Ultra-performance liquid chromatography-mass spectrometry for precise fatty acid profiling of oilseed crops

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Oilseed crops are one of the most important sources of vegetable oils for food and industry. Nutritional and technical properties of vegetable oil are primarily determined by its fatty acid (FA) composition. The content and composition of FAs in plants are commonly determined using gas chromatography-mass spectrometry (GS-MS) or gas chromatography-flame ionization detection (GC-FID) techniques. In the present work, we applied ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) technique to FA profiling of sunflower and rapeseed seeds and compared this method with the GC-FID technique. GC-FID detected 11 FAs in sunflower and 13 FAs in rapeseed, UPLC-MS appeared to be more sensitive, detecting about 2.5 times higher number of FAs in both plants. In addition to even-chain FAs, UPLC-MS was able to detect odd-chain Fas. The longest FA detected using GC-FID was a FA with 24 carbon atoms, whereas UPLC-MS could reveal the presence of longer FAs with the tails of up to 28 carbon atoms. Based on our results, we may conclude that UPLC-MS has a great potential to be used for the assessment of the FA profile of oil crops.

1 Ultra-Performance Liquid Chromatography Mass- Spectrometry

2 for precise fatty acid profiling of oilseed crops

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26 **Abstract**

27   Oilseed crops are one of the most important sources of vegetable oils for food and

28 industry. Nutritional and technical properties of vegetable oil are primarily determined by its

29 fatty acid (FA) composition. The content and composition of FAs in plants are commonly

30 determined using gas chromatography-mass spectrometry (GS-MS) or gas chromatography-

31 flame ionization detection (GC-FID) techniques. In the present work, we applied ultra-

32 performance liquid chromatography-mass spectrometry (UPLC-MS) technique to FA profiling

33 of sunflower and rapeseed seeds and compared this method with the GC-FID technique. GC-

34 FID detected 11 FAs in sunflower and 13 FAs in rapeseed, UPLC-MS appeared to be more

35 sensitive, detecting about 2.5 times higher number of FAs in both plants. In addition to even-

36 chain FAs, UPLC-MS was able to detect odd-chain Fas. The longest FA detected using GC-FID

37 was a FA with 24 carbon atoms, whereas UPLC-MS could reveal the presence of longer FAs

38 with the tails of up to 28 carbon atoms. Based on our results, we may conclude that UPLC-MS

39 has a great potential to be used for the assessment of the FA profile of oil crops.

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42 **Introduction**

43   Vegetable oils have been used by humans since ancient times. The main components of

44 the vegetable oil are triglycerides (about 95%) (Thomas, Matthäus & Fiebig, 2015). They are

45 composed of three fatty acids (FAs) attached to glycerol by ester bonds. FA profile is an

46 important characteristic of the vegetable oil (Zhang et al., 2014). The analysis of FA content is

47 one of the topical issues in plant metabolomics given the importance of deciphering lipid

48 biosynthesis pathways in plants. Oilseed crops are one of the most important sources of

49 vegetable oils for food and industry. Sunflower and rapeseed take the positions four and three in

50 the global production of vegetable oil after oil palm and soybean (Rauf et al., 2017). In oilseed

51 crops, the identification of FAs appears to be of special importance since nutritional and

52 technical properties of the oil extracted from these crops are primarily determined by its FAs

53 composition. For instance, unsaturated fatty acids contribute to decreasing cholesterol levels in

54 blood thus reducing the risk of heart diseases (Grundy, 1986).

55   Apart from their essential role in nutrition, sunflower and rapeseed oils have a number of

56   industrial applications: they can be used as basic components in polymer synthesis, serve as the

57   source of biofuel, or be used as emulsifiers or lubricants (Dimitrijević et al., 2017). Production of

58   selection hybrids with changed oil properties is one of the key objectives of the oilseed crop

59   breeding (Jocic´, Miladinovic´ & Kaya, 2015). Seed oil content and quality were subjected to

60   selection in all times in the history of sunflower and rapeseed crop improvement (Chapman &

61   Burke, 2012). One of the main directions in sunflower and rapeseed breeding is the selection for

62   high oleic oil. Such oil is characterized by higher degree of oxidative stability, which makes it

63   more suitable for frying, refining, and storage (Fuller, Diamond & Applewhite, 1967; Premnath

64   et al., 2016). This phenomenon finds many applications in food, automotive, and textile

65   industries.

66   Although sunflower and rapeseed breeding demonstrate certain similar traits, there exist

67   significant differences in the directions of the selection process in these two crops. The oil from

68   rapeseed naturally contains high quantities of erucic acid and glucosinolates. Due to this fact the

69   use of rapeseed oil in food industry was restricted, because of its physiopathological effect on

70   mammals(Borg, 1975). These limitations were overcome in the 1960s by the production of

71   rapeseed which oil was free from erucic acid. (Stefansson & Hougen, 1964).

72   Oilseed crop market constantly sets new trends in rapeseed and sunflower breeding which

73   stimulates plant breeders to develop new varieties with optimized FA content (Velasco &

74   Fernández-Martínez, 2002). Towards this end it appears necessary to elaborate efficient

75   protocols for precise and high throughput FA profiling. In the present time, the commonly used

76   techniques to measure FAs in plants are gas chromatography-mass spectrometry (GS-MS) or gas

77   chromatography-flame ionization detection (GC-FID) method (Li-Beisson et al., 2010). Prior to

78   GC analysis, FAs are usually converted into the corresponding methyl ester derivatives (FAMEs)

79   by methylation and *trans*-esterification (Liu, 1994).

80   By combining the advantages of fast, high-resolution chromatographic separation with

81   the high sensitivity, selectivity, and specificity of mass spectrometric detection UPLC-MS

82   became the technique of choice for a wide range of applications.(Pitt, 2009; Hummel et al.,

83   2011; Want et al., 2013) Bromke et al. (Bromke et al., 2013, 2015) suggested using liquid

84   chromatography high–resolution mass spectrometry (UPLC-MS) for FA profiling in Arabidopsis

85   *thaliana* and diatoms.

86   In the view of all mentioned above, the aim of the present study was to test the use of

87   ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) coupled with lipid

88   extraction using methyl tert-butyl ether for precise analysis of FA composition of oilseed crops

89   (sunflower and rapeseed).

90

91   **Materials & Methods**

92   *Plant Material*

93   Seeds from 50 sunflower (*Helianthus annuus*) and 50 rapeseed (*Brassica napus*) lines

94   (Table S1) from the Pustovoit All-Russia Research Institute of Oil Crops Collection (Russia,

95   Krasnodar) were used in the study.

96   *Reagents*

97   The following reagent were used: Methanol LC-MS (Scharlau, Spain), Methyl tert-butyl

98   ether HPLC grade (Scharlau, Spain), Chloroform HPLC grade (Fisher Chemical, USA), Heptane

99   LC/MS grade (Honeywell Fluka, USA), Water UHPLC-MS grade (Scharlau, Spain), Potassium

100   hydroxide solution 45% in water (Sigma Aldrich, USA), NaCl USP grade (Helicon, Russia),

101   HCl 37% (PanReac AppliChem,USA), Acetonitrile LC/MS grade (Fisher Chemical, USA),

102   Isopropanol LC-MS grade (Honeywell Fluka, USA), Ammonium acetate (Honeywell Fluka,

103   USA), Formic acid 98%-100% LC-MS grade (LiChropur Merck Millipore, USA), Acetic acid

104   Optima, LC-MS grade (Fisher Chemical, USA), and FA standards (Oleic acid-$^{13}C_{18}$ (Sigma

105   Aldrich), Palmitic acid-$^{13}C_{16}$ (Sigma Aldrich), and Stearic acid-$^{13}C_{18}$ (Sigma Aldrich)).

106   *GC-FID Analysis*

107   4-5 g of seeds were mixed together and homogenized and 0.5 g was taken for fatty acid

108   extraction with 4 ml of hexane.

109   To obtain the methyl esters of fatty acids, 2-3 ml of homogenized seed-hexane mixture was

110   transferred into the new tube and 0.1 ml of sodium methylate was added, and mixed intensively

111   for two minutes. The tube content was further transferred onto the paper filter with Na_2SO_4 on

112   the bottom. Obtained filtrate was then placed into the DAG-2M automatic dispenser tube.

113   GC-FID analysis was carried out using the «Chromateck-Crystall 5000» GC

114   chromatograph with the DAG-2M automatic dispenser. GC separation was performed in a

115   SolGelWax column with the dimensions of 30 m×0.25 mm×0.5 **µ**m; gas mobile phase - helium;

116   speed - 25 sm/sec; temperature range - 185-230 ºC.

117   UPLC-MS analysis

118   For lipid extraction, 10 mg (for each line) of *sunflower* (1 sample - 1 seed) and *rapeseed*

119   (1 sample - several seeds) seeds were homogenized using six 2.8 mm zirconium oxide beads

120   (Bertin Instruments, France) in the Precellys Evolution homogenizer (Bertin Instruments,

121   France) coupled with Cryolis filled with dry ice at the temperature not exceeding 10°C.

122   Homogenization parameters were as follows: 6800 rpm, 3* 20 sec, pause 30 sec. 400 uL of

123   methanol/methyl tert-butyl ether mixture (1 : 3 v : v) was added prior to homogenization. After

124   homogenization, another 400 **µ**l of methanol/methyl tert-butyl ether mixture was added, and the

125   sample was mixed by vortexing. After sonication for 10 min in the ice-cooled ultrasonic bath and

126   incubation at 4°C for 30 min with shaking, the sample was transferred into the new 1.5 ml

127   Eppendorf tube, and 560 ml of water/methanol mixture (3 : 1 v : v) was added. After the addition

128   of water/methanol the sample was mixed by vortexing for 10 min and centrifuged for 10 min at

129   4°C at 12700 rpm. This leads to the separation of two phases: the lipophilic phase and the polar

130   phase. The upper lipophilic phase was collected and vacuum dried in Concentrator plus

131   (Eppendorf) for 1.5 h at 30°C and the pellet was stored at - 80°C before FA measurement. This

132   protocol is based on the protocol described by Giavalisco et al. (2011) (Giavalisco et al., 2011) .

133   For FA quantification, three isotopically labeled internal FA standards were added to the

134   extraction mixture (3 µg of each FA per sample).

135   For the analysis of FAs, the extracts obtained at the previous step were hydrolyzed using

136   the protocol adopted from Bromke (Bromke et al., 2015). Lipid extracts were resuspended in

137   200 **µ**l of the mixture of methanol and 6% KOH (4 : 1, v : v). The tubes were incubated for 2 h at

138   60°C with continuous shaking (1800 rpm). After cooling to room temperature, 100 **µ**l of

139   saturated NaCl solution was added. The reaction mixture was acidified by the addition of 50 **µ**l

140   of 29% HCl. Tubes were thoroughly vortexed and spun for 30 s at full speed using a table

141   centrifuge. FAs were extracted with 200 **µ**l of chloroform/heptane mixture (1 : 4, v : v). After

142   mixing by vortexing and 15 sec centrifugation, the organic phase was collected. Extraction with

143   chloroform /heptane mixture was repeated the second time and the collected FA-containing

144   organic phases were combined. The extract was washed by the addition of 200 **µ**l of water

145   followed by short vortexing and 15 sec centrifugation at 12700 rpm, which resulted in the

146　separation of two phases. Finally, the organic upper phase was collected, dried under vacuum

147　conditions in Concentrator plus (Eppendorf) (30 min, 30°C) and stored at - 80°C before FA

148　measurement.

149　　　To prepare samples for the injection into the UPLC-MS system, the dried extracts were

150　first resuspended in acetonitrile/isopropanol mixture (70 : 30), vortexed for 10 sec, and incubated

151　for 10 min at 4°C with shaking, followed by sonication for 10min on ice and centrifugation for 7

152　min at 4°C at the speed 12700 rpm. After the completion of these procedures, final dilutions with

153　acetonitrile/isopropanol (70 : 30) were prepared in MS vials.

154　　　Samples were processed using mass spectrometry (UPLC-MS) coupled with reversed

155　phase ultra-performance liquid chromatography (ACQUITY UPLC System; Waters, USA) on Q-

156　TOF (Quadrupole-Time-of-Flight) Maxis Impact II mass spectrometer (Bruker Daltonic,

157　Germany). Parameters for analysis were set according to the negative ion mode with the spectra acquired

158　over a mass range from m/z 50 to 1200. The optimum values of the ESI-MS parameters were as follows:

159　capillary voltage, + 4.0 kV; drying gas temperature, 200 °C; drying gas flow, 6.0 L/min; nebulizing gas

160　pressure, 2 bar.

161　　　UPLC separation was carried out using the C8 Acquity Beh column (2.1mm x 100mm,

162　1.7- μm particle size; Waters) and the Acquity BEH C8 1.7 μm Vanguard precolumn (Waters) at

163　60°C. For UPLC gradient the mobile phases consisted of two solvents. Two different solvent

164　systems and gradients were tested.

165　　1)　Solvent A: 1% of 1 M NH_4Ac solution and 0.1% formic acid in water; and solvent B:

166　　　　acetonitrile/isopropanol (7 : 3, 1% of 1M NH_4Ac solution and 0.1% formic acid), with

167　　　　an injection volume of 3 μl. The following gradient profile was applied: 55% B, 1 min;

168　　　　linear gradient from 55% B to 80% B, 3 min; linear gradient from 80% B to 85% B, 8

169　　　　min; linear gradient from 85% B to 100% B, 3 min. After washing the column for 4 min

170　　　　50 sec with 100% B, the proportion of buffer B in the mixture is set back to 55%, and the

171　　　　column is re-equilibrated for 4 min 10 sec (24.5 min total run time), with the mobile

172　　　　phase flow rate of 400 **μ**l/min.

173　　2)　Solvent A: 1% of 1M NH_4Ac solution and 0.1% acetic acid in water; and solvent B,

174　　　　acetonitrile/isopropanol (7 : 3, 1% of 1M NH4Ac solution and 0.1% acetic acid), with

175　　　　an injection volume of 3 μl. The following gradient profile was applied: 55% B, 50 sec;

176　　　　linear gradient from 55% B to 75% B, 1 min; linear gradient from 75% B to 89% B, 5

177   min; linear gradient from 89% B to 100% B, 1 min 10 sec. After washing the column for

178   2 min with 100% B, the proportion of buffer B in the mixture is set back to 55%, and the

179   column is re-equilibrated for 1 min 50 sec (11.5 min total run time), with the mobile

180   phase flow rate of 400 **μ**l/min.

181   The sample final dilution was 5 (UPLC-5) and 400 times (UPLC-400) for the buffer systems

182   1 and 2, respectively.

183   *Method validation procedure*

184   Validation procedure included the evaluation of linearity, repeatability, reproducibility, and

185   calculation of the limit of detection (LOD) and the limit of quantification (LOQ).Linearity was

186   evaluated by building seven-point calibration curves (six replicates) in pure solvents. Solvent

187   (acetonitrile/isopropanol, 70/30) was spiked with a standard mixture of isotopically labeled fatty

188   acids (Oleic acid-13C18, Palmitic acid-13C16, and Stearic acid-13C18) in order to obtain all

189   standards in the concentrations of 5, 10, 50, 100, 500, 1000, and 2500 ng/mL. The mean

190   correlation coefficients (n = 6) of the calibration curves were 0.997 or higher for the target

191   analytes, indicating good linearity in the selected concentration range(Tables 1A and 1B).

192   The limit of detection (LOD) and the limit of quantification (LOQ) were determined from spiked

193   samples, as the minimum detectable concentration of analyte with a signal-to-noise ratio of 3 and

194   10, respectively.

195   For repeatability evaluation, MS technical repeats were performed. The results are presented in

196   the Table S2.

197   *Data processing analysis*

198   GC-FID: FAs detection was performed based on retention times using FA methyl ester

199   standards (Fluke). The percentage of each FA was calculated based on the peak area using GC

200   software.

201   Xcms package (Smith et al., 2006) was used for UPLC-MS data processing. Optimal

202   parameters were chosen with the aid of the IPO package (Libiseller et al., 2015). Peak intensities

203   were obtained using the peakTable function implemented in the xcms package with

204   method='maxint'. As a result, set of peaks characterized by the retention time, mass divided by

205   charge, and intensity for each sample was obtained. Isotopically labeled internal FA standards

206   were used for retention time correction and alignment.

207   To identify peaks that correspond to FAs, all possible chemical formulas that correspond

208   to FAs with the chain length varying between 10 and 28 carbon atoms (n) and with the number

209   of double bonds varying from 0 to 6 (k) defined as $C_nH_{2n-2k}O_2$ were generated. Since the method

210   does not distinguish between isomeric FAs, in what follows, the term 'FA' will be used to denote

211   the groups of isomers (example of isomeric pattern presented on Figure S1) defined by certain n

212   and k, for example, FA18:1 denote all fatty acids with 18 carbons and single double bond. The

213   masses of all these FAs in deprotonated state were calculated and all peaks matched masses

214   within 20 ppm (part per million, for two masses m1 and m2 ppm=abs(m1-

215   m2)/max(m1,m2)*10^6) were searched for.

216   Then peaks were manually selected based on the net-like patterns as described in [15].

217   Briefly, it was observed that the extension of the FA length increases retention time, while the

218   addition of the double bond reduces retention time, thus FA peaks form net-like pattern.

219   For the sunflower LC-5 FA18:3 two peaks with the ten second difference between their

220   retention times were obtained. Since the intensities of both these peaks exhibited high Pearson

221   correlation with the FA18:3 intensity in LC-400 data the sum of these peaks was used in the

222   analysis. Intensities of all detected FAs were at least three orders of magnitude higher in plant

223   samples than in blank samples. To obtain FA mass fractions, the intensities of all FAs were

224   divided by the sum of intensities of FAs identified using all three methods (GC-FID, UPLC-5,

225   and UPLC-400) and multiplied by 100.

226   Statistical analysis and data visualization was carried out using R (**R** Core Team, 2013).

227

228   **Results**

229   In the present work, we compared the application of GC-FID coupled with hexane

230   extraction and two UPLC-MS based approaches: 1:5 dilution in the buffer system with formic

231   acid (UPLC-5) and 1:400 dilution in the buffer system with acetic acid (UPLC-400) for FA

232   profiling in sunflower and rapeseed, both using MTBE extraction.

233   *GC-FID data*

234   Fifty lines of either of the two oilseed crops were analyzed using GC-FID. As a result we

235   have detected 11 and 13 FAs in sunflower and rapeseed, respectively (Figures 1C and 2C, Table

236   S3). The lists of FAs which were detected in the two crops differed by minor FAs, while C16:0,

237   C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, and C24:0, were common for both of

238   them. FA C20:2, C22:1, and C24:1 were detected in rapeseed only, while C14:1 FAs we found in

239   sunflower but were absent in rapeseed (Figures 1C and 2C).

240   In rapeseed oil, the most abundant FAs were C16:0, C18:0, C18:1, C18:2, C18:3, and

241   C20:1 which together made for 98.6 % of all FAs. In sunflower seed oil, the most abundant FAs

242   were C16:0, C18:0, C18:1, C18:2, C22:0, which constituted 98.9% of all FAs. In both species,

243   two FAs (18:2 and 18:1) constitute more than 80% of the total FA content. However, in

244   rapeseed, 18:1 is three times more abundant than 18:2, while in sunflower it is 18:2 which

245   dominates (Figure 1C and 2C).

246   The relative abundance of FAs was demonstrated to vary between samples (Table S3).

247   *UPLC-MS data*

248   For UPLC-MS fatty acids profiling we used two different buffer systems: the UPLC

249   buffer with formic acid and the buffer with acetic acid. The former buffer is the most common

250   buffer used in UPLC-MS experiments. In the latter buffer, ionization is known to be more

251   effective since acetic acid is a weaker acid, so lower sample amount, and, therefore, higher

252   sample dilution is required. For this reason for the system with acetic acid we used higher

253   dilution (1:400), and for the system with formic acid we used lower sample dilution (1:500).

254   Hydrolyzed extracts of all lines of both sunflower and rapeseed were analyzed using both

255   UPLC-MS-based methods.

256   At low dilution (UPLC-5), we are able to detect 29 FAs for sunflower and 35 FAs for

257   rapeseed. At high dilution (UPLC-400), 25 and 31 FAs were detected for sunflower and

258   rapeseed, correspondently (Figures 1C and 2C, Table S2). Hence, the use of lower dilution rates

259   proved to be a more efficient approach which allowed to reveal the highest number of FAs.

260   However, in this case it becomes impossible to perform the relative quantification of major FAs:

261   18:1 and 18:2, because they cause detector saturation (Figure 3A, B). Apart from even-chain

262   FAs, UPLC-MS also revealed odd-chain FAs (for example 17:0, 17:1, and 17:2). FAs with 18-

263   carbon chain (stearic, oleic, and linoleic) were the most abundant. A net-like pattern on the

264   retention time M/z-RT plots can be observed (Figure 4A, B and 5A, B).

265   FA content show a big variability between the lines (Table S2, Figure S2).

266   Results of both UPLC-MS methods show strong correlation: Spearman rho = 0.927 and

267   0,854 for sunflower and rapeseed, respectively (Figure 4C, 5C).

268

269   *Comparison of UPLC-MS with GC-FID*

270   Results of UPLC-MS were compared with results obtained using GC-FID. All 11 FAs

271   detected in sunflower and 13 FAs detected in rapeseed were also identified using UPLC-MS. In

272   the case of both plants, UPLC-MS proved to be more sensitive and detected about 2.5 times

273   higher number of FAs than GC-FID (Figure 1C, 2C). The majority of the minor FAs (mass

274   fraction below 0.5%) are missed by GC-FID. The longest FA detected by GC-FID was the FA

275   with 24-carbon chain, whereas UPLC-MS provided insight into the changes in longer FAs with

276   the chains containing up to 28 carbon atoms.

277   As it can be seen in Figures. 1A, B and 2A, B, although the relative amounts of FAs

278   measured by UPLC-MS and GC-FID are different, there exist significant correlation between

279   them. Spearman rho = 0.908 and 0,918 for sunflower GC-FID and UPLC-5 and GC-FID and

280   UPLC-400, respectively. Spearman rho = 0.979 and 0,947 for rapeseed GC-FID and UPLC-5

281   and GC-FID and UPLC-400, respectively.

282

283   **Discussion**

284   FA composition of seed oil from 50 sunflower and 50 rapeseed lines was analyzed by GC-FID,

285   which is traditionally used to measure FAs in plant oils, and two UPLC-MS-based approaches

286   (1:5 dilution in the buffer system with formic acid (UPLC-5) and 1:400 dilution in the buffer

287   system with acetic acid (UPLC-400)). GC-FID technique allowed to detect 11 and 13 FAs in

288   sunflower and rapeseed, respectively, all of them representing even-chain FAs.

289   GC-FID based FAs abundances in sunflower and rapeseed are in good agreement with

290   those obtained in previous investigations(Alpaslan & Gündüz, 2000; Pleite, Martínez-Force &

291   Garcés, 2006; Demurin & Borisenko, 2011; Bocianowski, Mikołajczyk & Bartkowiak-Broda,

292   2012; Sharafi et al., 2015).

293   UPLC-MS is a more sensitive technique compared with GC-FID, so it was not surprising

294   that we detected a considerable number of additional components in the FA profiles obtained

295   using this method. This result corresponds well with the data obtained by Bromke et. al(Bromke

296   et al., 2015) on *Arabidopsis thaliana* tissues.

297   In total, about 29 and 35 FAs were detected in sunflower and rapeseed samples,

298   respectively, by UPLC-MS. It is worth noting that utilizing the UPLC-5 approach we were able

299   to identify significantly more FAs in both crops compared with the UPLC-400 approach.

300   However with the 1:5 dilution implemented in this approach it was impossible to perform the

301   relative quantification of the two most abundant FAs, 18:1 and 18:2, due to detector saturation.

302   In view of this, we suggest that the UPLC-400 approach is more suitable for FA profiling of oil

303   crop samples.

304   We have demonstrated in our study that UPLC-MS method is appropriate for detection of

305   long FAs both in sunflower and rapeseed. The longest FA detected by GC-FID method was the

306   FA with 24-carbon chain, whereas UPLC-MS revealed FAs with the tails up to 28 carbon atoms

307   long. Generally, about half of the FAs identified by UPLC-MS belong to Very-long-chain fatty

308   acids (VLCFA, fatty acids with the chain length of at least 20 carbon atoms). According to the

309   previous investigations, VLCFAs are mainly located in the cuticular wax layer deposited at the

310   surface of plant aerial organs; they form part of triacylglycerides of seed oil and sphingolipids

311   and are essential for many aspects of plant development and apparently play role as signal

312   molecules governing both biotic and abiotic stress(Roudier et al., 2010; Bach & Faure, 2010; De

313   Bigault Du Granrut & Cacas, 2016).

314   Apart from even-chain FAs, odd-chain fatty acids were also detected by UPLC-MS. The

315   latter are present in the oil extracted from the analyzed plants only in minor quantities and

316   selection for these FAs is not currently included in breeding programs. However, beneficial

317   effects of these compounds on human health have been recently demonstrated. For example,

318   pentadecenoic and heptadecenoic acids contribute to reduced risks of developing multiple

319   sclerosis and act as anti-inflammatory and edema-inhibiting agents(Degwert, Jacob & Steckel,

320   1998; Jenkins et al., 2017). Additionally, odd-chain fatty acids inhibit the development of certain

321   plant pathogens and could be used as precursors for manufacturing agricultural and industrial

322   chemicals (Fitton & Goa, 1991; Avis, Boulanger & Bélanger, 2000; Avis & Bélanger, 2001;

323   Clausen, Coleman & Yang, 2010; Köckritz, Blumenstein & Martin, 2010). The chemical

324   properties and potential biological activities of odd-chain fatty acids are continuously under

325   investigation(Rezanka & Sigler, 2009). Due to the importance of odd-chain fatty acids efforts are

326   being made to produce yeast strains with increased content of FAs of this kind (Park et al.,

327   2018).

328   Therefore, the analysis of minor FA content in oil may be important to make a complete

329   assessment of the functional and nutritional properties of the oil. Our results suggest that UPLC-

330   MS has great potential as a precise tool for evaluation of the full FA profile in oilseed crops. It

331   can be essential for the creation of vegetable oils with increased nutrition value and/or new

332   technical characteristics and provide additional markers for agronomically important traits in

333   plants.

334   Considering the advantages of UPLC-MS and its applicability for FA profiling in oilseed

335   crops, we may observe that the overall result can be represented as a net-like pattern on the

336   retention time M/z-RT plots (Fig. 4A, B and 5A, B). This makes the process of results

337   interpretation and annotation easier compared to GC-FID results.

338   Another advantage of UPLC-MS is the possibility of analyzing thousands of samples per

339   month and small amount of plant material needed for the analysis. This technique requires only

340   5-10 mg of plant material per extraction, while GC-FID requires high amounts of material . For

341   plants with big seeds like sunflowers it is possible to take only a small part of the seed for the FA

342   profiling analysis and germinate the rest of the seed and plant it, which allows exact assignment

343   of phenotype to genotype in breeding programs.

344   It also worth mentioning that UPLC-MS technique involves no FA derivatization, which

345   allows identifying more FAs compared to the conventional GC-FID technique. The results of the

346   present study confirm this and they are in good correspondence with data obtained by Bromke et.

347   al. (Bromke et al., 2015) on Arabidopsis.

348   It is important to highlight that certain differences between the results obtained by GC

349   and UPLC-MS may be connected with the lipid extraction procedure. We used MTBE extraction

350   which extract both TAGs and phospholipids, which mean that some detected FAs may come

351   from phospholipids, compared to GS where extraction was performed with hexane which

352   normally extracts mostly TAGs.

353   Based on the results obtained in the present study, we conclude that UPLC-MS is a

354   promising technique which may be used for FA composition analysis in oil crops as it is highly

355   sensitive, scalable, and suitable for the individual seed analysis.

356   Currently gas chromatography with mass spectrometric or flame ionization detection is

357   the gold standard for quantitative assessment of FA composition of vegetable oils. Based on our

358   results, UPLC-MS has great potential to be used in evaluation of FA composition of oil crops as

359   highly sensitive, scalable, and suitable for the individual seed analysis technique. In our study, it

360   has been shown that GS-MS/GS-FID could be substituted by UPLC-MS in a number of cases.

361   However, additional comparative studies are required for UPLC-MS method to become a

362   standard technique for evaluation of oil FA composition both in breeding programs and for

363   industrial purposes.

364

365   **Conclusions**

366   In this work we compared the performance of hexane lipid extraction with GC-FID and

367   MTBE lipid extraction with UPLC-MS when measuring FA profiles in 50 sunflower and 50

368   rapeseed samples. Based on the obtained results we may conclude that UPLC-MS with MTBE

369   extraction has a great potential for the study of FA composition of the oilseed crops which may

370   be used both to evaluate the oil properties for nutritional and industrial needs and to perform

371   high-throughput analysis to assist oilcrop breeding as well as to search for new potential valuable

372   traits. The main advantages of this technique as it was demonstrated in this study are the high

373   sensitivity which detects 2.5 times more FA species both in sunflower and rapeseed than the

374   conventional GC-FID technique, including the minor fatty acids and odd-chain fatty acids,

375   which are usually omitted. Additionally UPLC-MS technique is able to detect FAs with long

376   carbon chains, including those with 28-carbon tails. UPLC-MS technique doesn't require FA

377   derivatization which also adds to its high sensitivity. It is worth mentioning, that using this

378   technique it appears possible to use small amount of the starting material which makes it very

379   useful in breeding programs since it appears possible to use a small part of the seed for analysis

380   and to germinate the remaining part and use it in breeding programs, for example for subsequent

381   phenotyping. Taken together our finding suggest that UPLC-MS provide a deep insight into the

382   oil FA content and may be applied for precise identification of FA profiles of oilseed crops,

383   although further comparative studies using larger samplings and further improvements and

384   optimization are required

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Table 1(on next page)

Linearity, LOD, LOQ, correlation coefficients (*R)*of the target compounds for LC-MS method validation (A-ammonium acetate with acetic acid addition, B- ammonium acetate with formic acid addition)

1   A.

Compound	Equation	Linearity, ng/mL	R	LOD ($ng*mL^{-1}$)	LOQ ($ng*mL^{-1}$)
Oleic acid-^{13}C18	y = 2424.2x - 4768.3	50-1000	0.999	15	50
Palmitic acid-^{13}C16	y = 5908.5x - 38971	10-2500	0.997	5	10
Stearic acid-^{13}C18	y = 2715.3x - 62204	50-1000	0.999	15	50

2   B.

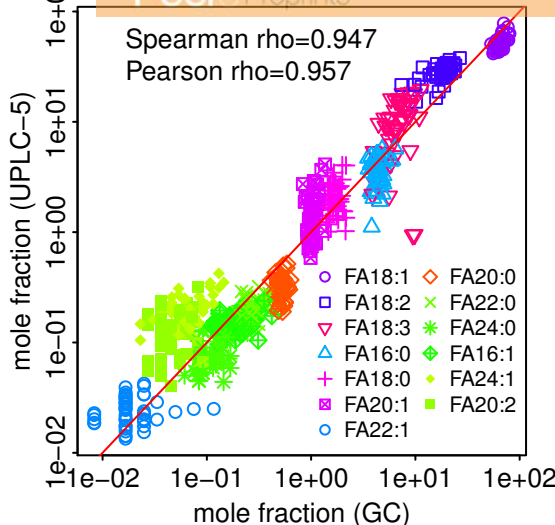| Compound	Equation	Linearity	R	LOD ($ng*mL^{-1}$)	LOQ ($ng*mL^{-1}$)
Oleic acid-^{13}C18	y = 273.87x - 12117	100-2500	1	40	100
Palmitic acid-^{13}C16	y = 354.89x - 14928	50-2500	0.999	20	50
Stearic acid-^{13}C18	y = 190.76x - 8817.5	100-2500	0.996	40	100

3

4

Figure 1(on next page)

Dependency between the mass fractions of FAs in sunflower (calculated for 11 FAs detected by GC-FID) estimated using different methods.

GC-FID vs UPLC-5 and GC-FID vs UPLC-400 are shown on panels (A) and (B), respectively. Each dot corresponds given FA (shown by color) in given sample (C) Mean mass fractions (relative to the total intensity of 11 FAs detected by GC-FID) for all FAs determined using the three techniques. FAs which were not detected by the indicated method are shown by white rectangles. FAs are ordered according to their intensities as obtained by UPLC-5.
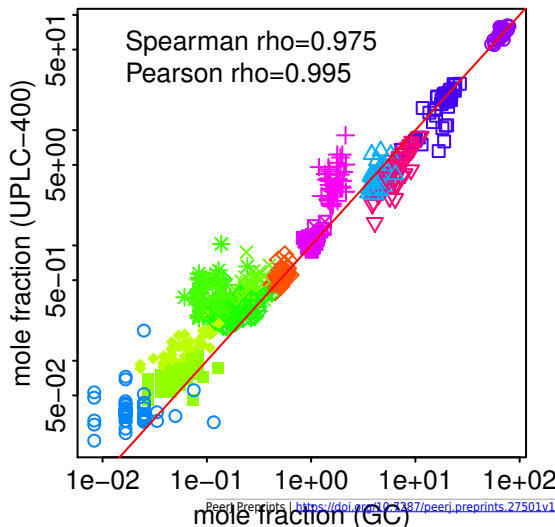
Figure 2(on next page)

Dependency between the mass fractions of FAs in rapeseed (calculated for 11 FAs detected by GC-FID) estimated using different methods.

GC-FID vs UPLC-5 and GC-FID vs UPLC-400 are shown on panels (A) and (B) respectively. Each dot corresponds given FA (shown by color) in given sample (C) Mean mass fractions (relative to the total intensity of 11 FAs detected by GC-FID) for all FAs determined using the three techniques. FAs which were not detected by the indicated method are shown by white rectangles. FAs are ordered according to their intensities as obtained by UPLC-5.

A

GC vs UPLC−5

Spearman rho=0.947
Pearson rho=0.957

mole fraction (UPLC−5)

FA18:1	FA20:0
FA18:2	FA22:0
FA18:3	FA24:0
FA16:0	FA16:1
FA18:0	FA24:1
FA20:1	FA20:2
FA22:1	

mole fraction (GC)

B

GC vs UPLC−400

Spearman rho=0.975
Pearson rho=0.995

mole fraction (UPLC−400)

mole fraction (GC)

C

	UPLC-5	UPLC-400	GC
FA18:1	52.4%	67.4%	66.1%
FA18:2	28.7%	17.1%	18.5%
FA18:3	10.4%	5.1%	7%
FA16:0	3.8%	3.8%	4.5%
FA18:0	2%	3.6%	1.7%
FA20:1	1.6%	1.1%	1%
FA20:0	0.3%	0.5%	0.5%
FA28:0		0.4%	
FA22:0	0.2%	0.4%	0.3%
FA16:1	0.2%	0.3%	0.2%
FA24:0	0.1%	0.4%	0.1%
FA24:1	0.2%	0.1%	0.1%
FA27:0		0.1%	
FA20:2	0.1%	0.1%	0.1%
FA14:0	0.02%	0.1%	
FA15:0	0.02%	0.1%	
FA17:0	0.04%	0.1%	
FA19:1	0.1%		
FA16:2	0.1%	0.1%	
FA17:1	0.04%	0.1%	
FA26:0	0.01%	0.1%	
FA16:3	0.04%	0.1%	
FA25:0	0.005%	0.1%	
FA23:0	0.02%	0.1%	
FA22:1	0.02%	0.04%	0.02%
FA15:1	0.02%	0.02%	
FA26:1	0.02%	0.02%	
FA23:1	0.02%	0.02%	
FA21:0	0.01%	0.03%	
FA25:1	0.01%	0.01%	
FA15:4	0.01%		
FA19:2	0.01%		
FA14:1	0.004%	0.01%	
FA20:3		0.01%	
FA19:0	0.004%		
FA17:2	0.004%		
FA12:0	0.002%		
FA14:2	0.001%		

Figure 3

LC chromatograms obtained for sunflower.

(A) UPLC-5. (B). UPLC-400. Chromatographic peaks corresponding to 18:1 and 18:2 FAs stay out of dynamic range in (A). Higher dilution rates aid in resolving this problem (B).
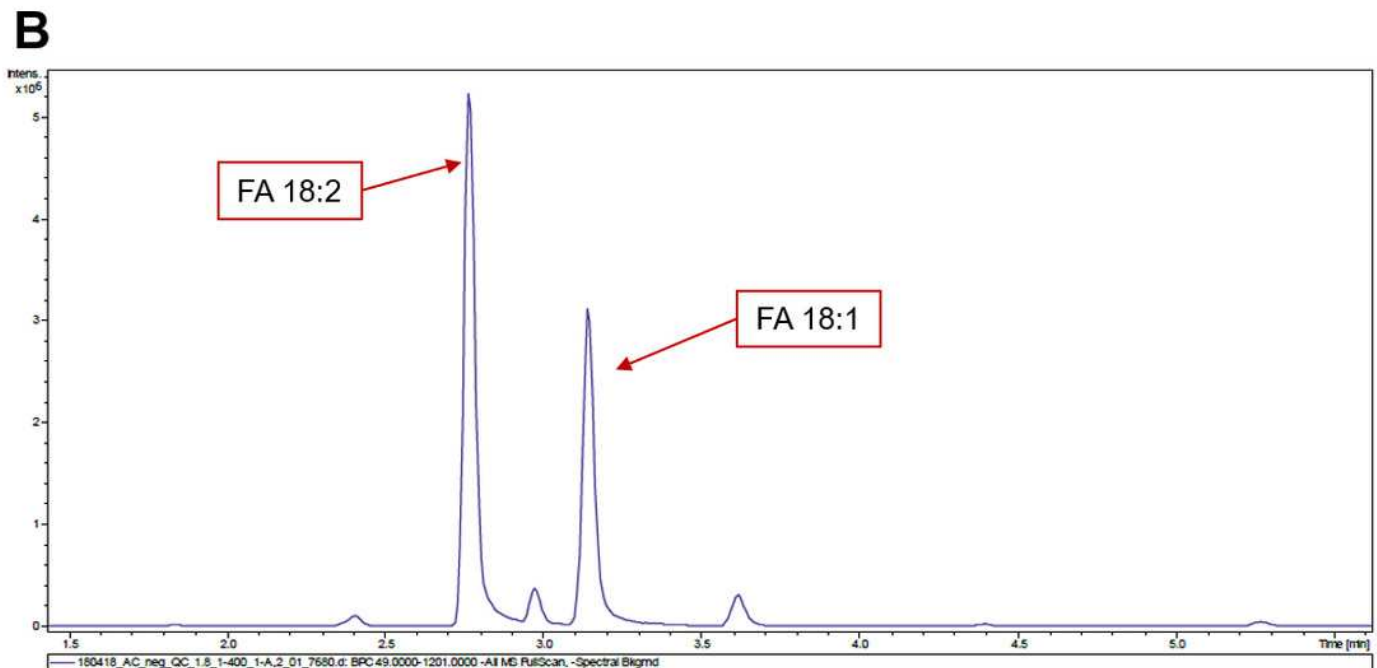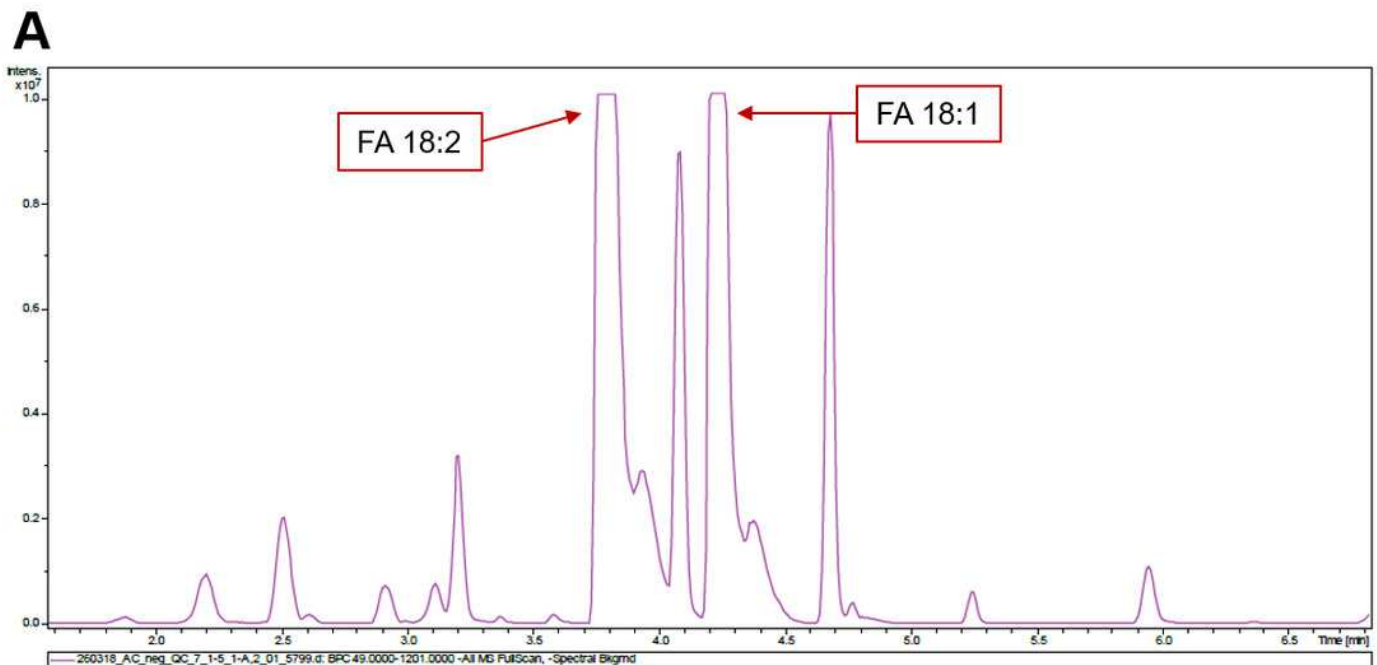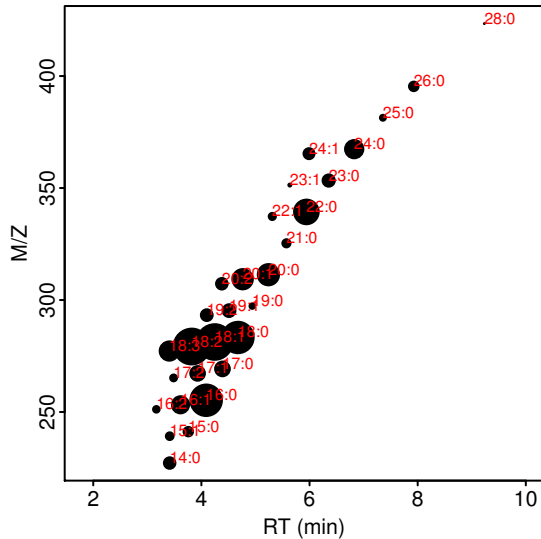
Figure 4(on next page)

Comparison of different techniques for quantitative assessment of FAs in sunflower

(A, B). Retention time (x-axis, RT) – m/z (y-axis) scatter plots for UPLC-5 (A) and UPLC-400

(B). Each dot corresponds to the individual FA, mean log intensity is indicated by the dot size.

Red color text indicates chain length and number of double bounds. (C) Dependency

between the mole fractions estimated using different methods, UPLC-5 vs UPLC-400.Pearson

correlation was calculated between logs of mole fraction.

A UPLC−5

B UPLC−400
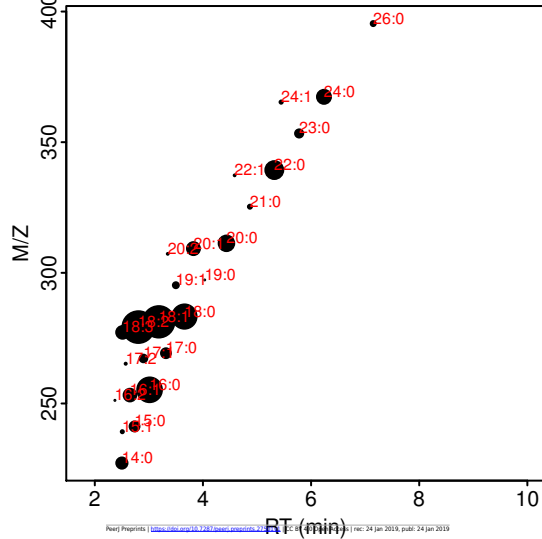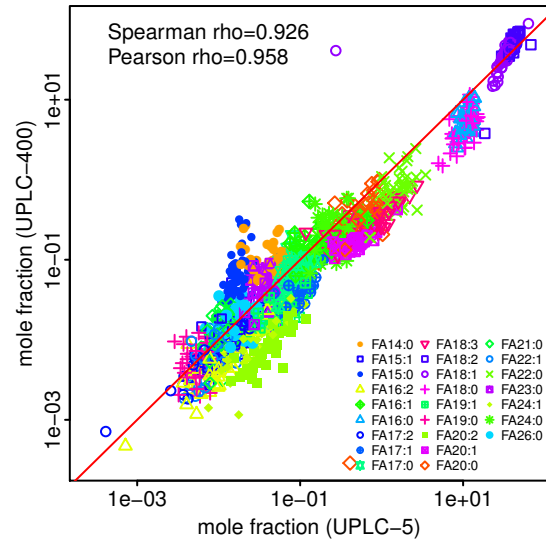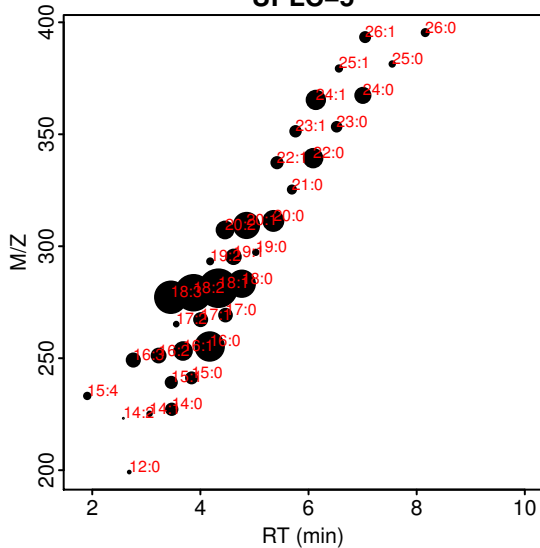
C UPLC−400 vs UPLC−5

Figure 5(on next page)

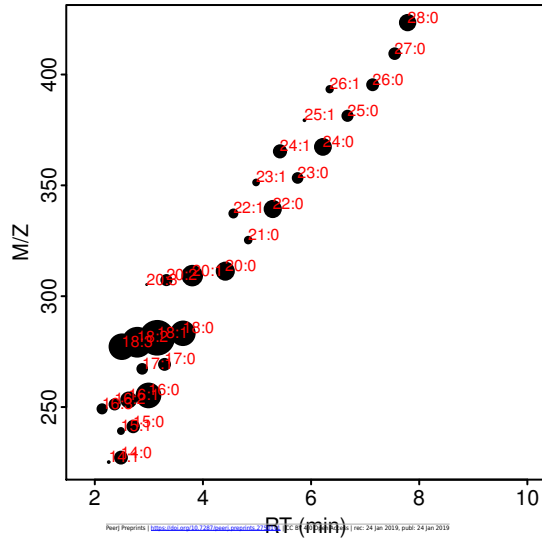Comparison of different techniques for FA quantification in rapeseed

(A, B) Retention time (x-axis, RT) – m/z (y-axis) scatter plots for UPLC-5 (A) and UPLC-400 (B).
Each dot corresponds to the individual FA, mean log intensity is indicated by the dot size.
Red color text indicates chain length and number of double bounds. (C) Dependency
between the mass fractions estimated using different methods, UPLC-5 vs UPLC-400. Each
dot corresponds to the individual sample

A UPLC−5

B UPLC−400

C UPLC−400 vs UPLC−5

Spearman rho=0.845
Pearson rho=0.95

mole fraction (UPLC−400)

mole fraction (UPLC−5)

FA18:1   FA20:0
FA18:2   FA22:0
FA18:3   FA24:0
FA16:0   FA16:1
FA18:0   FA24:1
FA20:1   FA20:2
FA22:1