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1 **Extraction methods of *Amaranthus* sp. grain oil isolation**

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25

26 **Abstract**

27 *BACKGROUND: Amaranthus* sp. is a fast-growing crop with well known beneficial
28 nutritional values (rich in protein, fat, dietary fiber, ash, and minerals, especially
29 calcium and sodium and contain a higher amount of lysine than conventional cereals).
30 *Amaranthus* sp. is an underexploited plant source of squalene, compound of high
31 importance in the food, cosmetic and pharmaceutical industries.

32 RESULTS: This paper has examined the effects of the different extraction methods
33 (Soxhlet, supercritical fluid and accelerated solvent extraction) on the oil and squalene
34 yield of three genotypes *Amaranthus* sp. grain. The highest yield of the extracted oil
35 (78.1 g kg^{-1}) and squalene (4.7 g kg^{-1}) in grain was obtained by accelerated solvent
36 extraction (ASE) in genotype 16. The post hoc Tukey's HSD test at 95% confidence
37 limit showed significant differences between observed samples. Principal Component
38 Analysis (PCA) and Cluster Analysis (CA), were used for assessing the effect of
39 different genotypes and extraction methods on oil and squalene yield, and also the fatty
40 acid composition profile. Using coupled PCA and CA of observed samples, the possible
41 directions for improving the quality of product can be realized.

42 CONCLUSION: The results of this study indicate that is very important to choose both
43 the right genotype and the right method of extraction for optimum oil and squalene
44 yield.

45 **Keywords**: extraction methods*;* *Amaranthus* sp.; oil yield; squalene; fatty acid.

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51 **INTRODUCTION**

52 *Amaranthus* sp. is gluten-free pseudocereal with beneficial nutritional properties
53 considering its nutritional quality and health benefits in terms of fiber content, high
54 amount of protein, tocols, squalene, and various bioactive compounds as well.[1-3]
55 Some previously performed studies reported that *Amaranthus* sp. grain contains a
56 naturally high concentration of minerals,[4-6] vitamins,[4] especially tocotrienols,[7] lysine,
57 sulfur-containing amino acids,[1,8] dietary fibre[9] and essential fatty acids.[10] *Amaranthus*
58 sp. grain contains 50 to 80 g kg^{-1} of oil, which is more than in the commercial
59 cereals.[11,12] Unsaturated fatty acids represent approximately 77% of *Amaranthus* sp.
60 grain oil, wherein a considerable amount of linoleic acid, which is necessary for human
61 metabolism and cannot be synthesized by the human body.[13] Generally, the
62 predominant fatty acids in *Amaranthus* sp. grain oil are consisted of palmitic acid (19.1-
63 23.4%), oleic acid (18.7-38.9%), and linoleic acid (36.7-55.9%), as it was reported by
64 He et *al*.[14] and Berganza et *al*.[15] *Amaranthus* sp. oil is a rich source of squalene,
65 unsaturated hydrocarbon triterpene, which is used in skin cosmetics and as a lubricant
66 for computer disks.[16] According to the results shown by Martinez-Correa et *al*.[17]
67 *Amaranthus* sp. squalene would significantly exert decreasing the risk for various
68 cancers and reducing plasma cholesterol levels. The healing effect was higher than one
69 obtained of shark fish squalene. Furthermore *Amaranthus* sp. grain is attractive as a
70 potential alternative to shark and whale liver oil as a source of squalene. It was reported
71 that squalene content in *Amaranthus* sp. grain oil ranges from 24 to 80 g kg^{-1}.[14,18,19]
72 Plant species, cultivars and accessions influence the content of all components in
73 *Amaranthus* sp. grains, whereas the content of lipids and their composition also depend
74 on their isolation procedure and applied solvent. The most common method used for the
75 oil isolation from *Amaranthus* sp. grains is well known solvent extraction in a Soxlet

76 apparatus with non-polar organic solvents.[20] Soxhlet extraction is often considered to be
77 the standard method when compared a range of oil extraction methods from grain.
78 These days versatile innovative eco-friendly thermal and non-thermal technologies are
79 developed and applied for usage in food technology.[21-26] In addition for extraction of
80 bioactive compounds from plants besides conventional technologies[27,28] researchers
81 used Ultrasound (US), High Hydrostatic Pressures (HHP), Microwave (MW), Infrared
82 (IR), Pulsed Electric Fields (PEF) and Supercritical Fluids.[29-32]
83 Supercritical fluid extraction (SFE) has attracted considerable attention as a suitable
84 alternative to conventional solvent extraction, especially due to safety and
85 environmental concerns.[33-35] Besides the ecological benefits of using supercritical fluids,
86 carbon dioxide would offer clear advantages such as non flammability, readily available
87 and easy removing from extracted materials.[36,37] Oil and squalene extraction from
88 *Amaranthus* sp. grain was investigated by supercritical carbon dioxide in comparison to
89 Soxhlet extraction reported by He et *al*.[38], Westerman et *al*.[39], Wejnerowska et *al*.[40] and
90 Kraujalis and Venskutonis.[41] Mentioned authors optimized the operational conditions,
91 including material pretreatment, extraction time, flow rate of carbon dioxide, process
92 temperature and pressure.
93 Accelerated solvent extraction (ASE) uses organic solvents at high pressures and
94 temperatures above the boiling point. The advantages of this extraction method are
95 considerable decrease in extraction time and in the volume of solvents, as well as a
96 possibility of using universal solvents and solvent mixtures differing in polarity.[42]
97 Kraujalis et *al*.[43] compared lipids from different particle size fractions of milled
98 *Amaranthus* sp. grains obtained by standard Soxhlet extraction and ASE.

99 The aim of the study presented in this paper was to examine and compare the oil and
100 squalene yield, such as fatty acid profiles of oil from three *Amaranthus* sp. genotypes
101 obtained by Soxhlet, SFE and ASE methods.
102 Pattern recognition techniques (Principal Component Analysis - PCA and Cluster
103 Analysis - CA) were applied to the experimental data (used as descriptors) to
104 characterize and differentiate among the observed samples.
105

106 **MATERIAL AND METHODS**

107 **Plant material**

108 The three genotypes of *Amaranthus* sp. (marked as 2, 16 and 31) were cultivated in the
109 field in Novi Sad, Serbia (45°19′40″ N, 19°49′41″ E). Grains were milled using
110 laboratory mill a "Knifetec" 1095 (Foss, USA). The particle size distribution was
111 performed by laser particle sizer (Malvern, UK). All measurements were performed in
112 triplicate. Moisture content of grain was determined using gravimetrical AOAC Method
113 950.46[44] , also known as „oven dry" method.
114

115 **Soxhlet extraction**

116 Soxhlet extractions were performed with a Büchi 810 Soxhlet fat extraction apparatus
117 (Soxtec system HT, 1043Extraction Unit, Foss Tecator AB, Höganäs, Sweden) in
118 accordance with manufacturer procedure and AOCS Method Ba 3-38.[45]Petroleum ether
119 (b.p. 40-60 ºC) was used as solvent for extractions. Procedure was carried out in
120 duration of 1.5 hours under temperature of 80 ºC and with sample mass of 3 g. After
121 removal from the apparatus, extracted lipids were cool at room temperature while
122 passing nitrogen over the samples for 1 min and then dried in the desiccant pouch until

123 they reached a constant weight (approximately 60 min). Fat content was expressed as
124 the percentage, by weight (gravimetrical method).
125
126 **Supercritical fluid extraction**
127 The supercritical fluid CO_2 extraction of milled *Amaranthus* sp. grains was performed
128 in a laboratory scale unit NOVA-Swiss, High Pressure Extraction Plant 565.0156 (Nova
129 Werke LTD, Effertikon, Switzerland). Extraction process was conducted at following
130 operating conditions: pressure 300 bar, temperature 40 °C, and CO_2 mass flow 0.194
131 kg/h. In order to study the dynamics of the separation process, extraction time
132 sequences were set at 30, 60, 90, 120, 150 and 180 min, as the extraction yield reached a
133 plateau after 3 h for each of the extraction procedures. Extract samples were collected in
134 the glass collector in the extractor and were weighted with a ±0.0001 g precision.
135 Thereafter samples were transferred into adequate glass vials using a Pasteur pipette and
136 stored at −20 °C for further use in GC-FID analysis.
137
138 **Accelerated solvent extraction (ASE)**
139 Accelerated solvent extraction (ASE) was performed on a Dionex ASE 350 system
140 (Dionex, Sunnyvale, CA, USA). Samples (6 g) were mixed with diatomaceous earth
141 (1:4) and placed in 22 ml cells. The cells were then closed with a stainless steel frit and
142 a cellulose filter at the ends of the cells to avoid solid particles in the collection vial.
143 Extractions were done using a *n*-hexane (J.T. Baker, USA). The following conditions
144 were used for the extraction: cells were preheated 5-6 min to ensure that the samples
145 reached thermal equilibrium at 10 MPa pressure before static extraction with 3 cycles of
146 extraction. Extraction time was 20 min at the temperature of 90 ºC. The solvent was

147 removed in a rotary vacuum evaporator at 30 ºC and the residue was weighed by
148 analytical balances.
149

150 **GC determination of fatty acid profile**

151 Fatty acid methyl esters were prepared from the extracted lipids using method based on
152 14% boron trifluoride/methanol solution, as recommended method for this type of
153 substrates.[46] Nitrogen gas was used for drying and removing solvents from fatty acid
154 methyl esters. Obtained samples were analyzed by a GC Agilent 7890A system with
155 FID, an autoinjection module for liquid, equipped with a fused silica capillary column
156 (DB WAX 30 m, 0.25 mm, 0.50 μm). Helium was used as a carrier gas (purity >
157 99.99%, vol., flow rate of 1.26 ml/min). The fatty acids peaks were identified by
158 comparison of retention times with retention times of standards from Supelco GRAIN
159 fame mix (Lot No. LB50766) and with data from the internal data library, based on
160 previous experiments. Results were expressed as mass of fatty acid or fatty acid group
161 (g) in 100 g of fatty acids (%, w/w).
162

163 **Squalene determination by HPLC**

164 The squalene content in extracted oil samples and purified samples were determined
165 using an Agilent 1200 system equipped with a Diode Array detector (DAD), binary
166 pump, a vacuum degasser and an auto sampler. System control and data analysis were
167 processed with Chemstation Software (Agilent Technologies). The chromatographic
168 column Zorbax eclipse XDB-C18 (1.8 μm particle size, 50 mm x 4.6 mm I.D.) was
169 obtained by Agilent. The column was eluted isocritically by acetonitrile at a flow rate of
170 1.0 mL/min. The injection volume of the sample was 5 μL. The total run time was 20
171 min. The spectra were recorded at a wavelength of a 195 nm. The compounds were

172 identified by comparing with authentic standards and against UV spectra comparison
173 using a DAD detector. Squalene (99% purity) was purchased from Fluka (Basel,
174 Switzerland). The retention time of squalene was 14.3 min. Quantification was based on
175 the use of a calibration curve, i.e. appropriate equation.

176

177 **Statistical analyses**

178 The data were processed statistically using the software package STATISTICA 10.0[47]
179 (StatSoft Inc., Tulsa, OK, USA). All determinations were made in triplicate, all data
180 was averaged, expressed by means ± standard deviation (SD). Analysis of variance
181 (ANOVA) and Tukey's HSD test for comparison of sample means were used to analyze
182 variations of the oil yield extracted from different *Amaranthus* sp. genotypes and
183 obtained by different extraction methods. Principal component analysis (PCA) was used
184 to discover the possible correlations among measured parameters, while Cluster analysis
185 (CA) is used to classify objects into groups.

186 Second order polynomial (SOP) models in the following form were developed to
187 relate responses (Y) and two process variables (X):

188
$$Y_k = \beta_{k0} + \sum_{i=1}^{2} \beta_{ki} \cdot X_i + \sum_{i=1}^{2} \beta_{kii} \cdot X_i^2 + \beta_{k12} \cdot X_1 \cdot X_2, \; k=1\text{-}2, \quad (1)$$

189 where: β_{k0}, β_{ki}, β_{kii}, β_{k12} were constant regression coefficients; Y_k oil yield (Y_1) or
190 squalene yield (Y_2), while X_1 is extraction method and X_2 is genotype. In this article,
191 ANOVA was conducted to show the significant effects of independent variables to the
192 responses, and to show which of responses were significantly affected by the varying
193 treatment combinations.

194

195 **RESULTS AND DISCUSSION**

196 **Comparison between Soxhlet, SFE and ASE methods**

197 Three genotypes of *Amaranthus* sp. grains (2, 16 and 31) were investigated for oil and
198 squalene yield and fatty acid profile of oil extracted by Soxhlet, SFE and ASE methods.
199 The several studies, grinding various grain samples were used to investigate particle
200 size effect on the extraction efficiency. [38,48,49]The grinding process disrupts cell walls of
201 grain, which led to an increase in the specific reactive surface of particles, reducing
202 mass transfer resistance and leaving the oil more accessible to the solvent, consequently
203 increasing the extraction rate and the oil yield. The oil yield increased with decreasing
204 average particle size because the intraparticle diffusion resistance is smaller for smaller
205 particles due to the shorter diffusion path. Particle size distribution parameters obtained
206 included specific surface area, volume weighted mean D[4,3], surface weighted mean
207 D[3,2], the largest particle size d(0.9), mean particle size d(0.5) and smallest particle
208 size d(0.1) are shown in Table 1.

209

210 **Table 1.**

211

212 The effect of extraction technique on oil yield of *Amaranthus* sp. grain was examined
213 (Table 2). The oil content of *Amaranthus* sp. grain is 2-3 times higher than that of other
214 cereals.[50] The results presented in Table 2 showed statistically significant differences in
215 oil content among various genotypes and applied methods of extraction. The oil content
216 of three *Amaranthus* sp. genotypes ranged from 54.6 g kg^{-1} in genotype 31 extracted by
217 SFE to 78.1 g kg^{-1} in genotype 16 extracted by ASE. These results are in accordance
218 with Gimplinger et *al*.[12], who considered that *Amaranthus* sp. grain usually contains
219 about 50 to 80 g kg^{-1} of oil. The highest oil yield was obtained by ASE for all
220 genotypes. It is evident that oil yield obtained by Soxhlet (Sox) and ASE were similar,

221 noting that the extraction rate in case of ASE was several times faster. Oil yield
222 obtained by SFE was significantly lower as compared to the other two methods
223 ($p<0.05$). In comparing different methods of extraction, beside yield of extracted
224 component, parameters such as relative capital cost, organic extraction solvent volume,
225 extraction time and sample weight should be taken into consideration.[51]

226

227 **Table 2.**

228

229 Comparison of the results for the squalene content of extracts obtained by Soxhlet, SFE
230 and ASE methods, shows that squalene content ranged from 3.3 to 4.7 g kg^{-1} of dry
231 grain, Table 3. The obtained results are in accordance with the investigations carried out
232 by Leon-Camacho et *al*.[48] and Bodroža-Solarov et *al*.[52] who reported a similar content
233 of squalene in oil from *Amaranthus* sp. grains. There were statistically significant
234 differences ($p<0.05$) in squalene yield for each genotype extracted by different methods
235 (Table 3). The highest squalene yield was obtained by ASE for all genotypes. It can be
236 concluded method of extraction has remarkably influenced to squalene yield. The
237 squalene yield is correlated to the oil yield ($r=0.793$, statistically significant at $p<0.01$
238 level, for $n=9$ observed samples). This indicates that squalene and oil components are
239 similarly extractable by different extraction methods, which can be caused by a similar
240 polarity squalene and other components of the oil.

241

242 **Table 3.**

243

244 ANOVA exhibits the significant independent variables as well as the interactions of
245 these variables. The analysis revealed that the quadratic terms of SOP model (Eq. (1))

246 were found significant in both models calculations. The ANOVA test shows the
247 significant effects of the independent variables to the responses and which of responses
248 were significantly affected by the varying treatment combinations (Table 4). The oil
249 yield evaluation was mostly affected by the quadratic term of extraction type in the SOP
250 model (statistically significant at $p<0.01$ level), while the quadratic and the linear term
251 of genotype were also very influential ($p<0.05$). The squalene yield was also mostly
252 affected by the quadratic term of extraction type in the SOP model ($p<0.01$ level), while
253 the linear term of extraction type and quadratic term of genotype significantly
254 contributed to squalene yield ($p<0.01$).
255
256 **Table 4.**
257
258 All SOP models had an insignificant lack of fit tests, which means that all the models
259 represented the data satisfactorily. A high coefficient of determination (r^2) is indicative
260 that the variation was accounted and that the data fitted satisfactorily to the proposed
261 model. The r^2 values for observed responses were found very satisfactory and showed
262 the good fit of the model to experimental results.
263
264 **Determination of fatty acids profile**
265 The fatty acid profile has a significant effect on the quality and stability of oil. The fatty
266 acid compositions of oil from three *Amaranthus* sp. genotypes were similar. The major
267 fatty acids were palmitic (18.68 to 21.29%), oleic (24.53 to 33.53%) and linoleic acids
268 (37.29 to 48.67%). Linolenic acid was present at low concentration. These results were
269 consistent with previous studies.[14] The composition of the *Amaranthus* fatty acids was
270 very similar to other cereals (corn, sesame, cottongrain) as was reported by León-

271 Camacho et *al*.[50] Oil from *Amaranthus* sp. grain were highly unsaturated, the total
272 unsaturated fatty acid content ranged from 66.79 to 75.01%. The S/U (where S is
273 saturated and U is unsaturated) fatty acid ratios of different genotypes ranged from 0.31
274 to 0.50. It is important indicators in evaluating the nutritional and functional value. The
275 lower S/U ratios, which includes a higher content of linoleic acid (C18:2n-6), indicates
276 a good nutritional properties of *Amaranthus* sp. grain. Linoleic acid is essential fatty
277 acid which is necessary for healthy brain function, skin and hair growth, bone density,
278 energy production and reproductive health.[53,54]

279

280 **Cluster analyses of fatty acids profile**

281 A dendrogram of fatty acid profile of oil extracted by different methods from
282 *Amaranthus sp.* grain using complete linkage as an amalgamation rule and the city
283 block (Manhattan) distance as a measure of the proximity between samples is shown in
284 Figure 1. The dendrogram based on GC data showed proper distinction between three
285 *Amaranthus* sp. genotypes. The variability among genotypes might probably be due to
286 genetic composition, cultural practices and environmental factors.[16] As expected,
287 extraction methods were not considerably influenced by fatty acid compositions of
288 *Amaranthus* sp. grain oil. As shown in Figure 1., there is similarity in the lipid fractions
289 of genotypes 2 and 16, while genotype 31 was placed into separate branches of
290 dendogram. The variability among extraction methods within genotype might be caused
291 by the degradation of the double bonds of polyunsaturated fatty acids during Soxhlet
292 extraction, probably due to oxidation.[55]

293

294 **Figure 1**

295

296 **PCA analysis**

297 The PCA of the presented data explained that the first three components accounted for
298 76.73% of the total variance (34.69, 26.04 and 15.88%, respectively) in the thirteen
299 variables (fatty acid contents). Considering the map of the PCA performed on the data,
300 the contents of oleic (which contributed 11.5% of total variance, based on correlations),
301 linolenic (8.6%), arachidic (10.5%) and erucic acids (9.3%) exhibited positive scores
302 according to first principal component, whereas myristic (13.2%), palmitic (10.3%),
303 stearic (8.4%) and linoleic (19.0%), showed a negative score values according to first
304 principal component (Figure 3). The positive contribution to the second principal
305 component calculation was observed for: palmitoleic (18.7% of total variance, based on
306 correlations), linolenic (11.4%), heptadecanoic (11.6%), arachidic (12.3%), eicosenic
307 (8.2%), and erucic acids (11.5%), while negative scores on second principal component
308 calculation was observed for behenic (8.7%).

309 Positive contribution to the third principal component was obtained for pentadecanoic
310 (12.8% of total variance, based on correlations) and behenic acids (28.13%), while
311 negative influence was observed for palmitic (20.78%), stearic (13.36%) and oleic acids
312 (10.98%).

313 The points shown in the PCA graphics, which are geometrically close to each other
314 indicate the similarity of patterns that represent these points. The orientation of the
315 vector describing the variable in factor space indicates an increasing trend of these
316 variables, and the length of the vector is proportional to the square of the correlation
317 values between the fitting value for the variable and the variable itself. The angles
318 between corresponding variables indicate the degree of their correlations (small angles
319 corresponding to high correlations). A positive correlation between oleic acid (C18:1)
320 and monounsaturated fatty acids (MUFA) was observed due to oleic acid is the

321 predominant monounsaturated fatty acid in the lipid compositionl of *Amaranthus* sp.
322 grain. The similar observations can be seen between linoleic acid (C18:2,ω-6) and total
323 content of polyunsaturated fatty acids (PUFA). Generally, *Amaranthus* oil provides an
324 excellent source of omega fatty acids. Due to its high content of linoleic acid, an
325 essential fatty acid, *Amaranthus* grains can therefore be recommended for consumption
326 by children since they need essential fatty acids for proper growth and development.[13,54]
327 Pentadecanoic acid (C15:0) was positively correlated to myristic (C14:0) and linoleic
328 acids (C18:2,ω-6) and polyunsaturated fatty acids, and negatively correlated to oleic
329 (C18:1) acid and monounsaturated fatty acids (statistically significant at $p<0.05$ level).
330 Palmitic acid (C16:0) was negatively correlated to behenic acid (C22:0), while
331 palmitoleic acid (C16:1) was positively correlated to heptadecanoic acid (C17:0). Oleic
332 acid (C18:1) was negatively correlated to myristic (C14:0), pentadecanoic (C15:0) and
333 linoleic acid (C18:2,ω-6) and polyunsaturated fatty acids. Linoleic acid (C18:2,ω-6) was
334 negatively correlated to oleic acid (C18:1) and monounsaturated fatty acids. Linolenic
335 acid (C18:3,ω-3) was positively correlated to eicosenoic (C20:1) and erucic acid
336 (C22:1). Eicosenoic (C20:1) and erucic acid (C22:1) were also related. Behenic acid
337 (C22:0) is positively correlated to saturated fatty acids and negatively correlated to
338 unsaturated fatty acids.

339

340 **Figure 2**

341

342 The squalene and oil yield was most intensive using ASE extraction, especially in
343 genotypes 2 and 16, as shown on Fig. 3. The larger amount of MUFA was obtained
344 when using Soxhlet extraction with genoptype 16 than by using ASE, which also makes
345 ASE method more applicable for oil extraction. It seems that Soxhlet method is better

346 for oil extraction from *Amaranthus* sp. grain, genotype 2 due to higher SFA and lower
347 UFA content. Genotype 31 gained the worst results in squalene and oil extraction,
348 regardless of the extraction method, with the highest PUFA content.
349
350 **Figure 3**
351
352 **CONCLUSION**

353 *Amaranthus* sp. grain is now attracting worldwide attention because of its an
354 exceptional nutritional and functional properties. Considering of the concern for marine
355 animal protection, *Amaranthus* sp. gained importance as crop sources of squalene,
356 alternative to shark and whale liver oil. The results of this investigation show that is
357 very important to choose both the right genotype and the right method of extraction for
358 optimum oil and squalene yield. In order to obtain high yields of oil and squalene, ASE
359 is the most favorable method of *Amaranthus* sp. grain oil isolation. On the other hand,
360 ASE and Soxhlet methods require large amounts of organic solvents as extractants and
361 from the aspect of green chemistry SFE is more suitable, an efficient and
362 environmentally friendly extraction method. Based on the differences between
363 extraction methods for each genotype separately, we can conclude that lipid fractions
364 obtained by ASE and SFE were close to each other.
365

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521 and Anti-Nutrient Content of Grain Amaranth (A. *albus*). *J Food Sci Qual Manage* ***25***:
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523

524 **List of tables:**

525 **Table 1.** Particle size distribution of investigated samples

Sample	Specific surface area m^2/g	D[3,2] µm	D[4,3] µm	d(0.1) µm	d(0.5) µm	d(0.9) µm
2	0.07±0.01^a	85.20±0.38^a	230.69±3.62^a	47.19±3.23^a	220.45±6.21^a	419.58±10.72^a
16	0.06±0.01^a	101.33±0.67^c	239.07±1.18^b	61.99±5.49^b	228.54±9.31^b	424.16±15.01^b
31	0.06±0.01^a	92.43±0.49^b	238.78±0.78^b	64.43±2.86^b	227.92±5.31^b	422.46±9.27^b

526 The results are presented as mean±SD, n=3; different letter within the same row

527 indicate significant differences ($p<0.05$), according to Tukey's HSD test

528 **Table 2.** Comparison of oil yield (g kg^{-1}) obtained by SFE, Soxhlet and ASE methods

Genotype	SFE	Sox	ASE
2	58.8±0.3^{c}	70.0±0.1^{b}	73.1±0.2^{a}
16	61.1±0.4^{c}	75.7±0.2^{b}	78.1±0.3^{a}
31	54.6±0.2^{c}	62.1±0.15^{b}	65.1±0.2^{a}

529 The results are presented as mean±SD, n=3; different letter within the same row

530 indicate significant differences (p<0.05), according to Tukey's HSD test.

531

532 **Table 3.** Comparison of squalene yield (g kg^{-1}) obtained by SFE, Soxhlet and ASE

533 methods

Genotype	SFE	Sox	ASE
2	3.3±0.02^{c}	3.8±0.04^{b}	4.4±0.03^{a}
16	3.8±0.04^{c}	4.2±0.01^{b}	4.7±0.06^{a}
31	3.4±0.01^{c}	4.0±0.01^{b}	4.4±0.03^{a}

534 The results are presented as mean±SD, n=3; different letter within the same row

535 indicate significant differences (p<0.05), according to Tukey's HSD test.

536

537 **Table 4.** ANOVA table of oil and squalene yield evaluation (sum of squares)

Term	df	Oil yield	Squalene yield
Extraction	1	0.066	0.005^{+}
$Extraction^2$	1	2.969^{+}	0.011^{+}
Genotype	1	0.673^{*}	0.000
$Genotype^2$	1	1.285^{*}	0.003^{+}
Extraction × Genotype	1	0.000	0.000
Error	3	0.178	0.000
r^2		0.966	0.992

538 $^{+}$Significant at *p*<0.01 level, *Significant at *p*<0.05, level 95 % confidence limit, error

539 terms have been found statistically insignificant, df - degrees of freedom

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Figure captions:

Figure 1. Complete-linkage dendrogram for oil extraction from *Amaranthus* sp. grain

Figure 2. PCA ordination of variables based on component correlations

Figure 3. PCA ordination of summarizing values based on component correlations

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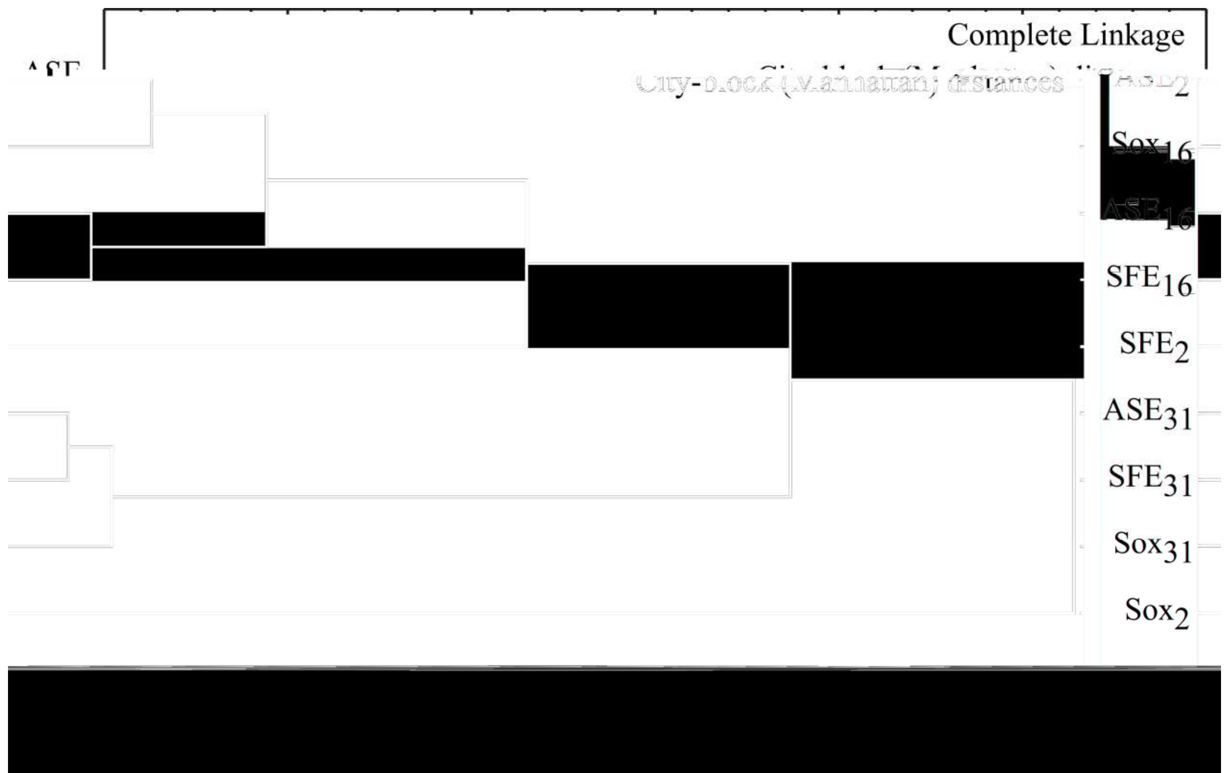

577 Figure 1

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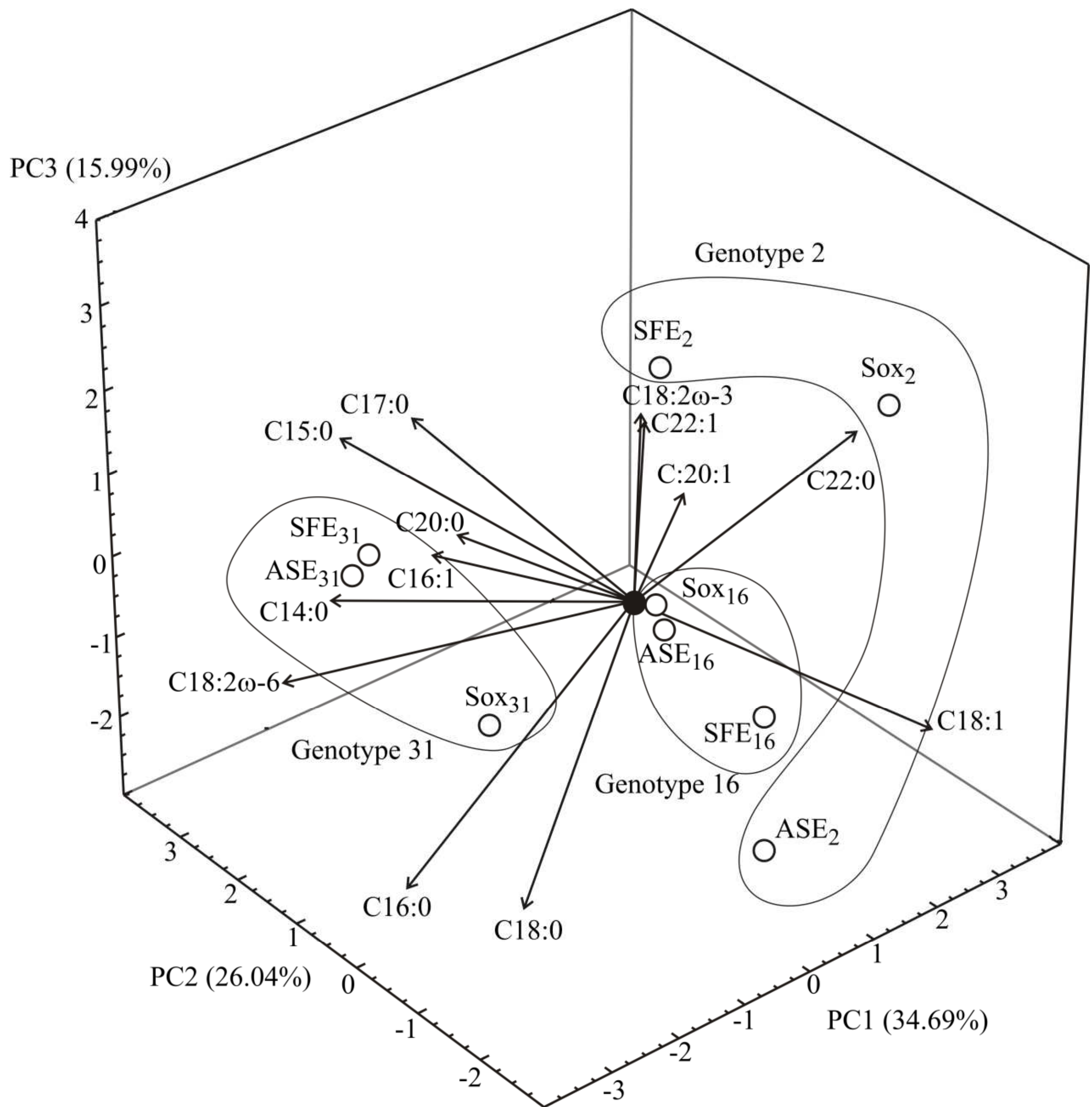

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592 Figure 2

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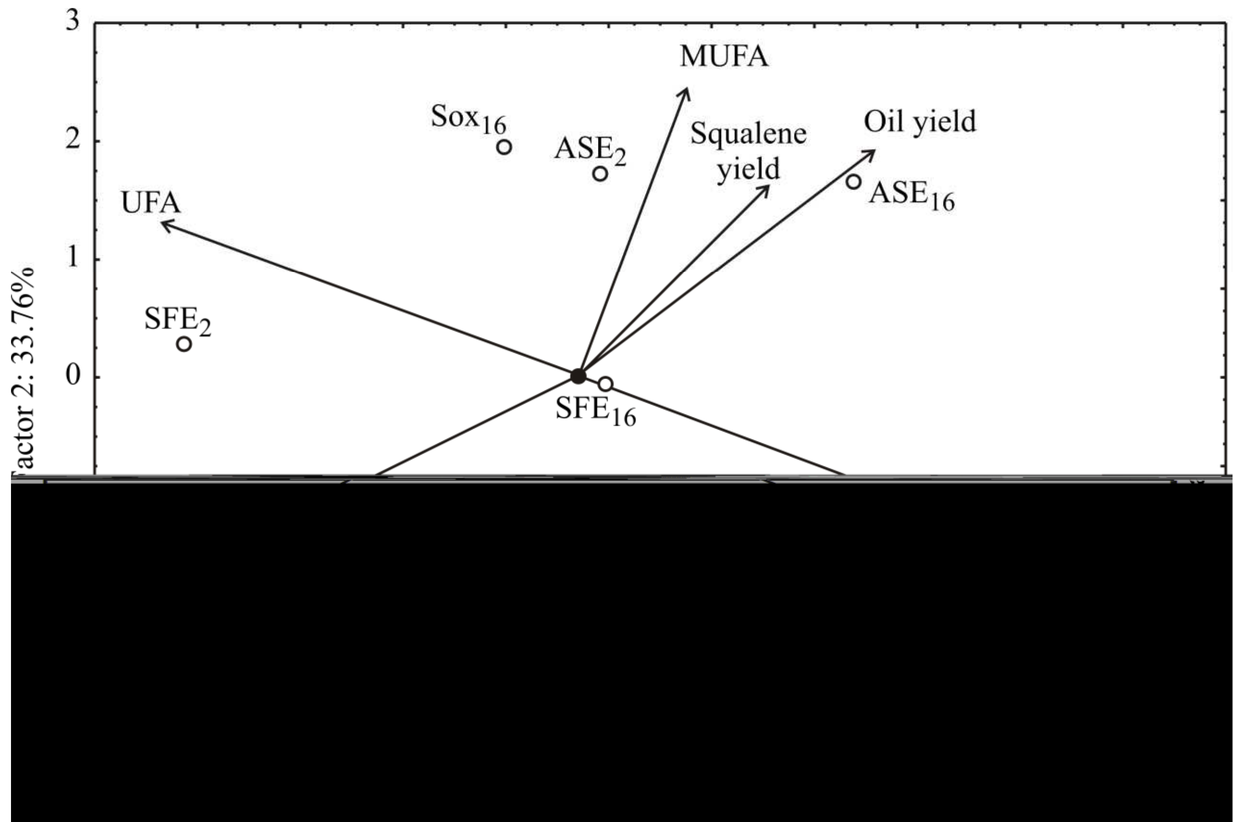

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600 Figure 3