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Nontargeted lipidomics of sorghum grain reveals novel fatty acid esters of hydroxy fatty acids and cultivar differences in lipid profiles

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Abstract

Sorghum, a globally grown gluten-free cereal, is used mainly as animal feed in developed countries regardless of its potential for human consumption. In this study, we utilized non-targeted lipidomics to thoroughly analyze, compare, and characterize whole-grain lipids in six sorghum cultivars grown in a single field trial in Australia: Buster, Bazley, Cracker, Liberty, MR43, and Tiger. In total 194 lipid molecular species representing five major lipid classes were identified. Multivariate analysis unveiled distinct lipid profiles among the cultivars. The most distinct lipid profile belonged to cv. MR43. The lower ω-6 to ω-3 ratio and optimal P:S ratio in cv. Bazley reflects this as a valuable source of balanced essential fatty acids in the diet. The novel bioactive lipids known as FAHFAs (fatty acid esters of hydroxy fatty acids) were identified and characterized in sorghum grains. These findings further emphasize the potential of whole-grain sorghum as the basis of new health-promoting food products.

Keywords: Sorghum, lipidomics, fatty acid ester of hydroxy fatty acids, liquid chromatography, mass spectrometry

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a widely consumed cereal staple in various regions across Africa and Asia[1], serving as a dietary cornerstone for over 500 million people in more than 30 countries[2]. Globally, it is the fifth most important cereal crop after wheat, maize, rice, and barley in terms of production and harvested area (https://www.fao.org/faostat/en/). Nigeria leads the world in sorghum production, accounting for ~12% of globally, followed by Sudan and Mexico, both for food and industrial processes (https://fas.usda.gov/home). In addition to its role as a dietary staple, sorghum grain has gained attention because of its potential health benefits. These benefits include protection against chronic health conditions prevalent in Western societies, such as inflammation, hypercholesterolemia, cancer, and type 2 diabetes(T2D)[3–6]. Furthermore, sorghum has emerged as a safe dietary alternative for people with celiac disease, as it lacks the specific peptide sequences present in wheat, barley, and rye which are highly detrimental to these individuals[7].

The value of sorghum in agriculture lies in its exceptional adaptability to challenging environments, making it a resilient crop. It can thrive in both drought-prone conditions and water-logged soils[8]. In addition, sorghum's status as a self-pollinating crop enhances its genetic stability[9]. The unique combination of characteristics of sorghum, ranging from adaptability to genetic stability, makes this crop a remarkable and multifaceted agricultural resource. The composition of sorghum grain is characterized by a subtle balance, with kernels typically comprising 3–6% pericarp, 84–90% endosperm, and 5–10% germ[10]. The pericarp of sorghum exhibits a spectrum of colors, including white, yellow, red, brown, and black. The major molecular components of sorghum grain are carbohydrates (75%), protein (12%), lipids (4%), fiber (3%), and ash (2%)[11]. An important feature that adds a unique nutritional dimension to sorghum grain is its relatively high lipid content, which includes valuable fatty acids like oleic and linoleic acid, compared to grains like rice and wheat[12]. A study highlights the unique potential of various *Sorghum bicolor* seed varieties as a new source of premium quality edible oil, particularly due to their significant content of clinically important saturated and unsaturated fatty acids, including the novel octanedioic (FA 8:0) and azelaic acids (FA 9:0) in some varieties [13]. A detailed study on the fatty acid composition of sorghum

68 revealed that, on average, the cultivars contained 14.8% saturated fatty acids, primarily
69 palmitic acid, 38% monounsaturated fatty acids, mainly oleic acid, and about 44%
70 polyunsaturated fatty acids (PUFAs), chiefly linoleic acid. Moreover, the studies are
71 specific to a particular class of lipids[14] .

72 The unique lipid distribution seen in sorghum kernels plays a major role in
73 determining the nutrient profile of the grain. The findings of Lee et al. highlight the
74 significant impact of sorghum lipids on mammalian health. Their study demonstrated that
75 supplementing high-fat hamster diets with sorghum whole-kernel oil significantly
76 reduced non-high-density lipoprotein (non-HDL) cholesterol levels in both plasma and
77 liver. Additionally, sorghum whole-kernel wax, while not directly affecting cholesterol
78 levels, increased bile acid and neutral sterol excretions, potentially enhancing cholesterol
79 excretion[5]. Another study highlighted the anti-tumor properties of sorghum lipids
80 containing phytosterols and unsaturated fatty acids[15]. Previous studies on sorghum grain
81 lipid composition have focused primarily on specific lipid classes. Desta et al. and
82 Trevino et al. used gas chromatography (GC) with a flame ionization detector (FID) to
83 identify and quantify the lipids present in sorghum. However, these studies were limited
84 to the major fatty acyl classes of lipids, such as palmitic, oleic, stearic, linoleic, and
85 linolenic acids[16,17]. Despite efforts to characterize the sorghum lipidome, investigations
86 of lipids have been limited to the fatty acyl class, and the GC technique used in these
87 studies has limited sensitivity in terms of detection and is time-consuming due to
88 derivatization procedures. Although previous studies have utilized liquid
89 chromatography-mass spectrometry (LC/MS) on sorghum grain extracts, this approach
90 was used primarily to identify phenolic compounds and anthocyanins[18,19].

91 Our approach here was to employ extremely sensitive liquid chromatography
92 combined with linear ion trap–Orbitrap mass spectrometry (LC/LTQ-Orbitrap-MS) to
93 analyze the whole-grain lipidome of six commercial sorghum cultivars grown in a single
94 field trial in Australia. We characterized bioactive lipids, such as fatty acid esters of
95 hydroxy fatty acids (FAHFAs), in these sorghum samples. Our study aimed to provide
96 insights into the variation in grain lipid profiles among sorghum cultivars and their
97 potential impact on health promotion.

2. Materials and methods

2.1 Sample information

Grains of six sorghum cultivars (all commercial hybrids)—Bazley, Buster, Liberty, Cracker, Tiger, and MR43 (mitch-rated)—were subjected to lipidomics analysis. Liberty has a white pericarp, whereas the other sorghums are red. Sorghum plants were grown in a field trial over the 2017-18 season at the University of Sydney's "Nowley" farm, situated in northern New South Wales (NSW), Australia (latitude 31°34'31" S; longitude 150°10'53" E). This enabled valid comparisons of the lipid profiles between genotypes because the environmental conditions (soil type, water availability, light, temperature, length of growing season, etc.) were the same for each genotype. Each cultivar was planted in three to five replicate plots, each measuring 1 × 2 m, in a randomized block design.

Data from the regional network of meteorological stations covering normal weather conditions in the area were used in the trial. The commercially coated seeds were sown in late October 2017 and harvested in March 2018. Following harvest, the grains were stored in sealed bags in a climate-controlled grain storage facility at the I. A. Watson Grain Research Centre (Narrabri, NSW) at 15 ºC and controlled relative humidity. The grains were transported to the main campus of the University of Sydney and stored in sealed plastic containers in a cold room at 4 ºC. The grains were then coarsely ground and immediately placed in sealed plastic containers under nitrogen gas before being shipped to Japan, where the whole-grain flour was kept at -80 ºC before lipid extraction.

LC/MS-grade solvents, including isopropanol, chloroform, and methanol, were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ammonium acetate solution (1 M), which served as an eluent additive for LC/MS, was obtained from Sigma-Aldrich (St. Louis, MO, USA). The EquiSPLASH LIPIDOMIX quantitative standard for mass spectrometry and oleic acid-d9 internal standards were acquired from Avanti Polar Lipids, Inc. (Alabama, AL, USA). N,N dimethylethylenediamine (DMED) was supplied by Sigma-Aldrich (Saint Louis, MO, USA). 2-chloro-1-methylpyridinium iodide (CMPI) and triethylamine (TEA) of analytical grade were obtained from Tokyo Chemical Industries Co., Ltd., (Tokyo, Japan). The reaction reagents, such as CMPI, TEA, and DMED, were prepared in LC-MS grade acetonitrile (ACN) at 20 mM concentration. The

FAHFAs standard 12-OAHOA (oleic acid esters of 12-hydroxy fatty acids) used in this study was synthesized in our laboratory according to the protocol mentioned in our previous study [20].

2.2 Lipid extraction

The modified Bligh-Dyer method was used to extract lipids from sorghum grains[21,22]. Initially, 150 mg of finely ground whole-grain sorghum powder (n=5) was weighed carefully and put into a 2 mL Eppendorf tube. The sorghum kernel structure along with images of powdered sorghum used for this study are illustrated in supplementary **Fig. S1 A and B** respectively. Subsequently, 1.5 mL of methanol was added to the solution, and the mixture was homogenized by using ceramic beads (1.4 mm, catalog no. 15–340-159, Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) for 1 min (two repeated 30 s cycles) using a Bead Mill 4 (Fisherbrand, Tokyo, Japan) homogenizer. A 200 µL aliquot of the homogenate was used for extraction. To this homogenate, 100 µL of MeOH and 100 µL of internal standard (IS) mixture (oleic acid-d9 (100 µg/mL) and EquiSPLASH Lipidomix (10 µg/mL) in methanol) was added. The solution was gently vortexed for 10 s. Then, 200 µL of chloroform and 40 µL of Milli-Q were added to the solution, which was vortexed at 3,500 rpm for 5 min (MSV-3500, Biosan Ltd.Riga, Lativia). The mixture was left at room temperature for 2 h, with an additional vortexing step (3,500 rpm for 1 min) after 1 h. After the 2 h incubation, the solution was vortexed at 3,500 rpm for 1 min and centrifuged at 15,000 rpm for 10 min. The resulting supernatant was carefully transferred to a new 2 mL Eppendorf tube. The residue was treated with 1 mL of methanol and chloroform (2:1 v/v), vortexed for 5 min, and centrifuged for 10 min. The combined extracts were transferred to a new 2 mL Eppendorf tube and subjected to evaporation using a centrifuge evaporator at 4 ºC. In the end, the dried extract was re-dissolved in 100 µL of MeOH, followed by centrifugation at maximum speed for 10 min, and then transferred to LC/MS vials for subsequent analysis with injection volume set at 10 µL for each sample.

2.3 Total lipid content analysis and DMED derivatization of FAHFAs

Total lipid content was determined using the Bligh-Dyer method[21]. First, 1 g of whole-grain flour of sorghum (n=3) was placed in a 15 mL falcon tube. Subsequently, 6 mL of methanol was added, and the mixture was vortexed for 10 s. Chloroform (3 mL)

and 600 µL of Milli-Q water were added, followed by another vortexing step at 3,500 rpm for 5 min. The solution was left at room temperature for 2 h and vortexed for 1 h (3,500 rpm for 1 min). Subsequently, the lipid extract was centrifuged at 15,000 rpm for 10 min and transferred to a new 15 mL Falcon tube. The residue remaining in the initial tube was mixed with methanol and chloroform (2:1 v/v), vortexed for 5 min, and centrifuged for 10 min. The organic layer from the combined extracts was transferred to the same new 15 mL Falcon tube. Crude extracts were obtained by drying overnight in a rotary evaporator. The total extracted lipid content was calculated by weighing the obtained extracts.

DMED derivatization for FAHFAs was performed as per our previous study, for the cv. MR43 (as MR43 exhibits an abundance of FAHFAs (**Table 1**))[23]. Before DMED derivatization, the lipid extracts were redissolved with 500 µL of ACN. 100 µL of sample with ACN was taken and 25µL of standard 12-OAHOA was added to the solution followed by 1 min vortex. Then, 20 µL of TEA with 10 µL of CMPI was added and vortexed for 3 min. Subsequently, 20 µL of DMED was added and vortexed for 30 min. Finally, the mixture was evaporated by using a centrifuge evaporator at 4 ºC. The dried extract was redissolved with 100 µL MeOH and transferred to LC/MS vials for analysis with an injection volume of 20 µL.

2.4 LC/MS analysis

The LC/MS conditions used were the same as those used in previous studies[24]. In brief, the LC system (Shimadzu Corporation, Kyoto, Japan) was used for chromatographic separation at a 200 µL/min flow rate, using an Atlantis T3 C18 column (2.1 × 150 mm, 3 µm, Waters, Milford, MA, USA) maintained at 40 °C oven temperature whereas for derivatized samples the oven temperature was maintained at 45 °C. The mobile phases (Pump A: 10 mM aqueous ammonium acetate; Pump B: isopropanol; Pump C: methanol) and elution gradient conditions were identical to those described in our previous report[20].

LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) was used under positive and negative ionization modes, the MS spectral scan ranges were set at *m/z* 150-1950, and *m/z* 160-1900 respectively. Collision-induced dissociation at 40 V collision energy was used to obtain MS/MS spectra. When processing

the raw data from the Orbitrap MS, MS-DIAL (ver. 4.9) was used to integrate and annotate lipids according to the set parameters reported in our earlier study.[24]. To ensure robust identification of lipid molecular species, both MS and MS/MS spectra were obtained using Xcalibur 2.2 (Thermo Fisher Scientific, Waltham, MA, USA). For the semi-quantification of annotated lipids, the guidelines established by the Lipidomics Standard Initiative (Level 2 or Level 3) were followed. The relative concentration of lipid metabolite was calculated based on the amount of added internal standard. The weight of the sample utilized for lipid extraction was used to normalize the concentration data. This comprehensive approach ensures the reliability and accuracy of lipid data interpretation. The accuracy of the LC/MS method was measured by performing intra-and inter-day assays. The resultant recovery (RE) and relative standard deviation (RSD %) are mentioned in supplementary **Table S1**.

2.5 Statistical analysis

The molecular species of lipids found in both the positive and negative ionization modes were included in the lipidomics dataset that was acquired. Microsoft Excel 2021 was utilized for data visualization, and the outcomes were presented as mean ± standard error of the mean (SEM). To gain a holistic understanding of data variation, MetaboAnalyst (version 5.0) (https://genap.metaboanalyst.ca/) was used to run a sparse partial least squares-discriminant analysis (sPLS-DA) and generate heatmaps. The results for the sPLS-DA plot are validated by the cross-validation method and the plot is illustrated in supplementary **Fig. S2** and the sPLSDA scores are shown in supplementary **Table S2**. These analyses provided a comprehensive overview of the nuances within the dataset, enhancing our ability to discern patterns and variations in molecular lipid species.

3. Results and Discussion

3.1 Determination of total lipid content and multivariate analysis of sorghum lipidome

The percentages of the total whole-grain lipid content of the six sorghum cultivars based on their fresh weight (FW) are represented as bar graphs (**Fig. 1A**). Although no significant variation was observed in the total percentage of lipid composition among the cultivars, Bazley exhibited the highest total lipid content of about 5.64 %, while Buster had the lowest of about 3.22 %. In comparison, a previous study on sorghum reported total lipid contents of 3.7 % based on dry weight (DW) [25]. In our investigation, Buster displayed a total lipid content of 3.22 %, aligning closely with previously reported sorghum values [25]. The other four cultivars, Cracker, Liberty, MR43, and Tiger, had higher total lipid contents of 4.66 %, 5.27 %, 4.92 %, and 4.96 %, respectively. These values surpass the reported total lipid content of sorghum and provide a detailed view of the variations in lipid composition among different sorghum cultivars grown in a single field trial [25]. It is important to note that our samples were stored at -80°C before extraction, and thus the reported lipid content is based on fresh weight rather than dry weight, which may account for the observed differences.

Total lipid content was further classified into five major classes: Fatty acyls (FAs), sphingolipids (SPs), glycerophospholipids (GPs), glycerolipids (GLs), and sterol lipids (STs) (https://lipidmaps.org/). Confirmation of the lipid molecular species was achieved through precise determination of their mass-to-charge ratios (*m/z* values) and retention times (RTs), as depicted in a scatter plot (**Fig. 1B**). This detailed classification and characterization contributes to an understanding of lipid composition across sorghum cultivars, complementing the variations in total lipid content. Further multivariate analysis was performed to differentiate lipids among the cultivars by sPLS-DA via the Metaboanalyst 5.0 web tool. This analysis classified the cultivars based on the first two principal components (PC1 and PC2), which collectively explained 64.7% of the group variance. The six cultivars were separated distinctly whereas repeated samples were gathered together (**Fig. 1C**). Notably, MR43 had a lipidome profile that was distinct from that of the other cultivars. The lipids responsible for the separation in the score plot are

shown in a loading plot (**Fig. 1D).** This loading plot reveals that the FAHFAs are the key differentiators for the observed separation. This divergence suggests potential differences in lipid biosynthesis pathways may contribute to the observed lipid variations.

The common lipid biosynthesis pathway for plants is depicted in **Fig. 2** (based on KEGG pathways (https://www.genome.jp/kegg/pathway.html)). Various enzymatic reactions govern the synthesis of different lipid classes. In brief, from Acetyl coenzyme A (Acetyl Co A), the free fatty acid (FFA) and FAHFAs (FAHFAs biosynthesis pathway is not known till now but believed to be synthesized from FFA) along with ST are synthesized. Acetyl Co A along with glycerol-3-phosphate (G3P) is responsible for the synthesis of lysophosphatidic acid (LPA), phosphatidic acid (PA), and GLs. Further, the PA combines Cytidine diphosphate diacylglycerol (CDP-DAG) to form phosphatidyl glycerol (PG) and lysophosphatidyl glycerol (LPG). Further addition of diacylglycerol (DG) resulted in the formation of other phospholipids belonging to the GP lipid class. Acetyl Co A also forms palmityl Co A which in combination with serine (Ser) amino acid results in the formation of the ceramides belonging to SP lipid classes. Based on our lipidomics results, we may predict the upregulation or downregulation of specific lipid biosynthesis pathways in different sorghum cultivars. For instance, in the cv. Buster and MR43 the FFA and FAHFAs concentration is higher than the other four cultivars. This pattern may indicate an upregulation of FA biosynthesis pathways from Acetyl CoA. Similarly, the concentration of ceramides (Cer) and hexosylceramides (HexCer) belonging to the SP lipid class is low in cv. Buster, Liberty, and Tiger compared to others. This may be due to the downregulation of the SP biosynthesis pathway which is indirectly linked to Acetyl CoA. The supporting information in **Table S3** provides a detailed list of the concentration of 194 lipid molecular species along with quality control (QC) samples to validate sample-to-sample deviation.

3.2 Distribution of lipid classes and fatty acyls composition in six sorghum cultivars

In our untargeted analysis of sorghum cultivars, we identified 43 fatty acids, which were classified into saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs. An SFA (such as palmitic acid FA16:0) has no double bond in its carbon chain, whereas MUFA (such as Oleic acid; FA 18:1) has one double bond mostly

between C9 and C10, and PUFA has more than one double bond. The PUFAs also have two families ω-3 (n-3), where the double bond is located on the C3 from the methyl end (such as linolenic acid; FA 18:3) and ω-6 (n-6) where the double bond is at C6 from the methyl end (such as linoleic acid; FA18:2)[26]. The distributions of SFAs, MUFAs, and PUFAs, are represented in **Fig. 3A**. The MR43 shows a significantly higher amount of SFA, MUFA, and PUFAs compared to other cultivars. This observation is in line with previous research on pigmented sorghum cultivars, which also identified the presence of PUFAs, MUFAs, and SFAs specifically in red sorghum[27]. The P:S ratio for the six cultivars is represented in **Fig. 3B**. This ratio is a crucial parameter for evaluating the nutritional value of dietary foods and their impact on cardiovascular health[28]. Our findings reveal distinct P:S ratios among sorghum cultivars, with cv. Buster exhibited the highest ratio at 2.15, followed by MR43 (1.88), Cracker (1.51), Bazley (1.44), Tiger (1.43), and Liberty with the lowest ratio at 1.36. Notably, a previous study on rats showed that a dietary P:S ratio within the range of 1.0-1.5 was favorable for mitigating the risk of cardiovascular disease (CVD)[28]. Our previous study, which showed a similarly high P:S value in fish and seaweed, further supports the significance of this ratio in evaluating the health impacts of dietary choice[29,30].

The relative abundances of the identified fatty acids, including FAHFAs (novel bioactive lipids of the PUFA sub-class of lipids), ω-6 and ω-3 PUFAs along with the ω-6 to ω-3 ratio are represented in **Fig. 3C and D** respectively. In the heat maps, deep red and blue colors represent high and low levels of the lipid molecular species. The heatmap revealed that primary fatty acids, such as palmitic, oleic, linoleic, and linolenic acids, were consistently present in all six sorghum cultivars, corroborating previously reported results[27]. The percentage distribution of SFAs, MUFAs, PUFAs, including ω-6 and ω-3 PUFAs, and FAHFAs is represented in **Table 1**. The Buster cultivar shows an abundance of oleic acid (OA), ω-6, and ω-3 PUFAs compared to others. These unsaturated fatty acids are known for their antioxidant and anti-inflammatory properties[31]. Specifically, studies have demonstrated that oleic acid can inhibit inflammation, while linoleic acid, an ω-6 fatty acid, can reduce the levels of TNF-α and IL-1β, thereby inhibiting the production of pro-inflammatory factors, reducing microglia activation, and playing a neuroprotective role [32,33]. Additionally, linolenic acid, an ω-3 fatty acid, is known to reduce the risk of

307 cardiovascular disease (CVD) and arrhythmias [34]. These omega-3 fatty acids can serve as
308 anti-cancer and anti-inflammatory agents [35]. Additionally, they improve mental, and
309 immune system health, and offer various benefits for nerves, eyes, bones, and muscles [36].
310 The structural confirmation of linolenic acid by MS spectral analysis is represented in
311 supplementary **Fig S3**. The ω-6 to ω-3 ratio in sorghum cultivars indicates that Bazley
312 has the lowest ratio of approximately 8:1 whereas Buster has the highest of about 10:1
313 ratio. This ratio has a positive relation with increased risk of autoimmune, inflammatory,
314 and allergic diseases. Although the appropriate ratio for human nutrition has not yet been
315 defined studies have shown to maintain a ratio of about 1-4:1 [37]. A detailed discussion
316 about FAHFAs is provided in section 3.4. The lipid profile of Bazley indicates its potential
317 for significant health benefits, making it a valuable component in dietary interventions
318 aimed at improving health outcomes.

319 **3.3 Correlation analysis of other grain lipids profiled in six cultivars of sorghum**

320 The heatmap offers a comprehensive visualization of the concentrations of the
321 major lipid classes, GP, SP, and ST, across the six sorghum cultivars, as shown in **Fig. 4A**,
322 **4B,** and **4C,** respectively. Interestingly, MR43, Bazley, and Cracker exhibited about 60-
323 fold and two-fold higher concentrations of GP and SP respectively, compared to Tiger,
324 Liberty, and Buster. Notably, within the GP major class, phosphatidylinositol (PI) and
325 phosphatidylcholine (PC) were the dominant components in Bazley, together accounting
326 for nearly 90%. However, a previous report showed that lysophosphatidylcholine (LPC)
327 and PC as the predominant components of GPs account for about 80% in sorghum[38].
328 Although our results deviate slightly from those of a previous study regarding GP
329 composition, this discrepancy underscores the diversity in sorghum lipidomes and the
330 potential influence of cultivar-specific factors. The SP major class of lipids especially
331 ceramides are abundant in Bazley, MR43, and Cracker compared to the other three
332 cultivars. The studies related to these SP classes (in plants termed phyto-ceramides) are
333 limited to sorghum. However, a recent study gives evidence of the presence of rare phyto-
334 ceramides in abundant food plant residues like wheat germs, ground coffee beans, and so
335 on. These ceramides are major components of skin and play an important role in
336 permeability barrier function [39].

337 Examining ST concentrations, we observed higher levels (of about two-folds) of

338 sitosterol (ST 29:1;O) in Bazley and MR43 grain compared to the other cultivars. This
339 corresponds with studies on sorghum Distillers Dried Grains (DDG) phytosterols
340 characterization, indicating that sitosterol constitutes the majority, comprising 50% or
341 more, of the total plant sterols in hexane extracts[40]. In addition, a previous study
342 underscored the significance of sorghum kernels, particularly in wet-milled fiber and
343 germ fractions (as opposed to the whole grains studied here), as a substantial source of
344 free phytosterols and fatty acyl phytosterol esters[41]. Studies also showed the ability of
345 these sterols to reduce low-density lipoprotein cholesterol (LDL-C) levels by decreasing
346 intestinal absorption [42]. These findings contribute to a deeper understanding of the
347 phytosterol composition of sorghum and its potential health-related implications.

348 A comprehensive visualization of the concentrations of the primary classes of
349 lipids in GL is shown in **Fig. 5A.** The results showed that cv. MR43 and Bazley had higher
350 abundances of GL than the other cultivars. In particular, triacylglycerol (TG) within the
351 GL major class was the most prevalent, with MR43 having the highest levels and Cracker
352 the lowest. This aligns with previous reports emphasizing that TGs are the predominant
353 lipid group in the oil fraction of grain sorghum (GS), with palmitic, oleic, and linoleic
354 acids being the most abundant fatty acids[5]. The TG 54 acyl chain carbons and two double
355 bonds (TG (54:2)) are not detected in all the six cultivars of sorghum. Studies showed
356 that TG (54:2) in plasma, is associated with the risk of CVD, which is one of the major
357 causes of mortality in patients with T2D [43] . Examining the role of ω-3 and ω-6 PUFAs
358 as primary structural components of GP and GL, our study reveals that in GL, the
359 concentration of ω-6 and ω-3 PUFAs is more than four-fold higher compared to GPs (**Fig.
360 5B** and **5C**). This result suggests that membrane lipids, particularly GL, are potential
361 sources of PUFAs in the human diet, offering fluidity and flexibility to cell membranes[44].

362 This observation builds upon existing knowledge that emphasizes the
363 importance of linoleic acid and linolenic acid as precursors of ω-6 and ω-3 PUFAs,
364 respectively. Cereal lipids are more valuable when they include essential fatty acids[45]. In
365 our study, the higher abundance of these essential fatty acids in the GL aligns with the
366 broader context of lipid composition in sorghum cultivars. This connection explains the
367 significance of considering lipid composition variations among cultivars, providing a
368 piece of information for understanding the potential health advantages associated with
369 the sorghum grain's lipid content.

370

371 **3.4 Identification of FAHFAs in sorghum**

372 FAHFAs are a relatively newly discovered family of bioactive lipids that are
373 becoming increasingly appreciated for their potential as therapeutics for lipid oxidation,
374 diabetes, obesity-related inflammation, and cancer. Many plant-derived foods, such as
375 vegetable oils, fruits, grains, cereals, tea, and fermented goods, have been found to include
376 different families and regioisomers of FAHFAs[46]. Among these foods, grains and grain
377 products are substantial sources of FAHFAs. Recent analyses have revealed the presence
378 of numerous FAHFA families and regioisomers in unfermented rice bran, fermented
379 brown rice, and rice bran with the fungus *Aspergillus oryzae* (FBRA)[47]. Liberati-Cizmek
380 et al found that whole-grain oats had a higher abundance of several FAHFA families than
381 coarse and fine flakes[48]. Although previous studies have documented the presence of
382 FAHFAs in various cereals, there is a lack of evidence regarding their presence in
383 sorghum. Here we identified five distinct FAHFAs in sorghum grain. The percentage
384 distribution of FAHFAs in sorghum cultivars is represented in **Table 1**, which shows that
385 MR43 has the highest amount of FAHFAs compared to others. The extracted ion
386 chromatograms (EIC) depicting these newly identified FAHFAs in sorghum cultivars,
387 along with their representative structures, are shown in **Fig. 6A.** MS and MS/MS analyses
388 were performed to confirm the presence of FAHFAs (**Fig. 6B**). Every lipid molecular
389 species in the FAHFA family underwent negative mode ionization, producing $[M-H]^-$ as
390 the precursor ion. The palmitic acid esters of hydroxy-linoleic acid (PAHLA) ionize in
391 the negative mode to give an $[M-H]^-$ ion at *m/z* 533.4563. The MS/MS spectra showed
392 subsequent ionization, resulting in *m/z* 295 and 255, indicating the loss of hydroxy-
393 linoleic acid (HLA) and palmitic acid (PA), respectively. Further fragmentation at *m/z*
394 295 produced *m/z* 277 with a neutral loss of H_2O, confirming that the compound was
395 PAHLA.

396 A recent study by Liu et al. linked elevated plasma PAHLA levels to early-stage
397 colorectal cancer (CRC), suggesting this as a biomarker for detecting CRC at early-stage[49].
398 Similarly, palmitic acid esters of hydroxy-oleic acid (PAHOA) were ionized to give an
399 $[M-H]^-$ ion at *m/z* 535.4718. The MS/MS spectra exhibited the characteristic losses of
400 hydroxy-oleic acid (HOA) and PA at *m/z* 297 and 255, respectively. Further neutral loss
401 of H_2O from HOA yielded *m/z* 279, ultimately confirming that the compound was

402 PAHOA. Interestingly, PAHOA has previously been detected in fruits, vegetables, and
403 cereals[48]. The oleic acid esters of hydroxy-oleic acid (OAHOA) ionized to produce an
404 $[M-H]^-$ ion at *m/z* 561.4875. The MS/MS spectra indicated the loss of HOA and oleic acid
405 (OA) at *m/z* 297 and 281, respectively. Further fragmentation of HOA revealed a neutral
406 loss of H_2O at *m/z* 279, confirming that the compound was OAHOA. Matsuzawa et al.
407 reported six FAHFA families in rice, with OAHOA being the most abundant[50]. In a similar
408 pattern, the linoleic acid esters of hydroxy-oleic acid (LAHOA) ionize to give an $[M-H]^-$
409 ion at *m/z* 559.4723 and its MS/MS spectra confirm the identity of the compound as
410 LAHOA, exhibiting characteristic losses of HOA and LA at *m/z* 297 and 279, respectively.
411 Further neutral loss of H_2O from HOA at *m/z* 279 confirmed that the moiety was LAHOA.
412 Interestingly, LAHOA was previously identified in walnut oil and raw almond oil[51].
413 Finally, linoleic acid esters of hydroxy-linoleic acid (LAHLA) were also ionized to give
414 an $[M-H]^-$ ion at *m/z* 557.4564, and its MS/MS spectra confirmed the identity of the
415 compound as LAHLA, revealing characteristic losses of HLA and LA at *m/z* 295 and 279,
416 respectively. Further neutral loss of H_2O from HLA gives *m/z* at 277, ultimately
417 confirming that the compound was LAHLA. A previous study on nut and oat oils revealed
418 that these are rich in unsaturated FAHFAs, particularly LAHLA, which is known for its
419 anti-inflammatory properties[52].

420 The derivatization of FAHFAs from DMED-labelled-lipid extracts of MR43
421 sorghum cultivar gives information about the position of the -OH group based on MS/MS
422 fragmentation of DMED-labelled-FAHFAs standard. The position-based fragmentation
423 was compared with the fragmentation pattern of standard 12-OAHOA as a reference. The
424 representative EIC and MS^n analysis spectra of standard DMED labeled 12-OAHOA and
425 sample DMED labelled OAHOA are shown in **Fig.7A**. MS^n analyses were performed to
426 confirm the position of -OH in the sample FAHFAs. Every lipid molecular species in the
427 DMED-labeled-FAHFA family underwent positive mode ionization, producing $[M+H]^+$
428 as the precursor ion. The standard 12-OAHOA ionizes in the positive mode to give an
429 $[M+H]^+$ ion at *m/z* 633.5934. The MS/MS spectra showed subsequent ionization,
430 resulting in *m/z* 588 indicating the neutral loss of dimethylamino group (45 Da) $[M+H-$
431 $C_2H_7N]^+$. The product ion at *m/z* 531 was due to the loss of the oleoyl group. Afterward,
432 *m/z* 306 represented the loss of the oleoyl group with the dimethylamino group. This
433 fragmentation for DMED-derivatized FAHFAs aligns with a previous study on the

434 derivatization of FAHFAs [53,54]. Further fragmentation at *m/z* 306 based on MS^3 analysis
435 produced *m/z* 208 (as loss of $C_{13}H_{22}NO^+$) with *m/z* 98 ($C_7H_{14}O^{\cdot+}$) and *m/z* 222
436 ($C_{14}H_{24}NO^+$), identifying the -OH position at C12 of HOA, confirming the structure to be
437 12-OAHOA. The exact similar fragmentation is observed in DMED labeled OAHOA in
438 sorghum cv. MR43 confirming the position of -Oh group in sorghum OAHOA at C12
439 position and named as 12-OAHOA.

440 Similar fragmentation has been observed for DMED-labelled-FAHFAs in the
441 sample except for LAHLA. The EIC and fragmentation pattern for MS^n analysis is
442 represented in **Fig. 7B**. In brief, DMED-labeled-PAHOA ionizes in the positive mode to
443 give an $[M+H]^+$ ion at *m/z* 607.5781. The MS/MS spectra showed subsequent ionization,
444 resulting in *m/z* 562 indicating the neutral loss of dimethylamino group (45 Da) [M+H-
445 C_2H_7N]+. The product ion at *m/z* 351 was due to the loss of the palmitoyl group.
446 Afterward, *m/z* 306 represented the loss of the palmitoyl group with the dimethylamino
447 group. Further fragmentation at *m/z* 306 based on MS^3 analysis produced *m/z* 208 (as loss
448 of $C_{13}H_{22}NO^+$) with *m/z* 98 ($C_7H_{14}O^{\cdot+}$) and *m/z* 222 ($C_{14}H_{24}NO^+$), identifying the -OH
449 position at C12 of HOA, confirming the structure to be 12-PAHOA as same as std 12-
450 OAHOA. The same fragmentation pattern is followed by other DMED-labeled-FAHFAs
451 such as OAHOA and LAHOA, where the position of the -OH group is identified at C12.
452 The representative. Whereas the DMED-labeled-FAHFAs such as LAHLA with HLA as
453 its hydroxy fatty acid follow a different fragmentation pattern and the position of -OH
454 group is identified at C14. In brief, the DMED-labeled-LAHLA produced $[M+H]^+$ as the
455 precursor ion $[M+H]^+$ ion at *m/z* 629.5635. The MS/MS spectra showed subsequent
456 ionization, resulting in *m/z* 584 indicating the neutral loss of dimethylamino group (45
457 Da) $[M+H-C_2H_7N]^+$. The product ion at *m/z* 531 was due to the loss of the linoleoyl.
458 Along with this *m/z* 349 indicates the loss of $C_{23}H_{41}O_2^+$ which confirms the position of -
459 OH to be at C14 in MS/MS spectral analysis. Afterward, *m/z* 306 represented the loss of
460 the linoleoyl group with the dimethylamino group as (45 Da). Further fragmentation at
461 *m/z* 306 based on MS^3 analysis produced *m/z* 208 ($C_{13}H_{22}NO^+$) with *m/z* 98 ($C7H14O^{\cdot+}$),
462 *m/z* 222 ($C_{14}H_{24}NO^+$), *m/z* 236 ($C_{15}H_{26}NO^+$) and *m/z* 250 ($C_{16}H_{28}NO^+$) which also identify
463 the -OH position at C14 of HLA, confirming the structure to be 14-LAHLA.

464 This study provides valuable information about the distribution and

465 concentration of FAHFAs in different sorghum cultivars and gives an in-depth description
466 of the potential variations of these bioactive compounds in sorghum. Although our
467 identification of FAHFAs in sorghum, as well as a thorough examination of their lipid
468 composition, have yielded insights, there are certain limitations in our data. Firstly, the
469 grain samples are from only one growing season and one location, and the lipid
470 composition varies depending on agronomic conditions, the stage of ripening, and post-
471 harvest conditions, which are not considered in this study. Our study uses single-year
472 cultivated sorghum samples, however, from the agronomic point of view multi-year
473 produced sorghum samples should be compared to draw better conclusions regarding
474 their lipidome variations. Furthermore, the quantities presented are semi-quantitative
475 rather than absolute, and internal standards were added after homogenization to avoid
476 their significant loss during the homogenization process. The lack of commercial
477 standards makes MS spectrum analysis necessary for the identification of new lipids like
478 FAHFAs. Additionally, due to the lower concentration of PAHLA, MS spectra for PAHLA
479 were not obtained for confirmation of the -OH position. Future studies should address
480 these limitations to gain a more thorough understanding of sorghum grain lipidomic
481 profiles.

482 Our in-depth examination of lipid molecular species in whole grains of six
483 distinct sorghum cultivars revealed rich diversity in their lipid profiles. Using multivariate
484 data analysis tools, we identified notable differences in the lipid contents of the cultivars,
485 underscoring the distinct biochemical signatures of each cultivar. The identification of
486 FAHFAs in sorghum grains through LC/MS analysis and derivatization represents a
487 significant advancement in our understanding of sorghum lipidomics. Among the six
488 cultivars, MR43 was particularly noteworthy, as it exhibited the highest degree of
489 variability in lipid composition, including bioactive lipid FAHFAs. The lowest ω-6 to ω-
490 3 ratio in cv. Bazley and optimal P:S ratio in cv. Cracker, Bazley, Tiger, and Liberty show
491 the importance of each sorghum cultivar in the human diet. By this sorghum grain
492 lipidomics research contributes to the broader trend toward unlocking the health-
493 promoting qualities inherent in everyday dietary choices. We aim to explore the broader
494 landscape of lipid molecules not limited to sorghum alone. Our research aims to identify
495 similar compounds in various cereals and food sources, thereby offering a detailed view
496 into the wider diversity and distribution of these lipid molecules in the food supply. Using

this multifaceted approach, we aimed to deepen our understanding of these unique lipids and their roles in agriculture, nutrition, and overall human health.

Supporting Information: Table S1: The RSD and recovery (RE) for internal standard used for lipid quantification. **Table S2:** The sPLSDA score plot scores of five principal components for validation of the sPLSDA plot. **Table S3:** Relative concentration (μg/g) of quantified lipid along with QC in six sorghum cultivars. **Fig S1:** The kernel structure and images of sorghum grains. **Fig S2:** The sPLSDA validation plot. **Fig S3:** The high-resolution mass Spectra (MS) and MS/MS of FA (18:3).

The Supporting Information is available free of charge at https://pubs.acs.org

Author contributions: L.R.N: Formal analysis, methodology, writing-original draft. S.G.B.G: Conceptualization, visualization, supervision, resources, writing-original draft. T.H.R.: Sorghum sample provider, supervision, formal analysis, writing review & editing. D.G.: Data curation, visualization, resources, writing-review & editing. A.K.: Sorghum sample provider, formal analysis, writing- review & editing. S-P.H.: Supervision, resources, writing- review & editing.

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Tables

Table 1: Major fatty acid with FAHFAs percentage composition of sorghum cultivars.

Fatty Acid	Buster (%)	Bazley (%)	Cracker (%)	Liberty (%)	MR43 (%)	Tiger (%)
Palmitic Acid	11.17	13.91	14.41	14.90	13.56	13.74
Stearic Acid	6.69	11.29	10.69	11.97	6.45	11.36
Oleic Acid	24.41	20.28	20.03	19.50	22.74	19.73
Linoleic Acid	34.89	31.31	32.98	31.13	31.65	31.92
Linolenic Acid	3.12	3.51	3.14	3.15	2.76	3.43
FAHFA	0.77	0.13	0.23	0.13	2.59	0.11
SFA	33.02	41.64	40.77	43.22	36.13	42.10
MUFA	26.46	22.18	21.34	20.62	25.40	21.31
PUFA	40.52	36.18	37.89	36.15	38.47	36.60

Figures

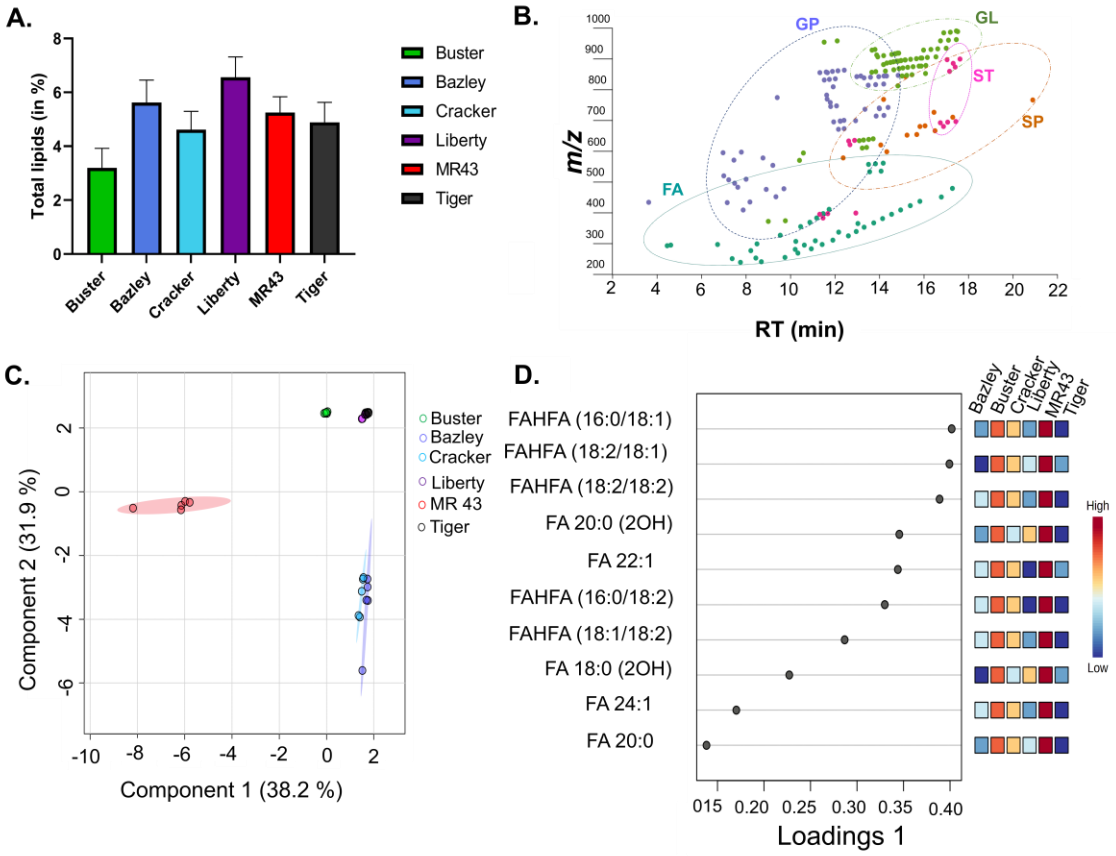

Fig. 1. Lipid Analysis of Sorghum Cultivars. (A) Percentage of total lipids in six sorghum cultivars on a fresh weight basis. (B) The plot of *m/z* vs RT (min) summarizing the lipid elution profiles for the sorghum cultivars. Different colored groups indicate major lipid classes: Fatty acyls (FAs), sphingolipids (SPs), glycerophospholipids (GPs), glycerolipids (GLs), and sterol lipids (STs). (C) Sparse Partial Least Squares-Discriminant Analysis (sPLS-DA) score plot showing distinct lipid profiles of the sorghum cultivars. (D) sPLS-DA loading plot indicates the most discriminating lipid molecular species of component 1. Data are presented as mean ± standard error of the mean (SEM) (n=5). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test.

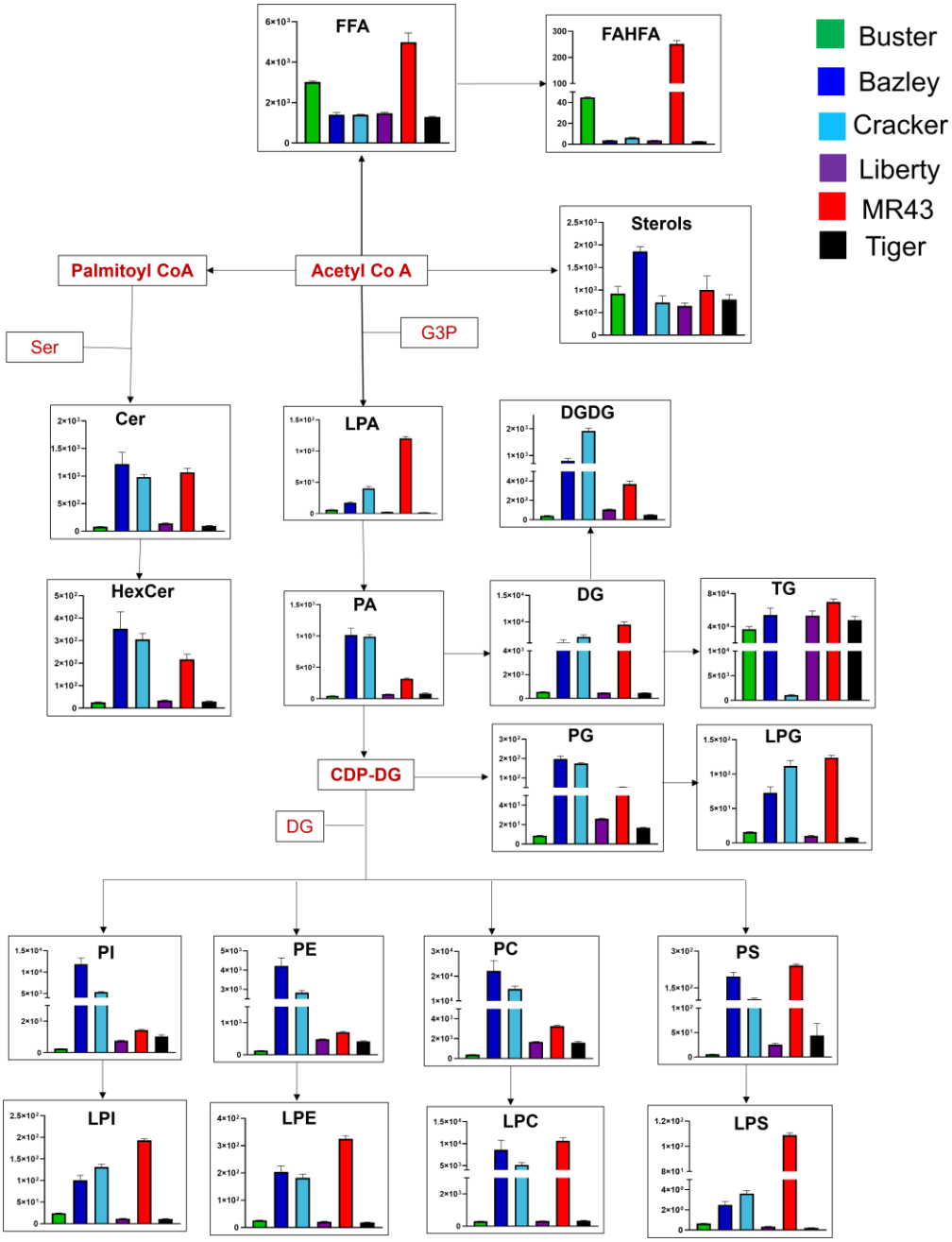

Fig. 2. Lipid mapping of six sorghum cultivars based on their possible biosynthesis. The Y-axis represents the concentration of the lipid sub-class in µg/g and the X-axis represents each cultivar. The data is represented as mean ± SEM (n=5). Where the abbreviations are as follows: Acetyl Coenzyme A (Acetyl Co A), Free Fatty Acids (FFA), Fatty Acid Easters of Hydroxy Fatty Acids (FAHFAs), Glycerol-3-Phosphate (G3P), Lysophosphatidic Acid (LPA), Phosphatidic acid (PA), Cytidine Diphosphate Diacylglycerol (CDP-DAG) Phosphatidylglycerol (PG), Lysophosphatidylglycerol (LPG), Phosphatidylinositol (PI), Lyso-phosphatidylinositol(LPI), Phosphatidylethanolamine(PE), Lyso-

phosphatidylethanolamine(LPE), Phosphatidylcholine(PC), Lyso-phosphatidylcholine(LPC), Phosphatidylserine(PS), Lysophosphatidylserine(LPS), Diacylglycerol (DG), Triacyl glycerol (TG), Digalactosyldiacylglycerol(DGDG), Serine (Ser), Ceramide(Cer), and hexosylceramide(HexCer).

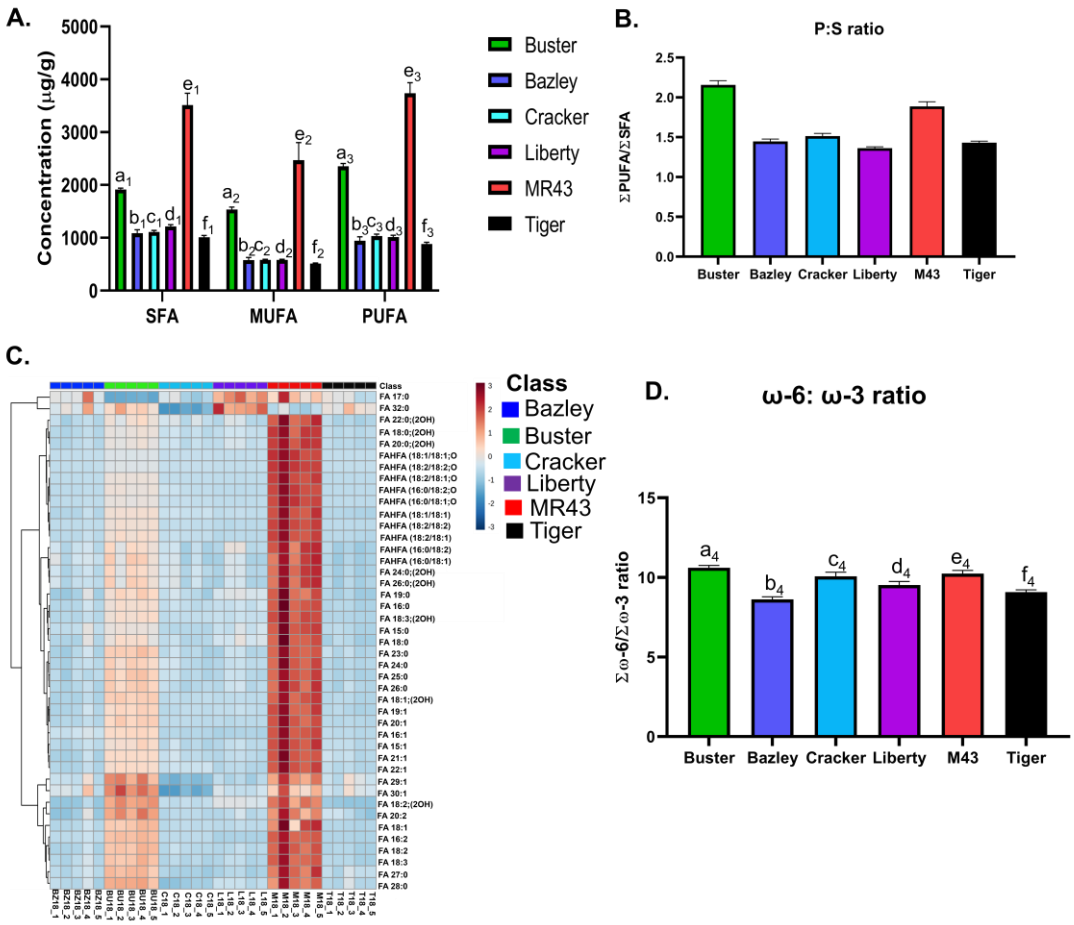

Fig 3. Free Fatty Acid (FFA) Distribution and Analysis in Six Sorghum Cultivars. (A) Distribution of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) across six sorghum cultivars. Significant group comparisons are as follows: SFA: a_1-[b_1,c_1,d_1,e_1,f_1] i.e. group a_1 is significantly different from the groups shown in a square bracket, b_1-[e_1], c_1-[e_1], d_1-[e_1], e_1-[f_1], MUFA: a_2-[b_2,c_2,d_2,e_2,f_2], b_2-[e_2], c_2-[e_2], d_2-[e_2], e_2-[f_2], PUFA: a_3-[b_3,c_3,d_3,e_3,f_3], b_3-[e_3], c_3-[e_3], d_3-[e_3], e_3-[f_3]. (B) The ratio of PUFA to SFA (P:S ratio) in six sorghum cultivars. (C) Heatmap representing the concentrations of FFAs in the sorghum cultivars. (D) ω-6 to ω-3 ratio in six sorghum cultivars. Significant group comparisons are as follows: a_4- [b_4, d_4, f_4], b_4- [c_4, d_4, e_4], c_4-[f_4], e_4-[f_4]. All the data are expressed as mean ± SEM (n = 5). Statistical significance was determined using two-way ANOVA with Tukey's multiple comparisons test. Different letters indicate significant differences at $p < 0.05$.

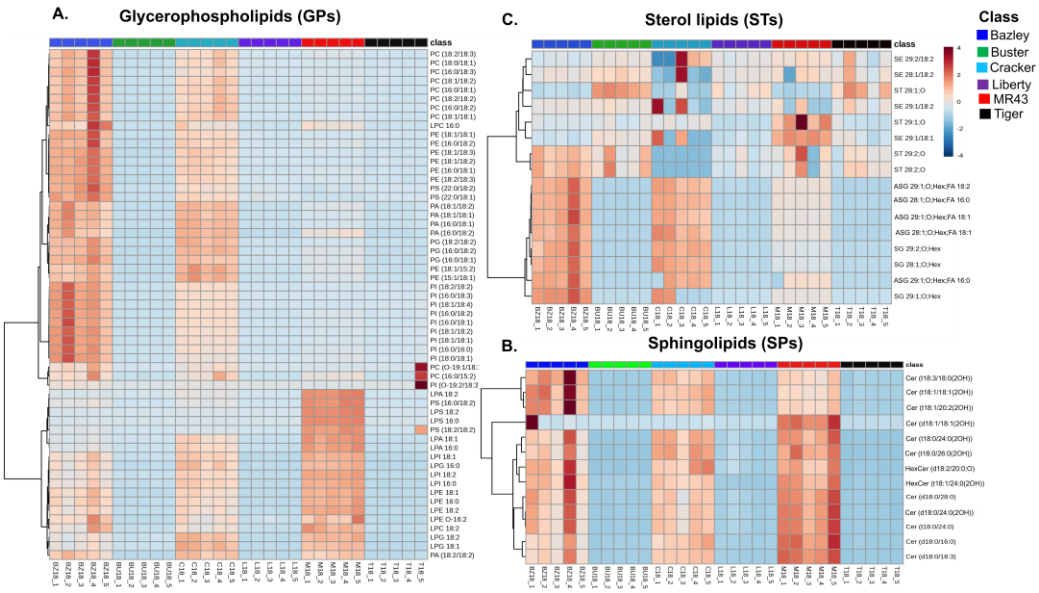

Fig. 4. Heatmap of three major lipid Classes in six sorghum cultivars. (A) Heatmap showing the concentration of glycerophospholipids (GPs) in six sorghum cultivars. (B) Heatmap displaying the concentration of sphingolipids (SPs) in the six cultivars. (C) Heatmap illustrating the concentration of sterol lipids (STs) across the six sorghum cultivars.

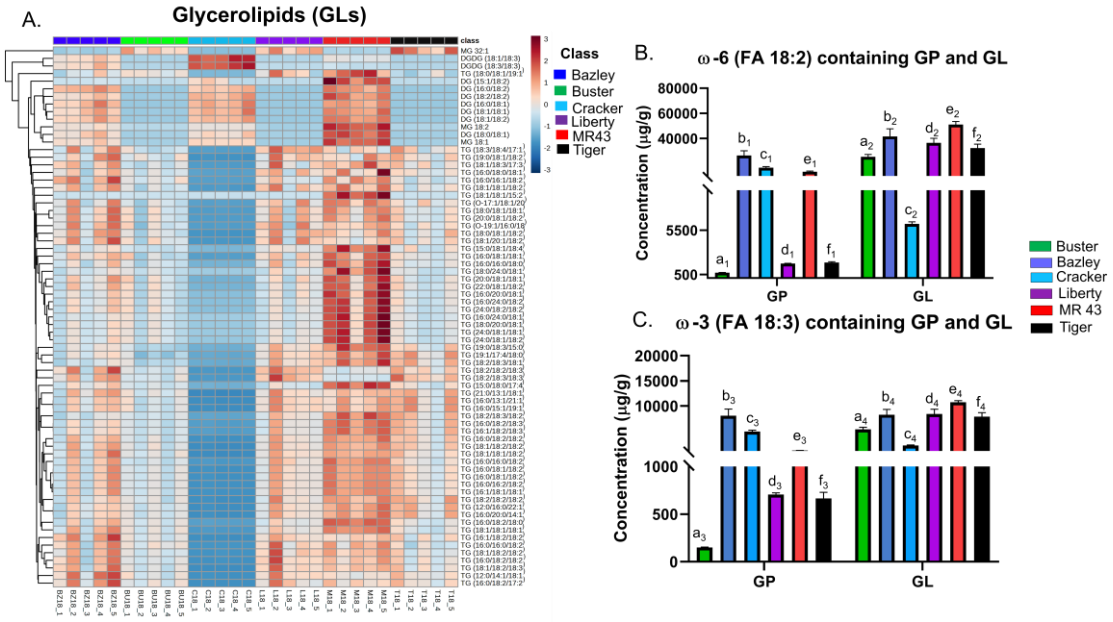

Fig. 5. Heatmap and statistical analysis of lipid concentrations in sorghum cultivars. (A) Heatmap displaying the concentration of glycerolipids (GL) in six sorghum cultivars. (B). Concentrations of ω-6 PUFAs (linoleic acid, FA (18:2)) in glycerophospholipids (GP) and GL. Significant groups for GP: a_1-[b_1, c_1], b_1-[d_1, e_1, f_1], c_1-[d_1, f_1]; for GL: a_2-[b_2, c_2, d_2, e_2], b_2-[c_2], c_2-[d_2, e_2, f_2], d_2-[e_2], e_2-[f_2]. (C). Concentrations of ω-3 PUFAs (linolenic acid, FA (18:3)) in GP and GL. Significant groups for GP: a_3-[b_3], b_3- [d_3, e_3, f_3], for GL: a_4- [b_4, c_4, d_4, e_4, f_4], b_4-[c_4], c_4- [d_4, e_4, f_4]. Data are expressed as mean ± SEM (n = 5). Statistical significance was determined using two-way ANOVA with Tukey's multiple comparison test. Values with different letters indicate significant differences at $p < 0.05$.

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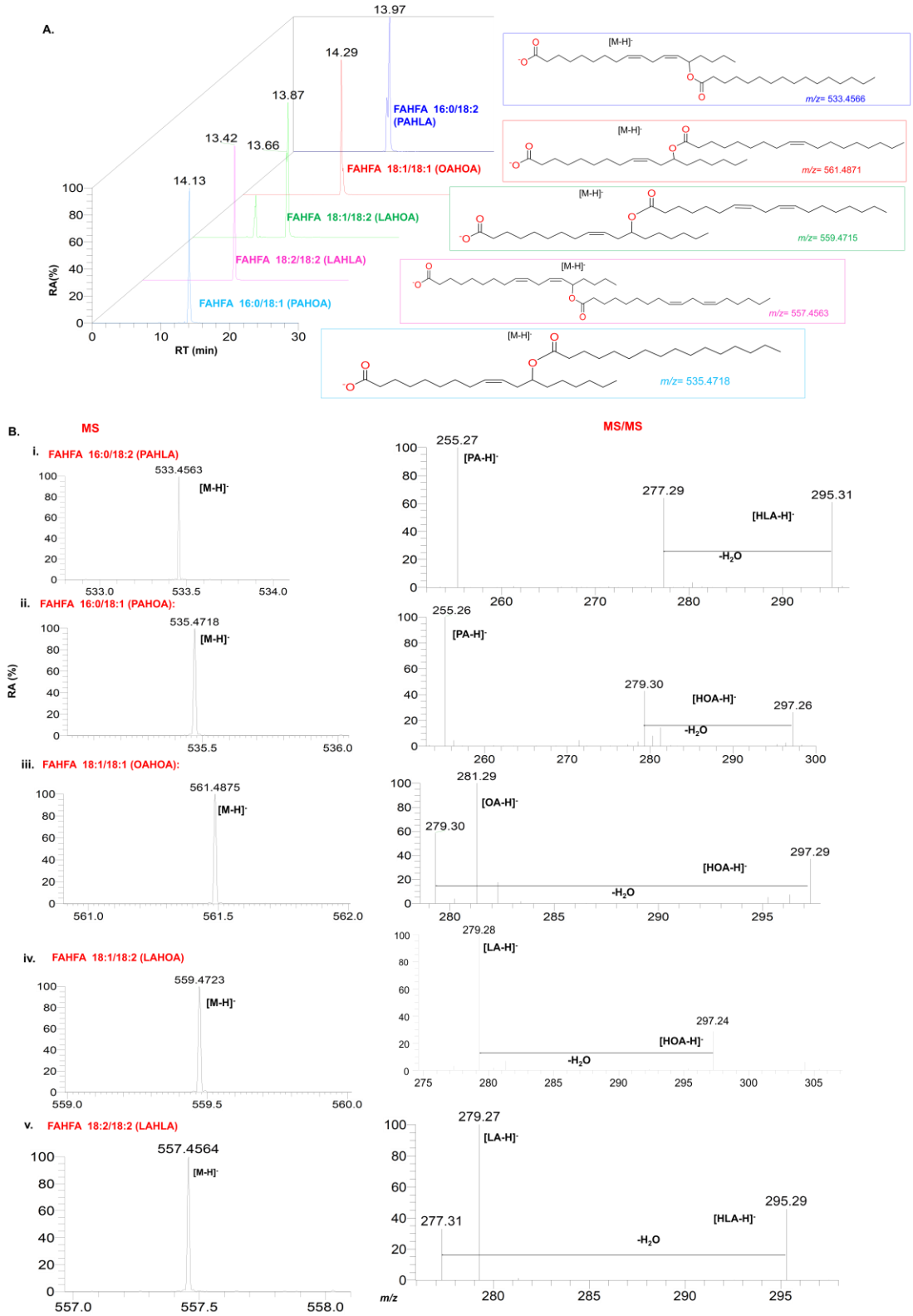

Fig. 6. Detection and Analysis of Fatty Acid Esters of Hydroxy Fatty Acids (FAHFAs) in Sorghum Cultivars (A) Extracted ion chromatograms (EICs) of FAHFAs identified

in the study, including palmitic acid esters of hydroxy-linoleic acid (PAHLA), palmitic acid esters of hydroxy-oleic acid (PAHOA), oleic acid esters of hydroxy-oleic acid (OAHOA), linoleic acid esters of hydroxy-oleic acid (OAHLA), and linoleic acid esters of hydroxy-linoleic acid (LAHLA). High-resolution mass spectra of representative FAHFAs detected in the study. (i) Mass spectra (MS) and MS/MS of PAHLA, where in the MS/MS spectra PA represents palmitic acid and HLA represents hydroxy-linoleic acid. (ii) MS and MS/MS of PAHOA, where in the MS/MS spectra PA represents palmitic acid and HOA represents hydroxy-oleic acid. (iii) MS and MS/MS spectra of OAHOA, where OA represents oleic acid and HOA represents hydroxy-oleic acid. (iv) MS and MS/MS spectra of OAHLA, where OA represents oleic acid and HLA represents hydroxy-linoleic acid. (v) MS and MS/MS spectra of LAHLA, where LA represents linoleic acid and HLA represents hydroxy-linoleic acid.

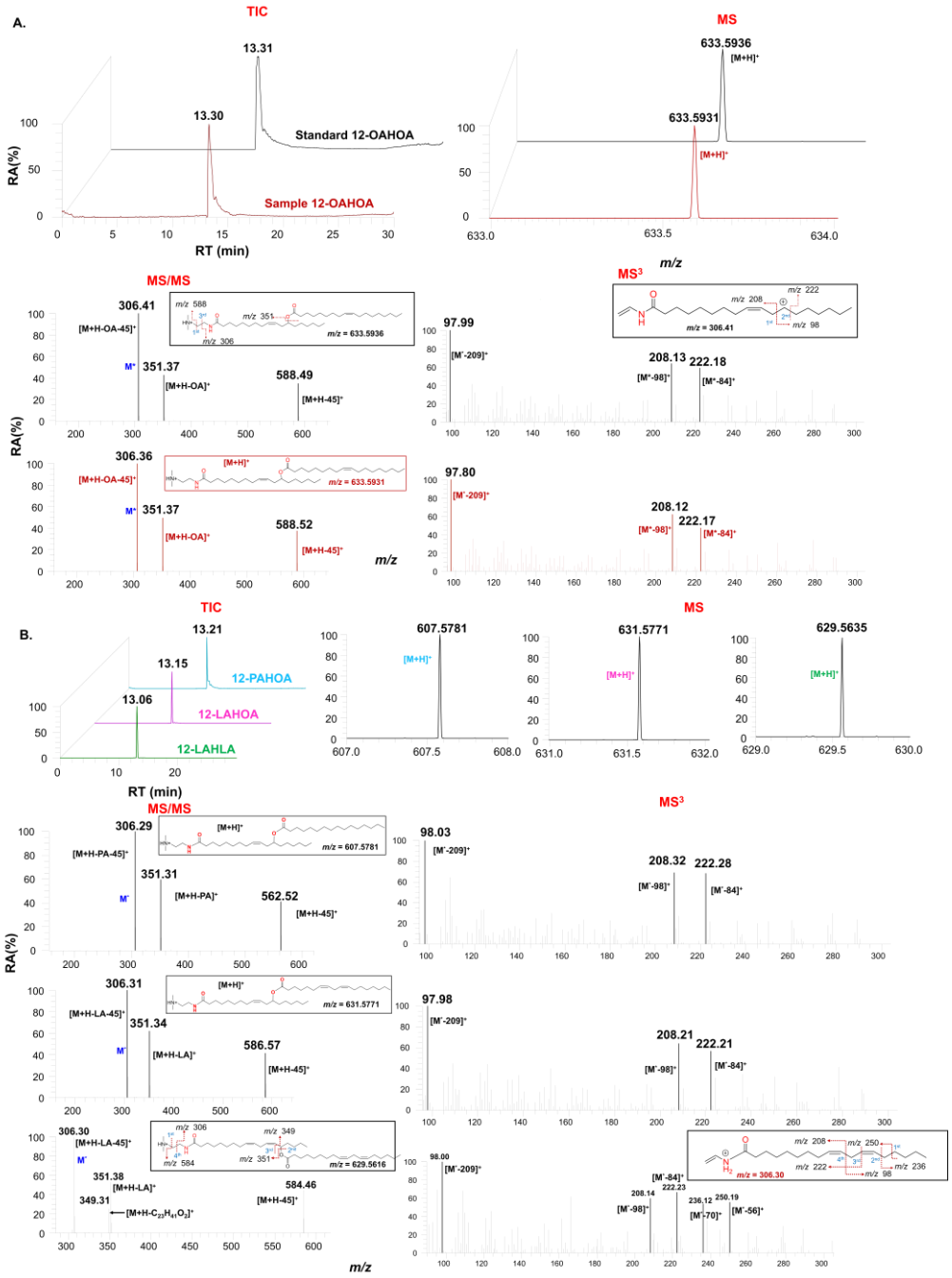

Fig. 7. Detection of DMED derivatized FAHFAs in MR43 sorghum cultivar. (A) EIC and MS^n analysis of DMED labeled standard (std) oleic acid esters of 12-hydroxy oleic acid (12-OAHOA) and DMED labeled sample 12-OAHOA. The standard spectra and sample spectra are represented in black and red color respectively. The fragmentation pattern for MS/MS and MS^3 spectra are shown in the rectangular box. The fragmentation spectra for both std and sample are the same. (B) EIC and MS^n analysis of MS^n analysis of DMED labeled sample FAHFAs, such as palmitic

acid esters of hydroxy-oleic acid (12-PAHOA), linoleic acid esters of hydroxy-oleic acid (12-LAHOA), and linoleic acid esters of hydroxy-linoleic acid (14-LAHLA) are shown. MS/MS and MS^3 spectra of DMED labeled sample 12-PAHOA, 12-LAHOA where the -OH position is identified as C12 is same as standard. Whereas, MS/MS and MS^3 spectra of DMED labeled sample 14-LAHLA the position of the -OH group is identified at C14. The fragmentation pattern of the representative FAHFAs is mentioned in the rectangular box where the cleavage for each fragment is mentioned with its respective *m/z*. The fragmentation of DMED labeled 12-OAHOA,12-PAHOA, and 12-LAHOA is not mentioned as the pattern of cleavage is the same as the std.

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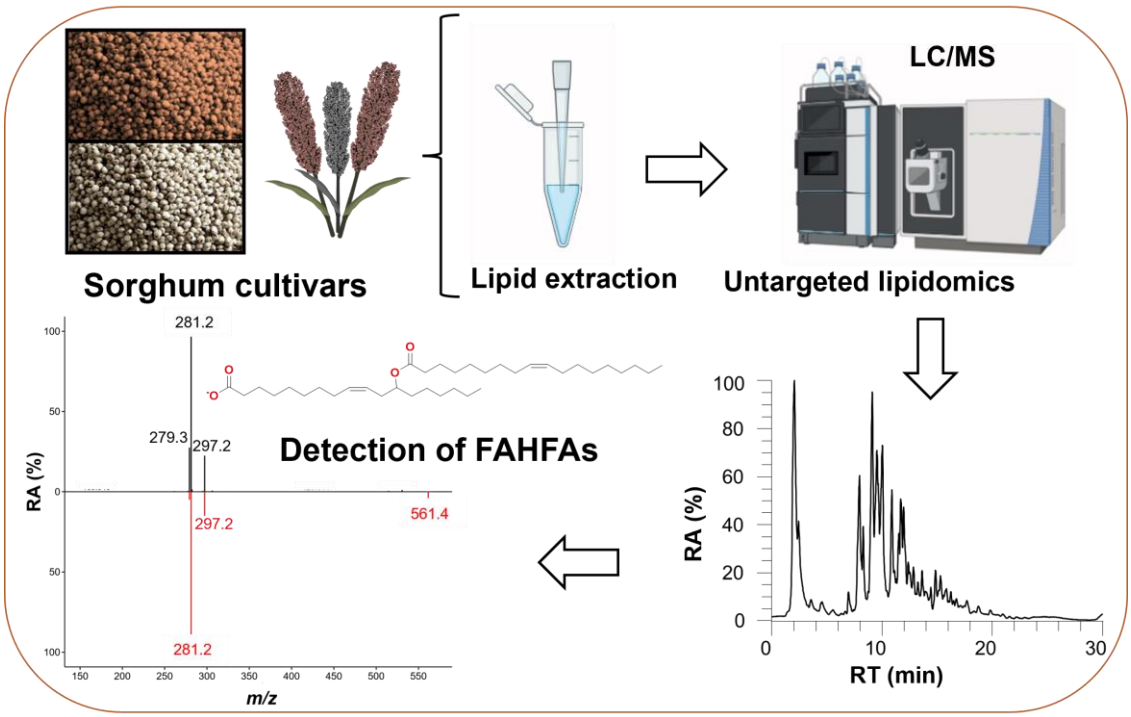
Sorghum cultivars
Lipid extraction
LC/MS
Untargeted lipidomics
Detection of FAHFAs
281.2
279.3
297.2
297.2
561.4
281.2
RA (%)
m/z
RA (%)
RT (min)