

Fatty Acid Profile-Based Chemometrics to Differentiate Metabolic Variations in Sorghum

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ABSTRACT: The extractive content and fatty acid profiles of Della and *REDforGREEN* (RG) sweet Sorghum varieties grown in two different seasons have been evaluated. The stalk internodes and nodes were quantitatively extracted with CH_2Cl_2 . The extracts were converted to their fatty acid methyl ester (FAME) derivatives and analyzed by gas chromatography-mass spectrometry (GCMS). The main fatty acids detected were azelaic (C9:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and eicosanoic acids (C20:1). Fatty acids were considered as chemical descriptors of varieties to evaluate metabolic variations, where principal component analysis (PCA) and linear discriminant analysis (LDA) multivariate analysis methods were applied. LDA allowed discrimination between Della and RG varieties with higher prediction accuracy, suggesting metabolic variations between them. The high predictive power suggests the use of a fatty acid composition as a fingerprint to reveal metabolic variations.

KEYWORDS: sorghum stalks, fatty acids, FAME, principal component analysis, linear discriminant analysis

1. INTRODUCTION

Sorghum is a commonly produced crop used for grain, forage, and bioenergy.^{1,2} Sorghum stalks are an important part of the crop that provide mechanical support to shoot components.³ The cell wall of lignocellulosic materials is mainly composed of structural biopolymer components such as cellulose, hemicellulose, and lignin.⁴ The cell wall compositions may vary considerably between species with respect to cellulose, hemicellulose, and lignin. The mechanical strength and rigidity of the cell wall are mainly attributed to the supramolecular structure between lignin, cellulose, hemicellulose, and proteins.⁵ Moreover, the composition, structure, and interactions of the biopolymers play several functions such as delivering nutrients, stabilizing the cell structure, and creating a protective environment.⁶ Additionally, there are also minor nonstructural components within the cell wall, such as extractives, ashes, and pectin. These components vary with species, tissue, maturity of plants, harvest times, and storage times and are primarily affected by environmental conditions.⁷ In lignocellulosic materials, lipophilic extractives are extracted using organic solvents such as CH_2Cl_2 , ethanol, or acetone. The nonpolar extractives are composed of mainly fatty acids, resin acids, and fatty acid esters.⁸ The variations of extractive content from species to species are the basis of chemotaxonomy.⁶

Breeding of the Sorghum crops has produced varieties that are easily digestible and produce more bioethanol. To serve different end uses, institutions have developed several Sorghum varieties. The *REDforGREEN* (RG) is a bioenergy Sorghum mutant developed by plant breeders⁹ through ethyl methanesulfonate (EMS) mutagenesis of the Della variety. The Sorghum stalks are shown in Figure 1, and RG varieties were



Figure 1. Groups of Sorghum stalks from Della and RG varieties grown over two different seasons.

screened through leaf color variation,⁹ which is apparent in the stalks as well (Figure 1). In relation to color variation, studies in wood^{10,11} show a strong association between color and lipophilic extractive contents. The chemical composition of Sorghum stalks has been used as a useful tool to characterize and differentiate varieties.^{9,12} It has also been reported that EMS-based mutation has significantly changed the fatty acid composition of different seeds.^{13–15} Despite fatty acids of

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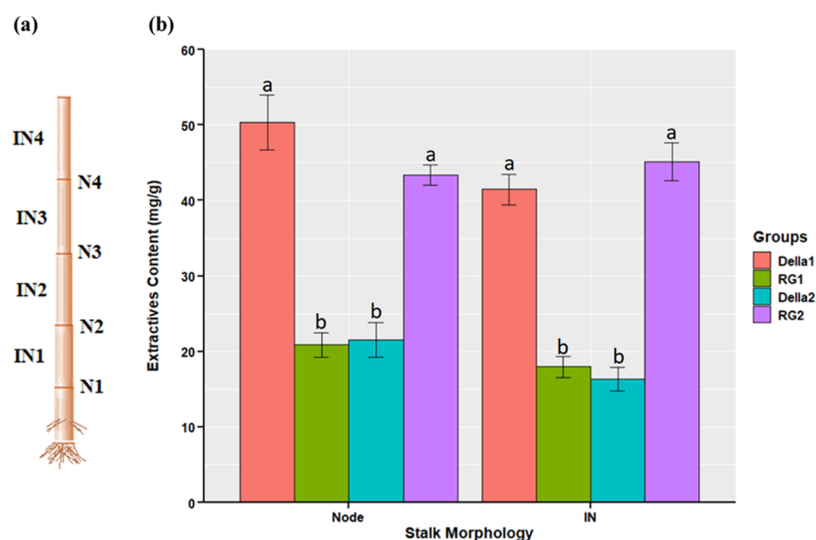


Figure 2. (a) Schematic depiction of Sorghum stalk and (b) average extractive content (mg/g dry biomass) for the Della1, RG1, Della2, and RG2 stalks at the nodes (N1–N4) and internodes (IN1–IN4). The same letters represent no significant difference.

extracts from the stems of Sorghum varieties having been published,^{16,17} the effect of EMS-based mutation on the biosynthesis of fatty acids and the use of fatty acids as a fingerprint to trace metabolic variations in Sorghum stalks are limited. Therefore, analysis of lipid fractions of extracts can be used as an alternative approach to reveal metabolic differences in the two Sorghum lines.

As the composition of the fatty acids of extractives depends on several factors such as variety and environmental factors, the objectives of this study were investigating the extractive content variation due to EMS-based mutation and growing season, identifying and quantifying fatty acid composition of Della and RG Sorghum stalk varieties grown in two different seasons. In addition, fatty acid profile-based chemometrics was applied using principal component and linear discriminant analysis to differentiate metabolic variations among the two lines of Sorghum.

2. MATERIALS AND METHODS

2.1. Plant Materials. Two varieties of sweet Sorghum, Della and RG, were grown in Lexington, KY, in 2018 and 2019 to characterize the ability of nonstructural fatty acid extractives to differentiate between Sorghum stalk features. Della is a common midseason cultivar of sweet Sorghum well adapted for Kentucky growing conditions and displays excellent drought tolerance and disease resistance to anthracnose pathogens.¹⁸ Chemical mutagenesis using ethyl methanesulfonate (EMS) was used to generate the previously characterized Della-derived RG mutant.⁹ Standard cultivation conditions were used to grow either variety to full physiological maturity across both years. Mature stalks were harvested using garden shears by cutting below the first elongated internode. Leaves and leaf sheaths were stripped from individual plants, and stalks were partitioned into subsets of nodes and internodes labeled sequentially starting from the base of a stalk; Figure 2a denotes the labeling convention used. To accumulate enough dry matter for testing, a stalk subsection from four to five individual plants was pooled for each variety in both years and ground using a Thomas–Wiley mill to pass through 1 mm screen. Prior to sample testing, residual moisture contents were determined using an HB43-S Halogen moisture analyzer (Mettler Toledo), and all subsequent analyses were performed across two technical replicates to ensure data reliability.

2.2. Extractive Content Analysis. Ground biomass samples at each node (N) (4.0 g) and internode (IN) (4.0 g) were Soxhlet

extracted using CH_2Cl_2 (150 mL) for 16–18 h, in duplicate, and the extractive content was determined gravimetrically according to ASTM D1108-96.

2.3. Fatty Acid Composition. The lipid extracts (2 mg, in duplicate) were trans-esterified into fatty acid methyl ester (FAME) derivatives by heating in a sealed 5 mL Reacti-vial for 90 min at 90 °C in a mixture of $\text{CH}_3\text{OH}/\text{H}_2\text{SO}_4/\text{CHCl}_3$ (1.7:0.3:2.0 v/v/v, 2 mL) as outlined by Osman et al.¹⁹ CHCl_3 contained 1-naphthaleneacetic acid as an internal standard (200 $\mu\text{g mL}^{-1}$). The FAME derivatives were analyzed by gas chromatography-mass spectrometry (GCMS) (ISQ-Trace1300, ThermoScientific) equipped with a ZB-5 (30 m $\times$ 0.25 mm $\varnothing$, 0.25 μm coating, Phenomenex) capillary column at a temperature gradient of 40 °C (1 min) to 320 °C at 5 °C min^{-1} . The eluted compounds were identified with authentic saturated and unsaturated fatty acid standards along with spectral matching to the NIST-2017 library.

2.8. Multivariate Analysis. To evaluate variation among two variety samples, analysis of variance (ANOVA) was performed using Microsoft Excel 2016 at a 95% confidence level. Pearson correlation, linear discriminant analysis (LDA), and principal component analysis (PCA) were performed using R-4.1.0 software based on 64 observations, 9 variables, 2 varieties (Della and RG), and 4 groups (Della1, RG1, Della2, and RG2). For LDA, samples were divided into training (70%) and test (30%) sets and scaled. Unless stated, all statistical comparisons of RG1 and RG2 were performed from Della1 and Della2, respectively. To maintain the natural variation of the fatty acids along the stalk, the multivariate analysis was performed directly from GCMS-detected concentrations.

2.8.1. Principal Component Analysis (PCA). PCA is a linear transformation method used for dimensional reduction, data visualization, and the exploration of multivariate data. As an unsupervised classification method, PCA is projecting multidimensional data into lower dimensions with a minimal loss of information and producing new orthogonal variables called principal components (PC), which are obtained as linear combinations of the descriptors. Thus, PCA was employed to understand fatty acid compositional variations in Sorghum stalks.

2.8.2. Linear Discriminant Analysis (LDA). LDA is a supervised machine learning technique that uses linear combinations of variables to build a model to classify multivariate data. LDA is intended to determine vectors that produce the maximum separation between classes by a projection of points from an original space.²⁰ Therefore, LDA was applied mainly for discrimination of fatty acid compositions for groups based on a combination of variety and growing seasons (Della1, RG1, Della2, and RG2).

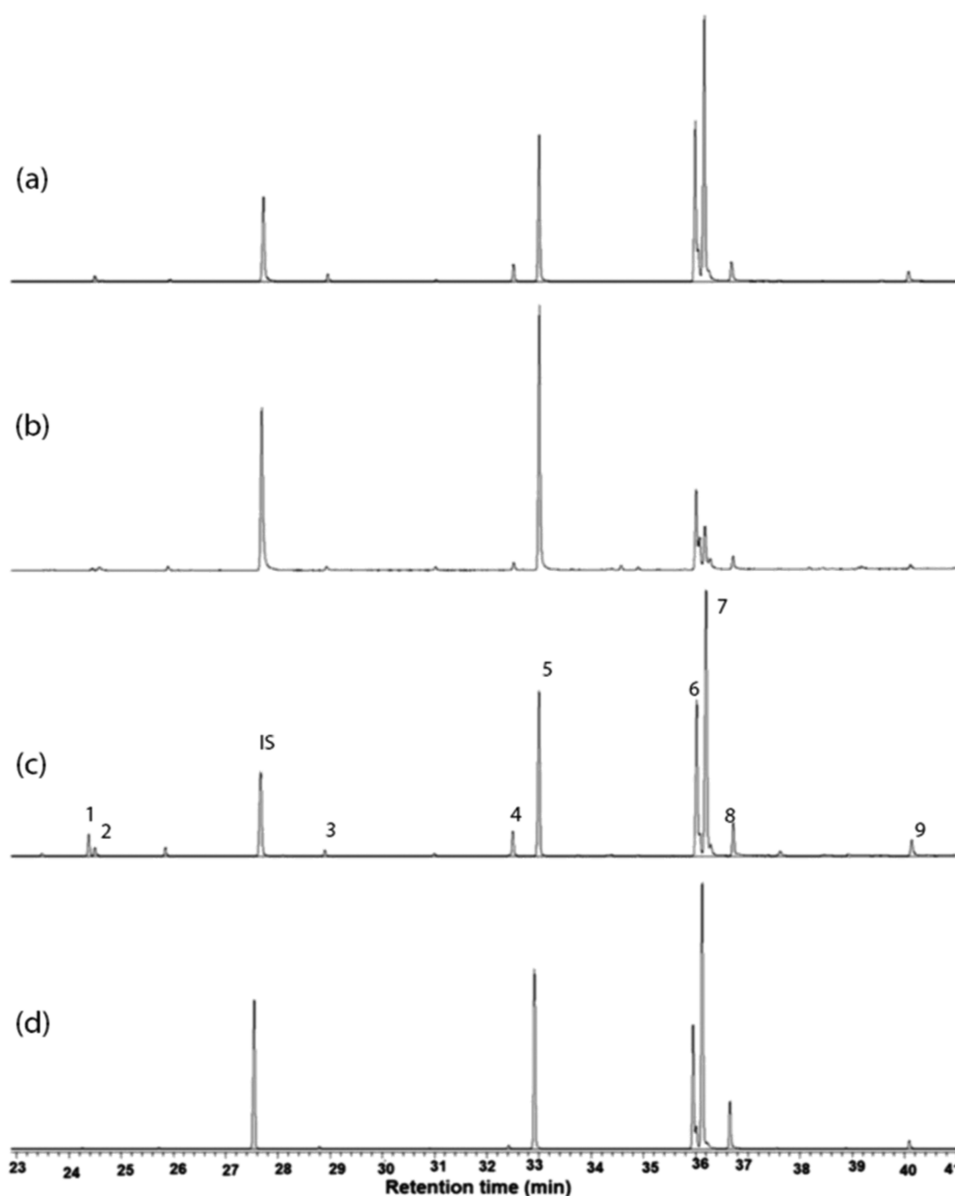


Figure 3. Representative of GCMS chromatograms of fatty acid methyl esters from IN3 of (a) Della2, (b) RG2, (c) Della1, and (d) RG1 Sorghum stalks. IS: internal standard, 1: lauric acid (C12:0), 2: azelaic acid (C9:0), 3: myristic acid (C14:0), 4: palmitoleic acid (C16:1), 5: palmitic acid (C16:0), 6: linoleic acid (C18:2), 7: oleic acid (C18:1), 8: stearic acid (C18:0), and 9: eicosanoic acid (C20:0).

3. RESULTS AND DISCUSSION

The RG and Della Sorghum stalk N and IN samples (Figure 2a) were characterized for extractives and their lipid contents to observe differences between varieties and tissue types.

3.1. Extractive Yields. Extractives are nonstructural components such as lipids, tannins, waxes, and aromatics.⁷ The CH₂Cl₂ extractive yields were Della1 (38.0–61.3 mg/g), RG1 (13.6–27.9 mg/g), Della2 (11.8–27.9 mg/g), and RG2 (39.3–53.6 mg/g). Sorghum stalk INs and Ns are represented in Figure 2a. The ANOVA test showed that the extractive content variation between INs and Ns was not significant in all varieties. The extractive content of each internode (IN1–IN4) and node (N1–N4) is given in Supporting Figure S1. On the other hand, Della1 has significantly more extractives than both RG1 and Della2, whereas Della2 and RG1 have similar contents (Figure 2b). The results also revealed that the extractive content of RG2 is (46–310%) more than Della2 but

RG1 has (36–70%) less than Della1. Across the growing seasons, notable variations have also been recorded; Della1 has 36–200% more than Della2, while RG1 has 40–63% fewer extractives than RG2. Combined analysis of variance for the entire stalk showed no significant difference between extractive contents of Della2 and RG1. Extractive content inhomogeneity of the stalks may suggest metabolic variations in the varieties.²¹ The variation of extractives in the same growing season reveals the effect of EMS mutation; on the other hand, variation across growing seasons demonstrates the effect of season of growth on extractives, verified by literature.²² Even though extractives constitute a minor fraction of the cell wall composition, they may have a profound role in determining the surface property of the cell walls.²³ Studies showed that extractives provide diffusion resistance toward the pith through blocking of miniature passages in cell walls and have antifungal and antioxidant properties to protect the cell wall against fungi and insects.²⁴ These functions, combined with the smaller variation

Table 1. Fatty Acid Concentrations of DCM Extracts from Della1, RG1, Della2, and RG2 Averaged at Nodes (N1–N4) and Internodes (IN1–IN4) in (mg/g of Extract) Determined by GCMS as FAME Derivatives^a

fatty acid	M ⁺ (m/z)	RT (min)	group	internode (n = 8)	node (n = 8)
lauric acid	214	24.2	Della1	6.1 ± 1.4 ^a	6.9 ± 2.3 ^a
			RG1	0.4 ± 0.0 ^a	1.0 ± 0.1 ^a
			Della2	20.0 ± 6.0 ^b	3.0 ± 1.0 ^a
			RG2	0.4 ± 0.1 ^a	5.6 ± 2.6 ^a
azelaic acid	216	24.3	Della1	3.3 ± 0.4 ^a	4.1 ± 0.5 ^a
			RG1	0.6 ± 0.1 ^{bc}	1.2 ± 0.2 ^{bc}
			Della2	0.8 ± 0.3 ^{bc}	0.1 ± 0.0 ^c
			RG2	0.7 ± 0.2 ^{bc}	1.6 ± 0.4 ^b
myristic acid	242	28.7	Della1	1.7 ± 0.2 ^c	2.2 ± 0.2 ^c
			RG1	1.3 ± 0.0 ^c	0.9 ± 0.1 ^c
			Della2	9.0 ± 1.1 ^a	7.2 ± 1.4 ^{ab}
			RG2	0.9 ± 0.1 ^c	4.1 ± 1.1 ^{bc}
palmitoleic acid	268	32.4	Della1	3.6 ± 0.4 ^{cd}	9.4 ± 1.5 ^b
			RG1	4.7 ± 0.6 ^{bcd}	3.8 ± 0.4 ^{cd}
			Della2	15.2 ± 1.5 ^a	8.9 ± 0.8 ^b
			RG2	1.6 ± 0.1 ^d	8.1 ± 2.0 ^{bc}
palmitic acid	270	32.8	Della1	54.4 ± 2.3 ^c	72.0 ± 6.8 ^c
			RG1	62.8 ± 0.5 ^c	81.9 ± 8.6 ^{abc}
			Della2	112.2 ± 9.4 ^a	108.7 ± 10.9 ^{ab}
			RG2	73.6 ± 2.8 ^{bc}	78.7 ± 1.4 ^{abc}
linoleic acid	294	34.7	Della1	91.5 ± 7.5 ^{cd}	154.7 ± 15.7 ^{ab}
			RG1	48.7 ± 1.0 ^d	51.1 ± 8.6 ^d
			Della2	143.5 ± 7.1 ^{ab}	172.6 ± 15.8 ^a
			RG2	93.5 ± 4.5 ^{cd}	120.4 ± 16.9 ^{bc}
oleic acid	296	36.1	Della1	151.4 ± 5.7 ^c	250.7 ± 19.5 ^{bc}
			RG1	162.6 ± 1.2 ^c	176.1 ± 23.1 ^c
			Della2	404.3 ± 27.1 ^a	423.1 ± 55.6 ^a
			RG2	270.7 ± 18.6 ^{bc}	335.4 ± 43.7 ^{ab}
stearic acid	298	36.6	Della1	10.3 ± 0.7 ^c	14.9 ± 1.3 ^{abc}
			RG1	21.3 ± 0.5 ^{bc}	13.9 ± 1.7 ^{bc}
			Della2	23.6 ± 3.0 ^{ab}	26.5 ± 3.2 ^a
			RG2	18.0 ± 1.5 ^{ab}	22.7 ± 3.9 ^{ab}
eicosanoic acid	326	40.0	Della1	9.2 ± 0.8 ^{bc}	10.4 ± 1.3 ^{bc}
			RG1	5.4 ± 0.3 ^c	4.1 ± 0.7 ^c
			Della2	19.2 ± 2.2 ^a	15.5 ± 4.2 ^{ab}
			RG2	12.4 ± 1.0 ^{abc}	8.5 ± 1.6 ^{bc}

^aDifferent letters represent significant differences of mean for each fatty acid at nodes and internodes for the varieties according to Tukey HSD test from eight number of samples.

and distribution of extractives within the stalk, may result in unique stalk behavior by defining stalk–environmental interaction and indicate disparity in cell wall chemistry.²³

3.2. Fatty Acid Composition. Fatty acid profiles of Sorghum stalk tissue extracts were determined as FAME derivatives from stalk node and internode subsections; a representative chromatogram of a sample at IN3 is given in Figure 3. Retention times and detailed composition of the fatty acids at each node (N1–N4) and internode (IN1–IN4) for the two varieties in two different growing seasons are given in Supporting Table S1, and the averaged results are summarized and shown in Table 1. The main fatty acids identified were oleic (C18:1), linoleic (C18:2), and palmitic acids (C16:0). Trace amounts of azelaic (C9:0), lauric (C12:0), myristic (C14:0), palmitoleic (C16:1), stearic (C18:0), and eicosanoic (C20:0) acids were also detected. Azelaic acid is a di-acid.

The results were consistent with the literature;¹⁷ similar fatty acids were also detected in two different lines of Sorghum grains.²⁵ It is found that the axial concentration variation of fatty acids within the pool of stalks between the N and IN was

not significant except for palmitoleic and linoleic acid. For palmitoleic acid, significant differences between N and IN were detected for Della1, Della2, and RG2. Similarly, linoleic acid variation across N and IN was significant for Della1. It is shown (Table 1) that fatty acids varied remarkably across varieties and growing seasons (Table 1), which could be an indication of metabolic variations.

It is worth mentioning that oleic acid varied in concentration in Della1 (139–295 mg g^{−1} of extract), RG1 (104–225 mg g^{−1} of extract), Della2 (298–537 mg g^{−1} of extract), and RG2 (233–449 mg g^{−1} of extract). Oleic acid is found in abundance in Sorghum grains.²⁵ Across the varieties, overall fatty acid concentration variations could be associated with seasonal effects on the fatty acid composition²⁶ and/or the impact of the mutation on lipid biosynthesis.^{13,27}

For better visualization of the fatty acid compositional variation between N and IN within the same variety, axial variation along the stalk and across the two varieties, mirror plots are given in Figure 4. It is shown that significant fatty acid compositional variation between Della and RG varieties within

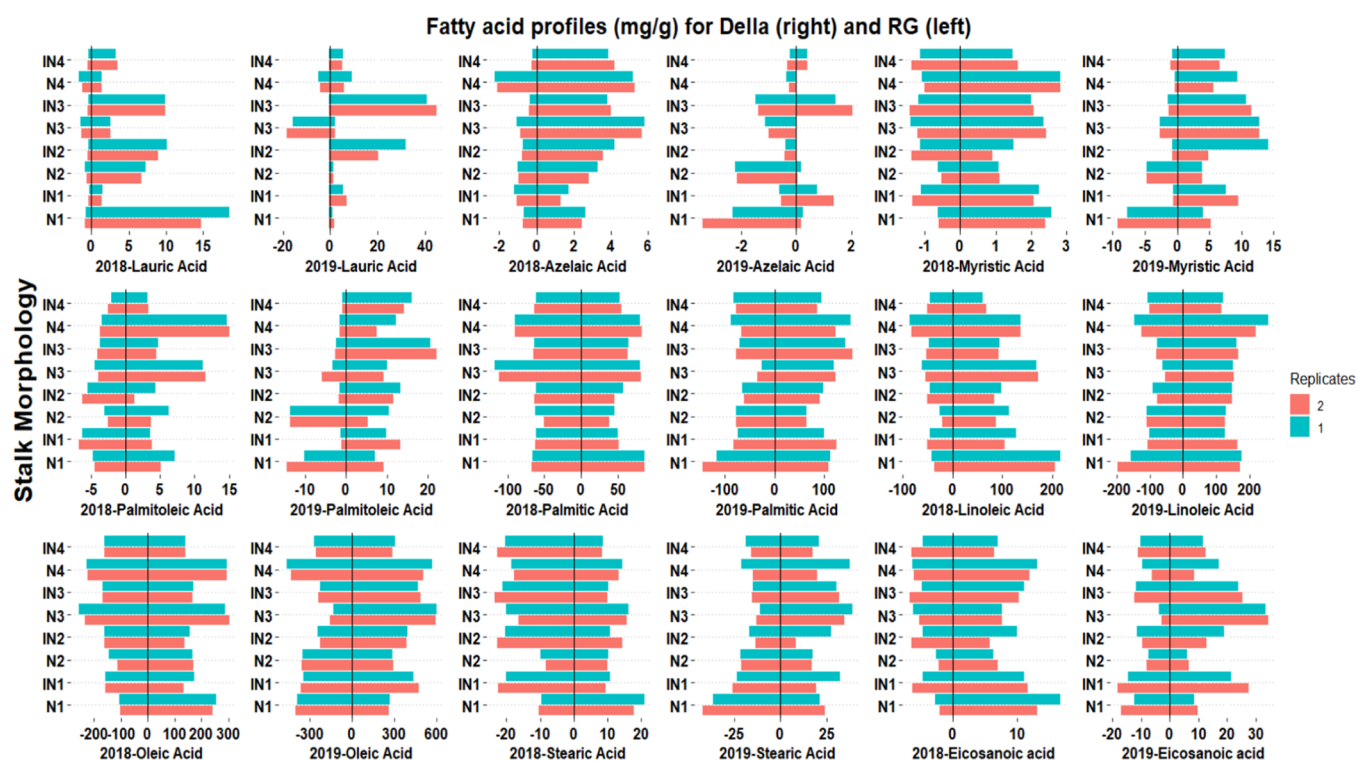


Figure 4. Mirror plots of fatty acid profiles for Della (right) and RG (left) varieties in mg/g at different internodes (IN) and nodes (N) across two growing seasons (2018 and 2019).

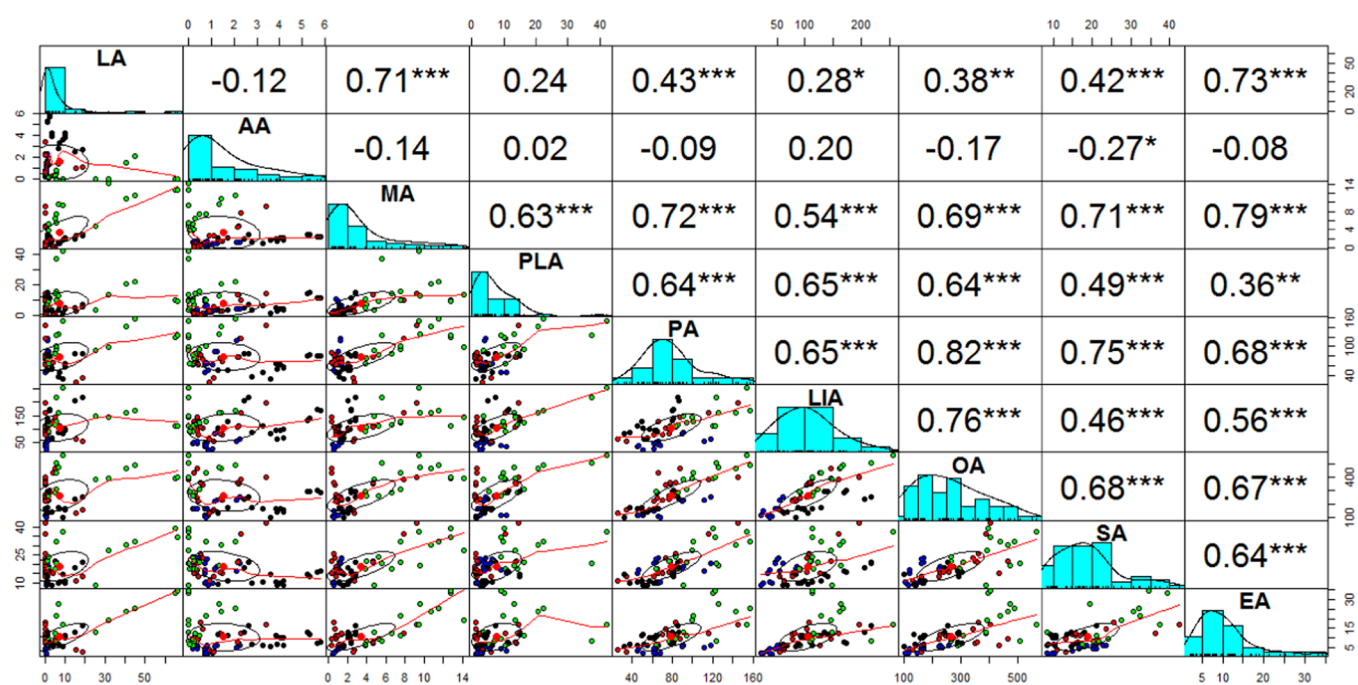


Figure 5. Scatter plots and Pearson correlation of fatty acid profiles from Sorghum stalks. LA: lauric acid, AA: azelaic acid, MA: myristic acid, PLA: palmitoleic acid, LIA: linoleic acid, OA: oleic acid, SA: stearic acid, and EA: eicosanoic acid. The symbols *, **, and *** correspond to significances at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

the same and different growing seasons have been detected. The C9:0 had significantly increased for Della in 2018, and Della varieties had more C14:0 and C18:2 than RG. The fatty acid compositional variation may be the metabolic differences between the two varieties and the impact of the growing seasons.

To understand the correlation of fatty acid accumulations in stalks, Pearson correlational analysis was conducted, and the result is shown in Figure 5. The Pearson correlation coefficients between fatty acids showed that there was better positive correlation between palmitic and oleic acids ($r = 0.82$, $p < 0.001$), myristic and eicosanoic acids ($r = 0.79$, $p < 0.001$), palmitic and stearic acids ($r = 0.75$, $p < 0.001$), and oleic acid

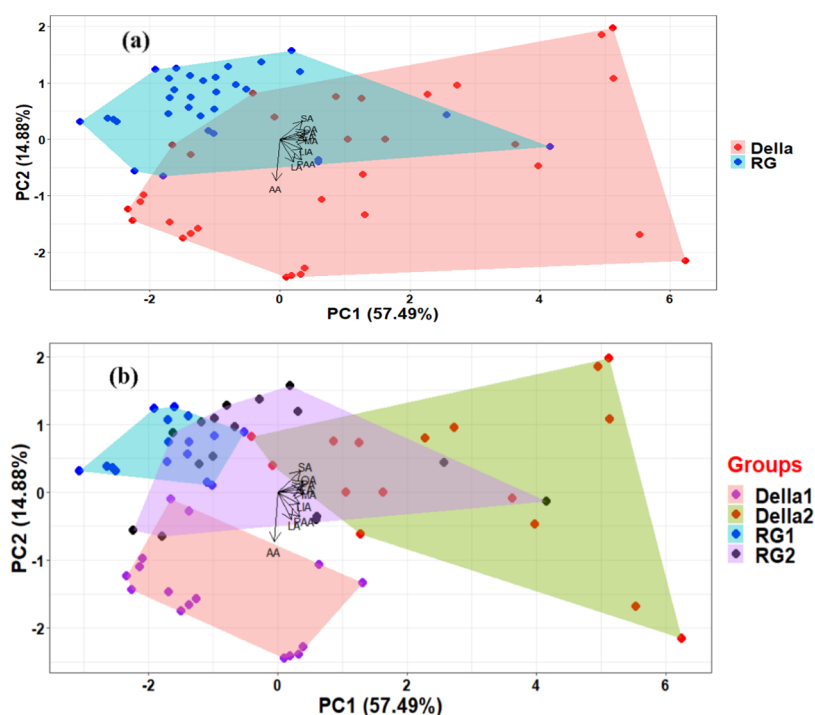


Figure 6. Principal components (a) based on variety and (b) based on groups.

and linoleic acids ($r = 0.76$, $p < 0.001$), which reveals the increasing of one fatty acid with the other. Detailed correlation coefficient values are shown in Figure 4, and the majority of the fatty acid concentrations are positively correlated. However, no correlation was observed between azelaic and all other fatty acids, except with stearic acid, as shown in Figure 5.

3.2.1. Principal Component Analysis (PCA). PCA was applied to the data matrix generated from GCMS of extracts from nodes (N1–N4) and internodes (IN1–IN4) in duplicates for Della1, RG1, Della2, and RG2 with a total of 64 observations with nine fatty acids (descriptors) and classed based on variety and groups (Figure 6). The analysis based on variety (Figure 6a, Della and RG) and groups (Figure 6b, Della1, RG1, Della2, RG2) showed that about 57.5 and 14.9% of the total metabolic variation was explained by the first and second principal components (PC1 and PC2), respectively, and 81.6% was explained by the first three components. The score plot obtained from eigenanalysis of the covariance matrix of scaled data for the first two principal components demonstrates the data distribution of the two Sorghum varieties along the two axes.

It is shown in Figure 6a that the majority of RG varieties are contributing toward positive PC2 and negative PC1, which indicates the possibility of discriminating Sorghum varieties based on their fatty acid compositions (Table 2).

3.2.2. Linear Discriminant Analysis (LDA). The LDA model was developed using the training set consisting of 45 samples, while 19 samples were used for validating the predictive properties of the model. The biplot of the LDA model developed from a scaled data set of fatty acid composition of Sorghum varieties is shown in Figure 7. It is shown that the model classified the groups with LD1 of about 64.4% and LD2 of 31.5%. The average accuracy for the prediction was found to be 97.6%. The high predictive power for the classification suggests the possibility of cultivar classification based on fatty

Table 2. Eigenanalysis of the Correlation Matrix Loadings of Principal Components (PC1–PC4)^a

fatty acid	PC1	PC2	PC3	PC4
LA	0.21	−0.41	0.74	−0.25
AA	−0.06	−0.73	−0.49	0.09
MA	0.40	−0.02	0.18	0.23
PAA	0.33	−0.36	0.07	0.56
PA	0.39	0.10	−0.14	0.11
LIA	0.34	−0.17	−0.32	−0.54
OA	0.39	0.16	−0.16	−0.25
SA	0.35	0.32	−0.15	0.35
EA	0.37	0.04	−0.01	−0.27

^aLA: lauric acid, AA: azelaic acid, MA: myristic acid, PLA: palmitoleic acid, LIA: linoleic acid, OA: oleic acid, SA: stearic acid, and EA: eicosanoic acid.

acid profiles, which can be used to reveal metabolic variations in the stems. The LDA, besides corroborating the fatty acid compositional variation of each group, also indicates the possibility of classifying sorghum stalks by fatty acid-based chemometrics. Findings showed the use of LDA of fatty acid composition as a fingerprint to identify among different cultivars.²⁸ The classification of groups with higher accuracy based on their fatty acid composition confirms the significant fatty acid profile variation between the varieties due to growing seasons and mutational effect, which ultimately influences the metabolic activities. Besides, the result also implies that the color variation on stalks might associate with the variation in extractives and their corresponding fatty acids. LDA has been applied to determine the geographical origin of hazelnuts based on their fatty acid compositions.²⁹ Fatty acids-based discrimination of the varieties also indicates the possibility of applying compositional chemometrics for evaluating metabolic variations.

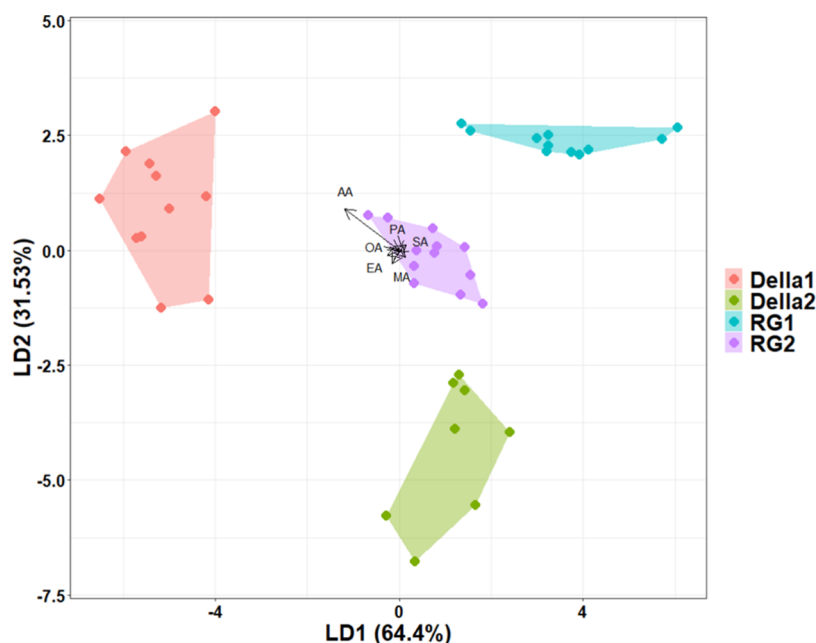


Figure 7. Biplot of linear discriminant analysis for the fatty acid composition from Della1, RG1, Della2, and RG2 stalks. AA: azelaic acid, MA: mristic acid, PLA: palmitoleic acid, LIA: linoleic acid, SA: stearic acid, and EA: eicosanoic acid.

In summary, the results suggest that the analytical GC of fatty acid data combined with chemometric approaches can be employed to provide information on the anticipated metabolic differences among the sorghum lines. The higher prediction accuracy of the LDA model also corroborates that those fatty acids can be used as a fingerprint and descriptors to reveal metabolic changes, which could be associated with seasonal change and the impact of EMS-induced mutation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsfoodscitech.1c00320>.

Extractive content (mg/g of dry sample) (Figure S1) and Fatty acid concentrations of DCM extracts (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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