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Effects of Genetics and Environment on the Metabolome of Commercial Maize Hybrids: A Multisite Study

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Supporting Information

ABSTRACT: This study was designed to elucidate the biological variation in expression of many metabolites due to environment, genotype, or both, and to investigate the potential utility of metabolomics to supplement compositional analysis for substantial equivalence assessments of genetically modified (GM) crops. A total of 654 grain and 695 forage samples from 50 genetically diverse non-GM DuPont Pioneer maize hybrids grown at six locations in the U.S. and Canada were analyzed by coupled gas chromatography time-of-flight-mass spectrometry (GC/TOF-MS). A total of 156 and 185 metabolites were measured in grain and forage samples, respectively. Univariate and multivariate statistical analyses were employed extensively to compare and correlate the metabolite profiles. We show that the environment had far more impact on the forage metabolome compared to the grain metabolome, and the environment affected up to 50% of the metabolites compared to less than 2% by the genetic background. The findings from this study demonstrate that the combination of GC/TOF-MS metabolomics and comprehensive multivariate statistical analysis is a powerful approach to identify the sources of natural variation contributed by the environment and genotype.

KEYWORDS: *metabolomics, metabolite profiling, GC-MS, multivariate analysis, safety assessment, substantial equivalence*

INTRODUCTION

The world's population reached 7 billion last year and is projected to grow to over 9 billion by 2050.[1] Genetically modified crops are expected to play a critical role in meeting the unprecedented demand for food around the world. The number of hectares cultivated with GM crops has increased sharply over the past decade.[2] GM crops have been documented to be often higher yielding, more nutritious, disease resistant, and drought tolerant in comparison to conventional crops.[3] However, introduction of GM crops has generated debate and controversy over their safety to humans and livestock and their long-term impact to the environment.[4−6] As a result, the Organization of Economic Cooperation and Development (OECD) introduced the concept of substantial equivalence, which has come to be accepted by various worldwide agencies as a means to evaluate GM crops.[7,8] This protocol, however, has not satisfied all concerns regarding the safety of GM crops.[9] Some studies suggested that metabolomics would be more appropriate to detect compositional changes since many analytes can be measured and unintended changes could therefore be more easily seen.

Metabolomics and metabolite profiling involve qualitative or semiquantitative detection of a high number of metabolites that are substrates, intermediates, and end products of cellular activities.[10−12] Metabolomics has been applied to a wide range of agricultural applications such as crop protection, plant biotechnology, and plant breeding.[13−19] A number of studies have attempted to use untargeted metabolomics or targeted metabolite profiling to access substantial equivalence and possible extrapolation for safety assessments of GM crops.[20−22] The potential utility of metabolomics for these purposes is due to the fact that metabolite concentrations are often altered more significantly compared to gene expression or protein levels, making detection of metabolites a sensitive and responsive measure of biological status. Metabolites are also "closer" to the end product of biological activities, thus presumably more reflective of a plant's phenotype, which is of prime value in substantial equivalence and safety assessments of new GM varieties.

There are several studies that have been conducted to investigate the effect of the environment and genotype on biochemical composition of maize grain.[23−29] Metabolite profiling has also been used to evaluate the effect of the environment and genetic modification in other crops, such as rice, potatoes, tomatoes, and wheat, among others.[20,30−34] These studies collectively underscore the need to understand and document the natural variation of metabolism due to the environment and genotype. Understanding the effect of the environment on genetically diverse non-GM crops is required before comparisons can be made with counterpart GM lines. However, to the best of our knowledge, no study to date has compared extensive metabolite profiles of both grain (kernels) and forage (leaves) from genetically diverse non-GM commercial maize lines from geographically diverse locations due to different genotypes, environments, or both.

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Table 1. Sample Information Summary Based On (a) Location and (b) GC Batch/Sequence

(a)

	forage			grain		
location	No. of samples	reference	QC	No. of samples	reference	QC
Illinois	120	15	12	117	15	12
Kansas	118	13	8	113	15	12
Minnesota	112	15	12	119	15	12
Nebraska	118	14	12	118	15	12
Ontario	66	12	10	108	15	12
Texas	120	15	11	120	15	12
Total	654	84	65	695	90	72

(b)

		forage			grain		
batch	locations	No. of samples	reference	QC	No. of samples	reference	QC
1	Illinois	40	5	4	39	5	4
2	Illinois	40	5	4	40	5	4
3	Illinois	40	5	4	38	5	4
4	Kansas	40	4	4	39	5	4
5	Kansas	40	5	0	39	5	4
6	Kansas	38	4	4	35	5	4
7	Minnesota	38	5	4	40	5	4
8	Minnesota	36	5	4	39	5	4
9	Minnesota	38	5	4	40	5	4
10	Nebraska	40	5	4	40	5	4
11	Nebraska	38	4	4	39	5	4
12	Nebraska	40	5	4	39	5	4
13	Ontario	26	5	4	36	5	4
14	Ontario	26	5	4	35	5	4
15	Ontario	14	2	2	37	5	4
16	Texas	40	5	3	40	5	4
17	Texas	40	5	4	40	5	4
18	Texas	40	5	4	40	5	4
Total All Sites		654	84	65	695	90	72

This study was designed to document the biological variation of many metabolites influenced by the environment, genotype, or both and to investigate the potential of metabolomics to support substantial equivalence assessments for GM maize. We applied a combination of gas chromatography coupled to time-of-flight-mass spectrometry (GC/TOF-MS), followed by univariate and comprehensive multivariate statistical methods principal component analysis (PCA), partial least-squares discriminant analysis (PLSDA), and hierarchical cluster analysis (HCA) to compare the metabolomic profiles of samples collected from grain and forage from fifty genetically diverse non-GM maize hybrids grown at six locations scattered throughout the maize-growing regions of North America.

MATERIAL AND METHODS

Plant Materials. Fifty genetically diverse non-GM maize hybrids from DuPont Pioneer were planted at six different locations. Five of the locations were in the U.S. (Illinois, Kansas, Minnesota, Nebraska, and Texas) and the sixth location was in Ontario, Canada. Planting locations for the hybrids were selected based on days to maturity such that each location had 20 unique genotypes, as shown in Table S1 of the Supporting Information. At every location, each genotype was planted in three randomized blocks (3 blocks and 2 replicates per block), as shown in Table S2 of the Supporting Information. Each block was separated by an alley at least 36 in. wide and surrounded on each end by two-row borders. Agronomic practices such as irrigation, fertilization, herbicide, and pesticide applications were applied uniformly across locations and were consistent with the normally acceptable practices for maize production.

Two forage samples were collected after flowering from three plants from each genotype and block and immediately placed on dry ice. Each forage sample represents the aerial portion of three entire plants. Collected frozen samples were stored temporally at less than or equal to −10 °C. Each grain sample was collected at physiological maturity from five hand pollinated ears from each genotype and block. For each sample, the resulting grain from five shelled ears were pooled and immediately placed on dry ice until transferred to a less than or equal to −10 °C freezer for temporary storage. The same harvesting protocol was adopted in all locations. Samples were shipped frozen to the DuPont Pioneer Regulatory processing laboratory in Ankeny, Iowa where they were lyophilized and ground. Lyophilized forage and grain samples were then shipped on dry ice to the DuPont Pioneer metabolomics laboratory in Johnston, Iowa, where they were stored at −80 °C until analyzed. Part a of Table 1 summarizes the number of samples obtained from each location.

Sample Preparation. For forage samples, metabolites were extracted from lyophilized tissue with dry weights between 4.0 and 6.0 mg (5.1 mg mean). Metabolites were extracted from grain samples with dry weights between 4.0 and 6.0 mg (5.0 mg mean). Five hundred microliters of chloroform:methanol:water (2:5:2, v/v/v) containing 0.015 mg ribitol internal standard were added to each sample in a 1.1 mL polypropylene microtube containing two 5/32 in. stainless steel ball bearings. Samples were homogenized in a 2000 Geno/Grinder ball mill (SPEX CertiPrep, Inc., Metuchen, NJ) at a setting of 1650 rpm for 1 min and then rotated on an end-over-end mixer (Glas-Col, LLC, Terre Haute, IN) at 4 °C for 30 min. Samples were then centrifuged at 1454*g* at 4 °C for 15 min. Next, 300 μL

aliquots were transferred to 1.8 mL high recovery autosampler vials and subsequently evaporated to dryness in a speed vac (Thermo Scientific, Waltham, MA). The dried residues were redissolved in 50 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine, capped, and agitated with a vortex mixer for approximately two seconds. Samples were then incubated in an orbital shaker at 30 °C for 90 min to form methoxyamine derivatives. Eighty microliters of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was added to each sample to form trimethylsilyl derivatives. To minimize sample variability as a result of different derivatization states, a CTC Combi PAL autosampler (Gerstel Inc., Linthicum, MD) controlled by Maestro software (Gerstel) was used to deliver the MSTFA through a diluter to each sample 30 min prior to injection.

To further minimize analytical errors and system bias, forage and grain samples were rearranged into 18 batches prior to sample preparation. For each batch, four quality control (QC) samples were prepared by pooling aliquots from each individual sample. The QC samples were used to minimize and correct within batch variation. In addition, we also ran five reference samples which were prepared from the same grain or forage material in every batch. The reference materials consisted of grain and forage samples originating from Illinois. Reference samples were used to minimize and correct across batch variability. A detailed summary of samples run in every batch is shown in part b of Table 1.

Instrumental Analysis. The derivatized samples were first separated by gas chromatography on a Restek 20m × 0.18 mm × 0.18 μm film thickness Rtx-5Sil MS column. One microliter injections were made with a 1:30 split ratio using the Gerstel autosampler. The Agilent 7890A gas chromatograph (Agilent, Palo Alto, CA) was programmed for an initial temperature of 60 °C for 0.5 min and then increased to 350 °C at a rate of 36 °C per minute, where it was held for 1 min before being cooled rapidly to 60 °C and held there for 2.5 min in preparation for the next run. The injector and transfer line temperature were set at 270 and 250 °C, respectively, and the source temperature was set at 200 °C. Helium was used as the carrier gas with a constant flow rate of 0.8 mL min^{-1} maintained by an electronic pressure control. Data acquisition was performed on a LECO Pegasus HT time-of-flight-mass spectrometer (Leco Corp, St Joseph, MI) at an acquisition rate of 20 spectra sec^{-1} in the mass range of 45 to 600 m/z. An electron beam of 70 eV was used to generate spectra and the detector voltage was set at 1575 V. The instrument was autotuned for mass calibration using PFTBA (perfluorotributylamine) prior to each gas chromatograph sequence.

Data Pretreatment. Raw LECO GC/MS .peg data files were converted into .netCDF (Andi) formats using the LECO ChromaTof software version 4.41. For each .netCDF file, retention times were converted into retention indices using an in-house program. Data preprocessing which consisted of chromatogram gridding in the m/z value and retention index dimensions, chemical noise subtraction, aligning the retention indices of each selected ion chromatogram, and detecting nominal mass peaks was performed with Genedata Refiner MS version 6.1 (Basel, Switzerland). The resulting matrix consisted of intensities for each m/z value and retention index combination for each sample. The aligned and denoised data matrix was then transferred to Genedata Analyst version 2.2, where each intensity value was normalized for both the ribitol internal standard signal and sample dry weight.

The data matrix was then subjected to a data reduction step using an in-house clustering program. During the data clustering process, all peaks within the same retention index window (~0.5 s) that corresponded to the same compound were grouped into one cluster/group based on the correlation between the individual intensity profiles across all samples. Each cluster was represented by the most abundant mass in that cluster/group. A total of 156 and 185 metabolites were identified from grain and forage samples, respectively, down from 5026 and 8015 unclustered signatures identified from peak detection in grain and forage samples, respectively.

Univariate Analysis. The relative standard deviation (RSD), also known as coefficient of variation (CV), for each metabolite was calculated as a measure of the variance in grain and forage metabolomics data due to different genotypes and environments. The mean RSD for all metabolites in plants grown in every location was used to evaluate the effect of the environment. The mean RSD for all metabolites from each genotype using samples grown in each location separately was used as a measure of the effect of genotypes on data variance.

To further investigate the effect of environment and genotypes on the metabolites, a paired student's t test with a Bonferroni adjustment was calculated for each metabolite cluster, and the number of metabolites that were highly significantly altered (p values < 0.01) were determined. To assess the effect of the environment, we calculated and compared the p values of every metabolite from one location to those from every other location; a total of 2046 (the number of metabolites times the number of locations) statistical comparisons were done at every location. To determine the number of metabolites whose expression were altered due to different genotype, we calculated and compared the p values of every metabolite in one genotype to that in every other genotype at every location; a total of 64 600 (the number of metabolites times the number of different genes combination) statistical comparisons were made.

Multivariate Analysis. Data matrix before and after clustering were imported into Matlab version R2010b (Mathworks) installed with the PLS toolbox version 6.0.1 (Eigenvector Research Inc., Wenatchee, WA) for modeling. For PCA and PLSDA, data were normalized and autoscaled prior to modeling.[35] An open source software R statistical package (version 2.12.1) was used for hierarchical cluster analysis to generate dendrograms and heat maps.[35,36]

RESULTS

Data Variability in Grain and Forage. To investigate the data variability in grain and forage samples due to different environment and genotypes, we first calculated and compared mean RSD for all metabolites. To evaluate the effect of the environment, we compared the mean RSD for the relative amounts of all metabolites in grain and forage, using samples grown at different locations (Table 2). The mean RSD for all metabolites in grain samples was higher than that for forage samples grown at the same location, with an exception of Texas. Grain samples from Texas had a mean RSD of about 60%, compared to 68% for that from the other locations. Furthermore, forage samples from Texas had a mean RSD of 61% compared to about 45% for the other locations. Apart from Texas, we did not observe any significant differences between the mean RSDs for all metabolites in grain or forage samples from one location to another. To further evaluate the data variability and measure the data reproducibility, we also compared the mean RSDs for all metabolites using grain and forage samples from each block (total of 3 blocks and 2 replicates per block) at every location. Mean RSDs for all metabolites from each block were very similar for grain or

Table 2. Mean RSD for Relative Abundance of Metabolites in Grain and Forage Samples from Different Locations

location	grain	forage
Illinois	69.12	43.26
Kansas	69.43	44.27
Minnesota	67.19	46.73
Nebraska	68.67	49.48
Ontario	66.38	44.07
Texas	59.96	61.29
ref samples	48.48	39.18

forage samples grown at the same location (Table S3 of the Supporting Information). The mean RSD for grain samples from each block was higher than that for forage samples from the same block at every location except Texas, consistent with what we observed using all samples. Texas forage samples from every block, especially block 2, had higher RSDs which accounted for the highest RSD from this location (Table 2).

To investigate the effect of different genotypes on data variability, we compared the data variability from grain and forage samples with different genotypes. Table 3 shows the mean RSD for the relative amounts of all the metabolites from different genotypes using samples from all locations. Mean RSDs for most of the genotypes were higher in grain samples compared to forage samples, similar to what we observed when we compared samples from different locations. To minimize any confounding effect of the environment, we recalculated the mean RSDs separately for different genotypes using samples grown at every location (Table S4 of the Supporting Information). Mean RSDs calculated from samples at any one location were significantly lower than that generated using samples from all locations. This result indicates that there was a confounding effect from the environment. It is worth noting that the mean RSD for most genotypes was very similar across different locations, also indicating good reproducibility in our data across locations.

Table 3. Mean RSD for Relative Abundance of All Metabolites in Grain and Forage Samples with Different Genotypes from All Locations

entry code	samples	grain	forage	entry code	samples	grain	forage
1	34	71.68	57.49	26	6	60.07	34.38
2	35	71.64	64.5	27	12	67.89	43.94
3	34	79.62	55.18	28	6	48.74	36.45
4	35	69.18	58.7	29	12	61.97	47.08
5	35	72.41	60.23	30	12	68.31	46.07
6	3	51.02	NA	31	6	53.09	34.17
7	6	51.02	41.55	32	12	63.64	56.8
8	6	70.28	40.38	33	23	69.06	60.23
9	11	58.09	50.87	34	24	63.05	58.81
10	9	61.19	47.86	35	18	68.88	55.3
11	9	59.19	NA	36	18	71.92	50.95
12	8	57.8	34.32	37	5	59.68	31.79
13	4	51.12	33.95	38	18	70.35	51.27
14	11	53.71	44.53	39	12	62.44	61.66
15	6	55.69	41.64	40	6	58.54	29.02
16	12	67.33	51.77	41	12	54.44	52.29
17	6	56.31	32.85	42	6	61.21	45.04
18	12	62.74	47.43	43	12	68.88	59.22
19	23	59.9	53.65	44	6	65.72	32.65
20	23	81.69	47.5	45	6	52.69	48.67
21	12	67.24	44.07	46	6	60.63	34.43
22	5	70.63	46.24	47	6	46.03	46.94
23	6	48.2	40.31	48	6	52.62	51.87
24	29	60.2	59.57	49	6	50.8	51.22
25	30	70.31	65.15	50	6	57.73	30.16

Univariate Analysis. To determine the number of metabolites that were affected by different genotypes, we calculated and compared p values for the relative amounts of every metabolite from one genotype to those in every other at every location; in total 2046 statistical comparisons were done. To minimize the effect of the environment, we compared the effect of different genotypes using samples grown at the same location. Parts a and b of Table 4 show the percentage of metabolites with statistically significant altered levels (p values < 0.01 after Bonferroni correction) from one genotype to another using Illinois grain and forage samples, respectively. Less than 2% of the metabolites were impacted by different genotypes in grain and forage samples. Similar results were obtained when we compared the impact of the genotype using samples from the other five locations (results not shown).

To determine the number of metabolites that were affected by the environment (location), we calculated and compared p values for the relative amount of every metabolite from one location to another for both grain and forage samples. Parts a and b of Table 5 show the percentage of metabolites with statistically highly significant altered levels (p values < 0.01 after Bonferroni correction) between different locations in grain and forage samples, respectively. From the results, approximately half of the metabolites were impacted by the environment in both the grain and forage samples. Forage samples originating from Kansas presented the highest percentage of metabolites with altered amounts compared to other locations. For example, close to 70% of all the metabolites in forage samples expressed altered levels between Kansas and Ontario, compared to only 44% between Illinois and Ontario (see part b of Table 5). Our results show that the environment had the biggest impact on both the grain and forage metabolomes, with almost 50% of the detected metabolites differentially expressed at one location compared to the others. However, less than 2% of the metabolites were altered as a result of different genotypes. Often, relative amounts of none of the detected metabolites were altered across genotypes in grain or forage samples (compare Tables 4 and 5).

Multivariate Analysis: Effect of Environment. To evaluate the effect of the environment, we visualized the metabolomic profiles of grain and forage samples from all locations using unsupervised PCA. Figure 1 (panels a and b) shows PCA score plots of grain and forage samples, respectively. No clear separation was observed for grain samples across the different locations (Figure 1a). The reference samples were tightly clustered right in the middle of the score plot, indicative of low variance which is consistent to what we observed from our univariate analysis. This result also indicates that technical error, if any, is lower than biological variability. However, for the forage samples, we observed a strong environmental effect. Forage samples clustered in the PCA score plot based on where they were grown (Figure 1b). In addition, forage samples from the same location were more tightly clustered compared to grain samples. Reference samples from forage were also tightly clustered in the middle of the score plot, again indicating that technical error is less than biology-derived error. The first two principal components accounted for 32% and 35% of the total variance in grain and forage samples, respectively.

To further evaluate the effect of the environment on the plant's metabolome, the same data from grain and forage samples were also subjected to HCA. Figure 1(panels c and d) shows heat maps and dendrograms from the grain and forage data, respectively. HCA allowed for the visualization of sample similarity across different locations. Grain samples from Kansas, Minnesota, and Texas clustered together, indicating that their metabolomes were very similar (Figure 1c). Likewise, grain samples from Ontario, Nebraska, and Illinois also clustered together and thus are deemed to possess some group similarity.

Table 4. Percentage of Metabolites with Relative Abundances that were Statistically Highly Significant ($p < 0.01$ After Bonferroni Correction) between Genotypes in (a) Grain and (b) Forage Samples from Illinois

(a)

	1	2	3	4	5	19	20	21	23	24	25	26	27	29	30	31	33	34	36	38
1	0																			
2	0	0																		
3	0	0	0																	
4	0	0	0	0																
5	0	0.54	0	0	0															
19	0	0	0	0	0	0														
20	0	0	0	0	0	0	0													
21	0	0	0	0	0	0	0	0												
23	0	0	0	0	0	0	0	0	0											
24	0	0	0	0	0	0	0	0	0	0										
25	0	0	0	0	0	0	0	0	0	0	0									
26	0	0	0	0	0	0	0	0	0	0	0	0								
27	0	0	0	0	0	0	0	0	0	0	0	0	0							
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
30	0	0.54	0	0	0	0	0	0	0	0	0	0	0	0	0					
31	0	1.62	0	0	0	0	1.08	0	0	0	0	0	0	0	0	0				
33	0	0	1.08	0	1.08	0	0.54	0	0	0	1.08	0.54	0	0.54	0	0	0			
34	0	0	0	0	0	0	0	0	0	0	0	0.54	0	0	0	0.54	0	0		
36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(b)

	1	2	3	4	5	19	20	21	23	24	25	26	27	29	30	31	33	34	36	38
1	0																			
2	0.54	0																		
3	0	0	0																	
4	0	0.54	0	0																
5	0.54	0	0	0	0															
19	0	0	0	0	0	0														
20	0	0.54	0	0	0.54	0	0													
21	0.54	0	0	0	0	0	0.54	0												
23	0	0	0	0	0	0	0	0	0											
24	0	0.54	1.08	0.54	0.54	0	0	1.08	0	0										
25	0	0	0	0	0	0	0	0.54	0	0.54	0									
26	0	0	0	0	0	0	0	0	0	0	0	0								
27	0.54	0	0.54	0	0	0	0	1.62	0	0	0	0	0							
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
30	0	1.62	0.54	0	0.54	0	0.54	0	0	0.54	0.54	0	0	0.54	0					
31	0	0	0	0	0	0	0	0	0	0	0	0	0.54	0	0	0				
33	1.08	1.62	1.08	0	0.54	0.54	1.62	0	1.08	1.08	0.54	0.54	0.54	0	0	0	0			
34	1.08	1.08	0	0	0	0.54	1.08	1.08	0.54	1.08	0.54	1.08	0.54	0	0.54	0	0	0		
36	1.08	0.54	0	0	0	0	1.62	0	0.54	0.54	0.54	0	0.54	0	0	0	0	1.08	0	
38	0	0	0	0	0	0	0	0	0	0	0	0	0.54	0	0	0	0	0	0	0

These relationships are also evident from the corresponding heat map. It is clear that the concentrations of most of metabolites in grain from Ontario, Nebraska, and Illinois were lower compared to those in the samples from Kansas, Minnesota, and Texas. Forage samples from Kansas and Illinois clustered together while the samples from the other locations also clustered together (Figure 1d). From the PCA of forage, we also saw some separation of Kansas and Illinois samples from those of the other locations along PC1, which accounted for 23% of the total variance.

Multivariate Analysis: Effect of Genotypes. To investigate the effect of genotype, metabolomics data derived from grain and forage samples collected from each location were subjected to unsupervised PCA separately to minimize any environmental effect. Figure 2 (panels a and b) shows PCA score plots from grain and forage using samples originating from Illinois. No separation was observed among grain samples with different genotypes. Grain samples with different genotypes were mixed and scattered along PC1 and PC2, which together accounted for about 40% of the total variance. Similarly, PCA of forage samples did not reveal any differences based on genotype. Reference samples were clustered together in the middle of the score plot, as expected. Similarly, we did not observe any differences between different genotypes using grain and forage samples from the other five locations (data not shown).

To further evaluate the effect of the genotype, we subjected the same grain and forage metabolomics data generated from the Illinois samples to HCA. Figure 2 (panels c and d) shows heat maps and dendrograms for grain and forage samples, respectively. Although PCA could not differentiate one genotype from any other, HCA allowed us to see how similar

Table 5. Percentage of Metabolites with Relative Abundances Statistically Highly Significant (p Values < 0.01 after Bonferroni Correction) between Locations in (a) Grain and (b) Forage Samples

(a)

	Illinois	Kansas	Minnesota	Nebraska	Ontario	Texas
Illinois	0					
Kansas	51.92	0				
Minnesota	58.33	44.23	0			
Nebraska	44.87	63.46	50.64	0		
Ontario	55.77	56.41	45.51	46.15	0	
Texas	57.05	47.44	45.51	50.00	44.87	0

(b)

	Illinois	Kansas	Minnesota	Nebraska	Ontario	Texas
Illinois	0					
Kansas	59.46	0				
Minnesota	54.05	65.41	0			
Nebraska	44.32	62.16	49.73	0		
Ontario	44.86	69.19	50.81	45.41	0	
Texas	48.65	55.68	52.97	44.32	47.03	0

metabolomes were among the genotypes. The metabolomic profiles of grain and forage samples from different genotypes did not show any significant differences, as seen in the heat map. HCA of samples from the remaining five locations (results not shown) did not reveal any genotype effect. These results support our conclusion that the environment and not genotype has the biggest impact on the metabolome of both grain and forage maize samples.

Effect of Genotype Times Environment Interaction (G × E). To investigate the genotype and environment interaction and understand the contribution of each, we considered metabolomics data derived from grain and forage samples with five different genotypes grown at all locations. We first calculated and compared the mean RSD for all metabolites for each genotype at every location using grain samples as shown in Table S4 of the Supporting Information. We then calculated the average of each of the five genotypes that were grown in all six locations and subjected the data to HCA. We reasoned that if the environment had a strong effect, we would expect to see samples from the same location clustered together

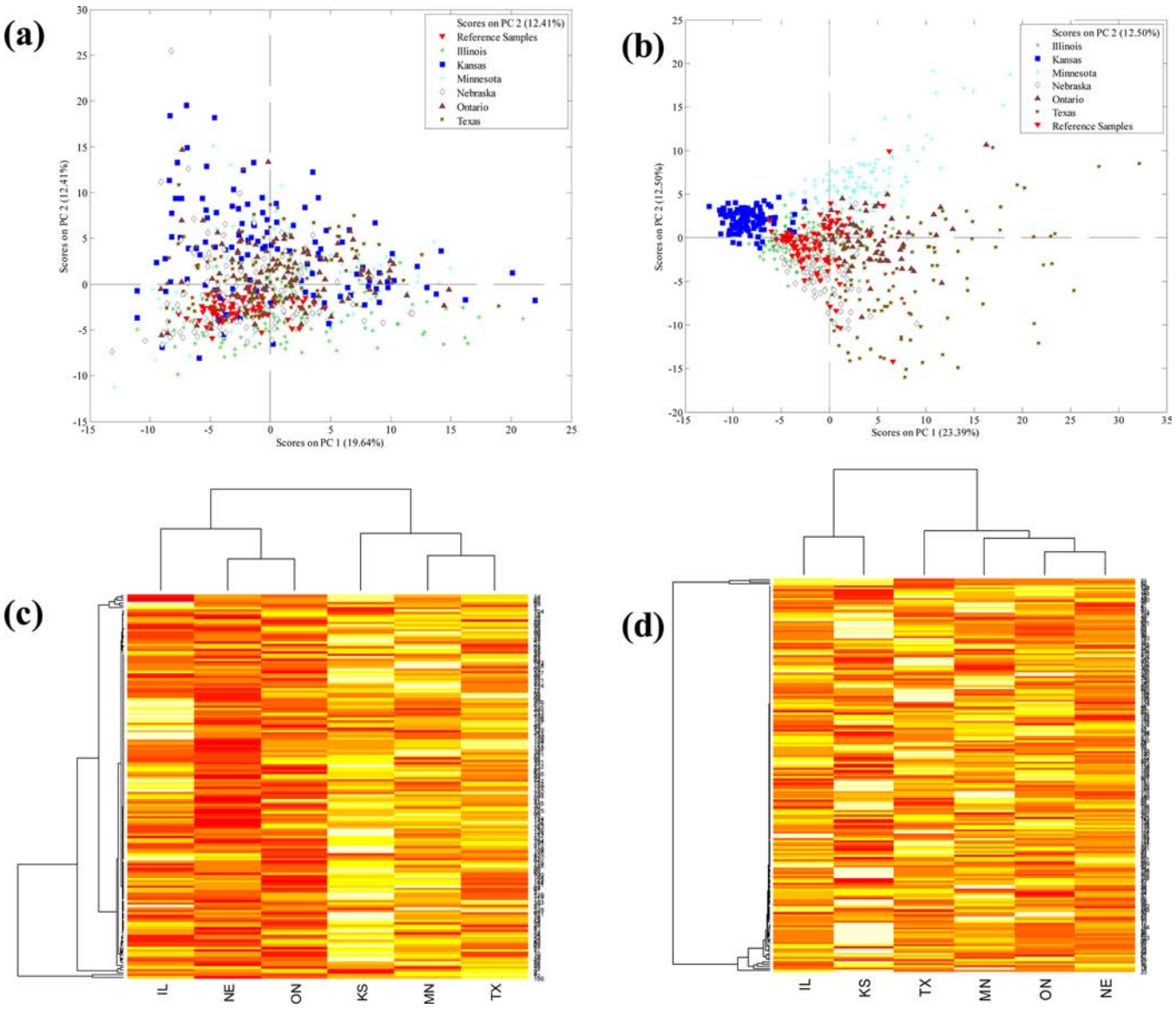

Figure 1. PCA score plots showing the effect of location on the metabolome of (a) grain and (b) forage samples. HCA and heat maps showing the effect of location on the metabolome of (c) grain and (d) forage samples.

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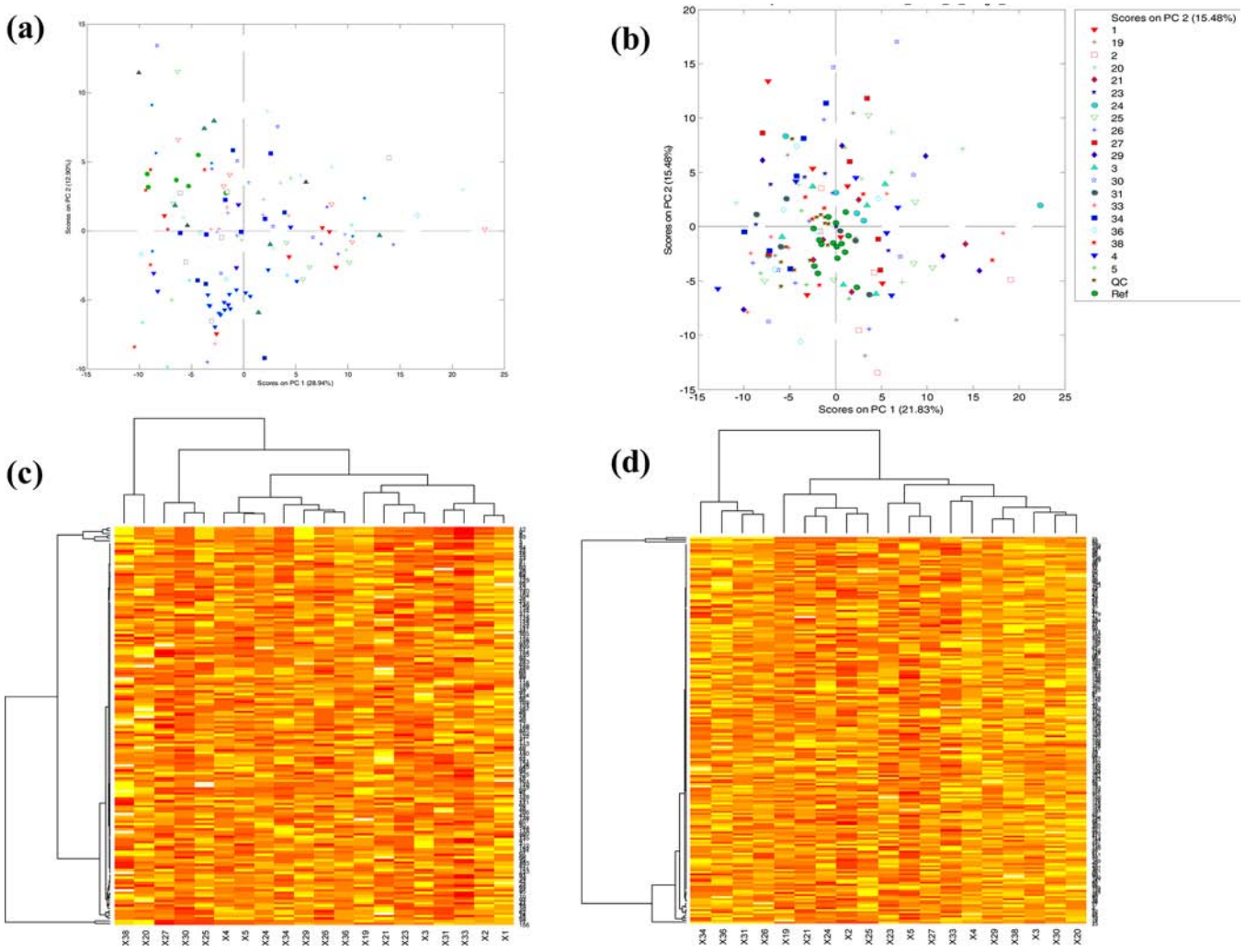

Figure 2. PCA score plots showing the effect of genotype on the metabolome of (a) grain and (b) forage samples from Illinois. HCA and heat maps showing the effect of genotype on the metabolome of (c) grain and (d) forage samples from Illinois.

regardless of genotype, and if the genotype had a strong effect, we would expect samples from the same genetic background to cluster together regardless of where they were grown. Figure 3 (panels a and b) shows dendrograms from grain and forage samples, respectively. The HCA of grain samples did not show any strong clustering of samples based on the where they were grown or their genotype. However, the HCA of forage samples revealed a strong environmental effect, especially for samples from Texas, Illinois, and Kansas. To further investigate the effect of the environment and genotype, the same data set was also subjected to unsupervised PCA. Figure S1 (panels a and c) of the Supporting Information shows the PCA score plot from the grain and forage sample, respectively, showing the effect of the environment. The first two principal components explained only 32% of the total variance in grain compared to 50% in forage samples. Forage samples from the same location were tightly clustered compared to grain samples, which is similar to what we observed using all fifty genotypes. From the PCA score plot shown in Figure S1 (panels b and d) of the Supporting Information from grain and forage samples, respectively, samples with different genotypes sourced from different locations were mixed together and thus processed metabolomes not specific to any one location.

To further investigate the effect of the genotype and environment on the metabolome of grain, samples collected from different locations were subjected to supervised PLSDA. The PLSDA score plot generated from the grain data from six different locations shows samples tightly clustered based on where they were grown (Figure 4a). Samples were clustered based on where plants were grown. Metabolites most responsible for driving the separation were identified based on the loadings and variable importance in the projection (VIP) scores. Figure 4b shows the PLSDA score plots for all genotypes. Similarly, those metabolites accounting for the separation were identified based on their loadings and VIP scores. Even with application of the supervised method, we still could not distinguish one genotype from another. This result also shows that the environment and not the genotype had the strongest effect on grain and forage metabolome. Table S5a of the Supporting Information shows the top VIPs with the highest scores based on the same cutoff for the genotype plotted against those with the highest scores for the environment. Metabolites along the *y* axis were more sensitive to different genotypes, while those along the *x* axis were more sensitive to different environments.

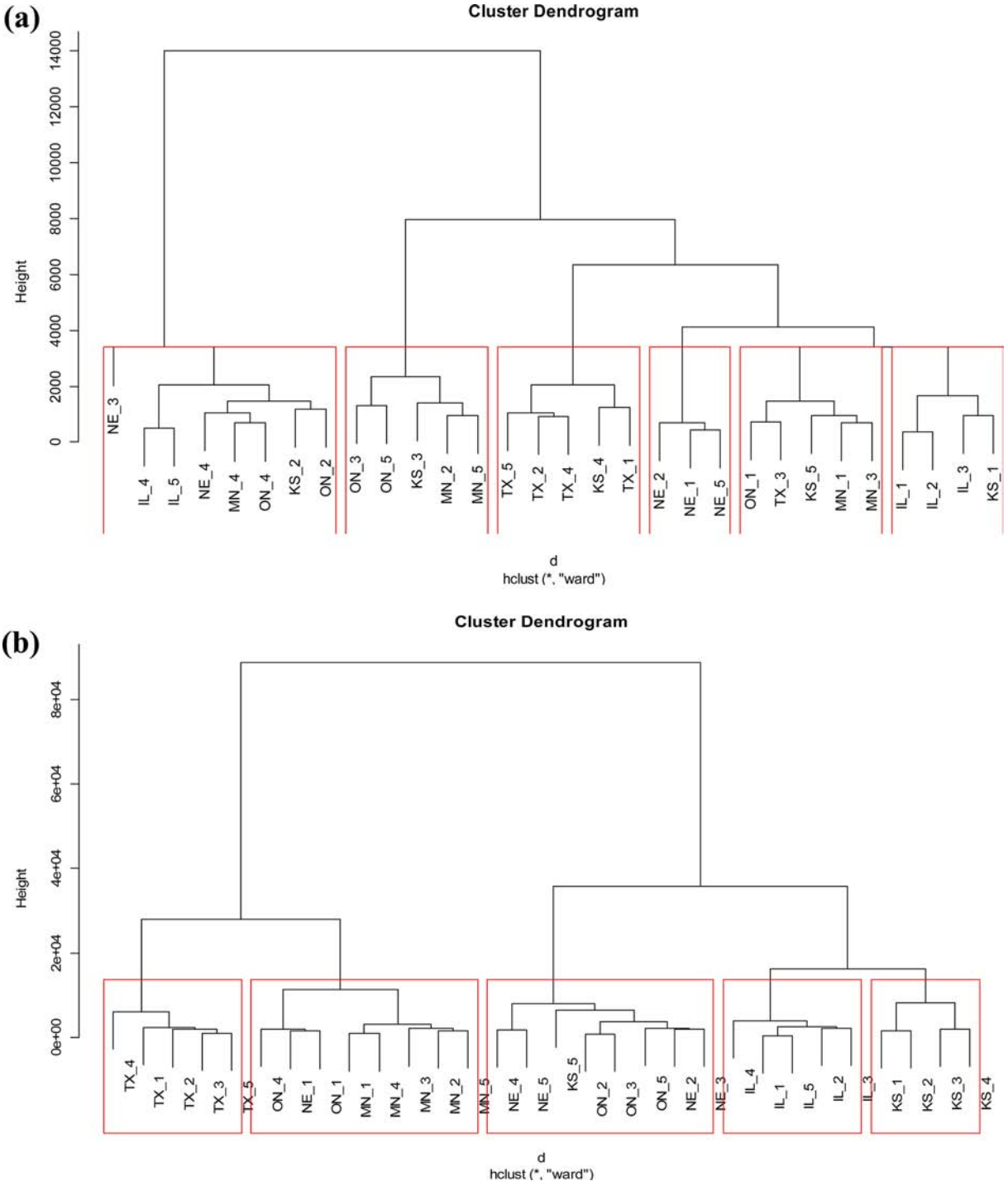

Figure 3. HCA on the metabolome of (a) grain and (b) forage samples of five genotypes (1−5) grown at six locations.

DISCUSSION

Various metabolomic studies have reported relative standard deviation (RSD) as a measure of data variability, reproducibility, and normalization.[37,38] In our study, we used RSD as a means to quantify data quality, variability, and reproducibility for both the grain and forage, as influenced by different environments and genotypes. We see more variability in grain samples compared to forage samples regardless of where the samples were grown or their genotype. The higher variability of many metabolites in grain is consistent with previous studies that have attributed this to the presence of very low abundant small metabolites (∼2−5% of grain biomass) in grain samples.[26,29] The levels of metabolites present in grain have been shown to vary widely based on the level of major macromolecular nutrients such as starch, protein, fat, and fiber.[26,29,39] We also found that the concentrations of most metabolites in grain were lower compared to those in forage from samples from the same plant. Furthermore, evaluation of the mean RSD for metabolites from grain and forage samples obtained from different blocks or field replicates in every location showed that our data was very reproducible. We used pooled QC samples to compare reproducibility within the same batch and the reference samples to compare the reproducibility across different batches.

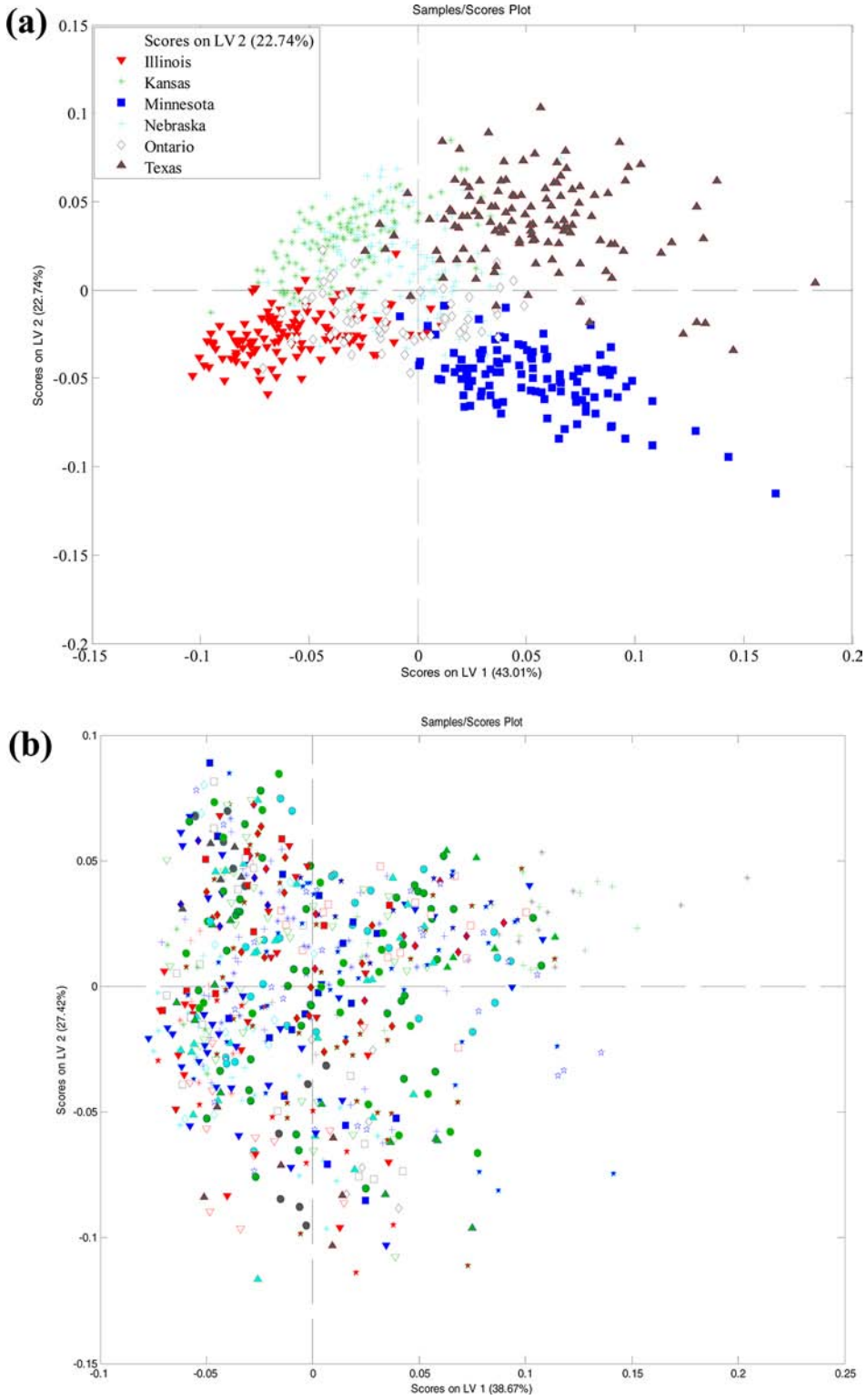

Figure 4. PLSDA score plots of the metabolome of grain samples showing effect of (a) location and (b) genotype.

From the results we also obtained very good reproducibility within and across different batches. This finding also indicated that our analytical and data processing techniques were stable over time.

A number of metabolomic or metabolite profiling studies have been conducted to investigate the effect of genetic modification and environment on maize grain, but to date there are no reports that have compared the effect on both the grain and forage samples using a diverse range of non-GM hybrids. We have shown that the environment had the highest impact on the relative amounts of metabolites in both grain and forage. The results of our study using grain samples are consistent with previous work. Frank et al. also observed that the environment has a greater effect on the metabolite profiles than either Bt or Roundup Ready genetic modifications.[22] From our univariate analysis, results comparing the relative amounts of metabolites

from one location to another, about 50% of the metabolites detected in both the grain and forage samples were affected by the environment compared to less than 2% of the metabolites affected by different genotypes. Although we did not compare our non-GM samples with a GM counterpart, a previous study revealed that the relative amount of 3 and 4% of the total peaks detected were different between GM and non-GM maize grown in Germany and South Africa, respectively.[24] There are other studies that have compared the natural variability of metabolite in maize grown in different locations and seasons.[28] They showed that the growing season was the most prominent factor in driving variation of the metabolite pool.

In conclusion, to further understand and validate the natural metabolome variability coverage contributed by environments and genotypes, additional analyses should be done on non-GM crop samples from more geographical locations, multiple years, and different growing seasons before comparison can be made between non-GM and GM counterparts. If a substantial equivalence assessment of transgenic lines is supplemented using metabolomics, it is best achieved with multivariate statistics with a focus on comparing samples (genotypes) of transgenic lines with their nontransgenic counterparts. It is imperative to differentiate between environmental and genotypic effects in respect to any qualitative and quantitative changes of the metabolites. We need to better understand the natural variation solely contributed by the genetics and if the change is biologically meaningful before metabolomics can be used to supplement compositional analysis for GM crop assessment. Metabolomics data sets contain many metabolites or metabolite signatures, and the assessment of individual metabolites using univariate analysis can lead to spurious conclusions, especially in nonvalidated (nonrepeated) experiments. It is for this reason that metabolomics data are best analyzed with multivariate statistical tools instead. We contend that if metabolomics is used to supplement compositional analysis for GM crop assessments by regulatory agencies, such experiments need to be correctly designed, and a proper data analysis approach needs to be selected, allowing for an appropriate interpretation of the results in the context of a safety assessment. Like with any analytical methodology, sample and technical variability associated with metabolomics measurements need to be quantified and ideally controlled with appropriate quality control protocols. This will be the subject of a subsequent study. This withstanding, before metabolomics or metabolite profiling are to be used to supplement compositional analysis for safety assessments of GM crops, it is necessary to understand the effect of the environment and genetic background with non-GM crops in order to place metabolomics data in proper context when applied to GM crops.

■ ASSOCIATED CONTENT

Supporting Information

Tables with detailed genotype distribution in each location and mean RSD from each block and Figure of PCA score plot of five genotypes planted in all six locations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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