

# **Metabolomic analysis by UAE-GC MS and antioxidant activity of *Salvia hispanica* (L.) seeds grown under different irrigation regimes**

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1 **Metabolomic Analysis by UAE-GC MS and antioxidant activity of *Salvia hispanica* (L.) seeds**  
2 **grown under different irrigation regimes**

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23 **Running Title:** Metabolomics and antioxidant activity of chia seeds and the effect of irrigation  
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25

26 **Abstract**

27 Chia (*Salvia hispanica* L.) is an emerging crop with a high content of  $\alpha$ -linolenic acid and metabolites  
28 of industrial and pharmaceutical interest but information on metabolome variations in response to  
29 agricultural management is scarce. We investigated the yield and metabolic profile of the seeds of  
30 two chia populations, one commercial black (B) and one long-day flowering genotype (G8), in  
31 response to two irrigation levels: replacement of 100% ET<sub>0</sub> (I) or rainfed (NI). Seed yield was higher  
32 in irrigated plots in G8 only (0.255 kg m<sup>-2</sup> for I vs 0.184 kg m<sup>-2</sup> for NI) while it was very low  
33 regardless of irrigation in B due to late flowering. Ultrasound assisted extraction (UAE) of seeds  
34 followed by gas chromatography-mass spectrometry (GC/MS) analysis showed differences in fatty  
35 acids and the major classes of organic compounds due to both genotype and irrigation, especially in  
36 the non-polar phase where irrigated samples showed a higher content of  $\alpha$ -linolenic and other fatty  
37 acids and a lower oleic/linoleic ratio (47.4 in NI vs. 39.6 in I). The antioxidant activity, expressed as  
38 trolox equivalent antioxidant capacity (TEAC), ranged from 1.317 ± 0.027 to 2.174 ± 0.010 mmol  
39 TEAC/g of defatted chia seed after 2 and 40 min respectively, and was negatively affected by  
40 irrigation. The total polyphenolic content (TPC) measured with the Folin-Ciocalteu method, also  
41 decreased with irrigation. According to our results irrigation can affect chia yield, metabolome and  
42 antioxidant behavior but some of the effects are genotype-dependent.

43

44 **Keywords:** Chia; Polyphenols; TPC; Management; Alpha-linolenic acid; Quality

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## 47 **1. Introduction**

48 Chia (*Salvia hispanica* L.) is an ancient short-day flowering crop with center of origin between  
49 Mexico and Guatemala (Cahill, 2004). It was one of the staple crops in pre-Columbian Central  
50 America but its diffusion was drastically reduced after the Spanish domination. Chia was re-  
51 discovered in the 90s by researchers intending to propose alternative crops to farmers (Coates, 1996;  
52 Gentry et al., 1990), and has thereafter spread to several areas of the world, at first due to favorable  
53 market placement linked to its nutraceutical properties, and more recently as a source of compounds  
54 of cosmetic, medical and industrial interest (Hermoso-Diaz et al., 2014; Lu and Foo, 2002; Muñoz et  
55 al., 2013). The *Salvia* genus includes more than 900 species, and in the New World around 500  
56 species belong to the subgenus *Calosphace* (Benth.) and may be grouped in complexes of species  
57 with common medicinal activity, mostly related to the content of terpenes in leaves. Jenks and Kim  
58 (2013) listed external application (antimicrobial, skin problems and rheumatisms) and internal uses  
59 (gastro-intestinal, gynecological and neurological) in the *mirto* complex, primary uses for the  
60 respiratory system in the *Nucchu* complex, and primary uses for biliary and kidney problem in the  
61 *Li'l ++*, *Cantueso* and *Manga-paqui* complexes. They also report that *S. divinorum* Epling and  
62 *Játiva-M.* are the most studied medicinal sage leaves within the *Calosphace* subgenus, due to their  
63 use as a hallucinogen by Mexican shamans and their selective kappa-opioid receptor agonist activity.  
64 The chia complex, including 18 species besides *S. hispanica* (2013), on the other hand, is the most  
65 renowned for the production of indehiscent dry fruits, commonly referred to as “seeds”, rich in oil.  
66 Chia seeds are one of the richest natural sources of omega 3 fatty acids (Ayerza and Coates, 2011;  
67 Ayerza, 1995) and show a high content of protein with a balanced composition in essential amino  
68 acids (Ayerza, 2013) and fiber (Capitani et al., 2012). A part of the fiber is located in the outer cells  
69 of the fruit and is extruded at the fruit surface upon hydration (Muñoz et al., 2012), forming a  
70 mucilaginous capsule with rheological properties that make it promising for industrial and medical  
71 uses: it is highly hygroscopic, viscous and adhesive (Švec et al., 2016). Many antioxidants have been  
72 identified in chia seeds, extracted oil and mucilage, especially phenolic acids and flavonoids, besides  
73 poly-unsaturated fatty acids (Amato et al., 2015; da Silva Marineli et al., 2014). Chia seeds or their  
74 products are therefore increasingly proposed not only as food but also as a component for  
75 biodegradable film (Capitani et al., 2016; Muñoz et al., 2012), thickening agents (Coelho and de las  
76 Mercedes Salas-Mellado, Myriam, 2015; Felisberto et al., 2015; Iglesias-Puig and Haros, 2013;  
77 Menga et al., 2017), anti-corrosive agents (Hermoso-Diaz et al., 2014), cosmetics (Muñoz et al.,  
78 2013) and medicaments (Vuksan et al., 2010). A strong variation in chia seeds’ composition has been  
79 reported: for instance, oil content ranges from little over 20% to over 36% (Ayerza and Coates, 2004;

80 Ayerza, 1995; Coelho and de las Mercedes Salas-Mellado, Myriam, 2014; da Silva Marineli et al.,  
81 2014; Ixtaina et al., 2011). Variability has mainly been researched in relation to genotype and  
82 environment: Ayerza (2009) reports a range in total fat content from 25.93% to 33.50% for the same  
83 genotype of chia grown in five different environments. The fatty acids profile, especially the content  
84 of  $\alpha$ -linolenic acid, is also affected by elevation in seeds of this species, even within the same  
85 genotype (Ayerza and Coates, 2011; Martínez-Cruz and Paredes-López, 2014). This is probably  
86 largely due to thermal effects of elevation, as reported by Ayerza and Coates (2004) who found a  
87 correlation between temperature and chia oil fatty acids measured across different environments; they  
88 concluded that levels of fatty acids' unsaturation in chia increase at cooler temperatures, as observed  
89 for other oil seed crops due to saturase-desaturase dynamics. In an experiment conducted across  
90 different countries in America, Ayerza (2009) reports a direct relationship between elevation and oil  
91 content and an inverse relationship between elevation and the content of proteins. Several  
92 environmental variables might be involved besides temperature, including soil properties and a  
93 negative correlation between oil and protein as found in other crops. Ayerza and Coates (2011)  
94 suggest that the relationships of oil and protein content and oil saturation with elevation are strong  
95 enough that they could be used to trace the growing environment of chia. Ayerza (2009) also found  
96 differences in protein content for the same genotypes grown in different environments, but could not  
97 prove differences among genotypes within a site, except for one variety at one site. In a further study,  
98 Ayerza (2013) could not find significant differences between two genotypes of different seed coat  
99 color for protein, oil, fiber, amino acids, and antioxidant content. (Silva et al., 2016) found that a  
100 white and a black seed crop with the same seed yield produced different amounts of unsaturated fatty  
101 acids: the white seed genotype yielded more linoleic and  $\alpha$ -linolenic acids (6.0 and 17.0 kg ha<sup>-1</sup>  
102 respectively) than the black seed one (4.4 and 16.7 kg ha<sup>-1</sup> respectively). De Falco et al. (2017b)  
103 studied the metabolic profile of the seeds of seven chia populations, including commercial and early  
104 flowering mutant genotypes, and showed significant differences in the metabolic fingerprinting of  
105 the different populations using nuclear magnetic resonance (NMR) and chemometrics. An  
106 investigation of the metabolome with gas chromatography-mass spectrometry has not yet been  
107 performed on chia seeds after agronomic management.

108 Very little information is available about the variation in chia seeds composition with agronomic  
109 management. Amato et al. (2015) compared nitrogen fertilization regimes on chia seed composition  
110 and found a higher *p*-anisidine value, content of phenols and oxidative stability in plots fertilized with  
111 organic nitrogen only, whereas the addition of mineral nitrogen in topdressing increased free acidity,  
112 chlorophyll and carotenoids content. De Falco et al. (2017b) reported that the effect of mineral  
113 nitrogen supply on chia positively affects the content of aliphatic free amino acids, and negatively

114 that of the main carbohydrates and flavonoids. Heuer et al. (2002) found that salinity of irrigation  
115 water decreases the oil content of chia seeds and increased their content of palmitic and linoleic acids.  
116 Irrigation is one of the major agronomical factors conditioning crop yield and composition, and  
117 namely that of oilseeds (Flagella et al., 2002). Silva et al. (2016) did not find a significant effect of  
118 irrigation on chia seed yield and content of linoleic and  $\alpha$ -linolenic acids. However authors point out  
119 that their experiment was conducted using short-day flowering genotypes at a latitude higher than  
120 optimal; due to photoperiod sensitivity flowering was delayed and temperatures during seed  
121 maturation were too low to allow complete grain filling. In this condition, temperature and not water  
122 was the limiting factor and even fully irrigated plots yielded poorly. More information is therefore  
123 needed on the response of chia to irrigation taking photoperiod sensitivity into account. The objective  
124 of this research was to study the response of chia to irrigation with the hypothesis that irrigation  
125 affects the yield of chia seeds, the fatty acid profile and the production of secondary metabolites found  
126 in the polar and non-polar extracts. The hypothesis that responses to irrigation are different in short-  
127 day and long-day flowering genotypes at high latitudes was also tested by using a short-day  
128 commercial chia seed source and a long-day flowering mutant.

## 129 **2. Materials and methods**

### 130 *2.1 Plant material*

131 Black chia (*Salvia hispanica* L.) seeds (B) were obtained from a commercial retailer (Eichenhain-  
132 Hofgeismar-DE) and seeds of one long-day flowering mutant genotype (G8) were obtained as  
133 described in Jamboonsri et al. (2012) and were kindly supplied through an agreement between the  
134 University of Basilicata and the University of Kentucky (US).

### 135 *2.2 Growth conditions*

136 Plants were grown in Basilicata (Southern Italy-Lat. N 40° 51' 37,59" Lon. E 15° 38' 49,43") on a  
137 Luvi-vertic Phaeozem (IUSS, 2007), loam soil (43.6% of sand, 34.2% silt and 22.1% clay) in the  
138 period June-December 2014. Soil water content was 0.279 g g<sup>-1</sup> at -0.03 MPa and 0.137 g g<sup>-1</sup> at -1.5  
139 MPa. A field factorial randomized block design with three replications was established to test the two  
140 genotypes B and G8 with two levels of irrigation:

141 NI = no irrigation

142 I = nonlimiting water supply. We provided 100% of ET<sub>0</sub> corresponding to the evaporative demand of  
143 the atmosphere (Allen et al., 1998) measured with a TE-ETG atmometer (Tecnoel, Rm Italy).

144

145 The irrigation system was drip with pre-installed emitter lines with drippers at 200 mm distance and  
146 maximum flow rate  $6.6 \times 10^{-5} \text{ m}^3 \text{ s}^{-1}$  at  $1 \times 10^5 \text{ Pa}$ . Plot size was 8 x 5 m with a density of 20 plants  $\text{m}^{-2}$ .  
147 The crop was sown on June 26, 2014. Precipitation and irrigation amounts are shown in Fig.1; total  
148 precipitation was 197 mm during the experiment and all plots received the same initial regime with  
149 52 mm of irrigation to help crop establishment. Treatments were differentiated at 51 DAS (day after  
150 sowing) when the I treatment was left in rainfed conditions and the I treatment was irrigated receiving  
151 172 mm of further irrigation. Evaporation was read daily and irrigation was performed when a set  
152 amount was reached. In order to cover the crop's needs for root establishment and full deployment of  
153 deep rooting potential, irrigation schedule and amount ranged from irrigation with around 4.5 mm  
154 every 4 days up to 15 DAS, to an irrigation amount of around 14 mm on average per irrigation  
155 thereafter. This corresponded to a number and timing of irrigations varying according to evaporation  
156 and the timing of precipitation: 5 irrigations in July (with a maximum of 10 days without irrigation  
157 due to precipitation), 10 in August (every 2-4 days), 2 in September (with a maximum of 26 days  
158 without irrigation due to precipitation) and October (with a maximum of 21 days without irrigation  
159 due to precipitation). Seeds of G8 and B were harvested at 132 and 173 DAS respectively, on one  
160 sample of 20 plants per replication. A trench dug after harvest showed roots up to 2 m and a total soil  
161 depth exceeding 3 m.

### 162 *2.3 Chemicals and reagents*

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

### 172 *2.4 Extraction procedure of the organic phase*

173 Chia seeds were strained in order to remove extraneous matter such as dust and straw. The clean seeds  
174 were blended in a laboratory mill (IKA Works MF10, Scotland, UK) in order to obtain a fine powder  
175 of the organic material. Subsequently, the powder (15g) was extracted with 80 ml of *n*-hexane for 2  
176 h under stirring. The mixture was centrifuged at 3700rpm for 10 min and the supernatant was

177 immediately stored at -80°C in the dark for later analysis. The pellet was washed twice with 20ml of  
178 *n*-hexane and then centrifuged at 3700rpm for 10 min. The supernatant was added to the previous  
179 fraction and the leftover pellet was left overnight in a fume hood in order to remove the excess solvent.

#### 180 *2.5 Ultrasound assisted extraction (UAE)*

181 The defatted chia seeds (10g) were extracted with 100ml of methanol/water (60:40). Sonication was  
182 performed at 20 kHz with 50% power using a Fischer Scientific Ultrasound (model FB705, 700W,  
183 2000 Park Lane, Pittsburgh, PA) with continuous stirring. The probe was a horn-type (model CL-  
184 334), which was kept at constant depth in the mixture using a 250ml glass beaker of standard  
185 dimensions. During the extraction, the temperature was monitored and kept constant (25°C ± 1) using  
186 a thermostatic bath. Samples (5ml) were collected after 2, 20, 40 min and centrifuged at 2500rpm for  
187 10 min. The supernatant was stored at -80°C for later analysis.

#### 188 *2.6 Total polyphenol content*

189 Total polyphenolic content (TPC) was determined by spectrophotometry according to the method  
190 described by Singleton and Rossi (1965) with some modifications: 125µl of diluted sample (1:10)  
191 were mixed with 500µl of distilled water and 125µl of Folin-Ciocalteu reagent. After 6 min, 1.25ml  
192 of a 7.5% sodium carbonate solution were added to the mixture and brought to a final volume of 3ml  
193 with distilled water. The test tubes were then allowed to stand in the dark for 90 min at room  
194 temperature. The absorbance was read at 760nm (Thermo Scientific Genesys 10S UV-Vis  
195 Spectrophotometer) and TPC was expressed in terms of gallic acid equivalents (GAE/g). A  
196 calibration curve ranging from 20 to 200µg ml<sup>-1</sup> was used to quantify the TPC content in the seed  
197 extracts. All determinations were performed in triplicate.

#### 198 *2.7 Antioxidant activity*

199 The free radical-scavenging activity was determined according to Re et al., (1999) using the reduction  
200 of radical cation 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS<sup>•+</sup>).  
201 A mixture of 2.5ml of 7 mM ABTS and 44µl of 140mM potassium persulfate was prepared and left  
202 overnight in the dark. The spectrophotometer wavelength was set at 734nm. The stock solution of  
203 ABTS was diluted to 1:80 until a final OD (optical density) reached a value between 0.7 and 0.8nm.  
204 Samples were diluted 1:10 and 100µl were added to 1ml of ABTS solution. After 2.5 min the  
205 reduction was measured as the percentage of inhibition. Results were expressed in mmol Trolox



206 equivalent antioxidant capacity (TEAC/g) and referred to a calibration curve ranging from 25 to  
207 250µM. All determinations were performed in triplicate.

## 208 2.8 *Derivatization and GC/MS analysis*

209 In order to obtain volatile and stable compounds, both polar and non-polar extracts were derivatized  
210 before analysis by GC/MS. For this purpose, the organic phase was dried under nitrogen stream and  
211 a stock solution (500ppm) was prepared. A subsample of 75µl was dissolved in 300µl of  
212 pyridine/BSTFA + 1% TMCS (1:1). The vials were vortexed, left at 25°C for 15 min and analyzed  
213 by GC/MS (Shareef et al., 2006). The metabolomic analysis of the polar extract needed a two-step  
214 process, starting with oximation to reduce tautomerism of aldehydes and ketones, followed by OH,  
215 SH and NH silylation (Gullberg et al., 2004). An aliquot (200µl) of diluted sample (1:50) was  
216 evaporated to dryness in a vacuum centrifuge (Eppendorf Concentrator 5301) and oxymated with  
217 50µl of methoxyamine hydrochloride (20mg ml<sup>-1</sup>) in pyridine at 60°C for 45 min. Samples were then  
218 silylated with MSTFA at 60°C for 45 min. Both polar and non-polar extracts were analyzed in a  
219 similar way by gas chromatography - mass spectrophotometry. Derivatized samples were injected  
220 (1µl) in a pulsed splitless mode into an Agilent-7820A GC system with 5977E MSD operating in EI  
221 mode at 70eV. The system was equipped with a 30m x 0.25mm id fused-silica capillary column with  
222 0.25µm HP-5MS stationary phase (Agilent technologies, UK). The injection temperature was set at  
223 270°C. Helium was used as carrier gas at a constant flow rate of 1ml min<sup>-1</sup>. Separation of the non-  
224 polar extract was achieved using a temperature program of 80°C for 1 min, then ramped at 10°C/min  
225 to 320°C and held for 1 min. The analysis of the polar compounds was performed under the following  
226 temperature program: 2 min of isothermal heating at 70°C, followed by a 10°C/min oven temperature  
227 ramp to 320°C, and a final 2 min at 320°C. The system was then temperature equilibrated for 1 min  
228 at 70°C before injection of the next sample. All spectra were recorded in the mass range 50 to 800  
229 *m/z*. Both chromatograms and mass spectra were evaluated using the MassHunter Qualitative  
230 Analysis B.07.00 (Agilent Technologies, CA, USA). Mass spectra of all detected compounds were  
231 compared with standard compounds and with spectra in National Institute of Standard and  
232 Technologies library NIST MS Search 2.2 (<https://www.nist.gov>). Data was processed with the  
233 AMDIS (Agilent Technologies, CA, USA) software to deconvolute co-eluting peaks. Artifact peaks,  
234 such as peaks due to derivatizing agents, were not considered in the final analyses. Peak areas of  
235 multiple peaks belonging to the same compound were summed together. The relative amount of  
236 separated metabolites was calculated from Total Ion Chromatography (TIC) by the computerized  
237 integrator and with the internal standard, malonic acid and 1-oleoyl-rac-glycerol, added to polar and  
238 non-polar extracts respectively.

## 239 2.9 Statistical analysis

240 Relative quantification was done by integrating the peak areas of the chromatographic profiles for  
241 each compound and normalizing the data to the internal standards. The effect of genotype, irrigation  
242 and their interaction on the chemical composition of chia seeds was evaluated through the analysis of  
243 variance with the R software (RDevelopment, 2012) after checking for normality and  
244 homoscedasticity of data.

## 245 3. Results and Discussion

### 246 3.1 Seed production and chemical properties

247 The genotype x irrigation interaction for seed yield is reported in Fig. 1. Yields of G8 were in the  
248 high yielding end of the range of productive data reported for chia worldwide (Bochicchio et al.,  
249 2015a) and were significantly higher than those of B. Irrigated plots yielded more than NI but  
250 statistical significance of this difference was reached in G8 only. Results confirm first reports of low  
251 yields from traditional genotypes of chia grown at high latitudes (Bochicchio et al., 2015b; Silva et  
252 al., 2016). Traditional genotypes are microthermal and short-day flowering, therefore are sown in late  
253 spring and cannot flower until late summer; seed ripening occurs in fall-winter and is hampered by  
254 low temperatures. Besides low yields, this explains the lack of significant effects of irrigation on the  
255 short-day flowering genotype since temperature during ripening becomes the limiting factor for this  
256 chia type and makes it difficult to detect yield differences due to other factors. This confirms the  
257 results of Silva et al. (2016) who also report non-significant irrigation effects on a short-day flowering  
258 genotype grown at high latitude.

259 Oil yield (Fig. 1) was 39.6% on average without significant differences between treatments. This  
260 value is in the high range of chia seeds oil content (Bochicchio et al., 2015a).

261 The TPC results, expressed as mg GAE/g of defatted chia seed, were in agreement with Amato et al.  
262 (2015) and with other previous reports (Coelho and de las Mercedes Salas-Mellado, Myriam, 2014;  
263 Reyes-Caudillo et al., 2008) but lower compared to data reported by da Silva Marineli et al. (2014).

264 The polyphenol content increased after 40 min of UAE ( $p < 0.01$ ). Although in many cases values were  
265 lower in irrigated treatments, differences were statistically significant ( $p < 0.01$ ) only for the  
266 commercial variety at 2 minutes, and sample variability did not allow significance to be reached in  
267 the other instances. The general trend of our data agrees with literature reports of a negative effect of  
268 irrigation on TPC for different species (Dag et al., 2008; Esteban et al., 2001).

### 269 3.2 Antioxidant activity

270 The antioxidant activity of chia seeds was also evaluated and Fig. 1 shows the TEAC results for Black  
271 chia and G8 seeds. The values ranged from  $1.317 \pm 0.027$  to  $2.174 \pm 0.010$  mmol TEAC/g of defatted  
272 chia seed, measured after 2 and 40 min, respectively. These results are in agreement with Sargi et al.  
273 (2013), but higher than those reported by other authors (Capitani et al., 2012; Vázquez-Ovando et al.,  
274 2009). Values were lower in irrigated treatments, but differences were statistically significant  
275 ( $p < 0.01$ ) only for G8 at 2 minutes. In many plant species, the amount of antioxidants and/or  
276 antioxidant activity is shown to increase (Wu et al., 2017) or remain unaffected (Kyrleou et al., 2016)  
277 with reduced water supply. Zhang and Kirkham (1996) show that the degree to which the activities  
278 of antioxidant enzymes and the amount of antioxidants change under drought stress is variable with  
279 plant species. No reports of chia antioxidant activity in response to irrigation are available, but Amato  
280 et al. (2015) found a reduction in oxidative stability and phenols in response to mineral nitrogen  
281 fertilization.

### 282 3.3 Metabolite profile

283 A whole metabolome profile of two different genotypes (G8 and Black chia) of *Salvia hispanica* L.  
284 seeds was evaluated by GC/MS analysis. All compounds from the polar and non-polar extracts with  
285 their respective retention times and  $m/z$  values are listed in Table 1, and representative total ion  
286 chromatograms (TIC) for both fractions are reported in Fig. 2. Our assignments from GC/MS are in  
287 agreement with those obtained using NMR and chemometrics by de Falco et al. (2017b) who  
288 performed a metabolomic fingerprinting of different populations of *S. hispanica* including G8 and  
289 Black chia seeds. The GC/MS approach, however, allowed us to identify compounds which were not  
290 detected by previous work such as the 10-heptadecenoic acid (17:1) and the comparison of irrigation  
291 treatments allowed us to detect responses (Table 2) which were not investigated by other experiments.

### 292 3.4 Non-polar phase

293 The main fatty acids detected in chia seeds were linolenic acid (18:3), linoleic acid (18:2), stearic acid  
294 (18:0), palmitic acid (16:0) and oleic acid (18:1), determined as trimethylsilyl (TMS) derivatives.  
295 These compounds were previously detected in other reports (Amato et al., 2015; Peiretti and Gai,  
296 2009). A preliminary analysis of chromatograms showed C18:3 as the most abundant fatty acid in all  
297 samples. On the contrary, 10-heptadecenoic acid (17:1) was detected in the lowest amount. This

298 compound was also reported as one of the least abundant by Segura-Campos et al. (2014) and da  
299 Silva Marineli et al. (2014).

300 MS spectrum of saturated fatty acids trimethylsilyl esters such as C16:0 and C18:0 have a base peak  
301 at  $m/z$  313 and 341 respectively, which represents the loss of methyl group from TMS ester group,  
302 while  $m/z$  132 represents the McLafferty rearrangement ion. In the MS spectrum of monounsaturated  
303 and polyunsaturated TMS fatty acids, such as C18:1, C18:2 and C18:3 characteristic peaks for each  
304 of them were detected at  $m/z$  339, 337 and 335, respectively. Also in these chromatograms, a base  
305 peak at  $m/z$  73, due to TMS, was always detected. As shown in Fig. 3, the most abundant fatty acid  
306 is  $\alpha$ -linolenic acid (C18:3) in all samples, and this is in agreement with those of other reports (Coelho  
307 and de las Mercedes Salas-Mellado, Myriam, 2014; de Falco et al., 2017a). Analysis of variance on  
308 the non-polar fraction (Table 2) shows that the main effects of genotype and irrigation were  
309 significant in many cases. In particular, the total amount of fatty acids was found higher in G8 than  
310 in B, except for C17:1 which was higher in B, and oleic acid (C18:1) and glycerol monostearate  
311 (GMS) which were not significantly different between genotypes. Although irrigation treatment  
312 affected the fatty acids composition of both genotypes of chia seeds, an interaction was significant in  
313 C18:1 where values of the irrigated treatment were higher than NI only for G8, and in GMS where  
314  $I < NI$  in G8 and  $I > NI$  in B. Silva et al. (2016) reported that irrigation did not affect significantly the  
315 content of linoleic and  $\alpha$ -linolenic acids of chia, but their experiment tested less extreme levels of  
316 irrigation, ranging between 40% and 100% of  $ET_0$ , whereas in our case 100% of  $ET_0$  is compared  
317 with rainfed conditions. No metabolomic study on the effect of irrigation on chia seeds is found in  
318 the literature, and few reports on the effect on single metabolites are available: Silva et al. (2016) did  
319 not find differences in linoleic and  $\alpha$ -linolenic acids content of chia seeds grown in different irrigation  
320 regimes, and Heuer et al. (2002) found an increase in the levels of palmitic and linoleic acids in  
321 response to salinity in irrigation water. In other species irrigation is reported to affect fatty acids  
322 composition but results are often contradictory. Erdemoglu et al. (2003) found a decrease in the  
323 content of linoleic and oleic acids with irrigation in sunflower seed oil. Sezen et al. (2011) found an  
324 increase in linoleic, palmitic and stearic acid concentrations with irrigation. Bellaloui et al. (2015)  
325 reported that irrigation affects soybean oil composition differently according to the degree and stage  
326 of differentiation of water treatments. Ayerza (2009) reports a negative correlation of the percentages  
327 of oleic and linoleic acid in chia samples from different environments. This is found in many crops,  
328 due to the dynamics of oleate desaturases: an increase of desaturation in cold conditions causes a  
329 decrease of the oleic/linoleic acids ratio in cooler environmental temperatures (Aparicio et al., 1994).  
330 In our data, although the amount of many fatty acids increases in response to irrigation, the response  
331 is proportionally lower in oleic acid, and therefore the oleic/linoleic ratio decreases from 47.4 in the

332 rainfed samples to 39.6 in the irrigated treatments. Flagella et al. (2002) observed a decrease in the  
333 oleic/linoleic acid ratio in sunflower in response to irrigation, and suggested that a possible thermal  
334 effect of irrigation may have affected the activity of oleate desaturase.

### 335 3.5 Polar phase

336 In the aqueous extracts sugars are the principal class of compounds and the disaccharide sucrose ( $m/z$   
337 361) represents the major component followed by methyl galactose as the major monosaccharide  
338 component (Table 1). Other sugars identified were glucose, galactose, fructose, mannitol and  
339 gluconic acids. In particular, TMS derivatives of monosaccharides such as glucose and galactose  
340 showed a very similar GC/MS profiles, due to their stereoisomery, with characteristic ions observed  
341 at  $m/z$  147, 205, 319 and 364. The final identification of these compounds was achieved by comparing  
342 their elution order with literature data (Gómez-González et al., 2010) and by injection of standard  
343 samples. Other compounds detected were the polyphenol caffeic acid, the polyol myo-inositol and a  
344 series of carboxylic acids and amino acids (Table 1). A base peak at  $m/z$  73, typical of silylated  
345 compounds, was always detected in the chromatograms due to the  $[(CH_3)_3Si]$  group. In the polar  
346 fraction (Fig. 4) sucrose (Sucr) and methyl-galactoside (mGal) are the most abundant sugars present  
347 in all samples, while within organic acids, lactic acid (LA) and citric acid (CI) have the highest value,  
348 followed by quinic acid (QUI).

349 Caffeic acid (CA) did not exhibit variation within genotype, but even if statistically not significant,  
350 its level is higher in Black chia than G8. Values were more variable than those of the non-polar phase,  
351 and statistical significance of the differences between genotype and irrigation treatments was not  
352 reached for many compounds (Fig. 4). A genotype effect was significant for some compounds (Fig.  
353 4): G8 showed a significantly higher amount of lactic acid (LA), benzoic acid (BE) and serine (Ser),  
354 while B showed a significantly higher amount of gluconic acid (GLUC), arabitol (Ara) and sugars, in  
355 particular mannose (Man). The overall effects of irrigation were not significant but interactions were  
356 found for some compounds (Fig. 4): irrigated samples showed significantly higher values of glycine  
357 (Gly) in G8 only, and significantly lower values of Asp and phenylalanine (Phe) in B only. No reports  
358 of chia polar extracts variation in response to irrigation are available in the literature. The only other  
359 data regarding agricultural management effects on chia seeds whole metabolome are related to  
360 nitrogen fertilization and point out a positive effect of mineral nitrogen topdressing on the content of  
361 aliphatic free amino acids, a reduction of carbohydrates and flavonoids and no effect on the pools of  
362 caffeoyl derivatives and organic acids (de Falco et al., 2017b).

#### 363 **4. Conclusion**

364 This work provides a high-throughput analysis of metabolomic fingerprinting including total  
365 polyphenolic content (TPC) and antioxidant activity (TEAC), on commercial black chia and early  
366 flowering G8 seeds. The analytical approach performed by UAE-GC MS allowed detection and  
367 quantification of a high number of metabolites.

368 The aim of the paper was to evaluate the difference in organic compounds between a commercial  
369 genotype, black chia, and the recently developed mutant G8. The species were grown at different  
370 levels of irrigation to evaluate the effect of water supply on the metabolite content. Results showed  
371 an increase of TPC and antioxidant activity (expressed as TEAC) in all samples relative to irrigation  
372 treatment or variety after 40 minutes of UAE. On the contrary a decrease of TPC and TEAC levels  
373 was observed after irrigation treatments. With regards to the non-polar phase, quantitative analysis  
374 showed a higher yield and content of many fatty acids including  $\omega$ -3 ( $\alpha$ -linolenic), in the early  
375 flowering G8 mutant, with a decrease of the ratio of oleic/linoleic acids. Concerning the polar fraction,  
376 sugars were found as the main metabolites with sucrose and methyl galactose as the major  
377 components. The genotype effect has more influence than the irrigation treatment on the aqueous  
378 extract. G8 showed significantly higher amounts of some organic acids and amino acids, such as LA,  
379 BE and Ser while GLUC, Ara and Mann are present in lower amounts.

380 Gas chromatography-mass spectrometry can provide a detailed metabolic profile of chia seeds  
381 extracted with ultrasound. Furthermore, this study highlighted for the first time the effects of  
382 irrigation on the metabolome of a late flowering and an early flowering mutant chia genotypes.

383 In addition, this approach proved that chia seeds of mutant genotypes can be cultivated at high latitude  
384 without loss in nutraceuticals. Within mutants, G8 can be proposed as excellent early-flowering  
385 genotype.

386 The obtained data indicate that metabolomics could be used as monitoring technique to control the  
387 agronomic management and its non-invasive features making it an ideal tool for crop production.

388

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394

#### 395 **Notes**

396 The authors declare no competing financial interest.

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400 **References**

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566 TABLES

567 **Table 1** Polar and non-polar metabolites assigned in Chia seeds by GC-MS

Peak	Detected metabolite	Abbreviation	RT (min)	Molecular formula	<i>m/z</i>
<i>Polar</i>					
1	Lactic Acid	LA	7.25	C <sub>9</sub> H <sub>22</sub> O <sub>3</sub> Si <sub>2</sub>	219, 191, 147, 133, 117, 73, 45
2	Methyl 2-ethyl malonate	Me-MA	7.32	C <sub>9</sub> H <sub>18</sub> O <sub>4</sub> Si	175, 89, 73
3	L-Alanine, N-methyl-N-(trifluoroacetyl)-, butyl ester	Ala, Nm-Tfa-Obu	9.99	C <sub>10</sub> H <sub>16</sub> F <sub>3</sub> NO <sub>3</sub>	154, 110
4	Benzoic Acid	BE	10.03	C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> Si	194, 179, 135, 105, 77
5	Glycerol	GLY	10.46	C <sub>12</sub> H <sub>32</sub> O <sub>3</sub> Si <sub>3</sub>	218, 205, 147, 117, 89, 73, 45
6	L-serine	Ser	11.74	C <sub>12</sub> H <sub>31</sub> NO <sub>3</sub> Si <sub>3</sub>	218, 204, 147, 100, 73
7	L-Threonine	Thr	12.14	C <sub>13</sub> H <sub>33</sub> NO <sub>3</sub> Si <sub>3</sub>	291, 218, 147, 117, 73
8	N-α-Acetyl-L-Lysine	AcLys	13.04	C <sub>17</sub> H <sub>40</sub> N <sub>2</sub> O <sub>3</sub> Si <sub>3</sub>	404, 287, 73
9	Malic acid	MA	13.52	C <sub>13</sub> H <sub>30</sub> O <sub>5</sub> Si <sub>3</sub>	245, 233, 147, 133, 73
10	L-Aspartic acid	Asp	13.97	C <sub>13</sub> H <sub>31</sub> NO <sub>4</sub> Si <sub>3</sub>	232, 218, 147, 100, 73
11	L-5-Oxoproline	PCA	14.00	C <sub>11</sub> H <sub>23</sub> NO <sub>3</sub> Si <sub>2</sub>	258, 230, 156, 133, 73, 45
12	L-Glutamic acid	Glu	15.21	C <sub>14</sub> H <sub>33</sub> NO <sub>4</sub> Si <sub>3</sub>	246, 147, 128, 73
13	Phenylalanine	Phe	15.35	C <sub>15</sub> H <sub>27</sub> NO <sub>2</sub> Si <sub>2</sub>	218, 192, 147, 100, 73
14	Tartaric acid	TA	15.54	C <sub>28</sub> H <sub>62</sub> O <sub>6</sub> Si <sub>4</sub>	549, 417, 389, 147, 73
15	Citric acid	CI	17.69	C <sub>18</sub> H <sub>40</sub> O <sub>7</sub> Si <sub>4</sub>	273, 147, 73, 45
16	Methyl galactoside	mGal	17.77	C <sub>19</sub> H <sub>46</sub> O <sub>6</sub> Si <sub>4</sub>	243, 217, 204, 133, 73
17	Quininic acid	QUI	18.24	C <sub>22</sub> H <sub>52</sub> O <sub>6</sub> Si <sub>5</sub>	345, 255, 191, 147, 73
18	D-Fructose MEOX	FRU	18.37	C <sub>22</sub> H <sub>55</sub> NO <sub>6</sub> Si <sub>5</sub>	217, 307
19	Arabitol	Ara	18.48	C <sub>20</sub> H <sub>52</sub> O <sub>5</sub> Si <sub>5</sub>	307, 277, 217, 189, 147, 103, 73

20	D-Galactose MEOX	Gal	18.61	C <sub>22</sub> H <sub>55</sub> NO <sub>6</sub> Si <sub>5</sub>	319, 205, 147, 103, 73
21	D-Glucose MEOX	Glc	18.67; 18.86	C <sub>22</sub> H <sub>55</sub> NO <sub>6</sub> Si <sub>5</sub>	364, 319, 205, 147, 73
22	D-Mannitol	MAN	19.04	C <sub>24</sub> H <sub>62</sub> O <sub>6</sub> Si <sub>6</sub>	421, 345, 319, 205, 147, 103, 73
23	D-Gluconic acid	GLUC	19.77	C <sub>24</sub> H <sub>60</sub> O <sub>7</sub> Si <sub>6</sub>	333, 292, 205, 147, 103, 73
24	Trimethylsilyl catechollactate tris(trimethylsilyl) ether	Cat	20.30	C <sub>21</sub> H <sub>42</sub> O <sub>5</sub> Si <sub>4</sub>	396, 267, 179, 147, 73
25	Myo-Inositol	Myo	20.69	C <sub>24</sub> H <sub>60</sub> O <sub>6</sub> Si <sub>6</sub>	305, 217, 147, 129, 73
26	Caffeic acid	CA	20.91	C <sub>18</sub> H <sub>32</sub> O <sub>4</sub> Si <sub>3</sub>	396, 381, 219, 191, 73
27	Sucrose	Sucr	25.66	C <sub>36</sub> H <sub>86</sub> O <sub>11</sub> Si <sub>8</sub>	437, 361, 319, 271, 217, 147, 103, 73
<i>Apolar</i>					
28	Palmitic Acid	C16:0	17.88	C <sub>19</sub> H <sub>40</sub> O <sub>2</sub> Si	328, 313, 145, 117, 73
29	Linoleic acid	C18:2	19.54	C <sub>21</sub> H <sub>40</sub> O <sub>2</sub> Si	337, 129, 95, 75, 73
30	α-Linolenic acid	C18:3	19.61	C <sub>21</sub> H <sub>38</sub> O <sub>2</sub> Si	335, 129, 95, 75, 73
31	Stearic acid	C18:0	19.80	C <sub>21</sub> H <sub>44</sub> O <sub>2</sub> Si	356, 341, 132, 117, 73
32	Oleic acid	C18:1	22.07	C <sub>21</sub> H <sub>42</sub> O <sub>2</sub> Si	354, 339, 129, 117, 73
33	10-Heptadecenoic acid	C17:1	24.05	C <sub>20</sub> H <sub>40</sub> O <sub>2</sub> Si	340, 325, 145, 129, 117, 73
34	Glycerol monostearate	GMS	24.34	C <sub>27</sub> H <sub>58</sub> O <sub>4</sub> Si <sub>2</sub>	487, 399, 147, 73

568

569

570 **Table 2** Analysis of variance on polar and non-polar fraction to evaluate the effect of genotype and  
 571 irrigation on Chia seeds

Compounds	Genotype	Irrigation	Genotype x Irrigation
<i>Polar</i>			
LA	p<0.05	n.s.	p<0.05
Me-MA	n.s.	n.s.	n.s.
Ala-Obu	n.s.	n.s.	n.s.
BE	p<0.05	n.s.	n.s.
GLY	n.s.	n.s.	p<0.05
Ser	p<0.05	n.s.	n.s.
Thr	n.s.	n.s.	n.s.
AcLys	n.s.	n.s.	n.s.
MA	n.s.	n.s.	n.s.
Asp	p<0.01	n.s.	p<0.05
PCA	n.s.	n.s.	n.s.
Glu	n.s.	n.s.	n.s.
Phe	n.s.	n.s.	p<0.05
TA	n.s.	n.s.	n.s.
CI	n.s.	n.s.	n.s.
mGal	n.s.	n.s.	n.s.
QUI	n.s.	n.s.	n.s.
FRU	n.s.	n.s.	n.s.
Ara	p<0.05	n.s.	n.s.
Gal	n.s.	n.s.	n.s.
Glc	n.s.	n.s.	n.s.
MAN	p<0.01	n.s.	n.s.
GLUC	p<0.01	n.s.	n.s.
Cat	n.s.	n.s.	n.s.
Myo	n.s.	n.s.	n.s.
CA	n.s.	n.s.	n.s.
Sucr	n.s.	n.s.	n.s.
GABA	n.s.	n.s.	n.s.
<i>Non-polar</i>			
C16:0	P<0.05	P<0.001	n.s.
C18:2	P<0.01	P<0.0001	n.s.
C18:3	P<0.01	P<0.0001	n.s.
C18:0	P<0.01	P<0.0001	n.s.
C18:1	n.s.	P<0.0001	P<0.01
C17:1	P<0.01	P<0.0001	n.s.
GMS	n.s.	n.s.	P<0.01

572

573

574 **FIGURE LEGENDS**

575

576 **Figure 1.** Growth environment data and interaction of genotype x irrigation for yield and chemical  
577 properties of Chia seeds (*Salvia hispanica* L.). Left: temperature, precipitation and irrigation amounts  
578 during crop growth (top); seed yield and oil content (bottom). Different lower case letters indicate  
579 significant differences ( $p < 0.05$ ) at the post-hoc Tukey's test. Right: Total polyphenol content (top)  
580 and Antioxidant activity (bottom) of defatted Chia seeds. Within each time different upper case letters  
581 indicate highly significant differences ( $p < 0.01$ ) at the post-hoc Tukey's test.

582

583 **Figure 2.** Representative TIC of the polar (A) and non-polar (B) fractions of Chia seeds extracts.  
584 Peaks correspond to numbering of compounds in Table 1

585

586 **Figure 3.** Metabolites belonging to different classes of compounds of non-polar extract of Chia seeds  
587 detected by GC/MS analysis. The Y-axis indicates the relative quantification obtained by integrating  
588 the peak areas of the chromatographic profiles for each compound and normalizing the data to the  
589 internal standards. Top left: main effects of genotype; top right: main effect of irrigation; bottom left:  
590 interaction of genotype x irrigation. Within each compound different upper case letters indicate highly  
591 significant differences ( $p < 0.01$ ) and different lower case letters indicate significant differences  
592 ( $p < 0.05$ ) at the analysis of variance for main effects and at the post-hoc Tukey's test for the  
593 interaction.

594

595 **Figure 4.** Metabolites belonging to different classes of compounds of non-polar extract of Chia seeds  
596 detected by GC/MS analysis. The Y-axis indicates the relative quantification obtained by integrating  
597 the peak areas of the chromatographic profiles for each compound and normalizing the data to the  
598 internal standards. Top left: overall average for compounds not significantly different between  
599 treatments; top right: main effect of genotype for compounds significantly different between G8 and  
600 B; bottom left: interaction of genotype x irrigation; within each compound different lower case letters  
601 indicate significant differences ( $p < 0.05$ ) at the post-hoc Tukey's test for the interaction.

602



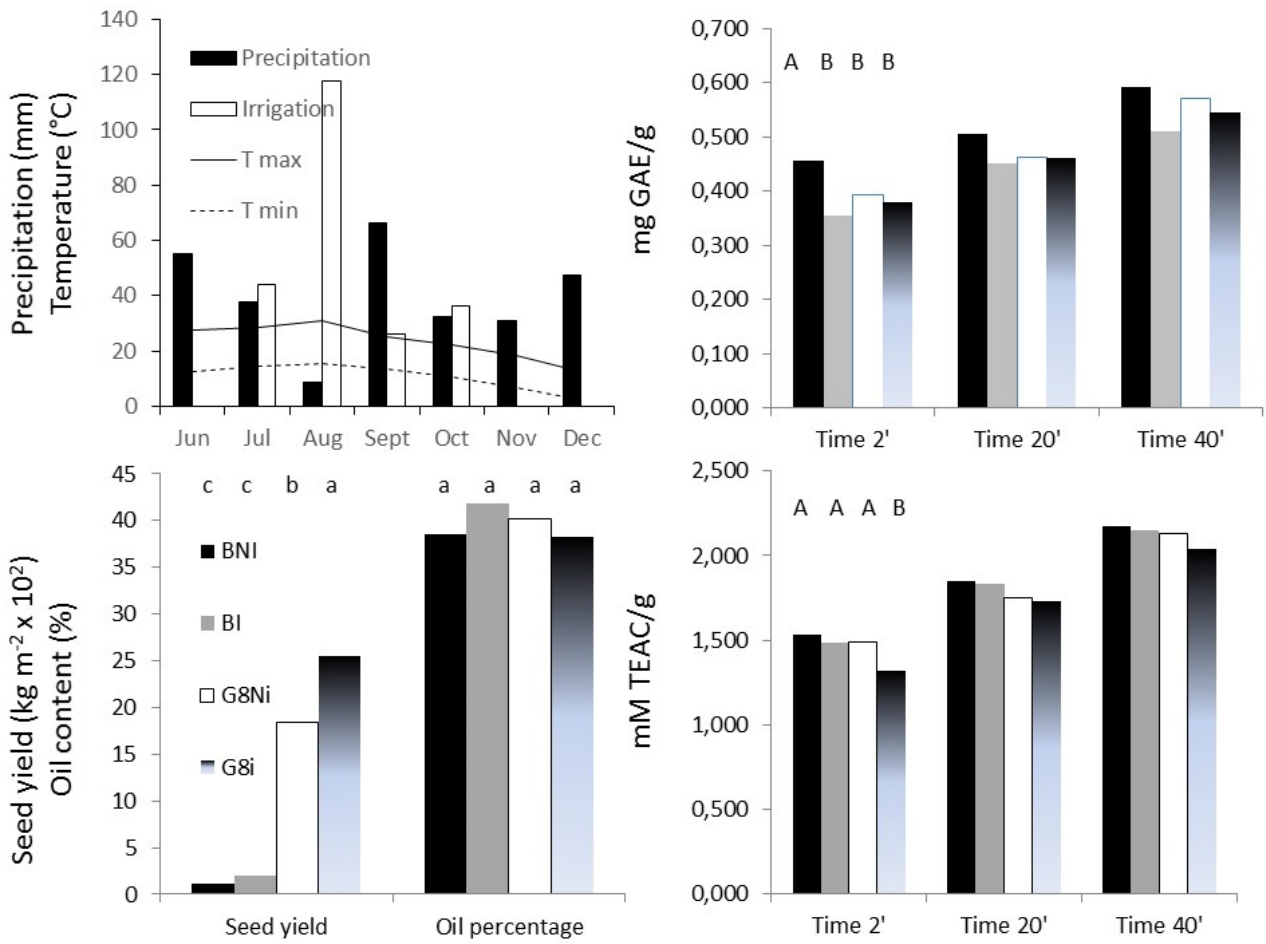
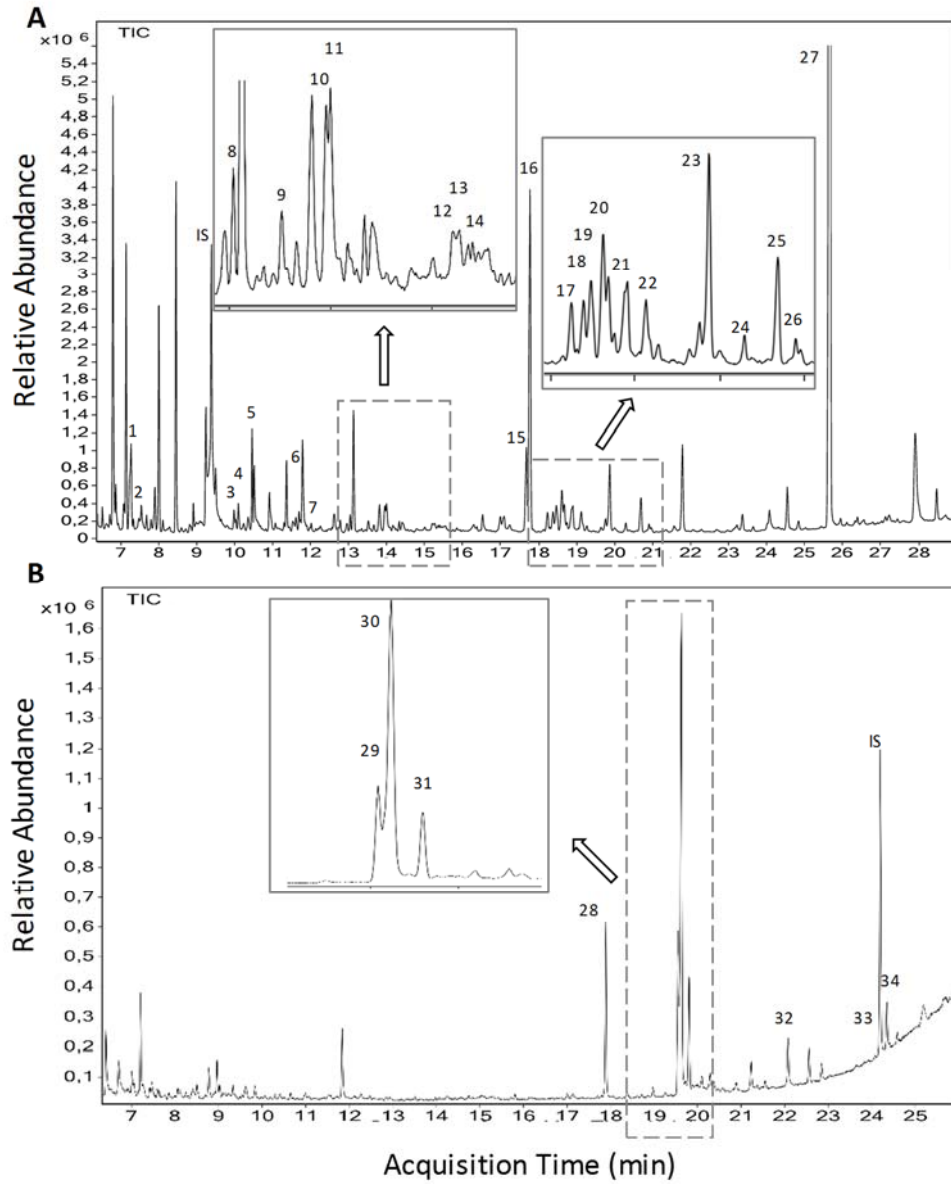
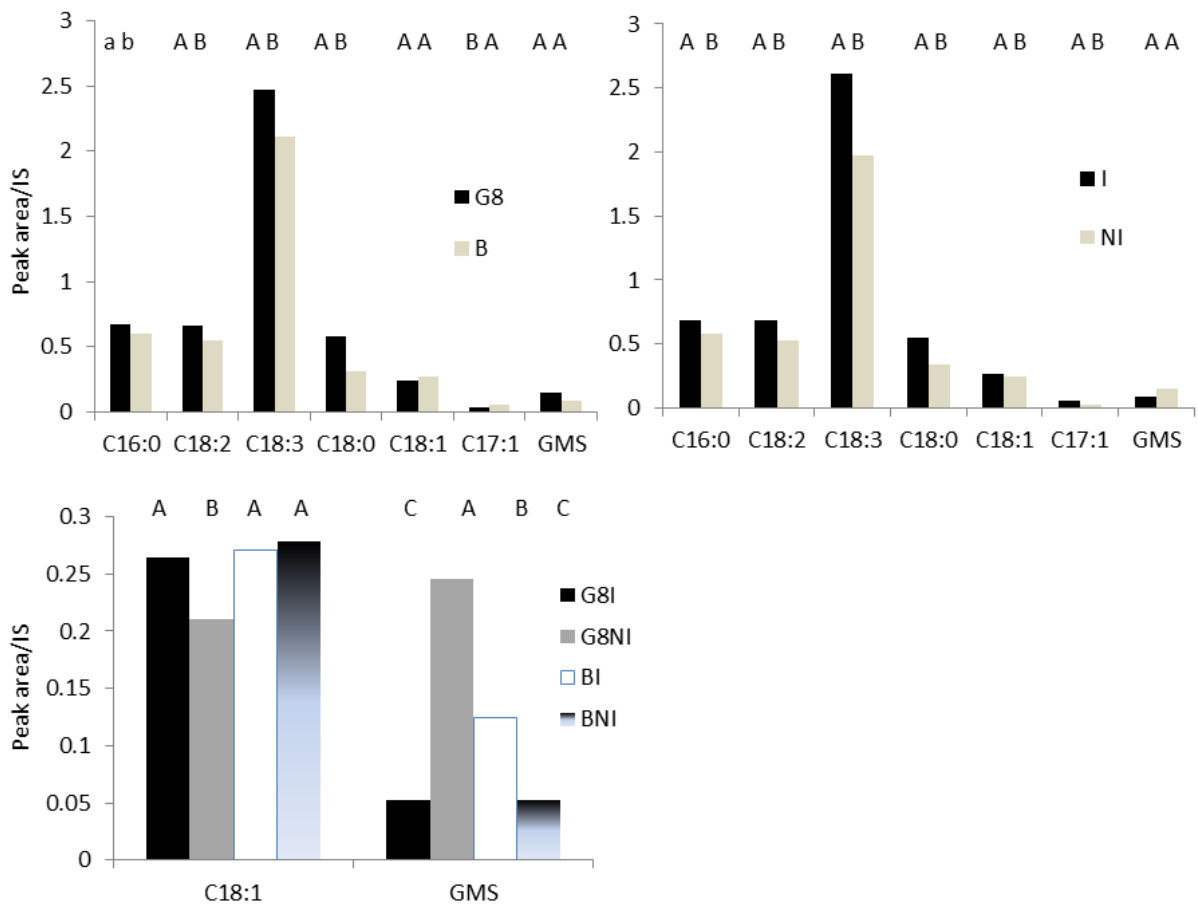


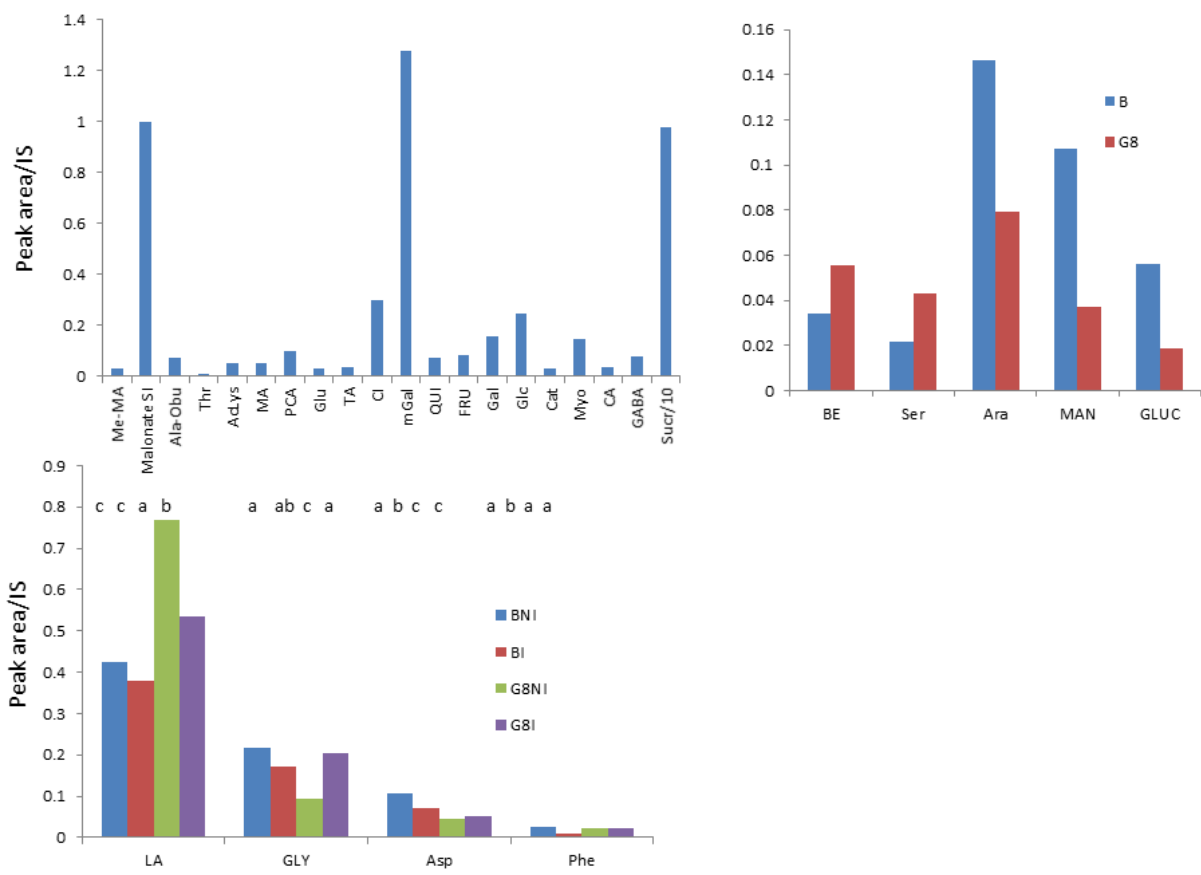
Figure 1



**Figure 2.**



**Figure 3**



**Figure 4**