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Metabolomics driven analysis by UAEGC-MS and antioxidant activity of Chia (*Salvia hispanica* L.) commercial and mutant seeds

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Running Title: Metabolomics and antioxidant activity of Chia seeds mutants.

Abstract

Chia is a food plant producing fruits called seeds which have seen an increase in interest due to their health benefits. This work is the first report on the metabolite profile, total polyphenols and antioxidant activity of chia seeds by ultrasound-assisted assisted extraction coupled with gas chromatography-mass spectrometry (UAE GC-MS). We compared different chia sources: two commercial (black and white) and three early flowering (G3, G8 and G17) mutant genotypes. Organic extracts were mainly composed of mono- and polyunsaturated fatty acids with alpha-linolenic being the most abundant. Polar extracts contained sucrose, methylgalactoside and glucose as main sugars. Antioxidant activity and total polyphenolic content were correlated. Chemical composition and yield potential of early flowering genotypes were different from commercial chia, and while white chia showed the highest content of omega-3 fatty acids, the high content of nutraceuticals of G17 and G8 suggests their potential source of raw materials for the food/feed industry.

Keywords: Early flowering genotypes; Ultrasound-assisted extraction; Total polyphenol content; Antioxidant activity; Alpha-linolenic acid

1. Introduction

Chia (*Salvia hispanica* L.), is an emerging crop of the *Lamiaceae* family with a wide range of potential uses in food technology due to the content of nutrients and nutraceuticals of its fruits and leaves (Amato et al., 2015, Ayerza, 1995), and to the antioxidant and rheological properties of whole fruits and extracts (Hermoso-Diaz, Velázquez-González, Lucio-Garcia & Gonzalez-Rodriguez, 2014, Lu & Foo, 2002, Muñoz, Cobos, Diaz & Aguilera, 2012, Er, 2013).

Research on Chia started only in the 1980s due to the long decline of the crop in Central America, the area of origin, after the Spanish conquest (Cahill, 2004). Early works focused on the fruit for its content of vitamin B (Bushway, Wilson, Houston & Bushway, 1984), proteins (up to 26%) (Ayerza & Coates, 2004, Ayerza, 2013, Coates, 2009, Sandoval-Oliveros & Paredes-López, 2012), and especially the oil for its composition of fatty acids. Alpha-linolenic acid was the main component, with one of the highest concentrations found in nature (Ayerza, 1995, Coates, 1996, Gentry, Mittleman & McCrohan, 1990). Later, a high amount of dietary fibre (33.9–39.9%) was reported (Capitani, Spotorno, Nolasco & Tomás, 2012). Part of this dietary fibre is is located in the epidermal cells of the fruit and may be extracted (Muñoz, Cobos, Diaz & Aguilera, 2012) and used as a thickening mucilage since it is highly hygroscopic, viscous and adhesive (Švec, Hrušková & Jurinová, 2016). The mucilage of chia can also be used as a basis for films to avoid food dehydration (Capitani, Corzo-Rios, Chel-Guerrero, Betancur-Ancona, Nolasco & Tomás, 2015, Capitani, Spotorno, Nolasco & Tomás, 2012, Muñoz, Cobos, Diaz & Aguilera, 2012), and as an additive to rice flour to improve both rheology and nutraceutical properties of gluten-free pasta (Menga, Amato, Phillips, Angelino, Morreale & Fares, 2017). Ground seeds of chia have been shown to increase the shelf-life of wheat bread, due to the reduction of starch retrogradation (Iglesias-Puig & Haros, 2013), to be a good substitute for fat in bread (Coelho & de las Mercedes Salas-Mellado, Myriam, 2015) or cakes (Felisberto, Wahanik, Gomes-Ruffi, Clerici, Chang & Steel, 2015) and, at the same time, to provide poly-unsaturated fatty acids (PUFA) and fibre.

Antioxidant compounds and good antioxidant activity have been documented in chia seeds and extracted oil (Amato et al., 2015) or mucilage (Menga, Amato, Phillips, Angelino, Morreale & Fares, 2017). Tocopherols, phytosterols, carotenoids, and phenols have been found, such as chlorogenic and caffeic acids, myricetin, quercetin, and kaempferol (Amato et al., 2015, da Silva Marineli, Moraes, Lenquiste, Godoy, Eberlin & Maróstica Jr, 2014, Reyes-Caudillo, Tecante & Valdivia-López, 2008). Poly-unsaturated fatty acids are also natural antioxidants found in chia oil, to the point that Hermoso-Diaz et al. (2014) proposed a methanol extract of *S. hispanica* as a substitute for toxic green corrosion inhibitors of carbon steel.

Due to a favourable market, the crop is spreading rapidly from the area of origin to new environments in North America, Africa, Australia and Europe (Bochicchio, Rossi, Labella, Bitella, Perniola & Amato, 2015). This is accompanied by breeding efforts to produce longer-day flowering genotypes in order to overcome limitations linked to the original short-day flowering genotypes, which cannot be viably grown for seed yield at high latitudes (Jamboonsri, Phillips, Geneve, Cahill & Hildebrand, 2012). Research on the characterization of such genotypes using the metabolomic approach is being undertaken in order to compare their seed quality with that of chia genotypes from the area of origin. A metabolomic study of commercial and mutant seeds flowering at between 13 and 16 hours day-length was performed (de Falco, Incerti, Bochicchio, Phillips, Amato & Lanzotti, 2017) through the identification of the major classes of organic compounds by NMR analysis and, from a quantitative point of view, by integration of the NMR spectra followed by chemometrics. However, by using ^{1}H NMR coupled with chemometrics, it was not possible to determine metabolites present in lower amounts or metabolites whose signals overlapped. De Falco, Amato & Lanzotti (2017) reviewed the literature on chemical composition of chia seeds and observed that a more thorough characterization of the metabolome was needed.

The aim of this work was to produce a thorough characterization of the metabolome of chia commercial seeds and some early-flowering mutants through a high-recovery technique for metabolites in polar and non-polar extracts. The work was conducted using ultrasound extraction

(UAE) of chia seeds followed by GC-MS analysis. This allowed the identification and quantification of a higher number of metabolites and the determination of the whole fatty acid profile of the non-polar extracts of the chia seeds.

2. Materials and methods

2.1. Chemicals and reagents

The reagents used for the extraction procedure and chemical characterization, namely anhydrous methanol (99.8%), anhydrous n-hexane (95%), 2,2`-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), derivatizating agents methoxyamine hydrochloride and N-methyl-N-trimethylsilyltrifuoroacetamide (MSTFA), were obtained from Sigma-Aldrich (Dorset, UK). N, O-Bis(trimethylsilyl)trifluoroacetamide, Trimethylchlorosilane (BSTFA with 1% TMCS) was purchased from Supelco Analytical (Bellefonte, PA). Pyridine, anhydrous sodium carbonate, Folin–Ciocalteu's reagent and gallic acid were obtained from Fischer Scientific (Loughborough, UK). Sugars, amino acids, organic acids, polyphenols used for identification and quantification were purchased from Sigma-Aldrich (Dorset, UK).

2.2. Plant materials

Two commercially available black and white Chia (*Salvia hispanica* L.) seeds were obtained from (Eichenhain– Hofgeismar–DE) and three long-day mutant genotypes, namely G3, G8 and G17, were obtained as described in (Jamboonsri, Phillips, Geneve, Cahill & Hildebrand, 2012) and were kindly supplied to the University of Basilicata (Italy) through an agreement with the University of Kentucky (US).

120 *2.3. Growth conditions*

121 Plants were grown at the latitude 40°51'46''80 N in 0.35m diameter pots with a mixture of 10:30:60
122 w:w:w of vermiculite, sand and wood litter compost. Plants were fertilized with commercial potting
123 liquid fertilizer, with a total amount corresponding to 0.53 g/plant N, and grown with a non-limiting
124 water supply.

125

126 *2.4. Extraction procedure*

127 Chia seeds were strained in order to remove extraneous matter such as dust and straw. The clean seeds
128 were blended in a laboratory mill (IKA Works MF10, Scotland, UK) in order to obtain a fine powder
129 of the organic material. Subsequently, the powder (15 g) was extracted with 80 ml of hexane for 2h
130 with stirring. The mixture was centrifuged at 3700 rpm for 10 min and the supernatant was
131 immediately stored at -80°C in the dark for later analysis. The pellet was washed twice with 20ml of
132 hexane and then centrifuged at 3700 rpm for 10 min. The supernatant was added to the previous
133 fraction and the leftover pellet was left overnight in a fume hood in order to remove the excess solvent.
134 Ten grams of defatted chia seeds were extracted with 100 ml of methanol/water (60:40). Sonication
135 was performed at 20 kHz with 50% power using a Fischer Scientific Ultrasound (model FB705,
136 700W, 2000 Park Lane, Pittsburgh, PA) with continuous stirring. The probe was a horn-type (model
137 CL-334), which was kept at constant depth in the mixture using a 250 ml glass beaker of standard
138 dimensions. During the extraction, the temperature was monitored and kept constant (25°C ± 1) using
139 a thermostatic bath. Five ml of each sample was collected after 2, 20, 40 minutes and centrifuged at
140 2500 rpm for 10 min. The supernatant was stored at -80 ºC for later analysis.

141

142 *2.5. Total polyphenol content*

143 Total polyphenolic content (TPC) was determined by spectrophotometry according to the method
144 described by Singleton and Rossi (1965) with some modifications described below: 125 µl of diluted

145 sample (1:10) was mixed with 500 µl of distilled water and 125 µl of Folin-Ciocalteu reagent. After
146 6 min, 1.25 ml of a 7.5% sodium carbonate solution was added to the mixture and brought to a final
147 volume of 3 ml with distilled water. The test tubes were then allowed to stand in the dark for 90 min
148 at room temperature. The absorbance was read at 760 nm (Thermo Scientific Genesys 10S UV-Vis
149 Spectrophotometer) and TPC was expressed in terms of gallic acid equivalents (GAE/g). A
150 calibration curve ranging from 20 to 200 µg/ml was used to quantify the TPC content in the seed
151 extracts. All determinations were performed in triplicate.

152

153 *2.6. Antioxidant activity*

154 The free radical-scavenging activity was determined according to (Re, Pellegrini, Proteggente,
155 Pannala, Yang & Rice-Evans, 1999) using the reduction of radical cation $ABTS^{\bullet+}$. A mixture of 2.5
156 ml of 7 mM ABTS and 44 µl of 140 mM potassium persulfate was prepared and left overnight in the
157 dark. The spectrophotometer wavelength was set at 734 nm. The stock solution of ABTS was diluted
158 to 1:80 until a final OD (optical density) reached a value between 0.7 and 0.8 nm. Each sample was
159 diluted 1:10 and then 100 µl were added to 1 ml of ABTS solution and after 2.5 min its reduction was
160 measured as the percentage of inhibition. Results were expressed in mmol Trolox equivalent
161 antioxidant capacity (TEAC/g) and referred to a calibration curve ranging from 25 to 250 µM. All
162 determinations were performed in triplicate.

163

164 *2.7. Sample preparation and GC-MS analysis*

165 In order to obtain volatile and stable compounds, both polar and non-polar extracts were derivatized
166 before analysis by GC-MS. For this purpose, 200 µl of the organic phase was dried under a nitrogen
167 stream and a stock solution prepared. A subsample of 75 µl (500 ppm) was dissolved in 300 µl of
168 pyridine/BSTFA + 1% TMCS (1:1). The vials were vortexed, left at 25°C for 15 min and analyzed

by GC-MS (Shareef et al., 2006). The metabolomics analysis of the polar extract needed a two step process, starting with oximation to reduce tautomerism of aldehydes and ketones, followed by OH, SH and NH silylation (Gullberg, Jonsson, Nordström, Sjöström & Moritz, 2004). An aliquot (200µl) of diluted sample (1:50) was evaporated to dryness in a vacuum centrifuge (Eppendorf Concentrator 5301) and oxymated with 50 µl of methoxyamine hydrochloride (20 mg/ml) in pyridine at 60°C for 45 min. Samples were then silylated with MSTFA at 60°C for 45 min. Both polar and non-polar extracts were analyzed in a similar way by gas chromatography - mass spectrophotometry. One µl of each derivatized sample was injected in a pulsed splitless mode into an Agilent-7820A GC system with 5977E MSD operating in EI mode at 70 eV. The system was equipped with a 30 m x 0.25 mm id fused-silica capillary column with 0.25 µm HP-5MS stationary phase (Agilent technologies, UK). The injection temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1 ml/min. Separation of the non-polar extract was achieved using a temperature program of 80°C for 1 min, then ramped at 10°C/min to 320°C and held for 1 min. The analysis of the polar compounds was performed under the following temperature program: 2 min of isothermal heating at 70°C, followed by a 10°C/min oven temperature ramp to 320°C, and a final 2 min at 320°C. The system was then temperature equilibrated for 1 min at 70°C before injection of the next sample. All spectra were recorded in the mass range 50 to 800 *m/z*.

2.8. Identification and quantification of polar compounds and fatty acids

Chromatograms and mass spectra of polar and non-polar phases, the latter containing fatty acids, were evaluated using the MassHunter Qualitative Analysis B.07.00. Mass spectra of all detected compounds were compared with standard compounds and with spectra in National Institute of Standard and Technologies library NIST MS Search 2.2. Data were processed with the AMDIS software to deconvolute co-eluting peaks. Artifact peaks, such as peaks due to derivatising agents, were not considered in the final analysis. Peak areas of multiple peaks belonging to the same

compound were summed together. The relative amount of separated metabolites was calculated from Total Ion Chromatography (TIC) by the computerized integrator and with the internal standard, malonic acid and 1-oleoyl-rac-glycerol, added to polar and non-polar extracts respectively. Relative quantification of polar compounds and fatty acids was done by integrating the peak areas of the chromatographic profiles for each metabolite and normalizing the data to the internal standards. The effect of mutation on the chemical composition of chia seeds was evaluated through the analysis of variance with SPSS software (IBM SPSS Statistics 23).

3. Results and discussion

3.1 Seed production

Total plant dry mass was higher ($p<0.05$) in commercial genotypes compared to mutants, with White chia (54.28 g/plant) > Black chia (46.83 g/plant) > G3 (34.68 g/plant) = G8 (33.06 g/plant) = G17 (35.62 g/plant). This was due to the short-day flowering behavior of commercial genotypes, which flowered 18-39 days later than the mutants at a latitude higher than 40°; their vegetative cycle was therefore longer and allowed to achieve a higher plant diameter, height and number of ramifications besides tpal and vegetative biomass (Fig. S1). The date of seed harvest ranged between 160 and 172 days after planting for the mutants G3, G8 and G17, and between 190 and 199 days for the commercial black and white seeds. The latest harvests recorded for Black and White chia occurred between November 21 and 30, at a time when average temperatures of less than 8°C did not allow a proper maturation of seeds. Therefore, in spite of a high number of spikes (> 54 per plant), the final seed yield in commercial genotypes was lower ($p<0.05$) than in mutants (11.03 and 11.33 g/plant in Black and White chia respectively vs. 12.85, 13.70 and 14.49 in G3,G8 and G17 g/plant respectively). This behavior resulted in a lower ($p<0.05$) ratio of seed/total biomass (harvest index) in the short-day flowering commercial Black and White chia vs. long-day flowering mutants (Fig. S1). Data on the comparison of commercial chia with photoperiod-mutant genotypes in the same environment is

scarce. De Falco et al. (2017) report that at high latitudes the mutant G8 produces high yields and responds well to irrigation, whereas the seed yield of commercial black chia is lower than potential due to late seed maturation, and does not respond to irrigation. Low yields at high latitudes are also found by Bochicchio et al. (2015). Our results confirm such findings and extend them to the other photoperiod mutants G3 nd G17. Moreover, we show that the whole plant architecture is involved in photoperiod response, with a higher overall growth and biomass in short-day commercial chia, due to a delay in flowering, and a lower seed yield and size because of grain filling happening too late in the season for complete maturation of all seeds in the spikes.

3.2 Total Polyphenol Content

The Total Polyphenol Content of chia seeds mutants in comparison to those of Black and White chia seeds is reported in Fig. 1 (top). For each of the genotypes, the polyphenol content increased with the time of extraction. With regard to the commercial chia seeds, results were in agreement with de Falco, Fiore, Bochicchio, Amato & Lanzotti, 2017 and with other previous reports (Amato et al., 2015, Coelho & de las Mercedes Salas-Mellado, Myriam, 2014, Reyes-Caudillo, Tecante & Valdivia-López, 2008) but lower compared to data reported by da Silva Marineli (2014). G3 had the highest content of polyphenols but differences were statistically significant ($p<0.05$) only after 2 and 20 minutes of UAE. G8 and G17 gave results that were between those for the commercial variety and G3 at each time of extraction.

3.3 Antioxidant activity

The antioxidant activity of defatted chia seeds (dcs) was also evaluated at different times and Fig. 1 (bottom) shows the TEAC results for each variety. The values of commercial seeds ranged from 1.474 ± 0.126 to 2.602 ± 0.193 mmol TEAC/g of dcs measured after 2 and 40 min, respectively. In general,

it was observed higher antioxidant activity in mutants samples compared to commercial varieties. An increase in the antioxidant activity was observed for all samples depending from the extraction time. Findings were in agreement with Sargi et al. (2013), but higher than those reported by other authors (Capitani, Spotorno, Nolasco & Tomás, 2012, Vázquez-Ovando, Rosado-Rubio, Chel-Guerrero & Betancur-Ancona, 2009). Interestingly, the Black Chia sample showed a higher antioxidant activity than that reported for the same variety in a previous work (de Falco, ,Fiore, Bochicchio, , Amato & Lanzotti, 2017), probably due to different soils and growth conditions of the plants. The highest value of antioxidant capacity (Fig. 1) was reached by G3 after 40 minutes of UAE with 3.237 ± 0.111 mmol TEAC/g of dcs, and was not significantly different from that of G17 at the same time point; the lowest value was found in White seeds after 2 min of extraction. Considering the obtained data, a relationship between the antioxidant activity and total polyphenols was observed for all samples at each time of extraction (Fig. 1). In addition, no evident correlation was shown for the content of other known antioxidant compounds, such as malic, citric and quinic acids, and the antioxidant activity (Table 3).

3.4 Metabolite profile

A metabolomics driven analysis was used to characterize a total of about 40 major and minor compounds present in polar and non-polar extracts of chia (*S. hispanica*) seeds in order to determine the effect of mutation on the metabolite composition. Representative total ion chromatograms (TIC) for both fractions are shown in Fig. 2 where each peak number corresponds to a single metabolite listed in Tables 1 and 2 with its retention time, molecular formula and *m/z* values. A base peak at *m/z* 73, typical of silylated compounds, was always detected in the chromatograms due to the $[(CH_3)_3Si]$ group. The relative quantification of each analyte was calculated from the peak area relative to that of the internal standard and reported in Table 3 and Fig. 3 for polar and non-polar extracts, respectively. Analysis, performed in triplicate, showed significant differences for some metabolites between the populations, however in other cases, the mutation did not produce such variation; this

could be due to the random mutation obtained by a chemical mutagen (Jamboonsri, Phillips, Geneve, Cahill & Hildebrand, 2012). With regard to the water extract, carbohydrates were the main metabolites, with sucrose being the most abundant one followed by methylgalactoside. This data is in accordance with our previous work (de Falco, Fiore, Bochicchio, Amato & Lanzotti, 2017, de Falco, Amato & Lanzotti, 2017, de Falco, Incerti, Bochicchio, Phillips, Amato & Lanzotti, 2017). Other sugars detected in the samples were fructose, galactose and glucose while arabinose and mannose were detected as sugar alcohols. These natural polyols are widely used in the food industry as thickeners and sweeteners, especially the mannitol that is a very important alternative treatment against diabetes due to its low glycemic index with high rate of controlling blood sugar levels (Shankar, Ahuja & Sriram 2013; Ibrahim, 2016)

Generally, G17 showed the highest content of all carbohydrates, excepted for Glc, which is more abundant in Black chia. Several organic acids were also identified in the water extract of chia populations with lactic, benzoic and citric acids as the major compounds. In particular, mutation increased the level of quinic acid in the samples, with the highest value in G17 and the lowest in white chia. G17 also showed the highest amount of lactic acid. These results are in line with another study on metabolomics of chia seeds in which an NMR spectroscopic approach was used (de Falco, Incerti, Bochicchio, Phillips, Amato & Lanzotti, 2017). On the other hand, the mutation increases the content of malic acid in G3 and G8 samples, while it produces a content of gluconic acid between that of black and white chia seeds, which have high and low content respectively. Citric acid is most abundant in G3 sample. Interestingly, black and white chia seeds have a low content of caffeic acid, whereas mutant samples showed high concentration of this metabolite, especially G8 and G17. In Chia seeds, caffeic acid can be bound to quinic acid at different positions to give rise to caffeoylquinic derivatives. However, we did not found in our extracts the caffeoylquinic derivatives but we found quinic and caffeic acids as two different metabolites. This could be due to the high temperature of the GC-MS analysis. Catechollactate, commonly named danshensu, was also found in all samples analyzed, with highest amount in G17 genotype. This is the second reporter of the finding of

294 danshensu in commercial chia seeds. Previous finding was observed very recently by Oliveira-Alves
295 et al. 2017 in chia seeds originating from Brazil and Chile.
296 Among the amino acids, Asp, Glu and GABA reached the highest value in G17, also as reported by
297 de Falco et al. (2017).
298 In relation to the organic extract, the main fatty acids detected by GC-MS analysis were linolenic acid
299 (18:3), linoleic acid (18:2), stearic acid (18:0), palmitic acid (16:0) and oleic acid (18:1), determined
300 as TMS derivatives and listed in Table 2. These results are in agreement with other published papers
301 (Amato et al., 2015, da Silva Marineli, Moraes, Lenquiste, Godoy, Eberlin & Maróstica Jr, 2014,
302 Peiretti & Gai, 2009). A preliminary analysis of chromatograms showed C18:3 as the most abundant
303 fatty acid in all samples, data also reported in previous works (Amato et al., 2015, Coelho & de las
304 Mercedes Salas-Mellado, Myriam, 2014, da Silva Marineli, Moraes, Lenquiste, Godoy, Eberlin &
305 Maróstica Jr, 2014, de Falco, Amato & Lanzotti, 2017). In the MS spectrum of saturated fatty acids
306 trimethylsilyl esters, such as C16:0 and C18:0, there are three characteristic *m/z* values at 313, 341
307 and 132 which correspond to the loss of methyl from the TMS ester group and the McLafferty
308 rearrangement ion. Analysis of variance on the non-polar fraction showed significant differences for
309 each metabolite at $p<0.05$. Our results, reported in Table S1, ranging from 37.24 to 39.02%, are in
310 line with many reports of Chia oil content (de Falco, Amato & Lanzotti, 2017).
311 The amount of fatty acids (Fig. 3) was found to be highest in White and G3 chia seeds except for
312 C18:1 which was higher in G8 and G17 samples. In particular, C16:0, C18:2, C18:3, C18:0 and C17:1
313 showed the highest value in White and the lowest in Black seeds with mutant genotypes giving
314 intermediate values within this range. In addition, the ω-3/ω-6 ratio found was not lower than 3:1 for
315 all analyzed samples (Table S1). The data obtained showed that within fatty acids ω-3 is the most
316 abundant component, in particular the content of α-linolenic acid (C18:3) is more than 50% of all
317 fatty acids. This is in accordance with previous work (Ayerza, 1995, Segura-Campos, Ciau-Solís,
318 Rosado-Rubio, Chel-Guerrero & Betancur-Ancona, 2014). Therefore, Chia seeds, differently from

other *Salvia* species (Özcan, 2015, Figueredo, 2012, Akgül, 1999) can be considered as a natural source of ω-3, which plays a very important role in human nutrition and health due to its anti-inflammatory, anti-arrhythmic and anti-thrombotic activity (de Falco, Amato & Lanzotti, 2017).

4 Conclusions

This work was aimed at characterizing the metabolome of chia through a high-recovery technique for metabolites in polar and non-polar extracts. Commercial chia populations were compared with short-day flowering mutants at a latitude higher than the area of origin, with the hypothesis that their performance would be different.

UAE proved an efficient extraction procedure and GC-MS analysis was used for the first time to detect differences in the metabolic profile of chia seeds of different sources.

Short-day genotypes flowered earlier and were able to produce a higher yield of chia seeds than commercial varieties, in spite of a lower total biomass. Results showed an increase of TPC correlated with antioxidant activity after 40 minutes of UAE and the mutants, especially G3 genotype, had the highest value. The G17 mutant showed a significantly higher amount of LA, Gly, Asp, Glu, QUI, Cat and CA.

Results allowed to identify the mutants G8 and G17 as overall better due to the high level of compounds with nutraceutical value, and therefore potentially interesting for food/feed industries besides their potential for production even outside the area of origin thanks to the ability to flower at shorter days than commercial chia sources.

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345 revision of manuscript.

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Highlights

- Chia metabolomics in response to mutation is reported for the first time
- Mutants showed higher seed yield than commercial seeds
- Antioxidant activity correlated with total polyphenolic content
- ω-3 was the most abundant component of all fatty acids
- Mutation affects chemical composition increasing nutraceutical properties

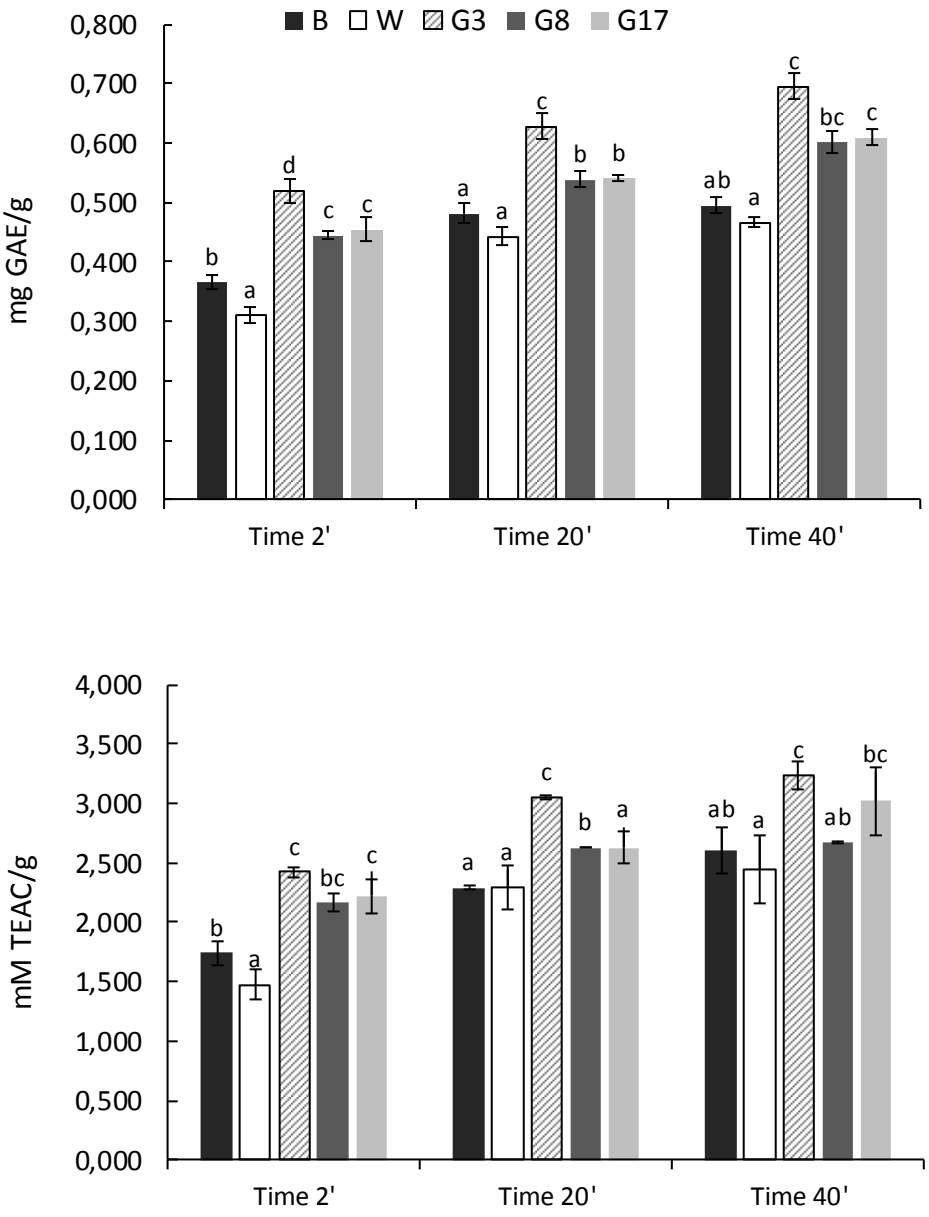

Figure 1. Total Polyphenol Content (top) and antioxidant activity (bottom) of defatted Chia seeds (*Salvia hispanica* L.) measured after 2, 20, 40 minutes of UAE. B, Black Chia; W, White Chia; G3, G8 and G17, mutant genotypes. Different letters indicate significant differences ($p<0.05$) at the post-hoc Tukey's test.

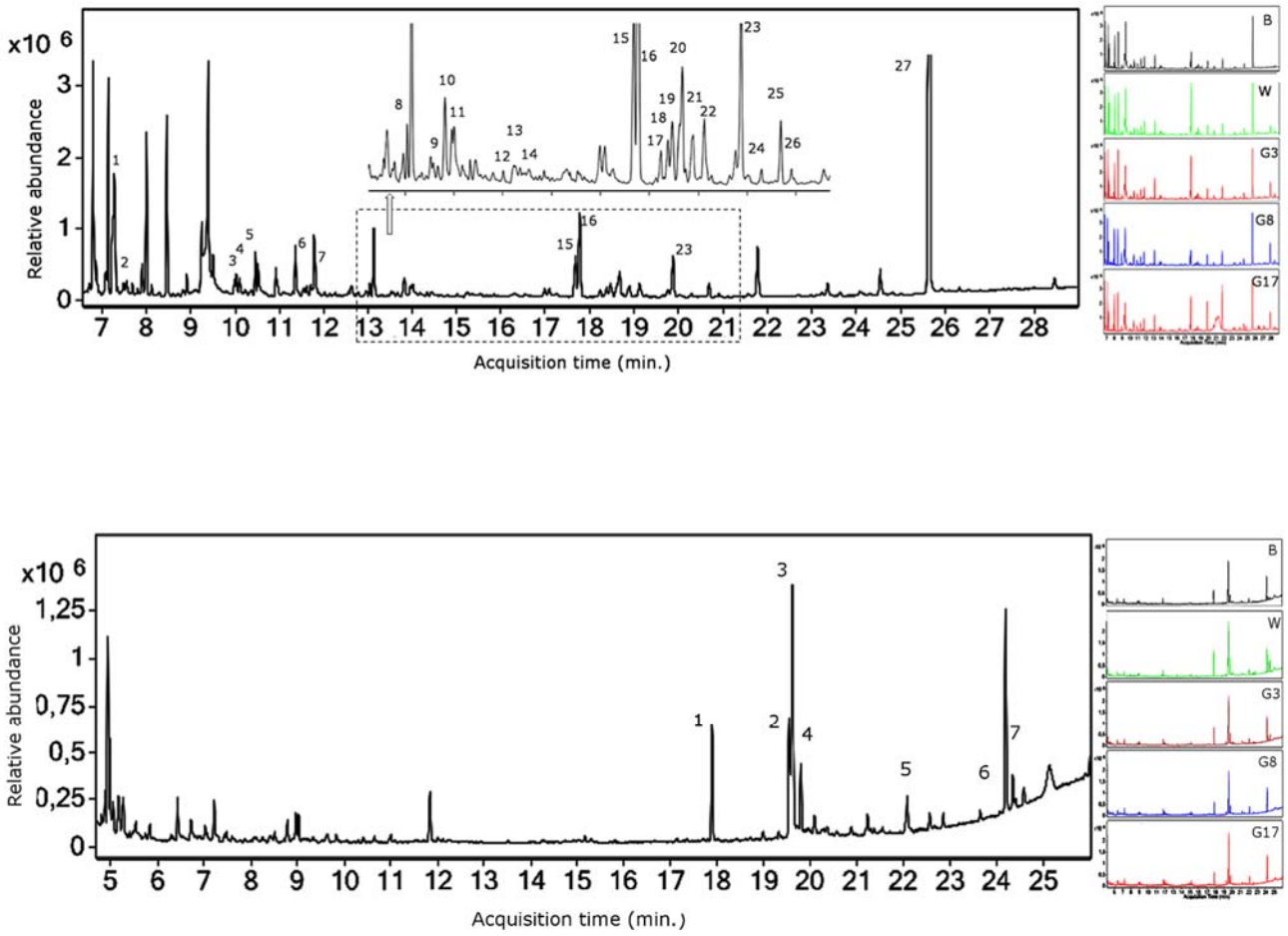

Figure 2. Total Ion Chromatograms (TIC) of polar fraction (Top) and non-polar fraction (bottom) of Chia seeds (*S. hispanica*). Chromatograms of Black Chia (B) are taken as representative model (left); chromatograms of all analysed samples B, W, G3, G8 and G17 (from top to bottom, right).

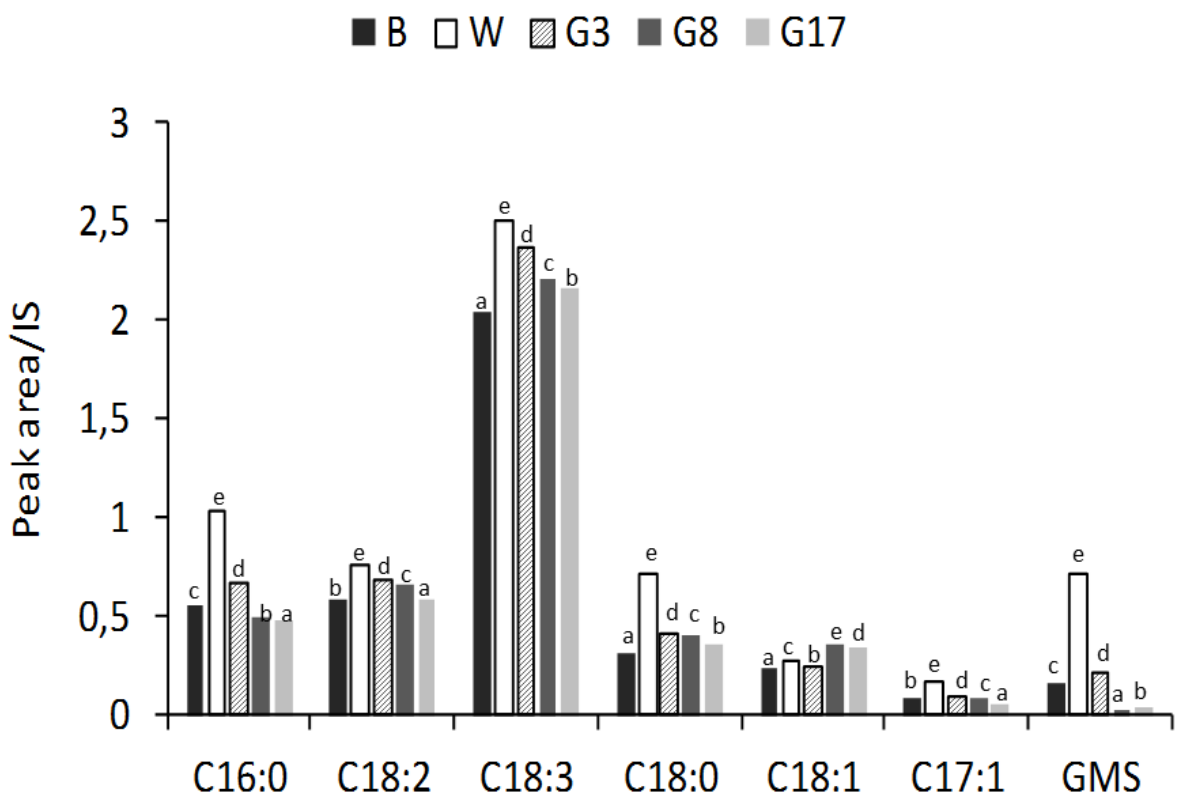

Figure 3. Relative amount of *non-polar* metabolites detected by GC-MS analysis. Different letters indicate significant differences ($p<0.001$) at the post-hoc Tukey's test. B, Black Chia; W, White Chia; G3, G8 and G17, mutant genotypes.

Table 1. Detected compounds, with their retention time, in chia seeds water extract after UAE. Peak number, molecular formula of trimethylsilyl derivatives and their *m/z* are also reported.

Peak number	RT (min)	Molecular formula	*m/z*	Detected compounds	Abbreviation
1	7.25	$C_9H_{22}O_3Si_2$	219, 191, 147, 133, 117, 73, 45	Lactic Acid	LA
2	7.32	$C_9H_{18}O_4Si$	175, 89, 73	Methyl 2-ethyl malonate	Me-MA
3	9.99	$C_{10}H_{16}F_3NO_3$	154, 110	L-Alanine, N-methyl-N-(trifluoroacetyl)-, butyl ester	Ala-Obu
4	10.03	$C_{10}H_{14}O_2Si$	194, 179, 135, 105, 77	Benzoic Acid	BE
5	10.46	$C_{12}H_{32}O_3Si_3$	218, 205, 147, 117, 89, 73, 45	Glycerol	GLY
6	11.74	$C_{12}H_{31}NO_3Si_3$	218, 204, 147, 100, 73	L-serine	Ser
7	12.14	$C_{13}H_{33}NO_3Si_3$	291, 218, 147, 117, 73	L-Threonine	Thr
8	13.04	$C_{17}H_{40}N_2O_3Si_3$	404, 287, 73	N-α-Acetyl-L-Lysine	AcLys
9	13.52	$C_{13}H_{30}O_5Si_3$	245, 233, 147, 133, 73	Malic acid	MA
10	13.97	$C_{13}H_{31}NO_4Si_3$	232, 218, 147, 100, 73	L-Aspartic acid	Asp
11	14.00	$C_{11}H_{23}NO_3Si_2$	258, 230, 156, 133, 73, 45	L-5-Oxoproline	PCA
12	15.21	$C_{14}H_{33}NO_4Si_3$	246, 147, 128, 73	L-Glutamic acid	Glu
13	15.35	$C_{15}H_{27}NO_2Si_2$	218, 192, 147, 100, 73	Phenylalanine	Phe
14	15.54	$C_{28}H_{62}O_6Si_4$	549, 417, 389, 147, 73	Tartaric acid	TA
15	17.69	$C_{18}H_{40}O_7Si_4$	273, 147, 73, 45	Citric acid	CI
16	17.77	$C_{19}H_{46}O_6Si_4$	243, 217, 204, 133, 73	Methyl galactoside	mGal
17	18.24	$C_{22}H_{52}O_6Si_5$	345, 255, 191, 147, 73	Quinic acid	QUI
18	18.37	$C_{22}H_{55}NO_6Si_5$	217, 307	D-Fructose MEOX	FRU
19	18.48	$C_{20}H_{52}O_5Si_5$	307, 277, 217, 189, 147, 103, 73	Arabitol	Arab
20	18.61	$C_{22}H_{55}NO_6Si_5$	319, 205, 147, 103, 73	D-Galactose MEOX	Gal
21	18.67	$C_{22}H_{55}NO_6Si_5$	364, 319, 205, 147, 73	D-Glucose MEOX	Glc
22	19.04	$C_{24}H_{62}O_6Si_6$	421, 345, 319, 205, 147, 103, 73	D-Mannitol	MAN
23	19.77	$C_{24}H_{60}O_7Si_6$	333, 292, 205, 147, 103, 73	D-Gluconic acid	GLUC
24	20.30	$C_{21}H_{42}O_5Si_4$	396, 267, 179, 147, 73	Catechollactate (Danshensu)	Cat
25	20.69	$C_{24}H_{60}O_6Si_6$	305, 217, 147, 129, 73	Myo-Inositol	Myo
26	20.91	$C_{18}H_{32}O_4Si_3$	396, 381, 219, 191, 73	Caffeic acid	CA
27	25.66	$C_{36}H_{86}O_{11}Si_8$	437, 361, 319, 271, 217, 147, 103, 73	Sucrose	Sucr

Table 2. *Non-polar* metabolites analysed by GC-MS as trimethylsilyl derivatives.

Peak number	RT (min)	Molecular formula	*m/z*	Detected compounds	Abbreviation
1	17.88	$C_{19}H_{40}O_2Si$	328, 313, 145, 117, 73	Palmitic Acid	C16:0
2	19.54	$C_{21}H_{40}O_2Si$	337, 129, 95, 75, 73	Linoleic acid	C18:2
3	19.61	$C_{21}H_{38}O_2Si$	335, 129, 95, 75, 73	α-Linolenic acid	C18:3
4	19.80	$C_{21}H_{44}O_2Si$	356, 341, 132, 117, 73	Stearic acid	C18:0
5	22.07	$C_{21}H_{42}O_2Si$	354, 339, 129, 117, 73	Oleic acid	C18:1
6	24.05	$C_{20}H_{40}O_2Si$	340, 325, 145, 129, 117, 73	10-Heptadecenoic acid	C17:1
7	24.34	$C_{27}H_{58}O_4Si_2$	487, 399, 147, 73	Glycerol monostearate	GMS

Table 3. Relative abundance (peak area/IS) of main metabolites detected by GC-MS analysis. Data refers to mean and standard deviation of 3 replicates. Results of ANOVA and post-hoc Tukey's test are also reported. Different case letters indicate significant differences ($p<0.05$).

	Chia seeds populations				
	B	**W**	**G3**	**G8**	**G17**
Metabolites					
LA	0,921 ± 0,04 ab	0,681 ± 0,35 b	0,861 ± 0,13 ab	0,985 ± 0,13 ab	1,235 ± 0,17 a
Me-MA	0,024 ± 0,00 ab	0,019 ± 0,01 ab	0,029 ± 0,00 ab	0,033 ± 0,00 a	0,013 ± 0,01 b
Ala-Obu	0,077 ± 0,00 bc	0,054 ± 0,00 d	0,085 ± 0,00 bc	0,068 ± 0,01 c	0,122 ± 0,00 a
BE	0,090 ± 0,01 a	0,045 ± 0,01 b	0,040 ± 0,01 b	0,043 ± 0,01 b	0,091 ± 0,02 a
GLY	0,171 ± 0,00 b	0,088 ± 0,00 c	0,141 ± 0,02 bc	0,172 ± 0,02 b	0,339 ± 0,04 a
Ser	0,028 ± 0,00 ab	0,032 ± 0,00 a	0,028 ± 0,00 a	0,021 ± 0,00 c	0,022 ± 0,00 bc
Thr	0,006 ± 0,00 b	0,007 ± 0,00 ab	0,007 ± 0,00 ab	0,007 ± 0,00 ab	0,010 ± 0,00 a
AcLys	0,043 ± 0,00 b	0,050 ± 0,00 b	0,048 ± 0,00 b	0,052 ± 0,01 b	0,078 ± 0,01 a
MA	0,024 ± 0,00 a	0,029 ± 0,00 a	0,017 ± 0,00 b	0,016 ± 0,00 b	0,024 ± 0,00 a
Asp	0,059 ± 0,00 b	0,077 ± 0,01 b	0,054 ± 0,01 b	0,064 ± 0,01 b	0,112 ± 0,02 a
PCA	0,088 ± 0,01 b	0,108 ± 0,00 b	0,111 ± 0,02 b	0,110 ± 0,01 b	0,174 ± 0,03 a
Glu	0,040 ± 0,00 ab	0,044 ± 0,03 ab	0,027 ± 0,01 b	0,024 ± 0,00 b	0,133 ± 0,07 a
Phe	0,018 ± 0,00 ab	0,016 ± 0,00 ab	0,013 ± 0,00 b	0,018 ± 0,00 ab	0,023 ± 0,01 a
TA	0,038 ± 0,00 b	0,011 ± 0,01 b	0,048 ± 0,01 b	0,049 ± 0,01 ab	0,104 ± 0,04 a
CI	0,002 ± 0,00 b	0,003 ± 0,00 b	0,309 ± 0,04 a	0,002 ± 0,00 b	0,207 ± 0,20 ab
mGal	0,535 ± 0,00 b	1,631 ± 0,10 a	1,530 ± 0,21 a	0,520 ± 0,07 b	1,886 ± 0,25 a
QUI	0,040 ± 0,01 bc	0,017 ± 0,00 c	0,056 ± 0,01 b	0,040 ± 0,01 bc	0,104 ± 0,02 a
FRU	0,063 ± 0,01 b	0,095 ± 0,00 ab	0,110 ± 0,01 ab	0,082 ± 0,01 b	0,139 ± 0,04 a
Arab	0,104 ± 0,00 ab	0,085 ± 0,01 b	0,108 ± 0,01 ab	0,090 ± 0,01 b	0,155 ± 0,05 a
Gal	0,094 ± 0,00 b	0,227 ± 0,02 a	0,212 ± 0,02 a	0,083 ± 0,01 b	0,256 ± 0,02 a
Glc	0,215 ± 0,01 a	0,135 ± 0,00 bc	0,155 ± 0,02 bc	0,121 ± 0,02 c	0,156 ± 0,01 b
MAN	0,001 ± 0,00 c	0,003 ± 0,00 c	0,022 ± 0,00 b	0,024 ± 0,01 b	0,040 ± 0,01 a
GLUC	0,044 ± 0,00 a	0,007 ± 0,00 c	0,022 ± 0,01 b	0,027 ± 0,00 b	0,027 ± 0,00 b
Cat	0,261 ± 0,00 b	0,164 ± 0,15 b	0,285 ± 0,15 b	0,265 ± 0,12 b	1,302 ± 0,47 a
Myo	0,017 ± 0,00 e	0,145 ± 0,12 a	0,035 ± 0,01 d	0,036 ± 0,00 c	0,055 ± 0,01 b
CA	0,021 ± 0,00 d	0,013 ± 0,01 e	0,025 ± 0,02 c	0,657 ± 0,64 b	1,585 ± 1,37 a
Sucr	5,86 ± 0,07 c	9,80 ± 0,67 b	11,19 ± 1,57 ab	8,08 ± 1,08 bc	13,70 ± 1,68 a
GABA	0,082 ± 0,00 c	0,107 ± 0,00 bc	0,117 ± 0,01 ab	0,084 ± 0,01 c	0,141 ± 0,02 a