)	0.014 (0–0.033)	0.014 (0–0.043)	0.2077	0.0014	0.7157	
sucrose	28.90 (12.91–89.60)	30.47 (21.08–94.62)	29.50 (15.91–91.93)	26.81 (8.32–39.82)	24.59 (7.38–33.42)	26.59 (8.39–38.28)	0.8089	0.0207	0.2203	

[a] Response units (dry weight). [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [c] ILSI (*10*). [d] Parts per million. [e] Percent dry weight.

Table 6. Organic Acid Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana				
component[a]	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction[b]
citric acid	33.23 (20.01–74.28)	23.76 (14.58–39.67)	33.92 (18.01–60.53)	27.10 (18.85–42.43)	43.10 (19.42–73.10)	30.99 (18.61–74.64)	<0.0001	<0.0001	0.1543
fumaric acid	0.13 (0–0.30)	0.16 (0.086–0.24)	0.15 (0.077–0.39)	0.17 (0.066–0.31)	0.12 (0.043–0.22)	0.16 (0–0.30)	<0.0001	0.0020	0.4199
glutaric acid	0.033 (0–0.055)	0.035 (0.011–0.076)	0.036 (0.021–0.066)	0.031 (0–0.075)	0.028 (0–0.044)	0.033 (0.011–0.054)			0.0095
isocitric acid	0.12 (0–0.19)	0.080 (0.043–0.15)	0.14 (0.054–0.28)	0.095 (0.043–0.13)	0.14 (0.075–0.24)	0.098 (0–0.19)	<0.0001	0.0005	0.9336
malic acid	2.36 (0–4.68)	2.93 (1.76–4.28)	3.31 (1.70–7.04)	3.54 (1.72–5.20)	2.84 (0.81–5.73)	3.42 (1.99–4.99)	<0.0001	<0.0001	0.3248
succinic acid	0.45 (0–0.68)	0.60 (0.41–1.03)	0.58 (0.30–1.00)	0.69 (0.37–1.09)	0.39 (0.17–0.70)	0.60 (0.30–1.12)			0.0406

[a] Response units, dry weight. [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$).

mannitol, and stachyose were observed in most samples, but values were typically close to the assay limit of quantitation. Trehalose was not detected by this method in any sample, although there is evidence from other studies that it may be present at very low levels (*19*). Sucrose, derived from leaf tissue, is used by maize kernels as a building block for starch synthesis, and the high levels present, relative to other sugars, could therefore be reasonably anticipated. Our study highlights that whereas the concentration of sucrose is extremely variable, a location effect could be observed. Non-interacting tester and location effects were observed for glucose, fructose, and sorbitol/mannitol.

Organic Acid Composition. Organic acids are rarely measured in mature grain harvested from maize. In this study, a comparative screening approach was adopted to facilitate rapid measurement of the large sample set, to maximize organic acid metabolite coverage, and because semiquantitative data were deemed to be adequate for the statistical analyses. This LC-MS/MS-based method demonstrated that few organic acids were present at significant levels (**Table 6**). The only acids detected in most samples at levels consistently within the calibration curve range were citric acid (the most abundant organic acid detected), succinic acid, and malic acid. Acids observed in most samples but at very low levels included isocitric acid, fumaric acid, and glutaric acid (responses fell close to the lowest calibration standard). Acids not observed included chorismic acid, shikimic acid, anthranilic acid, homogentisic acid, α-ketoglutaric acid, 4-hydroxyphenylpyruvic acid, phenylypyruvic acid, prephenic acid, pyruvic acid, saccharopine, and salicyclic acid. Variation in recorded levels was very high with most organic acids. Of the abundant organic acids, citric acid and malic acid showed non-interacting tester and location effects. For citric acid mean levels were typically lower in the hybrids from the Iodent group, whereas for malic and succinic acid levels were lower in the hybrids from the C103 group.

The organic acid analysis did not include the phenyl cinnamic compounds, ferulic acid and *p*-coumaric acid, secondary metabolites implicated in lignification and which are included in the OECD consensus documents. Compositional analyses of these metabolites were suggested by OECD as significant changes in their levels may be indicative of unintended effects of trait modification on metabolism, and considerable information on natural variation in their levels is provided in the ILSI Crop Composition Database (*11*).

Table 7. Glycerol, Glycine Betaine, and Abscisic Acid Composition of Grain Harvested from All Three Sites

	Cambridge		Huxley		South Amana				
component	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	C103 mean (range)	Iodent mean (range)	*p*-value tester	*p*-value location	*p*-value interaction[b]
abscisic acid[a,c]	10.82 (2.62–21.47)	13.31 (4.80–50.02)	12.55 (5.43–41.37)	13.31 (4.84–46.98)	13.97 (2.71–58.58)	13.75 (5.87–54.97)	0.1550	0.2146	0.5877
glycerol[d]	96.34 (10.22–358.26)	132.11 (43.19–366.60)	143.38 (7.99–390.22)	141.75 (47.63–273.52)	90.52 (14.59–308.66)	119.90 (25.29–481.60)	0.0097	0.0006	0.1379
glycine betaine[c,d]	9.49 (3.60–27.80)	14.33 (1.80–46.30)	7.83 (2.70–23.80)	12.99 (1.50–40.90)	20.84 (9.30–66.30)	19.87 (1.40–79.20)			0.0012

[a] Parts per million × 1000, dry weight. [b] If the interaction between the effects of test substance and treatment is significant, then the test substance and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the test substance and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [c] To fit the assumptions of the statistical models used, analyte was treated as a log-transformed variable (see Materials and Methods). [d] Parts per million.

Total Glycerol, Glycine Betaine, and Abscisic Acid Composition. Free glycerol is often considered to be an important osmolyte in many organisms and tissues (*20*, *21*). Total glycerol content measured here showed extensive variability (>59-fold), ranging from 7.99 to 481.6 ppm (**Table 7**). Levels were shown to be dependent on both genetic background and growing location.

Little prior literature on glycine betaine levels in maize is evident (see refs *22*−*26*). One survey (*22*) highlights that glycine betaine levels in maize are low when compared to other foodstuffs such as wheat and spinach. The data presented here show a huge range (>55-fold) for glycine betaine, with values from 1.4 to 79.2 ppm (**Table 7**). Significant genotypic variation in glycine betaine levels in leaf has been recorded for maize lines (*23*, *24*), although it has been demonstrated that levels in maize lines with initially high content (3−10 μmol per gram of fresh weight) can increase further in growing seasons that experience drought (*25*, *26*). This is consistent with the proposed role of glycine betaine as an osmoprotectant. A significant interaction between tester and location was noted, highlighting the importance of genetic background and growing location on this metabolite.

Abscisic acid has long been proposed to play a role in maize kernel development (*27*−*31*). Levels of abscisic acid are also known to increase with water deficit (*27*−*31*); however, concentrations of this hormone are typically very low. This was confirmed by our LC-MS, where we found that the concentrations for most samples were close to the assay limit of quantitation (**Table 7**).

Conclusion. A major focus of this study was to further define the metabolite composition of grain harvested from maize by surveying a wide genetic range of maize hybrids. We expanded metabolite coverage beyond the OECD components (*9*) typically used in maize compositional analyses (proximates, total amino acids, free fatty acids) to include free amino acids, organic acids, sugars, total glycerol, abscisic acid, and glycine betaine, metabolites that might reasonably be expected to be affected by new traits addressing, for example, nutritional enhancement or improved stress tolerances. The study was also intended to provide additional insights into the effect of genetic by environmental interactions on nutrient and metabolite composition in grain, and hybrids were therefore grown at three different sites in the United States. It is apparent from this survey that these additional metabolites (free amino acids, organic acids, sugars, and stress metabolites) are either extremely variable or at low levels. They would most likely not add useful information to a safety and/or nutritional assessment of a new product unless they were the intended endpoint for improvement.

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