Lithospermum officinale L. is a versatile source of γ-linolenic acid- and stearidonic acid-rich oils

Running title: GLA and SDA from *Lithospermum officinale*

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Abstract

Seeds of *Lithospermum officinale* L. from different climatic zones were analyzed looking for new sources γ-linolenic acid (GLA, 18:3*n*-6) and stearidonic acid (SDA, 18:4*n*-3). Cultured *B. officinalis* was also analyzed with comparative purposes. Analyses were conducted for fatty acid (FA) profiles in the whole seeds and in the neutral and polar lipids by GC; lipid classes by open column chromatography and preparative TLC; and tocopherols, sterols and phenolic compounds by HPLC-DAD, and the later compounds were confirmed by LC-MS. The richest GLA sample was *L. officinale* from St. Petersburg Botanical Garden (17.9% of total FA), while wild-growing *L. officinale* from the Rostov region showed the highest percentage of SDA (17.2% of total FA). Total FA content ranged from 11.3 to 20.8% of seed weight. Neutral and polar lipids accounted for ~98 and 2.27% of total lipids. Five neutral lipid classes were identified (% of NL): triterpene esters, 1.3; triacylglycerols, 93.1; free FA, 1.8; diacylglycerols, 1.4; and monoacylglycerols, 2.4. Tocopherols and sterols reached 35.7 and 83.8 mg/100 g seeds; γ-tocopherol was the main tocopherol detected, and Δ^5-avenasterol was the predominant sterol. *L. officinale* seeds contain high amounts of phenolic compounds (389.9 mg/100 g as upper limit), in which rosmarinic acid was the main component. Overall, all data suggest the possibility of using *L. officinale* seed oil in pharmaceutical and cosmetic formulae and as functional food.

Keywords: *Lithospermum officinale*; gamma-linolenic acid; stearidonic acid; neutral lipids; polar lipids

1. Introduction

Polyunsaturated fatty acids (PUFA) constitute one of the most valuable components of vegetable oils, which contributes to the regulation of several physiological processes (Guil-Guerrero, 2007). Among PUFA, γ-linolenic acid (GLA, 18:3*n*-6) and stearidonic acid (SDA, 18:4*n*-3) are unusual in the plant kingdom, although both compounds reaches largest amounts in the seeds of some species of the family Boraginaceae Juss. These PUFA display well-documented health benefits: GLA exercises anti-inflammatory, antimicrobial, and antiplatelet activities (Menghini et al., 2019), while clinical experiments have shown that the consumption of GLA-containing foods could be useful in the treatment of local eczema, diabetes, virus infections and some type of cancer (Asadi-Samani et al., 2014). Regarding SDA, is the biosynthetic precursor of very long-chain PUFA (eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), from which the production of anti-inflammatory lipid mediators takes place (i.e. eicosanoids). Moreover, SDA has been described as a potent inhibitor of cancer cell growth, and its efficiency against skin inflammation and dermatitis has been highlighted (Guil-Guerrero, 2007). Besides their pharmaceutical and nutraceutical interest, both PUFA provide valuable information on taxonomic and phylogenetic relationships among *Boraginaceae* species (Velasco & Goffman, 1999; Guil-Guerrero et al., 2001).

Borage seeds are one of the richest sources of GLA and SDA found in nature, and their oils contain 21-23% GLA of total FA (Guil-Guerrero et al., 2018). Meanwhile, SDA occurs in large amounts in the seeds of *Buglossoides arvensis* (L.) I.M.Johnst. (syn. *Lithospermum arvense* L.) (Ahiflower® oil), which is cultured for the commercial production of SDA-rich oils (~18% SDA of total FA) (Guil-Guerrero et al., 2013). Moreover, GLA and SDA were reported in *Lithospermum officinale* L. up to a ceiling

of 16.6 and 13.3% of total FA, while high amounts of γ-tocopherol were reported in this species (Velasco & Goffman, 1999). *L. officinale* comprises approximately 40 species, with a center of diversity in the Southwestern United States and Mexico. Due to its medicinal properties, *L. officinale* is cultivated nearly all over Europe and Western parts of Asia and acclimated in China and North America (Cohen & Davis, 2009). The actives principles of this plant are included in shoots and roots, and besides shikonin, which have antimicrobial, anticancer, wound healing, anti-inflammatory, and antithrombotic uses, it contains allantoin, rutin, p-hydroxybenzoic, hydrocaffeic, chlorogenic and rosmarinic acids (Dresler et al., 2017).

L. officinale seed oil was reported as a GLA-source; however, there is a lack of data on the phytochemicals contained in this oil. The occurrence of suitable amounts of phytochemicals (i.e. tocopherols (Tp), sterols (St) and phenolic compounds) in oils is desirable for both oil preservation and to maintain human health. Taking into account the interest about discovering new GLA and SDA sources for use as functional oils and as cosmetic and pharmaceutical components, the goals of this study were: i) to study the FA profiles of *L. officinale* seeds growing in different climatic areas; ii) to establish the FA profiles of the various lipid classes of *L. officinale* seed oil; iii) to characterize Tc, St and phenolic compounds of *L. officinale* seeds.

2. Experimental Procedures

2.1 Samples

The characteristics of analyzed samples are detailed in Table 1. Some *L. officinale* seeds were supplied by several botanical gardens, while other seeds were collected at mature stages from their natural habitats. *Borago officinalis* seeds were collected from plants cultured in the *University of Almería*. Each wild species was collected from three subpopulations in each collection location. Upon arrival to the laboratory, the seeds were labeled, weighed, measured and placed in a glass desiccator until analysis. Just prior to analysis, seeds were ground to powder with a mortar, then they were immediately analyzed. ~2 g of each sample was used for moisture analysis. This was carried out in a forced air oven at 105 ºC for 8 h, and all results are expressed on dry weight (d.w.) basis. Moisture content for al seeds was ~7.5±0.5.

2.2 Isolation of neutral and polar lipids

Neutral lipids (NL) were extracted using the standard method of exhaustive extraction by *n*-hexane in a Soxhlet apparatus, and the polar lipids (PL) were extracted from the residual cake using a $CHCl_3$:MeOH (2:1) solution. Water and alcohol-soluble components (carbohydrates, amino acids, etc.) were extracted with $CHCl_3$:MeOH, and the condensed PL extract was treated with a 0.1% NaCl solution and dried over anhydrous Na_2SO_4. The amounts of all lipid classes were estimated gravimetrically (Yunusova et al., 2017). Equipment at the Khimiya CUC, UfIC, RAS, was used for analysis. To isolate FA from NL, PL, and acyl-containing NL fractions, an alkaline hydrolysis was effected with KOH (10%) in MeOH (1:10) at 60°C for 30 min. The hydrolysis of triterpene esters was carried out using KOH (20%) in MeOH (1:10). The resulting solution was diluted with hot water purified in a ratio of 1:2, acidified with 5% sulfuric acid solution and extracted three times with diethyl ether. The ether extracts

were combined, washed with water purified until neutral pH values, dried over Na_2SO_4 anhydrous, and the solvent was removed on a rotary evaporator (Yunusova et al., 2017).

2.3. FA analysis

FA determination was carried out after derivatization of FA to FA methyl esters (FAME) by methylation with diazomethane in diethyl ether (diazomethane). To obtain FAME, diazomethane (DAM) (0.5-1 ml) was mixed with FA (10–20 mg). The reaction takes place within 10-15 min. The completeness of the reaction was monitored by TLC in a *n*-hexane-diethyl ether system (9:1); if necessary, a new portion of DAM was added. At the end of the reaction, the remaining unreacted DAM was removed on a rotary evaporator. DAM was obtained from nitrosomethylurea in diethyl ether. GC analyses of the FA methyl esters were effected in a GC-2014 chromatograph (Shimadzu) with an Omegawax TM 250 capillary column (30.0 m × 0.25 mm, Supelco), poly(ethylene glycol) L stationary phase (0.25 μm). The temperatures were: column 205 °C, vaporizer 250 °C, detector 260 °C. The carrier gas was He at a flow rate of 30 mL/min. The peak area of the internal standard was used as reference to calculate the mass of each FA in the resulting chromatograms, and results were computed as FA percentages of total FA. Peaks were identified by retention times obtained for known FA standards (PUFA No. 1, 47033; methyl γ-linolenate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma-Aldrich, Steinheim, Germany.

2.4 NL classes

NL were fractionated into lipid classes using an open column chromatography (CC) and preparative thin layer chromatography (PTLC) (Yunusova et al., 2017). Then, analytical TLC (ATLC) was used to obtain homogeneous fractions and to monitor the purity of the obtained classes. Analytical TLC was carried out on “Alugram” Si G plates (KG,

141"Macherey-Nagel" GmbH Co.) for separation, purification, and identification of NL, 142and the solvent systems were *n*-hexane- diethyl ether 9.5:0.5 (1), 9:1 (2), 8:2 (3), and 1437:3 (4). NL were fractionated by CC, which was developed using silica gel grade L 144(100–160 m, "Chemapol", Czechoslovakia) as stationary phase, the extract–adsorbent 145ratio was 1:50 (w/w), and the column cross-section area to adsorbent layer height ratio 146was 1:20. The column was eluted using *n*-hexane–diethyl ether (0–100%). Fractions 147were purified, if necessary, by PTLC on glass plates (20×20 cm) by means of a MN-148Kieselgel G silica gel (KG, Macherey-Nagel GmbH & Co.) using the solvent systems 1491–4. The amount of each lipid class was estimated gravimetrically. Lipid classes were 150identified as previously reported (Yunusova et al., 2017).

151*2.5 Extraction of tocopherols and sterols*

152The extraction of tocopherols (Tp) and sterols (St) was carried out according to 153Fabrikov et al. (2019). Approximately 0.2–0.3 g of *L. officinale* seeds were weighed in a 154100 mL screw cap flask. Solutions of ascorbic acid (0.1 M, 5 mL) and potassium 155hydroxide (2 M, 20 mL) were added. The mixture was heated for 45 min at 60 °C. 156Extraction of unsaponifiable components was carried out by filtration of the initial 157mixture, followed by addition of 10 mL of saturated NaCl solution and 10 mL of n-158hexane with BHT (5 mg/L). The sample was stirred for 1 min in Vortex and the organic 159layer was collected in a 50 mL round bottom flask. The aqueous layer was re-extracted 160with 5 mL of *n*-hexane and then the hexane layer obtained was combined with the first 161organic phase. The obtained *n*-hexane solution was evaporated in a rotavapor at 60 °C 162to dryness. The resulting residue was dissolved in 1 mL of 2-propanol:*n*-hexane (50:50, 163v/v).

164*2.4. Determination of tocopherols*

Tp were identified by chromatographic comparison with authentic standards, by co-elution and by their UV spectral characteristics (DAD). Peaks purity evaluation on the DAD measurements was based on spectral comparison at three different peak heights. Tp were determined using RP-HPLC/DAD (Agilent 1100 series, Palo Alto, CA, USA) equipped with a ProntoSIL C30 column (4.6 × 250 mm, 3 μm; Bischoff Chromatography, Leonberg, Germany) cooled at 15 °C. Mixtures of methanol:acetonitrile (95:5, v/v, phase A) and 2-propanol:*n*-hexane (50:50, v/v, phase B) were used as mobile phase at a flow rate of 0.8 mL/min. The following sequence was used to elute each sample: 25 min of phase A (100 %) followed by 20 min of phase B (100 %). Additional 15 min of phase A (100%) were used to re-equilibrate the column. Phase B was used as a washing solution. The wavelength selected for DAD was 290 nm. Limit of detection (LOD), limit of quantification (LOQ), linear range and recoveries rates were obtained as previously reported (Fabrikov et al., 2019). For α-Tp, LOD and LOQ were 0.40 and 1.80 mg/L; linear range – (R^2) was 4−400 mg/L (0.9993); and the recovery rate was 102.6±1.4%. For δ-Tp, LOD and LOQ were 0.25 and 1.20 mg/L; linear range – (R^2) was 4−400 mg/L (0.9990); and the recovery rate was 101.8±1.7%. For γ-Tp, LOD and LOQ were 0.35 and 1.80 mg/L; linear range – (R^2) was 4−400 mg/L (0.9994); and the recovery rate was 99.0±2.1%.

2.5. Determination of sterols

St were determined by RP-HPLC/DAD using a Luna C18 column (250 × 4.6 mm, 5 μm; Phenomenex) at a fixed temperature of 30 °C. The mobile phase was programmed in isocratic mode, containing methanol:acetonitrile (70:30, v/v), and the flow rate was 0.8 mL/min for 55 min. The wavelength selected for DAD was 210 nm. St were quantified by means of a calibration curve made with pure standards: 99.0%-purity Δ5-avenasterol, 90.0%-purity Campesterol, and ≥96%-purity β-Sitosterol (Sigma-Aldrich,

Barcelona, Spain). For Δ5-avenasterol, LOD and LOQ were 1.3 and 3l5 mg/L; linear range – (R^2) was 3−190 mg/L (0.959); and the recovery rate was 98.7±1.9%. For Campesterol, LOD and LOQ were 1.8 and 3.9 mg/L; linear range – (R^2) was 5−140 mg/L (0.981); and the recovery rate was 95.7±2.1%. For β-Sitosterol, LOD and LOQ were 1.2 and 2.0 mg/L; linear range – (R^2) was 3−240 mg/L (0.9994); and the recovery rate was 91.5±1.2%.

2.6 Extraction and determination of phenolic compounds by HPLC-DAD

~0.2 g of dry seed powders were extracted three times at room temperature with methanol:water (60:40, v/v) as solvent and adjusted to pH 3.2 with formic acid, under stirring for 4 h. MeOH was of HPLC grade. The mixture was centrifuged at 3500 rpm for 10 min, and the supernatants were collected, combined and vacuum-evaporated at 60 °C to dryness, and then the obtained residue was dissolved in 1 mL of the previous methanolic solution.

Phenolics were analyzed using a HPLC Finnigan Surveyor LC Pump Plus Thermo Scientific system equipped with a Diode Array Detector (DAD) Thermo Scientific and Hypersil Gold column (250 x 4.6mm, 5µm). The compounds were separated with gradient elution using aqueous acetic acid (acetic acid: H2O, 4:96, v/v) (A) and methanol (B) as eluents at ambient temperature. The elution system was as follows: 0-30 min, 80% B; 30-50 min, 25% B; 50-60 min, 80% B, all them at a flow rate of 0.65 mL/min. The mobile phase was filtered through a 0.45 µm membrane filter (Millipore, Durapore®, Ireland), and the injection volume was 10 µL. Chromatograms were recorded at 320, 300 and 280 nm. A typical chromatogram is displayed in Fig. 1. Phenolic compounds were identified by congruent retention times compared with pure standards. Quantification of the compounds was made using external calibration curves

obtained from pure standards of phenolic compounds (Sigma Aldrich, Barcelona, Spain) in the HPLC system.

2.7 Characterization of phenolic compounds by LC-MS

The confirmation of the structure of phenolic compounds by HPLC-DAD was accomplished by LC-MS. The chromatographic separation of the phenolics was performed on a Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific TranscendTM, Thermo Fisher Scientific, San Jose, CA, USA) by using a Hypersil Gold column (250 x 4.6mm, 5 μm). A flow rate of 0.65 mL/min was set. The compounds were separated with gradient elution using aqueous acetic acid (acetic acid: H2O, 1:99, v/v) (A) and methanol (B) as eluents at ambient temperature. The step gradient was as follows: 0–20 min 80% of A; then, it was linearly decreased to 25% in 10 min and remained constant during 10 min. Later, it was increased to 80% in 10 min and remained constant during 5 min. The total running time was 55 min. The column temperature was set at 25 °C and the injection volume was 10 μL. The LC system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (ExactiveTM, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N2>95%), 35 (adimensional); auxiliary gas (N2>95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time =

2390.10 s, (3) full MS, ESI- using the aforementioned settings and (4) AIF, ESI- using the 240settings explained for (2). Mass range in the full scan experiments was set at m/z 50–241700. LC chromatograms were acquired using the external calibration mode and they 242were processed using XcaliburTM version 3.0, with Qualbrowser and Trace Finder 4.0 243(Thermo Fisher Scientific, Les Ulis, France). Unknown analysis was carried out with 244Compound DiscovererTM version 2.1.

245*2.11 Statistical analysis*

246Data correspond to the analyses effected to seeds received in a single shipment. All data 247in tables were analyzed using one-way ANOVA (Statgraphics Centurion XVI.I, 248Warrenton, VA, USA) and expressed as the average ± SD of three replicates. 249Differences among mean values were tested using Duncan's test at $P<0.05$.

3. Results and Discussion

3.1 FA profiles

Total FA content in *L. officinale* seeds ranged from 10.4 in wild E-05 to 20.8 g/100 g d.w. in cultivated A-17 (Table 2). Such figures are not too high, considering oil content in other *Boraginaceae* species, but it was quite similar to *B. officinalis* analyzed in this work (18.4 g/100 g d.w.); however, both oil content and bioactive PUFA in Boraginaceae species can be easily increased trough agrotechnological strategies and culture lines selection (Berti et al., 2007). Palmitic acid (PA, 16:0) was the main saturated FA (SFA) in all samples (3.2-5.8% of total FA) which is the most common SFA found in animals and plants. Such percentage was followed by those of stearic acid (SA, 18:0) (1.6 to 2.4%). As for unsaturated FA (UFA), the *n*-3 PUFA fraction (SDA and ALA) ranges between 42.0 (F-18) and 53.3% (E-14), while the *n*-6 one (LA and GLA) ranged between 27.2 (E-17) and 35.7% (B-17). GLA was found in all analyzed samples, ranging from 11.5 (E-17) to 17.9% (F-18), and such percentages were slightly lower than values obtained for borage seeds (19.4%), but higher than other figures reported for blackcurrant and evening primrose seed oils (10.4 and 9.24% GLA, respectively) (Guil-Guerrero, 2007). As for SDA, it ranged from 9.2 (F-18) to 17.2% (E-17), and in some cases such figures are below to SDA percentages reported for *E. plantagineum* seed oil (12–14%). However, E-17 and E-14 samples showed higher amounts of SDA (~17%) than *E. plantagineum*, which are similar to values reported for commercial Ahiflower® oil (18-20% SDA) (Guil-Guerrero et al., 2013). Such differences in individual FA contents could be related to the existence of different chemotypes, given that GLA and SDA percentages changes marginally year-on-year within culture locations. This way, in cultivated A-16 and A-17 samples (RAS MBG, Moscow), GLA percentages slightly changed (17.4 and 17.8%), and the same is true for

SDA (10.6 and 11.3%). Moreover, wild growing *L. officinalis* samples from Orlovsky (E-17, E-15 and E-14) reached similar GLA percentages in 2014, 2015, 2017 (11.5, 11.8 and 12.3%, respectively). However, environmental parameters (temperature, humidity, soil composition and other abiotic factors) can marginally affect PUFA percentages (Guil-Guerrero et al., 2017).

The GLA/SDA ratio widely changed in the different samples. Most samples showed higher GLA percentages than those of SDA. However, wild samples contained similar GLA and SDA percentages (13.8 and 13.5% in G-16; 15.8 and 15.9% in H-18, respectively). This fact might be related to environmental factors, as these seeds were collected from plants growing in subarctic climatic conditions, with average annual temperature ~-1ºC. Specifically, G-16 sample grew up in Dfc climate, which is characterized by strong cold without dry season and cold summer, while H-18 seeds were collected under monsoon-influenced subarctic climate (Beck et al., 2018). Only E-17 and E-14 samples contained higher SDA% than that of GLA% (both ~17 SDA and ~12% GLA). The highest PUFA content (86.4%) was reached in H-18 sample, which was collected in the Republic of Buryatia (Eastern Siberia, Russia), located on the southern boundary of permafrost distribution and characterized by a very cold environment (Badmaev et al., 2019). Similar conclusions on the influence of climate parameters on GLA content were obtained when checking blackcurrant cultivars from the Republic of Sakha (Yakutia, Siberia) (Lyashenko et al., 2019). These findings are consistent with earlier reports indicating that seeds of plants growing under cold climate tends to produce higher amounts of UFA in comparison with those produced in warmer climates. This is due to that the unsaturation level of membrane lipids and the position of double bonds within the FA structure regulates membrane fluidity, which provides tolerance and acclimatization to environmental stresses (Özcan, 2010). According to

300data summarized in Table 2, *L. officinalis* seed oil can be divided into 3 groups: i) 301higher GLA% than that of SDA% (~GLA ≥15%, ~SDA ≥9%); ii) GLA% lower than 302that of SDA% (~GLA ≥11%, ~SDA ≥16%); and iii) GLA% and SDA% at similar 303values (~GLA and SDA ≥12%). Such variations in the FA profiles of *L. officinalis* seed 304oil lead us to consider to this species as a versatile source of GLA and SDA, with a wide 305variety of applications. For instance, it can be useful for obtaining different plant 306chemptypes having selected GLA/SDA ratios, thus, adapted to the needs of the various 307consumer.

308In addition to GLA and SDA, *L. officinale* seed oil showed high amounts of LA and 309ALA (15.7-19.8 and 32.8-37.4%, respectively). The highest LA percentage was 310detected in G-16 sample (19.8), while A-17 sample showed the highest ALA percentage 311(37.4). Such ALA percentages were higher than those found in common commercial 312ALA sources: canola and rapeseed oil (9%), and soybean oil (7%) (Barceló-Coblijn, & 313Murphy, 2009).

314The major difference between *L. officinale* and *B. officinalis* seeds is that the latter 315contains larger fractions of *n*-6 PUFA, SFA and MUFA, while it practically lacks *n*-3 316PUFA.

317The amounts of ALA and LA detected in *L. officinale* have nutritional interest, since 318these are the starting molecules for the biosynthesis of EPA and arachidonic acid (ARA, 31920:4*n*-6), from which the synthesis of eicosanoids and prostaglandins takes place 320(Barceló-Coblijn, & Murphy, 2009). Interestingly, *E. plantagineum* (Purple Viper's 321Bugloss) seed oil have similar FA profiles to some *L. officinale* chemotypes detailed in 322this study. The former is considered as ‘‘the plant-based alternative for marine oils’’, 323and it contains GLA (~10%), SDA (~13%) and high ALA percentages (~30%) (Mir, 3242008). This oil was approved as Novel Food (approval 2008/558). According to the

EU's legislation and policy on food and Novel Foods Regulation (EU), foods that are considered as "substantially equivalent" to an existing food being already marketed within the EU, have a simplified procedure for marketing (Regulation (EU) 2015/2283). Thus, considering a very similar nutrient and bioactive compounds composition between *E. plantagineum* and *L. officinale* seed oils, the latter would be marketed as functional oil, to achieve the physiological benefits related to bioactive GLA and SDA.

3.2 Lipid classes

To study the composition of the lipid classes (Table 3), C-15 was used, since it was the only sample containing enough material to carry out this type of analysis. As expected, NL accounted for nearly 98% of total lipids, and PL (2.3%) was a minor fraction of the total lipids. These data agree with those of *Symphytum officinale* (Yunusova et al., 2017) and *B. officinalis* (Napal Senanayake, & Shahidi, 2000), in which NL was the main lipid class. In the NL fraction five lipid classes were identified: triterpene esters (TE), 1.3; triacylglycerols (TAG), 93.1; free FA (FFA), 1.8; diacylglycerols + sterols (DAG), 1.4; and monoacylglycerols (MAG), 2.4 (% of NL). NL contained mainly TAG, as is commonly found in the NL of ripe plant seeds, which act as lipids storage to be used as an energy source during seed germination and seedling growth (Shimada, et al., 2017). The remaining NL classes were detected in negligible amounts. The percentage of TAG in *L. officinale* NL (93.1 %) was higher than that previously described for *S. officinale* (86.6 %) (Yunusova et al., 2017), but lower than that detected in *B. officinalis* seeds (99.1%) (Napal Senanayake, & Shahidi, 2000). The FA composition of the TAG fraction was quite similar to that of NL. PA was the main SFA in NL (5.6-18.0%), and the main PUFA in all NL classes was LA (18.4-26.8%), except in the TAG fraction, in which ALA was the main PUFA (36.1%). The highest GLA percentages were detected in TAG and MAG (17.0 and 14.2%), while the higher SDA percentages were detected

in the TAG and DAG fractions (10.5 and 9.5%). The PL fraction contained PA (18.1%), OA (17.5%), LA (22.2%), GLA (7.3 %), and ALA (10.9%), while SDA was found in small amounts (2.4%). In total, PL reached lower PUFA percentages (42.8%) than that of NL (>77%). PUFA contained in the different lipid classes have different degree of bioavailability, and this fact may have practical applications. For instance, PUFA contained in MAG and DAG are highly bioavailable (Meynier & Genot, 2017). On this basis, GLA- and SDA-based MAG may offer a clinical advantage to patients struggling with fat malabsorption/maldigestion problems, for instance, patients with cystic fibrosis (Cuenoud et al., 2020). On the other hand, *n*-3 PUFA are better protected against oxidation when they are included in polar lipids, while the bioavailability of *n*-3 PUFA included in the PL fraction is higher than that of TAG (Jiménez Callejón et al., 2020). These lipid fractions were not too high in the studied sample, but it is expected that these fractions could be higher in other unanalyzed *L. officinale* chemotypes, which will be checked as soon as possible.

3.3 Unsaponifiable fraction

These analyses were accomplished in E-17 and F-18 samples, in which SDA and GLA reached the highest relative percentages (Table 4). Tp reached the highest amount in F-718 sample, which contained 35.7 mg/100 g d.w. Such figure was slightly lower than that of *B. officinalis* seeds (44.1 mg/100 g d.w.). The predominant Tp in *L. officinale* was γ-Tp (33.0 mg/100 g d.w. in F-18 sample), which was in good agreement with data reported by Velasco and Goffman (1999). Overall, the main difference between *L. officinale* and *B. officinalis* seeds is that the later contains δ-Tp and the former γ-Tp as predominant Tp, while *B. officinalis* lacks α-Tp.

As for sterols, the total amounts detected in *L. officinalis* (83.8 mg/100 g d.w. in E-17) was higher than that found in borage seeds (65.1 mg/100 g d.w.). In *L. officinalis* Δ^5-

avenasterol was the predominant sterol (56.0 mg/100 g seed in E-17 sample), followed by campesterol (23.5 mg/100 g d.w. in E-17 sample) and β-sitosterol (8.8 mg/100 g d.w. in F-18 sample). For St, no large differences were found between *L. officinale* and *B. officinalis* seeds.

A 280-nm HPLC-DAD chromatogram of phenolic compounds of *L. officinale* seed extract is shown in Figure 1, the calibration data on phenolic compounds determined by HPLC-DAD and LC-MS ion parameters are summarized in Table 5, and the amounts detected of phenolics are detailed in Table 6. Gallic, protocatechuic, p-hydroxybenzoic, caffeic, salicylic, rosmarinic and lithospermic acids were identified in *L. officinale*. Rosmarinic acid was the main compound (323.4 mg/100 g seeds in E-17 sample), and this amount was higher that than detected in borage seeds (96.1 mg/100 g). Rosmarinic acid accumulation is a rather consistent feature of species within the *Boraginaceae* family (Dresler et al. 2017). The main differential characteristic of *B. officinalis* with respect to *L. officinale* is that the former contains DL-p-Hydroxyphenyl lactic acid, 3,4-Dihydroxyhydrocinnamic acid, p-coumaric acid, ferulic acid and 2-hydroxy-4-methoxy benzoic acid; while *L. officinale* contains p-hydroxybenzoic acid, which is absent in *B. officinalis*. On the other hand, the total content of phenolic compounds is much higher in *L. officinale* (389.9 and 367.2 mg/100 g d.w. for E-17 and F-18 samples) than in *B. officinalis* (22.6 mg/100 g d.w.).

5. Conclusions

Some chemotypes of *L. officinalis* seeds analyzed in this work contained high GLA and SDA amounts, and intraspecific variations of *L. officinalis* lead to considerer this species as a suitable candidate for cultivation and functional oils obtainment. Until now, GLA- and SDA-oils have been obtained from a small number of species; however, using plant chemotypes belonging to the same species for obtaining both GLA- and SDA-rich oils would be advantageous, since the agronomic knowledge applied for a single species cultivation would be applied to any plant chemotype. Some of the chemotypes analyzed here show advantageous FA profiles with respect to other Boraginaceae species currently cultivated for obtaining GLA- or SDA-rich oils. The high variability detected for GLA and SDA percentages indicates a high genetic variability of the Δ6-destaturase enzyme within this taxon, and this fact could be advantageously used to obtain plant chemotypes hyperproducers of GLA and/or SDA by culture lines selection. This way, the current cultured *B. arvensis* used for SDA-rich oils production might be exchanged by others chemotypes of *L. officinale* detected in this work, which produces similar SDA percentages. Future actions should be devoted to study the health benefits associated with *L. officinale* seed oils intake, including *in vivo* studies, while genetic and agronomic variables should be explored to allow an efficient cultivation of the various *L. officinale* chemotypes. Considering that *L. officinale* roots are a source of shikonin, this species could be used to produce functional GLA- and SDA-rich oils, and simultaneously the roots could be used for the obtention of shikonin.

Conflict of Interest: The authors declare that they have no conflict of interest.

References

Asadi-Samani, M., Bahmani, M., & Rafieian-Kopaei, M. (2014). The chemical composition, botanical characteristic and biological activities of *Borago officinalis*: a review. Asian Pacific Journal of Tropical Medicine, **7**:S22-S28.

Badmaev, N., Bazarov, A., Kulikov, A., Gyninova, A., Sympilova, D., Shakhmatova, E., Badmaeva, N., Gonchikov, B., & Mangataev, A. (2019). Global climate change: wild fires and permafrost degradation in the Republic of Buryatia (Eastern Siberia, Russia). Earth and Environmental Science, **320**:012017. https://doi.org/10.1088/1755-4281315/320/1/012017.

Barceló-Coblijn, G., & Murphy, E. J. (2009). Alpha-linolenic acid and its conversion to longer chain n−3 fatty acids: Benefits for human health and a role in maintaining tissue n−3 fatty acid levels. Progress in Lipid Research, **48**:355–374. https://doi.org/10.1016/j.plipres.2009.07.002.

Beck H, Zimmermann N, McVicar T, Vergopolan, N., Berg, A., & Wood, E.F. (2018). Present and future Köppen-Geiger climate classification maps at 1-km resolution. Scientific Data, **5**:180214. https://doi.org/10.1038/sdata.2018.214.

Berti, M., Johnson, B. L., Dash, S., Fischer, S., Wilckens, R., & Hevia, F. (2007). Echium: A source of stearidonic acid adapted to the northern Great Plains in the US. In J. Janick & A. Whipkey (Eds.), Issues in new crops and new uses (pp. 120–125). Alexandria: ASHS Press.

Cohen, J.I., & Davis, J.I. (2009). Nomenclatural changes in *Lithospermum (Boraginaceae)* and related taxa following a reassessment of phylogenetic relationships. Brittonia, **61**:101–111. https://doi.org/10.1007/s12228-009-9082-z.

Cuenoud, B., Rochat, I., Gosoniu, M. L., Dupuis, L., Berk, E., Jaudszus, A., & Cruz-Hernandez, C. (2020). Monoacylglycerol form of omega-3s improves its bioavailability

in humans compared to other forms. Nutrients, **12**:1014. https://doi.org/10.3390/nu12041014.

Dresler, S., Szymczak, G., & Wójcik, M. (2017). Comparison of some secondary metabolite content in the seventeen species of the *Boraginaceae* family. Pharmaceutical Biology, **55**:691–695. https://doi.org/10.1080/13880209.2016.1265986.

Guil-Guerrero, J.L., Rincón-Cervera, M.Á., Gómez-Mercado, F., Ramos-Bueno, R.P., Venegas-Venegas, E. (2013). New seed oils of *Boraginaceae* rich in stearidonic and gamma-linolenic acids from the Maghreb region. Journal of Food Composition and Analysis, **31**:20-23. https://doi.org/10.1016/j.jfca.2013.02.007.

Guil-Guerrero, J. L., Maroto, F. G., & Gimenez, A. G. (2001). Fatty acid profiles from forty-nine plant species that are potential new sources of γ-linolenic acid. Journal of the American Oil Chemists' Society, **78**:677-684.
https://doi.org/10.1007/s11746-001-0325-9

Guil-Guerrero, J.L. (2007). Stearidonic acid (18:4n-3): Metabolism, nutritional importance, medical uses and natural sources. The European Journal of Lipid Science and Technology, **109**:1226–1236. https://doi.org/10.1002/ejlt.200700207.

Guil-Guerrero, J. L., Gómez-Mercado, F., Ramos-Bueno, R. P., González-Fernández, M. J., Urrestarazu, M., & Rincón-Cervera, M. Á. (2017). Sardinian *Boraginaceae* are new potential sources of gamma-linolenic acid. Food Chemistry, **218:**435–439. https://doi.org/10.1016/j.foodchem.2016.09.111.

Jiménez Callejón, M., Robles Medina, A., González Moreno, P., Cerdán, L., Orta Guillén, S., & Molina Grima, E. (2020). Simultaneous extraction and fractionation of lipids from the microalga *Nannochloropsis* sp. for the production of EPA-rich polar lipid concentrates. Journal of Applied Phycology, **1**:1-12. https://doi.org/10.1007/s10811-020-02037-z.

Lyashenko, S., González-Fernández, M. J., Gómez-Mercado, F., Yunusova, S., Denisenko, O., & Guil-Guerrero, J.L. (2019). *Ribes* taxa: A promising source of γ-linolenic acid-rich functional oils. Food chemistry, **301**:125309. https://doi.org/10.1016/j.foodchem.2019.125309.

Menghini, L., Ferrante, C., Zengin, G., Mahomoodally, M. F., Leporini, L., Locatelli, M., Cacciagrano, F., Recinella, L., Chiavaroli, A., Leone, S., & Brunetti, L. (2019). Multiple pharmacological approaches on hydroalcoholic extracts from different parts of *Cynoglossum creticum* Mill.(Boraginaceae). Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, **153**:633-639.

https://doi.org/10.1080/11263504.2018.1527790.

Meynier, A., & Genot, C. (2017). Molecular and structural organization of lipids in foods: their fate during digestion and impact in nutrition. Oilseeds and fats, Crops and Lipids, **24**:1-12. https://doi.org/10.1051/ocl/2017006.

Mhamdi, B., Wannes, W. A., Bourgou, S., & Marzouk, B. (2009). Biochemical characterization of borage (*Borago officinalis* L.) seeds. Journal of Food Biochemistry, **33**:331–341. https://doi.org/10.1111/j.1745-4514.2009.00221.x.

Mir, M. (2008). *Echium* oil: A valuable source of n-3 and n-6 fatty acids. Oléagineux, Corps Gras, Lipides, **15**:252–256. https://doi.org/10.1051/ocl.2008.0203.

Namal Senanayake, S. P. J., & Shahidi, F. (2000). Lipid components of borage (*Borago officinalis* L.) seeds and their changes during germination. Journal of the American Oil Chemists' Society, **77**:55–61. https://doi.org/10.1007/s11746-000-0009-5.

Peel, M. C., Finlayson, B. L., & McMahon, T. A. (2007). Updated world map of the Köppen-Geiger climate classification. Hydrology and Earth System Sciences Discussions, European Geosciences Union, **4**:439-473.

https://hal.archives-ouvertes.fr/hal-00298818

Özcan, T. (2010). Differentiation of *Symphytum* Species Using RAPD and Seed Fatty Acid Patterns. Natural product communications, **5**:587-596. https://doi.org/10.1177/1934578X1000500419

Shimada, T. L., Hayashi, M., & Hara-Nishimura, I. (2017). Membrane dynamics and multiple functions of oil bodies in seeds and leaves. Plant Physiology, **176**:199–207. https://doi.org/10.1104/pp.17.01522.

Velasco, L., & Goffman, F. (1999). Chemotaxonomic significance of fatty acids and tocopherols in *Boraginaceae.* Phytochemistry, **52**:423-426. https://doi.org/10.1016/S0031-9422(99)00203-4.

Yunusova, S. G., Lyashenko, S. S., Fedorov, N. I., Yunusov, M. S., & Denisenko, O. N. (2017). Lipids and lipophilic constituents of comfrey (*Symphytum officinale* L.) seeds. Pharmaceutical Chemistry Journal, **50**:728–731. https://doi.org/10.1007/s11094-017-5071521-7.

508Figure Legend:

509Figure 1. 280 nm-RP-HPLC chromatogram of *Lithospermum officinale* seeds. Legend:

5101 Gallic acid (6.8 min), 2 Protocatechuic acid (9.4 min), 3 p-Hydroxybenzoic acid (13.1

511min, 4 caffeic acid (21.9 min), 5 Salicylic acid (31.0) min, 6 ferulic acid (32.3 min), 7

512Rosmarinic acid (35.2 min), 8 Unknown.

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