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Exploration of the lipid profile of edible oleaginous microgreens by mass spectrometry-based lipidomics

A. Castellaneta[1], M. Höring[2], I. Losito[1,3,*], B. Leoni[4], P. Santamaria[3,4], C.D. Calvano[1,3], T.R.I. Cataldi[1,3], S. Matysik[2], G. Liebisch[2]

[1]Dipartimento di Chimica, Università degli Studi di Bari Aldo Moro, 70126 Bari, Italy.
[2]Institut für Klinische Chemie und Laboratoriumsmedizin, Universitätsklinikum Regensburg, 93053 Regensburg, Germany.
[3]Centro Interdipartimentale SMART, Università degli Studi di Bari Aldo Moro, 70126 Bari, Italy.
[4]Dipartimento di Scienze del Suolo e degli Alimenti, Università degli Studi di Bari Aldo Moro, 70126 Bari, Italy.

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*Author for correspondence, email: ilario.losito@uniba.it

(ORCID code: 0000-0003-0025-3350)

Abstract

The spreading awareness of the health benefits associated with the consumption of plant-based foods is fuelling the market of innovative vegetable products, including microgreens, recognized as a promising source of bioactive compounds. To evaluate the potential of oleaginous plant microgreens as a source of bioactive fatty acids (FA), gas chromatography-mass spectrometry was exploited to characterize the total fatty acid content of five microgreens, namely chia, flax, soy, sunflower and rapeseed (canola). Chia and flax microgreens appeared as interesting sources of α-linolenic acid (ALA), with a total concentration of 2.6 and 2.9 g/100 g of dried weight (DW), respectively. Based on these amounts, approximately 15% of the ALA daily intake recommended by the European Food Safety Authority (EFSA) can be provided by 100 g of the corresponding fresh products. Flow-injection analysis (FIA) with high-resolution Fourier transform Single and Tandem Mass Spectrometry (FTMS, FTMS/MS) enabled a semi-quantitative profiling of triacylglycerols (TGs) and sterol esters (SEs) in the examined microgreen crops, confirming their role as additional sources of fatty acids like ALA and linoleic acid (LA), along with glycerophospholipids. The highest amounts of TGs and SEs were observed in rapeseed and sunflower microgreens (ca. 50 and 4-5 μmol/g DW, respectively), followed by flax (ca 20 and 3 μmol/g DW). TG 54:9, 54:8 and 54:7 prevailed in the case of flax and chia, whereas TG 54:3, 54:4 and 54:5 were the most abundant TGs in the case of rapeseed. β-sitosteryl linoleate and linoleate were generally prevailing in the SE profiles, although campesteryl oleate, linoleate and linolenate exhibited a comparable amount in the case of rapeseed microgreens.

Keywords: microgreens, fatty acids, triacylglycerols, sterol esters, gas chromatography, flow-injection analysis, high-resolution mass spectrometry

1. Introduction

Fatty acids (FAs) are the building blocks of a large variety of lipid molecules, with triacylglycerols (TGs) representing the common storage of fatty acyl chains (FACs) storage. Along with sterol esters (SEs), TGs are hosted in the core of lipid droplets (LD) in the cytoplasm of most eukaryotic cells. FAs released from LD can be employed either as metabolic fuel or building material for lipid membranes[1]. More than 400 different fatty acids have been identified in the plant kingdom[2,3], with palmitic (hexadecanoic acid, or FA 16:0), oleic (*cis*-9-octadecenoic acid, or FA 18:1 (n-9)), linoleic (9,12-octadecadienoic acid, or FA 18:2(n-6)), and α-linolenic (9,12,15-octadecatrienoic acid, or FA 18:3(n-3)) acids being generally recognized as the most abundant[3,4]. Linoleic (LA) and α-linolenic (ALA) acids, with all *cis* double bond configurations, are biosynthetic precursors of important omega-6 (n-6) and omega-3 (n-3) fatty acids, namely arachidonic (5,8,11,14-eicosatetraenoic acid, or FA 20:4(n-6)), eicosapentaenoic (5,8,11,14,17-eicosapentaenoic acid, or FA 20:5(n-3)), and docosahexaenoic (4,7,10,13,16,19-docosahexaenoic acid, or FA 22:6 (n-3)) acids (although the conversion rate of ALA to docosahexaenoic acid (DHA) is limited in mammalians, since less than the 2% of the amount of ALA that reaches rat liver per unit of time is converted to DHA; the remaining amount is subjected to β-oxidation[5]). Both LA and ALA cannot be synthesized in the human body and need to be introduced with the diet[6,7]. Antiatherogenic and anti-inflammatory properties were also recognized to ALA [8].

Plant-based foods and oils are common sources of LA and ALA[5,6]. Among others, flax and chia seeds are known to be rich in ALA[9], a feature that has stimulated their use for the extraction of vegetal oils and even for direct consumption as a diet supplement. On the other hand, the possibility of using chia and flax seeds to produce microgreens, thus leading to a more

72 traditional, vegetable-like, edible product with potential health benefits, represents an
73 interesting further application. Microgreens represent an intriguing culinary novelty, that
74 found various applications in the preparation of salads and as garnishments of drinks,
75 appetizers, main and second courses in the world's finest restaurants[10–12]. Despite no legal
76 definition has been provided for microgreens[10], they can be described as edible seedlings
77 with fully developed cotyledons, usually harvested after the appearance of the first true
78 leaves by cutting the stem at few millimetres from the soil level [11,13,14]. Differently from
79 sprouts (*i.e.*, germinated seeds), the radicles are thus not considered as an edible part in the
80 case of microgreens. Moreover, unlike sprouts, the production of microgreens requires light,
81 the use of a growing medium, and a longer growth cycle [10].
82 The growth as microgreens may represent an innovative and alternative use of the most
83 common oleaginous crops, including also soybean, sunflower, and rapeseed, along with chia
84 and flax. Consistently with data reported in our previous works[15,16], dealing with
85 glycerophospholipids (GPLs) and sterols, respectively, these ready-to-eat small vegetables
86 might also represent a good source of health-promoting lipids.
87 To the best of our knowledge, those studies provide the only information currently available
88 about lipids in microgreens. Interestingly, the tandem MS (MS/MS) structural
89 characterization of GPLs showed that the FAC 18:3 (*i.e.*, a fatty acyl chain with 18 carbon
90 atoms and 3 double bond equivalents) was the most diffused fatty acyl unit in the main GPL
91 classes encountered in chia and flax microgreens[15]. However, no information was retrieved
92 for less polar lipid classes, such as TGs and SEs, that might also represent a source of that
93 fatty acyl chain. Moreover, despite the recognition of 18:3 fatty acyl chains suggested the

possible presence of ALA, a confirmation could not be obtained, since no additional evidence was provided to unveil the position of carbon-carbon double bonds.

All these limitations were addressed in the present work. The hyphenation of gas chromatography with mass spectrometry was exploited for the quantitative and qualitative characterization of the fatty acid (FA) content in chia, flax, soybean, sunflower and rapeseed microgreens. Indeed, gas chromatography (GC) is considered the primary analytical tool for the separation of FAs in several types of samples[17,18]. Moreover, the coupling with mass spectrometry (MS) represents a powerful alternative for FA identification when compared to the more traditional use of flame ionization detectors (FID)[19]. The knowledge of the fatty acid profile obtained by GC-MS was exploited to interpret the outcomes of high-resolution (HR) MS-based characterization of complex lipid species as TGs and SEs. To this purpose, the versatility of the electrospray ionization (ESI) was combined with the performances of the Q Exactive quadrupole-orbitrap mass spectrometer. Notably, the use of orbitrap mass analysers is considered the golden standard for HRMS lipidomic studies[20]. The high mass resolution provided by such platforms allows the distinction of lipid ions that exhibit very slight differences in their theoretical *m/z* ratio[21], and accurate *m/z* values can be employed to identify lipid molecules at the species level[22].

As shown in our previous study[15], the reliability of HRMS-based studies can be supported by the adoption of a preliminary chromatographic separation. In the case of glycerophospholipids, hydrophilic interaction liquid chromatography (HILIC) separations, based on polar stationary phases, represent a very effective approach, since they provide a class-related separation[23]. As a result, lipids belonging to the same class experience the same ESI ionization environment, thus a limited number of non-endogenous internal standards

(ISTDs) is required to compensate for the effects of mobile phase composition, ion source fluctuations and ion suppression/competition phenomena, that are known to affect the ionization process[24–26]. Actually, HILIC is not suitable for the separation of non-polar lipids like TGs and SEs, for which reverse phase liquid chromatography (RPLC) represents a more effective approach. However, due to the dependence of retention mainly on the fatty acyl chain hydrophobicity, lipids belonging to the same class are usually spread across a wide retention time window in the case of RPLC and a higher number of ISTDs per class is usually required for a reliable quantification.

As an alternative to RPLC, promising analytical methods based on flow injection analysis (FIA) coupled to ESI-HRMS have been recently developed for the characterization of TGs and SEs in human biological samples[27,28]. The use of FIA-ESI-HRMS methods is also highly suitable for the reliable quantification of lipids, due to the existence of the same ionization conditions in the ESI source[20,29], although a more extensive ion suppression effect might be observed in respect to approaches relying on liquid chromatography. In the present study, a previously developed FIA-ESI-HRMS workflow[30] was thus implemented for the quantitative characterization of TGs and SEs in chia, flax, soybean, sunflower, and rapeseed microgreens. By comparing different solvents, such as methanol, isopropanol, water, and methanol/chloroform 2:1 (v/v), the most useful homogenization medium was recognized. TGs and SEs were characterized in terms of fatty acyl chain composition using a semi-untargeted approach, in which species corresponding to all the possible combinations of FAs identified by the preliminary GC-MS analysis and, for SEs, of sterols emerged from the GC-MS/MS characterization of the total (free and esterified) sterol content[16] were considered. Targeted MS/MS analyses were also performed to obtain a confirmation.

2. Materials and methods

2.1 Chemicals

Acetyl chloride, methanol (LC-MS grade), hexane (HPLC grade) and calcium carbonate used for the extraction and derivatization of fatty acids were purchased from Merck (Darmstadt, Germany). The commercial Supelco FAME 37 standard mixture of fatty acid methyl esters (FAME) was purchased from Sigma Aldrich (Taufkirchen, Germany). The tridecanoic (FA 13:0) and 19-methyleicosanoic (FA 21:0) fatty acids were purchased from Larodan (Solna, Sweden) and were employed as internal standards for the GC-MS analysis. Chloroform (HPLC grade), 2-propanol (HPLC grade) and water (HPLC grade) were purchased from Roth (Karlsruhe, Germany) and were employed, along with methanol, for both plant tissue homogenization and lipid extraction. 1,2,3-triheptadecanoylglycerol (TG 51:0) and 1,2,3-trinonadecanoylglycerol (TG 57:0) were purchased from Larodan (Solna, Sweden). Cholesteryl heptadecanoate (CE 17:0) and cholesteryl docosanoate (CE 22:0) were purchased from Sigma Aldrich (Taufkirchen, Germany). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC 28:0), 1,2-dibehenoyl-sn-glycero-3-phosphocholine (PC 44:0), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (PE 28:0), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (PE 40:0), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (PG 28:0), 1,2-diphytanoyl-sn-glycero-3-phosphoglycerol (PG 40:0) and epta-deuterated 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-phosphoinositol (d7-PI 33:1) were purchased from Avanti Polar Lipids (Alabaster, Alabama).

2.2 Production of microgreens

The production of microgreens was based on seeds of chia (*Salvia hispanica* L.), flax (*Linum usitatissimum* L.), and sunflower (*Helianthus annuus* L.), produced in the Veneto region

(Italy) and purchased from Selex (Trezzano sul Naviglio, Italy); seeds of soybean (*Glycine max* (L.) Merr.), produced in the Lombardy region (Italy) and purchased from Soia Italiana (Lodi, Italy), and seeds of rapeseed (canola, *Brassica napus* L., cv PR44D06, double-zero variety), produced in France and purchased from Dupont-Pioneer (Johnston, IA, USA). All the seeds were produced in 2021 and certified to belong to the European common catalogue of varieties of agricultural plant species (whose last version is available as Supplement 2021/6, published in the *Official Journal of the European Union* 2021, C211/1).

The germinating ability of the seeds was preliminarily assessed in Petri dishes. For microgreen production, the seeds were sown on a substrate consisting in a mixture of peat (50% white–50% black peat mixture, Brill 3 Special, Brill Substrates, Georgsdorf, Germany) contained into a 150 cm^2 cultivation plastic tray (with holes on the bottom), with a density of 3 seeds/cm^2 for flax and rapeseed, 1 seed/cm^2 for sunflower, 2 seeds/cm^2 for soybean and 4 seeds/cm^2 for chia. The sowing bed was irrigated with nebulized distilled water and each tray was covered with a black polyethylene film, to keep internal humidity constant, and transferred into a growth chamber (Bertagnin, Imola, Italy) at 20 °C and 80% relative humidity. Total germination was assessed after three days, then the black film was removed, relative humidity was lowered to 60% and neon lights were turned on to activate photosynthesis. Specifically, the seedlings were exposed to a light irradiance of 40 µmol m^{-2} s^{-1} (determined by using a LICOR LI-190 quantum sensor - Li-Cor Inc., USA) for a 16 h photoperiod. The seedlings were fertigated daily using a nutrient solution containing all the essential macro- and micro-nutrients with the following concentrations expressed in mg/L: N 105, P 15, K 117, Ca 100, Mg 24, B 0.25, Cu 0.01, Fe 2.5, Mn 0.25, Zn 0.025, and Mo 0.005[31]. Notably, both the nature of substrate and the composition of the nutrient solution have been

186 shown to influence relevant parameters of microgreens cultivation, such as population and
187 fresh weight density (*i.e.*, the number of shoots and the amount of fresh weight of product
188 per surface unit, respectively), and the final content in specific components of the harvested
189 plants (see Ref. 10 and references cited therein). The substrate and nutrient solution
190 adopted in the present case for the five oleaginous microgreens were thus optimized during
191 previous experiments on this type of product[10].
192 After ten days, once the development of seedlings and the appearance of the first true leaves
193 were assessed, harvesting was carried out by cutting the microgreens just above the surface
194 of the growing medium. The fresh cut microgreens were stored for three days at -20°C, then
195 they were freeze-dried for four days in a ScanVac CoolSafe 55-9 Pro freeze-dryer (LaboGene
196 ApS, Lynge, Denmark).

197 **2.2 Plant tissue homogenization**

198 For each microgreen crop, the lyophilized seedlings were weighted and transferred into a
199 tube (2 mL) for bead-beating applications. The tube was prefilled with ceramic beads. The
200 plant material was dispersed in a volume of homogenization solvent to a nominal
201 concentration of 0.05 mg/µL and subjected to tissue homogenization using a Precellys 24
202 tissue homogenizer from Bertin Instruments (Berlin, Germany). Methanol, isopropanol,
203 water, and the Bligh-Dyer mixture (methanol/chloroform 2:1 v/v) were chosen as alternative
204 homogenization media. The homogenizer was operated at 5000 rpm and two
205 homogenization session were performed. Each session consisted in two cycles of 15 s run
206 time. A 60 s break interval was introduced between the two cycles.

207

208 **2.3 Sample preparation for GC-MS analysis**

209 FA derivatization and extraction were performed following the protocol suggested by Ecker
210 *et al.* [19]. Prior to the GC separation, the free and esterified FA need to be converted into their
211 methyl esters (FAME) to reduce their polarity and enhance their volatility [18]. A 10% (v/v)
212 methanolic solution of acetyl chloride (derivatizing solution) was employed for the latter
213 purpose. A volume of 5 µL of microgreen methanol homogenate was mixed with 50 µL of a
214 methanolic solution of the FA 13:0 (20 µg/mL) and FA 21:0 (20 µg/mL) internal standards.
215 Thereafter, 2 mL of the derivatizing mixture were added, and the resulting system was
216 vigorously stirred by using a vortex mixer. The overall mixture was incubated in a thermal
217 shaking bath at 95°C for 60 min. The FAME were extracted with 500 µL of hexane and the
218 resulting mixture was washed (vortexing) with 5 mL of a calcium carbonate 6% (m/v) solution
219 to neutralize the excess of hydrochloric acid (*i.e.*, the by-product of the reaction between
220 methanol and acetyl chloride). A stable phase separation was obtained after centrifugation
221 (4000 rpm, 10 min). Eventually, 150 µL of the organic phase (supernatant) were transferred
222 in 1.5 mL amber glass vials prior to the GC-MS analysis.

223 **2.4 Sample preparation for FIA-ESI-FTMS analysis**

224 The Bligh-Dyer protocol[32] was adopted for the extraction of TG and SE from plant tissue
225 homogenates. To the latter purpose, 10 µL of homogenate were diluted in 790 µL of water.
226 For each microgreen crop, methanol, methanol/chloroform 2:1 v/v, isopropanol, and water
227 homogenates were considered to evaluate the influence of the homogenization medium on
228 lipid extraction. The resulting 800 µL volume was spiked with 50 µL of a mixture of internal
229 standards containing TG 51:0 (18 µg/mL), TG 57:0 (18 µg/mL), CE 17:0 (10 µg/mL), CE 22:0
230 (10 µg/mL), PC 28:0 (25 µg/mL), PC 44:0 (25 µg/mL), PE 28:0 (10 µg/mL), PE 28:0 (10 µg/mL),

PE 40:0 (10 µg/mL), PG 28:0 (2.5 µg/mL), PG 40:0 (2.5 µg/mL), d7-PI 33:1 (5 µg/mL). The overall sample volume was mixed with 3 mL of the Bligh-Dyer extraction mix (*i.e.*, methanol/chloroform 2:1 v/v), followed by vortexing. Thereafter, the mixture was kept quiescent in the dark for 60 min at room temperature. The phase separation was induced after the addition of 1 mL of water and 1 mL of chloroform and a stable phase separation was obtained after 10 min centrifugation at 4000 rpm. A 500 µL aliquot of the chloroform-rich phase was transferred in a 1.5 mL amber glass vial by a pipetting robot (Tecan Genesis RSP) and vacuum dried. The dry residue was dissolved in 1.2 mL of a chloroform/methanol/2-propanol (1:2:4 v/v/v) mixture containing ammonium formate 7.5 mM. The resulting solution was subjected to FIA-ESI-FTMS analysis.

2.5 GC-MS instrumentation and operating conditions

The GC separation of fatty acids was performed using a highly polar SGE BPX70 column (10 m length, 0.1 mm internal diameter) coated with 70% cyanopropyl polysilphenyl-siloxane (0.2 µm film thickness) stationary phase (Thermo Fisher Scientific, Bremen, Germany). The column was mounted on a GC-2010 gas chromatograph coupled to a GCMS-QP2010 single-quadrupole mass spectrometer (Shimadzu Deutschland GmbH, München, Germany). The detector temperature was hold at 250 °C. A 1 µL sample volume was injected in a programmed temperature vaporizer (PTV). The PTV operated in the split mode (split ratio 1:20) for 3 s, in the splitless mode for 1.3 min and again in split mode, with a split ratio of 1:100, until the end of the run. The initial injection temperature was 72 °C. After 3 s it was increased to 250 °C at a 240 °C/min rate and hold for 15 min. The liner was packed with CarboFrit (Restek GmbH, Bad Homburg vor der Höhe, Germany). The temperature program for the GC separation is summarized as follows: the initial oven temperature was set to 50°C

and hold for 0.75 min. Thereafter, the temperature was raised to 155 °C at 40 °C/min, then to 210 °C at 6 °C/min and to 250°C at 15°C /min. Eventually the temperature was held at 250°C for 2 min. Helium was used as carrier gas with a constant linear velocity of 50 cm/s.

2.6 FIA-ESI-FTMS instrumentation and operating conditions

Lipid quantification was performed by direct flow injection with MS detection based on a Q Exactive hybrid quadrupole-Orbitrap Fourier-transform mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. The ion source was operated using the following settings: spray voltage of 3.5 kV, S-lens RF level 50, capillary temperature of 250 °C, aux gas heater temperature of 100 °C, sheath gas flow rate of 15 a.u. and auxiliary gas flow rate of 5 a.u. All spectra were acquired in profile mode. The resolving power was set to 140,000 units (at *m/z* 200). A sample volume of 50 µL was injected by a PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with an UltiMate 3000 isocratic pump (Thermo Fisher Scientific, Waltham, MA). A chloroform/methanol/isopropanol (1:2:4 v/v/v) mixture was delivered at an initial flow rate of 100 µL/min for 0.25 min. The flow was reduced to 10 µL/min for 2.5 min. A final washout was performed at 300 µL/min for 0.5 min. FTMS spectra were acquired in positive ion mode in the *m/z* range 500 -1000 for 1 min. A normalized collision energy (NCE) of 26 units was adopted for the acquisition of higher-energy collision dissociation (HCD) Fourier Transform tandem mass spectra (FTMS/MS).

2.7 Annotation of lipid species

In the present work, the shorthand notation suggested by Liebisch *et al.* [22,33] was adopted for lipid molecules identified through FIA-ESI-FTMS and FIA-ESI-FTMS/MS analysis[22]. An

exception was introduced for the annotation of SE at the species level. The knowledge of the accurate *m/z* value is not sufficient to distinguish the contribution of the sterol backbone and of the fatty acyl moiety to the total number of carbon atoms and double bonds (DB) in the SE molecule. MS/MS experiments are required to acquire this information and were performed for major SEs during the present study. For this species, the overall number of carbons and DB for the steryl moiety and the acyl chain were reported separately (*e.g.*, SE 29:2/18:3). In other cases, the overall numbers of C atoms and C=C bonds (*e.g.*, SE 47:2) were reported.

3. Results and discussion

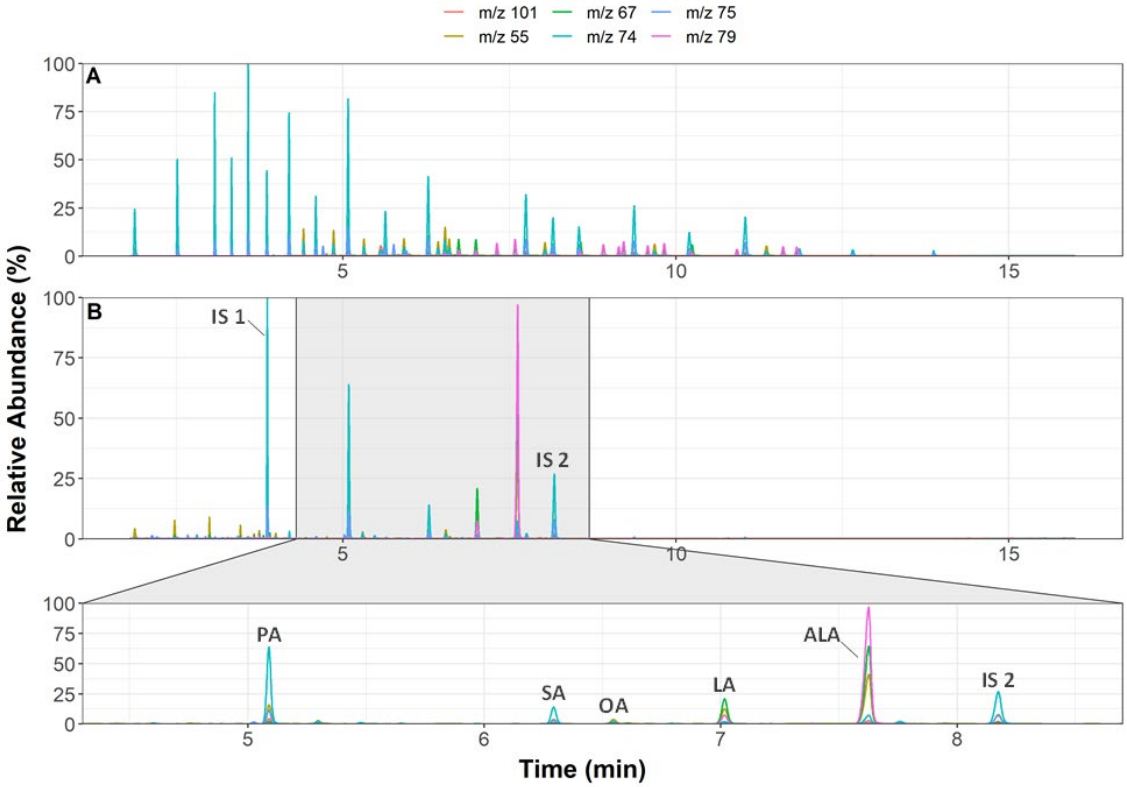
3.1 GC-MS Characterization of fatty acids in microgreens

As anticipated, all FAs in the microgreens under study were identified as the corresponding FAMEs, exploiting the intensity of diagnostic peaks in the corresponding electron ionization (EI) mass spectra, known to depend on the number of C=C bonds in the fatty acyl chain[19]. Specifically, the *m/z* 74 ion has higher intensity in the EI-MS spectra of saturated FAME, the *m/z* 55 and *m/z* 67 ions are characteristic of mono- and doubly unsaturated FAME, respectively, and the *m/z* 79 ion is dominant for polyunsaturated FAME[34]. During the present study, the cited ions were monitored during GC separations of FAME mixtures by adopting multiple selected ion monitoring (SIM) events. **Figure 1** shows the overlap of SIM chromatograms referred to the available FAME standard mix (panel **A**) and to the chia microgreen sample (panel **B**).

The identification of FAMEs obtained from each microgreen sample was based on the retention time and supported by the information about the double bond (DB) number

299 provided by MS. Notably, the reliability of this analytical approach requires that
300 chromatographic peaks of FAME sharing the same DB number are fully resolved in the SIM
301 chromatogram.

302 The results of the quantitative analysis of the most abundant fatty acids identified in chia,
303 flax, soybean, sunflower and rapeseed microgreen samples are shown in **Figure 2** (numerical
304 values have been also reported in **Table S1** in the Supporting Information). The means and
305 standard deviations referred to four homogenization, extraction and derivatization
306 replicates produced for each of the five investigated microgreen crops are reported in the
307 figure.



308
309 **Figure 1**
310 Selected Ion Monitoring (SIM) chromatograms obtained for the Supelco FAME standard mix
311 (A) and the chia microgreen sample (B). The peaks in the magnified region of panel B are
312 labelled with the acronyms of the fatty acids corresponding to the identified FAME, *i.e.*, PA
313 (Palmitic Acid), SA (Stearic Acid), OA (Oleic Acid), LA (Linoleic Acid), and ALA (α-Linolenic
314 Acid). Peaks referring to the two internal standards are labelled as IS 1(methyl tridecanoate)
315 and IS 2 (methyl 19-methyleicosanoate).
316

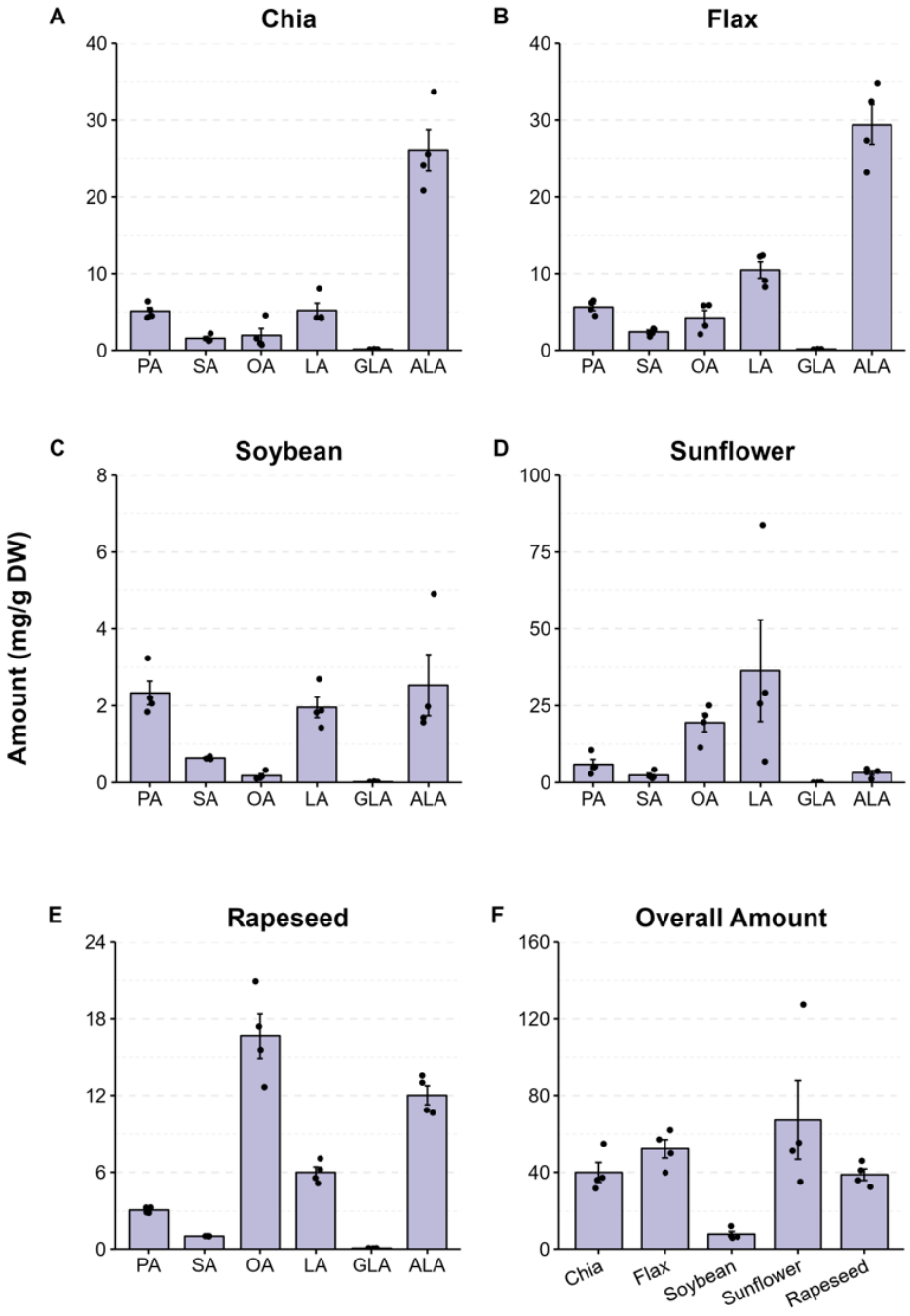


Figure 2

Bar charts showing the average amount of the main fatty acids (FA) detected in chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**) microgreens, expressed as milligrams per gram of dry weight (DW) plant material. The overall FA content is also shown (**F**). Error bars refer to the standard deviation calculated for the four homogenization/extraction/ derivatization replicates (see the Materials and Methods section for details) performed for each microgreen sample. Data points indicate the values observed from each replicate. The acronyms shown on the x-axis refer to the following fatty acids: palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), and α-linolenic acid (ALA).

Chia and flax microgreens exhibited very similar FA profiles, both dominated by the occurrence of ALA in comparable amounts (26±5 and 29±5 g/kg DW, respectively), corresponding to the highest contents among the investigated microgreen crops. Data were in excellent agreement with estimates based on our previous investigation on GPLs in these two microgreens [15]. Notably, the amount of the (n-6) isomer of the 18:3 FA, *i.e.*, γ-linolenic acid, was generally low in all the investigated microgreens (see **Figure 2** and **Table S1**). As for LA, the most relevant (n-6) fatty acid detected in microgreen samples, its amount in flax was slightly higher than in chia and rapeseed, yet the maximum amount, by far, was obtained for sunflower microgreens, reaching 40±20 g/kg DW. This result was somewhat surprising since estimates based on the quantification of GPL suggested sunflower as the third microgreen in terms of LA content [15]. As discussed in the next section, TGs are likely responsible for the majority of LA in sunflower microgreens. This might also explain the remarkable variability observed in LA content in the case of sunflower, evidenced in **Figure 2**. Indeed, this variability seemed to be mainly associated to the extraction of TG from the sunflower homogenates (*vide infra*). As for other FAs, sunflower microgreens were also rich in oleic acid (OA, 19±6 g/kg DW) and a comparable amount was found in rapeseed microgreens (17±3 g/kg DW), whereas it was much lower in the case of the remaining microgreens, especially soybean (0.18±0.10 g/kg DW). As inferred from **Figure 2**, soybean was the microgreen providing the lowest amount of FA (7.6±1.8 g/kg DW) among the products under study, whereas sunflower was the richest one (67±30 g/kg DW). This result was confirmed also for saturated FA, *i.e.*, palmitic (PA) and the stearic (SA) ones; in any case, PA prevailed over SA in all the microgreens under study (see **Figure 2** and **Table S1**).

Data reported in **Figure 2** and **Table S1** represent a very useful guide to evaluate the potential of microgreens obtained from oleaginous plants as sources of bioactive fatty acids in the daily diet. If ALA is considered, the total amounts estimated for chia and flax microgreens would correspond to 2.6 and 2.9 g per 100 g of dry weight. Considering that the latter was found to account approximately for the 10% of fresh weight in the investigated microgreens[15], a 100 g portion of fresh chia or flax microgreens would provide ca. 0.3 g of ALA. This amount would represent the 15-30% of the recommended daily intake of this fatty acid, according to the reference values adopted European Food Safety Authority [35] and the U.S. Food and Nutrition Board [36], thus emphasizing the role that both microgreens might play as sources of ALA. They could represent convenient and more appealing alternatives to seeds or oils obtained from the same plants.

As for LA, the amount estimated for sunflower microgreens would correspond to ca. 0.36 g per 100 g of fresh product, which is rather limited compared to the daily intake suggested by the European Food Safety Authority (EFSA), *i.e.*, 10 g [35]. Notably, the amounts of ALA for chia and flax estimated in our previous study focusing on microgreen GPLs corresponded to 0.16 and 0.078 g per 100 g of dry weight, respectively [15]. A 0.06 g amount was estimated for the of GPL-related LA content in 100 g of dry weight of sunflower microgreens. The remarkable difference observed for both ALA and LA between the GPL-related amount and the total one inferred by GC-MS for a specific microgreen suggests that TG might contribute relevantly to provide both ALA in chia/flax microgreens and LA in sunflower microgreens. For this reason, TGs, along with SEs, were considered as additional analytical targets in this study, after evaluating the effect of the medium adopted for microgreen homogenization on their extraction yields.

3.2 FIA-ESI-FTMS analysis of complex lipids: a focus on TGs and SEs

Effect of the homogenization solvent on the extraction yield for complex lipids. The FAME-based GC-MS analysis of the total FA content in the five microgreens of interest was performed starting from methanolic plant homogenates (see the Materials and Methods section for details). Preliminary FIA-ESI-FTMS measurements enabled us to assess whether the solvent used for homogenization affected the extraction yield of complex lipids occurring in microgreens, and then the measured FA content. The study focused on TGs, SEs and on the main GPLs classes previously recognized in the same microgreens using HILIC-ESI-FTMS[15], namely phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG) and phosphatidylinositols (PI). While a targeted approach, based on the previously identified GPL species[15], was adopted for the quantification of GPLs, a semi-untargeted approach was implemented for the quantification of TGs and SEs. A list of the TG sum compositions (fatty acyl chain carbon atoms : fatty acyl chain DB) that were hypothesized on the basis of the results of the GC-MS characterization of FAs for all the oleaginous microgreens under study is reported in **Table 1**. These data were merged with those obtained by GC-MS/MS on sterols in the same microgreen crops[16]. The SEs sum compositions (overall carbon atoms : overall number of DB) accounting for all the possible fatty acid/sterol combinations are summarized in **Table 2**.

The identification of GPLs, TGs and SEs at the species level was based on the accurate *m*/z values arising from FIA-ESI-FTMS spectra and the use of the *Alex*^{123} software[37]. All the measured intensities of lipid MS peak signals were subjected to the *Type I* isotope correction. The *Type II* isotope correction was also introduced for TGs, using the LIPIC R package[29]. Assuming comparable ionization yields for the internal standards (IS) and the analytes, the

concentrations of the latter were estimated, and this semi-quantitative approach was introduced to assess the possible effect on the lipid extraction of the use of methanol, isopropanol, water, and the methanol/water 2:1 v/v mixture (*i.e.*, the Bligh-Dyer extraction medium) as homogenization media.

Isopropanol is known to inhibit the activity of phospholipases, including phospholipase D (PLD), which catalyses the hydrolysis of GPL to phosphatidic acids. However, if alcohols are present in the reaction medium, the conversion to phosphatidic acid esters (transphosphatidylization) may also occur. The key role of PLD in the formation of methylated phosphatidic acids (MPA) was demonstrated in our recent work[38], in which the reaction occurred when plant tissue homogenization was performed in water prior to Bligh-Dyer lipid extraction. Conversely, the activity of PLD was heavily inhibited when isopropanol was adopted as homogenization medium. This finding can be invoked to explain the results shown in **Figure S1** (see Supporting Information) for flax microgreens. In the present case, a remarkable decrease of PC and PE concentrations occurred when the bead-beating homogenization was performed in water rather than in isopropanol. A substantial decrease in the content of PG and PI was observed as well (data not shown). On the other hand, TGs and SEs were found in comparable amount in both water and isopropanol homogenates. Therefore, the decrease in GPL levels cannot be attributed to a reduced yield of lipid extraction from water homogenates. It is also worth noting that only slight differences, if any, were observed in the estimated amounts of GPL, TG and SE species when methanol or the Bligh-Dyer mixture, rather than isopropanol, were employed as homogenization media. This outcome greatly supports the reliability of the determination of total FA, that was performed on methanol homogenates.

Characterization of TGs in oleaginous microgreens. The generally low impact of the homogenization solvent on the extraction of TGs from the five microgreens under study can be easily inferred from **Figure 3**. Given the low extraction yields of TGs and SEs, water was not considered as a homogenization solvent during further analyses focused on these lipids. Chia and soybean showed the lowest TG content among the investigated microgreen crops, with the soybean one being almost negligible. This outcome is consistent with the low number of FAs found for the same microgreen. Higher amounts of TGs were measured for flax, sunflower and rapeseed. Notably, data for sunflower microgreens revealed a high variability, by analogy with those referred to FAs discussed before. However, this was not recognized as a lipid extraction issue, since the same variability was not observed in other lipid classes (*e.g.*, PCs and SEs, see **Figure S2** in the Supporting Information and **Figure 4**, respectively). Hence, it can be attributed to the lack of repeatability of TG extraction from the corresponding homogenates, although the reasons for this outcome are not clear.

It is worth noting that the similarities observed for the TG profiles of the five microgreen crops recall those observed for their FA content (see **Figure 2**). This was further confirmed through the tandem mass spectrometry-based determination of the fatty acyl composition of the most abundant TG species identified in each plant extract. Notably, the collision-induced dissociation of TG $[M+NH_4]^+$ adduct ions determines the formation of diacylglyceride(DAG)-like ions through the formal loss of a fatty acid ammonium salt. This process occurs from each of the three *sn* positions of the glycerol backbone[39]. The evaluation of these neutral losses was exploited to retrieve the fatty acyl composition of the TG. The identification of the FACs was corroborated by the detection of the corresponding acylium ions. The latter were generated by the further fragmentation of the DAG-like ions in the HCD

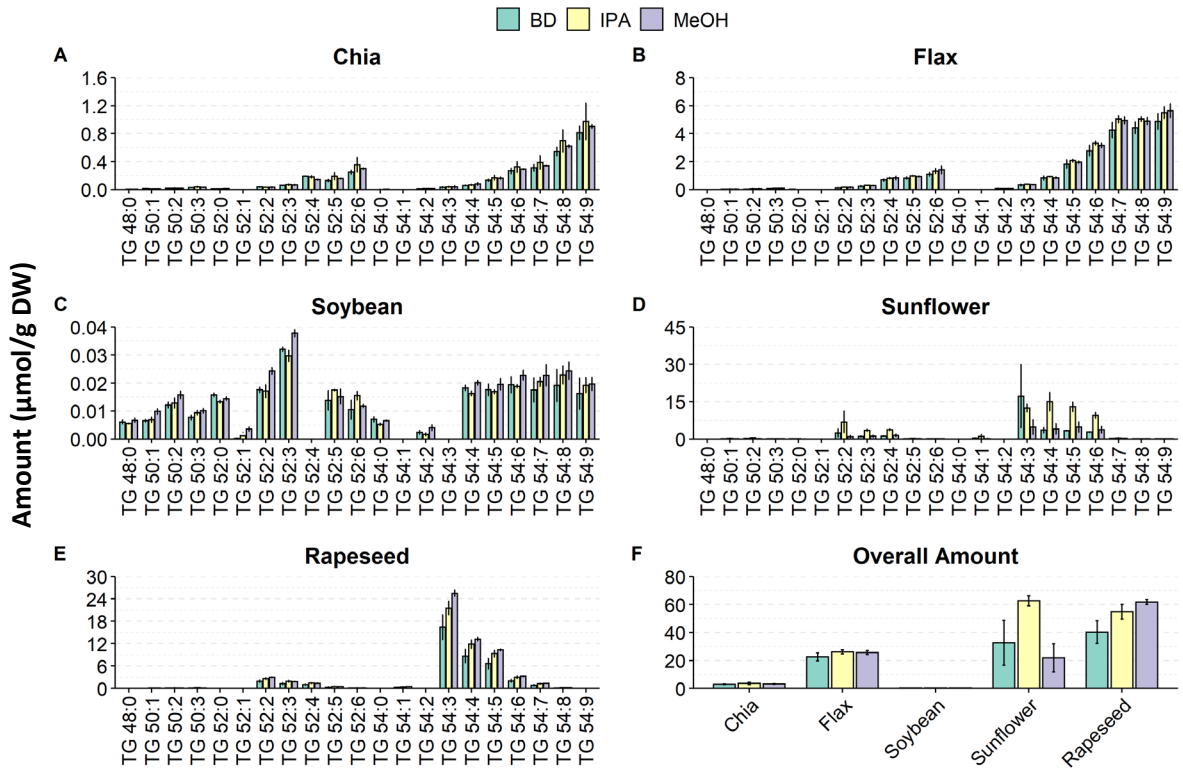


Figure 3

Bar charts showing the outcomes of the semi-quantitative FIA-ESI-FTMS analysis of TG in five oleaginous microgreen crops, namely chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**). Bar colours refer to the different homogenization media that were adopted, *i.e.*, methanol (MeOH), isopropanol (IPA) and the Bligh-Dyer mixture (BD). The error bars indicate the standard deviation calculated for three homogenization/extraction replicates. The overall TG amount estimated for each microgreen crop is also shown (**F**).

cell of the Q Exactive mass spectrometer. **Figures S3A** and **B** offer an example of how FIA-ESI(+)-FTMS/MS experiments were successfully applied to retrieve information about the fatty acyl composition shown in **Table 1** for the most abundant TGs detected in the five oleaginous microgreen crops.

Figure S3A shows a portion of the FIA-FTMS spectrum acquired for sunflower microgreens. This magnified view reflects the quadrupole isolation window that was adopted for the isolation of the TG 54:4 $[M+NH_4]^+$ precursor ion before MS/MS analysis. No interference was observed by ions sharing the same nominal mass. The two main MS/MS signals at *m/z*

601.5813 and *m/z* 603.5334 (see **Figure S3B**) were consistent with the loss of FA 18:1 and FA 18:2 ammonium salts, respectively. The presence of these two FACs was further confirmed by the detection of the respective acylium ions at *m/z* 265.2523 (18:1) and 263.2365 (18:2). Therefore, TG 54:4 could be annotated as TG 18:1_18:1_18:2 at the molecular species level[22].

ALA was recognized as the most abundant FA in both flax and chia microgreens materials. This is consistent with the prevalence of TG 18:3/18:3/18:3 (TG 54:9) in the corresponding TG profiles. Likewise, the higher amount of LA detected in flax compared to chia microgreens can be tentatively attributed to the higher content of TG 18:3_18:2_18:2 (TG 54: 7) and TG 18:3_18:3_18:2 (TG 54:8) in flax seedlings. TG 18:1/18:1/18:1 (TG 54:3) was the most abundant species in rapeseed microgreens, which is consistent with the outcomes of the FA analysis by GC-MS for this product. Notably, ALA contributed significantly to the overall FA content of rapeseed (see **Figure 2**). However, the impact of TG 54:9 on the rapeseed TG profile was negligible.

Turning to sunflower microgreens, TG 54:3 was attributed to triolein (TG 18:1/18:1/18:1) also in this case. It exhibited a relevant abundance, along with TGs 54:4 (TG 18:1_18:1_18:2), 54:5 (TG 18:1_18:2_18:2) and 54.6 (TG 18:2/18:2/18:2 and TG 18:1_18:2_18:3). These three TGs included from one to three 18:2 acyl chains, respectively. LA represents the major FA in sunflower microgreens (see **Figure 2**), despite a limited occurrence was observed in GPLs extracted from them [15]. As far as soybean is concerned, TGs including 48 and 52 carbon atoms in their acyl chains were recognized, thus suggesting the presence of FAC 16:0, along with 18:x ones. However, as mentioned before, the amounts of TGs were always quite low in soybean microgreens, in accordance with the low FA concentration.

Table 1

Sum compositions of TG species that can be assembled from the main fatty acids identified in the microgreen plant tissue, *i.e.*, palmitic (FA 16:0), stearic (FA 18:0), oleic (FA 18:1), linoleic (FA 18:2) and linolenic (FA 18:3) acids. TGs were detected as adducts with ammonium ions through FIA-ESI(+)-FTMS analysis. The corresponding theoretical *m/z* ratios are reported. The fatty acyl chain composition, retrieved using FIA-ESI(+)-HCD-FTMS/MS analysis of TGs ammonium adducts, is also shown for the most abundant TGs detected in each of the five oleaginous microgreen crops.

		Annotation of the most abundant TGs at the molecular species level				
Sum composition	***m/z***	**Chia**	**Flax**	**Soybean**	**Sunflower**	**Rapeseed**
TG 48:0	824.7702					
TG 50:1	850.7864					
TG 50:2	848.7707					
TG 50:3	846.7551					
TG 52:0	880.8333					
TG 52:1	878.8177					
TG 52:2	876.8020				TG 16:0_18:1_18:1	TG 16:0_18:1_18:1
TG 52:3	874.7864				TG 16:0_18:1_18:2	TG 16:0_18:1_18:2
TG 52:4	872.7707	TG 16:0_18:2_18:2 TG 16:0_18:1_18:3	TG 16:0_18:2_18:2 TG 16:0_18:1_18:3		TG 16:0_18:2_18:2	TG 16:0_18:1_18:3 TG 16:0_18:2_18:2
TG 52:5	870.7551	TG 16:0_18:2_18:3	TG 16:0_18:2_18:3			
TG 52:6	868.7394	TG 16:0_18:3_18:3	TG 16:0_18:3_18:3			
TG 54:0	908.8646					
TG 54:1	906.8490					
TG 54:2	904.8333					
TG 54:3	902.8177				TG 18:1/18:1/18:1	TG 18:1/18:1/18:1
TG 54:4	900.8020				TG 18:1_18:1_18:2	TG 18:1_18:1_18:2
TG 54:5	898.7864	TG 18:1_18:2_18:2 TG 18:1_18:1_18:3 TG 18:0_18:2_18:3	TG 18:1_18:2_18:2 TG 18:1_18:1_18:3 TG 18:0_18:2_18:3		TG 18:1_18:2_18:2	TG 18:1_18:1_18:3 TG 18:1_18:2_18:2
TG 54:6	896.7707	TG 18:2/18:2/18:2 TG 18:1_18:2_18:3 TG 18:0_18:3_18:3	TG 18:2/18:2/18:2 TG 18:1_18:2_18:3 TG 18:0_18:3_18:3		TG 18:2/18:2/18:2 TG 18:1_18:2_18:3	TG 18:1_18:2_18:3
TG 54:7	894.7551	TG 18:2_18:2_18:3 TG 18:1_18:3_18:3	TG 18:2_18:2_18:3 TG 18:1_18:3_18:3			TG 18:1_18:3_18:3 TG 18:2_18:2_18:3
TG 54:8	892.7394	TG 18:2_18:3_18:3	TG 18:2_18:3_18:3			
TG 54:9	890.7238	TG 18:3/18:3/18:3	TG 18:3/18:3/18:3			

Characterization of SE in oleaginous microgreens.

SEs were considered as potential further sources of bioactive FAs in microgreens of oleaginous plants. By analogy with TGs, the choice of the homogenization solvent did not significantly affect the measured content of SEs for all the microgreen samples under study (see **Figure 4**).

Possible assignments of specific SEs, based on the accurate *m/z* ratios of the corresponding ammonium adducts, are reported in **Table 2**. As for TGs, targeted FIA-ESI(+)-FTMS/MS experiments were performed to retrieve more detailed structural information about the most abundant SE detected in the five oleaginous microgreen crops. Indeed, the collision-induced dissociation of SE $[M+NH_4]^+$ ions determines the loss of the FA residue in the form of its ammonium salt. The *m/z* of the resulting resonance-stabilized carbocation returns important information about the sterol backbone[39]. **Figures S3C** and **D** offers an example of how the FIA-ESI(+)-FTMS/MS analysis of the sunflower microgreen extract was employed to retrieve the annotation at the molecular species level for SE 47:4. Despite some minor ion interferents were isolated with the precursor ion of interest (see **Figure S3C**), the signals pertaining to two sterol carbocations at *m/z* 397.3824 and *m/z* 395.3666 were easily detected in the FTMS/MS spectrum in **Figure S3D**. These were also consistent with the loss of FA 18:3 and FA 18:2 as ammonium salts. Conversely to the case of TGs, the MS/MS signals of acylium ions could not be exploited to corroborate the identification of the FACs in SEs. Indeed, the formation of acylium ions is expected only in the case of SEs bearing an hydroxylated fatty acyl residue[39]. Nonetheless, the presence of two constitutional isomers (*i.e.*, SE 29:1/18:3 and SE 29:2/18:2), pertaining to the same SE 47:4 annotation retrieved from the accurate *m/z* of the precursor ion, could be inferred from MS/MS data.

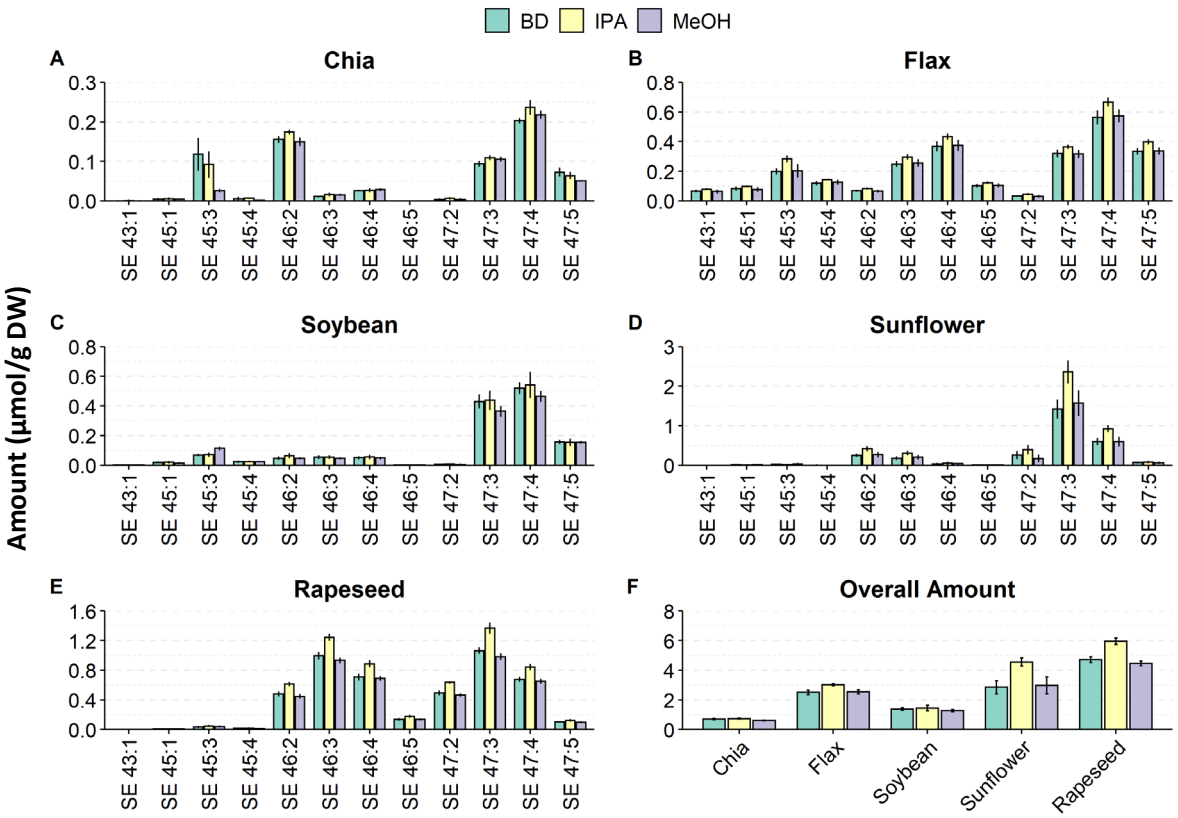


Figure 4

Bar charts showing the outcomes of the quantitative FIA-FTMS analysis of SE in five oleaginous microgreen crops, namely chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**). Bar colours refer to the different homogenization media that were adopted, *i.e.*, methanol (MeOH), isopropanol (IPA) and the Bligh-Dyer mixture (BD). The error bars indicate the standard deviation calculated for three homogenization/extraction replicates. The overall SE amount estimated for each microgreen crop is also shown (**F**).

β-sitosterol (having a 29:1 composition in terms of carbon atoms:DBs) was recognized as the most frequently occurring sterol in chia, soybean, sunflower and rapeseed microgreens. Interestingly, SE 29:1/18:2 (SE 47:3) and SE 29:1/18:3 (47:4) were among the prevailing SEs in the four products. These are likely to correspond to β-sitosteryl linoleate and linolenate, respectively. Conversely, flax microgreens exhibited a more balanced distribution of β-sitosterol, campesterol and stigmasterol. Isofucosterol and cholesterol were also identified as important contributors to their sterol profile[16]. These features can be promptly inferred from the SE profile of the flax microgreens, considering the assignments reported in **Figure**

4 and **Table 2**. Notably, flax microgreens were found to have the highest amount of cholesterol among the studied crops [16], thus SE 27:1/18:3 (SE 45:4, likely corresponding to cholesteryl linolenate) was more abundant than other microgreen crops. In chia microgreens, the amount of campesterol and stigmasterol was found to be much lower than β-sitosterol. Hence, the abundance of the corresponding ALA esters, namely SE 28:1/18:3 (SE 46:4) and SE 29:2/18:3 (SE 47:5), was significantly lower than that of SE 29:1/18:3 (SE 47:4).

Along with chia, soybean microgreens showed the lowest SE content among the investigated crops, in accordance with data previously described for FAs, TGs and GPLs. Nevertheless, PA, ALA and LA were recognized as the most abundant FAs in soybean seedlings, while β-sitosterol and stigmasterol were prominent in their total sterol profile[16]. These data can support the SE distribution observed in **Figure 4C**. SE 29:1/18:2 (β-sitosteryl linoleate), along with SE 29:2/18:2 (stigmasteryl linoleate) and SE 29:1/18:3 (β-sitosteryl linolenate), emerged as the most abundant SE species, followed by SE 29:2/18:3 (SE 47:5). The latter more likely corresponds to stigmasteryl linolenate than isofucosteryl linolenate, given the lower abundance of isofucosterol in the sterol profile of soybean[16]. Despite PA's prominence in the FA profile of soybean microgreens, it was not recognized as a major component of SE in this plant material. This observation suggests a prevailing esterification of PA in other lipids, such as TGs or GPLs. As reported in **Figure 4D**, SE 47:4 (corresponding to both SE 29:1/18:3 and SE 29:2/18:2) and SE 47:3 (SE 29:1/18:2) were recognized as the major SE species also in sunflower microgreens. Notably, sunflower exhibited a sterol profile similar to the one of soybean [15]. However, the impact of ALA on the FA profile of sunflower was much lower than

552 **Table 2**

553 Sum compositions (SC) and corresponding possible attributions to SE species that can be assembled from palmitic (FA 16:0), stearic (FA 18:0), oleic
554 (FA 18:1), linoleic (FA 18:2) and linolenic (FA 18:3) acids and the main sterols detected in the five oleaginous microgreen crops[16], namely β-sitosterol,
555 campesterol, stigmasterol, brassicasterol and isofucosterol. SEs were detected as adducts with the ammonium ion by FIA-ESI(+)-FTMS analysis. The
556 corresponding theoretical *m/z* ratios are also reported. The annotations at the molecular species level (*i.e.*, the sum composition of the fatty acyl and
557 sterol moieties) are shown for the most abundant SEs detected in each of the five oleaginous microgreen crops. The possible matching attributions
558 are shown in brackets.

			Annotation of the most abundant SEs at the molecular species level				
SC	***m/z***	**Possible attributions**	**Chia**	**Flax**	**Soybean**	**Sunflower**	**Rapeseed**
SE 43:1	642.6184	*cholesteryl palmitate*					
SE 44:1	656.6340	*campesteryl palmitate*					
SE 44:2	654.6184	*brassicasteryl palmitate*					
SE 45:1	670.6497	*cholesteryl stearate* *β-sitosteryl palmitate*					
SE 45:2	668.6340	*cholesteryl oleate* *stigmasteryl palmitate* *isofucosteryl palmitate*					
SE 45:3	666.6184	*cholesteryl linoleate*		SE 27:1/18:2 [cholesteryl linoleate]			
SE 45:4	664.6027	*cholesteryl linolenate*		SE 27:1/18:3 [cholesteryl linolenate]			
SE 46:1	684.6653	*campesteryl stearate*					
SE 46:2	682.6497	*campesteryl oleate* *brassicasteryl stearate*	SE 28:1/18:1 [*campesteryl oleate*]			SE 28:1/18:1 [*campesteryl oleate*]	SE 28:1/18:1 [*campesteryl oleate*]
SE 46:3	680.6340	*campesteryl linoleate* *brassicasteryl oleate*		SE 28:1/18:2 [*campesteryl linoleate*]		SE 28:1/18:2 [*campesteryl linoleate*]	SE 28:1/18:2 [*campesteryl linoleate*] SE 28:2/18:1 [*brassicasteryl oleate*]
SE 46:4	678.6184	*campesteryl linolenate* *brassicasteryl linoleate*	SE 28:1/18:3 [*campesteryl linolenate*]	SE 28:1/18:3 [*campesteryl linolenate*]			SE 28:1/18:3 [*campesteryl linolenate*]

							SE 28:2/18:2 [*brassicasteryl linoleate*]
SE 46:5	676.6027	*brassicasteryl linolenate*					
SE 47:1	698.6810	β-*sitosteryl stearate*					
SE 47:2	696.6653	*stigmasteryl stearate* *isofucosteryl stearate* β-*sitosteryl oleate*				SE 29:1/18:1 [β-*sitosteryl oleate*]	SE 29:1/18:1 [β-*sitosteryl oleate*]
SE 47:3	694.6497	*stigmasteryl oleate* *isofucosteryl oleate* β-*sitosteryl linoleate*	SE 29:1/18:2 [β-*sitosteryl linoleate*]	SE 29:1/18:2 [β-*sitosteryl linoleate*]	SE 29:1/18:2 [β-*sitosteryl linoleate*]	SE 29:1/18:2 [β-*sitosteryl linoleate*]	SE 29:1/18:2 [β-*sitosteryl linoleate*]
SE 47:4	692.6340	*stigmasteryl linoleate* *isofucosteryl linoleate* β-*sitosteryl linolenate*	SE 29:1/18:3 [β-*sitosteryl linolenate*] SE 29:2/18:2 [*stigmasteryl linoleate* *or* *isofucosteryl linoleate*]	SE 29:1/18:3 [β-*sitosteryl linolenate*] SE 29:2/18:2 [*stigmasteryl linoleate* *or* *isofucosteryl linoleate*]	SE 29:1/18:3 [β-*sitosteryl linolenate*] SE 29:2/18:2 [*stigmasteryl linoleate* *or* *isofucosteryl linoleate*]	SE 29:1/18:3 [β-*sitosteryl linolenate*] SE 29:2/18:2 [*stigmasteryl linoleate* *or* *isofucosteryl linoleate*]	SE 29:1/18:3 [β-*sitosteryl linolenate*] SE 29:2/18:2 [*stigmasteryl linoleate* *or* *isofucosteryl linoleate*]
SE 47:5	690.6184	*stigmasteryl linolenate* *isofucosteryl linolenate*	SE 29:2/18:3 [*stigmasteryl linolenate* *or* *isofucosteryl linolenate*]	SE 29:2/18:3 [*stigmasteryl linolenate* *or* *isofucosteryl linolenate*]	SE 29:2/18:3 [*stigmasteryl linolenate* *or* *isofucosteryl linolenate*]		SE 29:2/18:3 [*stigmasteryl linolenate* *or* *isofucosteryl linolenate*]

that observed for soybean, being OA and LA the most abundant FAs. This is in accordance with the predominance of SE 29:1/18:2 (β-sitosteryl linoleate) over SE 29:1/18:3.

As depicted in **Figure 4E**, the profile of SEs in rapeseed microgreens exhibited a bimodal distribution, with species containing 46 and 47 carbon atoms dominating the profile. Notably, β-sitosterol, campesterol and brassicasterol were recognized as the primary sterols in rapeseed seedlings [16]. On the other hand, OA and LA were the most abundant FA in this plant material. Based on these observations, SE 28:1/18:1, SE 28:2/18:1, and SE 28:2/18:2 can be reasonably attributed to campesteryl oleate, brassicasteryl oleate and brassicasteryl linoleate, respectively, while SE 29:1/18:1, SE 29:1/18:2 are likely corresponding to β-sitosteryl oleate and β-sitosteryl linoleate, respectively.

The five investigated microgreens offered a diverse range of SE profiles, with sunflower, rapeseed, and flax displaying higher content compared to chia and soybean (see **Figure 4F**). Comprehensive graphs like those reported in **Figure 4** might represent a convenient approach to compare different microgreens as sources of SEs.

3.3 Oleaginous microgreens as a source of bioactive lipids

The GC-MS analysis of FAs in the five oleaginous microgreen crops under study in this work provided valuable insights, complementing previous findings obtained for GPLs in the same products [15]. Unsaturated FAs, namely ALA, LA and OA, generally prevailed over saturated ones, namely PA and SA. Soybean was the only exception, with PA levels comparable to those of LA and ALA. However, soybean microgreens exhibited the lowest total FA content among the five products, indicating their limited potential as a source of bioactive FAs. Among the latter, ALA was the primary FA with an 18:3 sum composition and chia, flax and rapeseed microgreens were recognized as major sources of it. Calculations based on the concentration

583 obtained for ALA using GC-MS and accounting for the contribution of water to the weight of
584 those microgreens led to estimate that a 100 g serving of fresh chia or flax microgreens could
585 provide up to the 15-30% of the recommended daily uptake, according to the European Food
586 Safety Authority [35] and the U.S. Food and Nutrition Board [36]. Sunflower microgreens were
587 recognized as the richest source of LA, followed by flax. The contribution to the
588 recommended uptake of LA potentially provided by sunflower microgreens appeared
589 significant, although it was lower than the ALA one provided by chia and flax microgreens.
590 The FIA-ESI-FTMS characterization of the microgreen lipidome clarified that TG play an
591 important contribution to total FA content of flax, sunflower and rapeseed microgreens. In
592 particular, the amount of ALA-based TGs (TG 54:9, TG 54:8, and TG 54:7) was higher in flax
593 than in chia microgreens. Conversely, our previous study showed that the amount of ALA
594 embedded in GPL was higher in chia than in flax. As a result, these two opposite trends led
595 to an overall similar ALA amount in both chia and flax microgreens.[15]. The FIA-ESI-FTMS
596 analysis also enabled the identification and quantitative characterization of several sterol
597 esters. According to the total SE concentrations, the five microgreens under study could be
598 classified into high content (> 4 μmol/ g DW, rapeseed), medium content (4-2 μmol/ g DW,
599 sunflower and flax) and low content (<2 μmol/g DW, chia and soybean) products.
600 Correlations between the investigated lipid classes, *i.e.*, TG and SE, and the overall FA
601 content could be also inferred through the accurate evaluation of the corresponding class-
602 related profiles. For every microgreen crop, the contribution of each lipid species to the
603 profile was calculated by dividing its absolute amount by the overall amount of all lipids
604 pertaining to the same class. These data were subjected to Principal Component Analysis
605 (PCA). As inferred by the score plot and the loading plot for the first two principal

606 components, reported in **Figure S4** (see the Supporting Information), the high abundance of
607 ALA was clearly correlated to the predominance of highly unsaturated TGs and SEs, such as
608 TG 18:2_18:2_18:3 and TG 18:1_18:3_18:3 (TG 54:7), TG 18:2/18:3/18:3 (TG 54:8), TG
609 18:3/18:3/18:3 (TG 54:9), and SE 29:2/18:3 (SE 47:5) for both chia and flax microgreens. In
610 the case of rapeseed microgreens, the contribution of ALA to the FA profile came only after
611 the one of OA. The latter was found to be the main building block of TG and SE species,
612 namely TG 18:1/18:1/18:1 (TG 54:3) and SE 29:1/18:1 (SE 47:2), that mainly differentiated
613 rapeseed microgreens from the other products. Similarly, the predominance of distinctive
614 lipid species, like TG 18:2/18:2/18:2 (TG 54:6) and SE 29:1/18:2 (SE 47:3), reflected the
615 remarkable impact of LA in the FA profile of sunflower seedlings. On the other hand, the
616 separation of soybean microgreens in the score plot was mainly ascribable to the relevant
617 contribution of PA to the FA composition. This determined the strong influence of TG 52:3
618 on the TG profile. Conversely, the SE profile was mainly shaped by the incorporation of
619 longer and unsaturated fatty acids, such as FA 18:3 and 18:2, as in the case of SE 47:3 and
620 47:4.
621 The results described so far complement those previously obtained by HILIC-ESI-FTMS on
622 glycerophospholipids in the same vegetal products. Notably, most of the plants under study
623 are mainly employed for oil production from their seeds, or eventually for the direct
624 consumption of the latter, known for their ability to provide bioactive fatty acids to
625 consumers. The fact that some of them might contribute to the daily intake of essential fatty
626 acids even in the form of microgreens might stimulate the cultivation and production of
627 these innovative vegetal products. Consumed as fresh vegetables, like in salads, microgreens
628 of oleaginous plants would offer complex flavour profiles, along with visual appeal, while

629 delivering a non-negligible amount of beneficial lipids. Moreover, their potential for
630 cultivation in vertical greenhouses, eventually in the presence of artificial light sources, or
631 even in indoor domestic contexts, might make them a readily accessible source of nutrients.
632

Abbreviations used

ALA: α-linolenic acid

CE: Cholesteryl Ester

DB: Double Bond

DW: dried weight

EFSA: European Food Safety Authority

EI: Electron Ionization

ESI: ElectroSpray Ionization

FA: Fatty Acids

FAC: Fatty Acyl Chains

FAME: Fatty Acid Methyl Esters

FIA: Flow-Injection Analysis

FID: Flame Ionization Detectors

FTMS: Fourier-Transform Mass Spectrometry

GPL: GlyceroPhosphoLipids

HCD: Higher-energy Collision Dissociation

HESI: Heated ElectroSpray Ionization

HILIC: Hydrophilic Interaction Liquid Chromatography

HRMS: High Resolution Mass Spectrometry

IS: Internal Standards

LA: linoleic acid

LD: Liquid Droplets

MPA: Methylated Phosphatidic Acid

NCE: Normalized Collision Energy

OA: Oleic Acid

PA: Palmitic Acid

PC: PhosphatidylCholines

PE: PhosphatidylEthanolamines

PG: PhosphatidylGlycerols

PI: PhosphatidylInositols

PLD: PhosphoLipase D

664 PTV: Programmed Temperature Vaporizer

665 SA: Stearic Acid

666 SE: Sterol Esters

667 SIM: Selective Ion Monitoring

668 TG: TriacylGlycerol

669 TIC: Total Ion Current

670

671

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678

679 ## Supporting Information description

680

681 **Table S1.** Amounts (mean ± standard deviation, n = 4) of fatty acids detected in each
682 oleaginous microgreen under study, expressed in milligrams per gram of dry weight (DW)
683 plant material; **Figure S1**. Bar charts indicating the effect of the homogenization medium
684 on the yield of the extraction (Bligh-Dyer) of phosphatidylcholines,
685 phosphatidylethanolamines, triacylglycerols, and sterol esters from flax microgreen
686 homogenates; **Figure S2**. Bar charts showing the average content of phosphatidylcholines
687 obtained by FIA-ESI-FTMS analysis of the microgreens under study; **Figure S3**. Mass
688 spectrometric characterization of TG 54:4 and SE 47:4 ammonium adduct ions detected in
689 sunflower microgreens. **Figure S4.** Score plot (panel **A**) and loading plot (panel **B**) for the

690 first two principal components obtained after performing Principal Component Analysis
691 (PCA) on fatty acid (FA), triacylglycerol (TG) and sterol ester (SE) profile data related to chia,
692 flax, soybean, sunflower, and rapeseed microgreens.

693

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Figure Captions

Figure 1

Selected Ion Monitoring (SIM) chromatograms obtained for the Supelco FAME standard mix (A) and the chia microgreen sample (B). The peaks in the magnified region of panel B are labelled with the acronyms of the fatty acids corresponding to the identified FAME, *i.e.*, PA (Palmitic Acid), SA (Stearic Acid), OA (Oleic Acid), LA (Linoleic Acid), and ALA (α-Linolenic Acid). Peaks referring to the two internal standards are labelled as IS 1(methyl tridecanoate) and IS 2 (methyl 19-methyleicosanoate).

Figure 2

Bar charts showing the average amount of the main fatty acids (FA) detected in chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**) microgreens, expressed as milligrams per gram of dry weight (DW) plant material. The overall FA content is also shown (**F**). Error bars refer to the standard deviation calculated for the four homogenization/extraction/derivatization replicates (see the Materials and Methods section for details) performed for each microgreen sample. Data points indicate the values observed from each replicate. The acronyms shown on the x-axis refer to the following fatty acids: palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), and α-linolenic acid (ALA).

Figure 3

Bar charts showing the outcomes of the semi-quantitative FIA-ESI-FTMS analysis of TG in five oleaginous microgreen crops, namely chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**). Bar colours refer to the different homogenization media that were adopted, *i.e.*, methanol (MeOH), isopropanol (IPA) and the Bligh-Dyer mixture (BD). The error bars indicate the standard deviation calculated for three homogenization/extraction replicates. The overall TG amount estimated for each microgreen crop is also shown (**F**).

Figure 4

Bar charts showing the outcomes of the quantitative FIA-FTMS analysis of SE in five oleaginous microgreen crops, namely chia (**A**), flax (**B**), soybean (**C**), sunflower (**D**) and rapeseed (**E**). Bar colours refer to the different homogenization media that were adopted, *i.e.*, methanol (MeOH), isopropanol (IPA) and the Bligh-Dyer mixture (BD). The error bars indicate the standard deviation calculated for three homogenization/extraction replicates. The overall SE amount estimated for each microgreen crop is also shown (**F**).

833 **Graphic for Table of Contents**

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835