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α-Linolenic acid and product octadecanoids in Styrian pumpkin seeds and oils: How processing impacts lipidomes of fatty acid, triacylglycerol and oxylipin molecular structures

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ABSTRACT

Styrian pumpkin seed oil is a conditioned green-colored oil renowned for nutty smell and taste. Due to α-linolenic acid (ALA) contents below 1% of total fatty acids and the prospect of nutritional health claims based on its potential oxidation products, we investigated the fate of ALA and product oxylipins in the course of down-stream processing of seeds and in oils. Lipidomic analyses with Lipid Data Analyzer 2.8.1 revealed: Processing did not change (1) main fatty acid composition in the oils, (2) amounts of triacylglycerol species, (3) structures of triacylglycerol molecular species containing ALA. (4) Minor precursor ALA in *fresh* Styrian and normal pumpkins produced 6 product phytoprostanes in either cultivar, quantitatively more in the latter. (5) In oil samples 7 phytoprostanes and 2 phytofurans were detected. The latter two are specific for their presence in pumpkin seed oils, of note, quantitatively more in conditioned oils than in cold-pressed native oils.

1. Introduction

Nutty taste, smell and the dark-green color are the sensory and optical characteristics of Styrian pumpkin seed oil. The brand originates from pumpkins having 'naked' or hull-less/thin-coated seeds, a specific variety grown in the southeastern region of Austria, in particular in the state of Styria (Fruhwirth & Hermetter, 2008). From the nutritional point of view this oil has a minute amount of ω3 fatty acid, i.e. α-linolenic acid (ALA or FA 18:3), whereas the main fatty acid constituent is ω6 linoleic acid (LA or FA 18:2). None-the-less, due to the non-fatty acid features, Styrian pumpkin seed oil is considered a delicacy like olive oil, due to their own non-fatty acid components. Styrian pumpkin seed oil is not a native, cold-pressed oil. It is a conditioned oil produced by drying the seeds, their subsequent grinding, adding of salted water, toasting of resulting paste at high temperature, and finally pressing the oil out of the paste at enhanced temperature. The fact that

Abbreviations: ALA also (FA 18:3), α-linolenic acid; PhytoP, phytoprostane; PhytoF, phytofuran; LD, lipid droplet; FA, fatty acid / fatty acyl; FAME, fatty acid methyl ester; ACN, acetonitrile; RT, room temperature; IS, internal standard; GC, gas-chromatography; FID, flame ionization detector; SPE, solid phase extraction; LC, liquid chromatography; UHPLC, ultra-high-performance liquid chromatography; qTOF, quadrupole time-of-flight; MS, mass spectrometry; ESI, electrospray ionization; LDA, Lipid Data Analyzer; CARS, coherent anti-Stokes Raman Scattering; TPE, two-photon excitation; suppl., supplementary.

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ALA does occur possibly as free fatty acid (FA) or as constituent of triacylglycerols (TGs), raises the following questions: On the one hand, whether such down-stream processing effects the profiles and structures of FAs and ALA-containing TG species and molecular species. On the other hand, if ALA is a precursor of oxylipins, then their occurrence in the oil could be a beneficial attribute with respect to nutritional value.

In the scientific literature, oxylipins like phytoprostanes (PhytoPs) and phytofurans (PhytoFs) have been reported in several plant matrices, such as rice, almonds, macroalgae, cacao, among others (Pinciroli et al., 2017; Ruesgas-Ramón et al., 2019; Vigor et al., 2018; Łuczaj, Jarocka-Karpowicz, Bielawska, & Skrzydlewska, 2015). But as far as we know, no previous information exists on the levels of these metabolites in pumpkin seeds. Indeed, it is known that, in addition to enzymatic oxidation in plants, ALA may be precursor of PhytoPs and PhytoFs which are oxidized metabolites derived from non-enzymatic autoxidation of this ω3-polyunsaturated fatty acid (Cuyamendous, Leung, Durand, Lee, Oger, & Galano, 2015; Imbusch & Mueller, 2000). Their production is stimulated by the enhanced exposure to reactive oxygen species and are even biomarkers of oxidative stress in plants (Cuyamendous et al., 2016; Galano et al., 2017). PhytoP and PhytoF studies are also interesting because they contribute to defensive signaling and thus to the prevention of cellular damage (González Roldán et al., 2019; Loeffler et al., 2005). Moreover, by analogy with human isoprostanes and prostaglandins found in the mammalian kingdom, which are known for their anti-inflammatory activity, such plant oxylipins are also biologically active in vivo in humans (Campillo et al., 2021; Minghetti et al., 2014).

Lipidomic approaches (Carrasco-Del Amor et al., 2017) and imaging (Waschatko et al., 2016) are the methods of choice in this investigation. In a nutshell, we ask the questions (i) what is the fate of ALA as such and as a component in TG molecular species during the steps in down-stream processing of Styrian and normal pumpkin seeds and in the oils, (ii) do PhytoPs and PhytoFs – the potential non-enzymatic oxidation products of ALA – occur in seeds and oils processed therefrom in view of their assumed health potential. The emphasis is on changes in precursor ALA and product octadecanoid contents.

2. Materials and methods

2.1. Plant sources

Mature seeds from pumpkin and rapeseed plants were harvested 2016 by local plant growers and delivered to the oil mill in Styria (Ölmühle Schalk, 8262 Kalsdorf bei Ilz, Austria) for processing. Pumpkin variety *Cucurbita pepo* subsp. *pepo* var. *styriaca* (Styrian oil pumpkin) produces dehulled (or 'naked') seeds giving rise to the green and tasty oil, commercially protected by official PGI (protected geographical indication). Controls were the seeds from 'normal' hulled pumpkins (*Cucurbita pepo* sp.) for production of colorless oils, rapeseeds (*Brassica napus*) known for high ALA contents in the colorless oil (Woodfield, Cazenave-Gassiot, Haslam, Guschina, Wenk, & Harwood, 2018). A fresh Styrian oil pumpkin and a fresh 'normal' garden pumpkin from harvest 2017 served as further control.

2.1.1. Down-stream processing of seeds

In a first step seeds from Styrian oil pumpkins are dried at 40 and 60 °C, respectively (samples 1A and 1B). The lower temperature preserves the ability of seeds to sprout and to use them as seedlings for the next crop, the higher temperature enables long-term storage of the seeds. Then, batches of 65 kg dried seeds are subjected to grinding and ground seeds are mixed with 11 L tab water containing 200 g table salt. The mixture is heated under stirring up to 125 °C maximum, which took around 65 min. This step is called 'toasting' and green pastes thus produced (samples 2A and 2B) emanate already the nutty smell and have a taste characteristic for the final Styrian pumpkin seed oil. In the final step pastes are placed into a stamping press containing two components, a ram and a fitting colander built in the 1950ies. Here, green oil is squeezed out of the paste at 38 °C (measured by a pyrometer). In this down-stream process each seed batch furnishes around 23 L of green oil, of which samples 3A and 3B are taken. From the residual press cakes obtained, we investigated the one starting with seeds dried at 60 °C (sample 4B).

For production of native pumpkin seed oils from hull-less seeds, seeds are dried at 40 and 60 °C (samples 5A and 5B, respectively) as before. Now, dried seeds are directly 'cold-pressed' without external heat in an extrusion press in a process, in which seeds are pushed through a fixed colander with a screw shaped, rotating component. The component is leaving less and less space for the seeds at the end of the process, while the oil leaks through the colander, whereas the solid fraction is held back, thus separating oil from residual press cake. The resulting native oils (samples 6A and 6B) have a green color, yet do not smell and taste like the Styrian pumpkin seed oil.

Rapeseeds are physically cleaned (sample 5C) and subjected to the same process as used for production of native pumpkin seed oils; resulting native rapeseed oil (sample 6C) has its own specific taste.

For testing pumpkin seeds without any prior treatment, fresh Styrian oil pumpkin and 'normal' pumpkin from harvest 2017 are used. Respective fresh seeds (samples 7 and 8) are subjected to immediate lipid extraction for further investigation or to imaging. The samples investigated and their description and *commercial labels* are as follows:

2.1.2. Samples for production of conditioned pumpkin seed oils

1A: Styrian pumpkin seeds dried at 40 °C (*Bio Österreichische Kürbiskerne max. 40 °C*)

2A: Paste from 40 °C-Styrian pumpkin seeds (*Bio Österreichisches Kürbiskernröstgut max. 40 °C*)

3A: Styrian pumpkin seed oil pressed from 40 °C-paste (*Bio Steirisches Kernöl g.g.A. Bio Austria, max. 40 °C*)

1B: Styrian pumpkin seeds dried at 60 °C (*Bio Österreichische Kürbiskerne max. 60 °C*)

2B: Paste from 60 °C-pumpkin seeds (*Bio Österreichisches Kürbiskernröstgut max. 60 °C*)

3B: Styrian pumpkin seed oil pressed from 60 °C-paste (*Bio Steirisches Kürbiskern Öl*)

4B: Presscake from 60 °C-paste (*KÜRBISKERN PROTEIN PULVER*)

2.1.3. Samples for production of native pumpkin seed oils

5A: Styrian pumpkin seeds dried at 40 °C (*Bio Österreichische Kürbiskerne* max. *40 °C*)

6A: Styrian pumpkin seed oil, cold-pressed from 40 °C-seeds (*Bio Österreichisches Kübiskernöl nativ,* max. *40 °C*)

5B: Styrian pumpkin seeds dried at 60 °C (*Bio Österreichische Kürbiskerne* max. *60 °C*)

6B: Styrian Pumpkin seed oil, cold-pressed from 60 °C-seeds (*BIO & NATIV KÜRBIS KERN ÖL*)

2.1.4. Further control samples

5C: Rapeseeds (*Rapssaat*)

6C: Rapeseed oil, cold-pressed from seeds (*Bio & NATIV RAPS ÖL*)

7: Fresh Styrian oil pumpkin seeds, harvest 2017, producing green oil

8: Fresh 'normal' pumpkin seeds, harvest 2017, producing colorless oil

2.1.5. Fatty acid and triacylglycerol analysis

2.1.5.1. Preparation of lipid extracts. Total lipids of either 300 mg (seed samples cut with Stanley knife and ground with mortar and pestle) or 10 mg (oil) are extracted twice with 4 mL chloroform/methanol (2:1, v/v) (Folch, Lees, & Sloane Stanley, 1957) containing 500 pmol butylated hydroxytoluene, 1 % acetic acid. Extraction is carried out under constant shaking for 90 min at room temperature (RT). After addition of 1 mL $MgCl_2$ (0.034 % in dH_2O) and further incubation for 30 min at RT,

samples are centrifuged at 1,000 × g for 15 min at RT to establish phase separation. The lower organic phase is collected, 2.5 mL chloroform added to remaining aqueous phase and second extraction is performed as described above (30 min, RT, with subsequent centrifugation).

2.1.5.2. Analysis of fatty acids by gas-chromatography - flame ionization detection (GC-FID). FA species are analyzed by a GC-FID instrument by established protocol (Sattler, Puhl, Hayn, Kostner, & Esterbauer, 1991) with following modifications: For transesterification, 2 mL BF_3 are added to 300 μL lipid extract as described above, containing 250 nmol FA 17:0 as internal standard (IS) and are incubated for 1 h at 110 °C. Reactions are stopped by addition of 1 mL ice-cold H_2O. FA methyl esters (FAMEs) are extracted twice by addition of 2 mL hexane/chloroform (4:1, v/v) and shaking for 10 min at RT. After centrifugation (1,000 × g, 10 min, RT) the upper phase is collected. Combined phases are evaporated under nitrogen and FAMEs are dissolved in 100 μL hexane. GC conditions are set to split injection (split flow 15 mL/min, split ratio 1/5, injection volume 2 μL) at injector temperature of 230 °C, using a wall-coated open tubular fused silica column (25 m, 0.32 mm inner diameter, free fatty acid phase-coated, film thickness 0.3 μm; Agilent Technologies, Santa Clara, CA, USA) and helium as carrier gas. The temperature gradient rises first with 5 °C/min from 150 to 250 °C and stays there for 2 min, and from there with 10 °C/min to 260 °C followed by a hold for 5 min. Conditions for FID (Trace-GC 2000 series instrument, ThermoQuest Corp., Atlanta, GA, USA) are as follows: Base temperature 150 °C, gas flow 200 mL/min air, 30 mL/min hydrogen for FID, and 20 mL/min helium as carrier gas. Data acquisition and analysis are done with Xcalibur 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA). For quantitative analysis corresponding FAME peaks are integrated and peak areas calculated in relation to FA 17:0 peak (IS). FAME concentrations are calculated as percentage of total FAMEs in a given sample and/or as amounts per sample weight (nmol/mg).

2.1.5.3. Analysis of triacylglycerol molecular species by ultrahigh-performance Orbitrap mass spectrometry (UHPLC-Orbitrap MS). One volume lipid extract (see *2.1.5.1*) is diluted with 14 volumes isopropanol/chloroform/methanol (90:5:5, v/v/v) and subjected to liquid-chromatography – mass spectrometry (LC-MS) analysis for TG quantification, modifying a method described earlier (Triebl, Trötzmuller, Hartler, Stojakovic, & Köfeler, 2017). The Dionex Ultimate 3000 RS UHPLC system (Thermo Fisher), thermostated to 50 °C, with a BEH C8 column (100 × 1 mm, 1.7 μm) from Waters (Milford, MA, USA) serves in chromatographic separation. Mobile phase A consists of de-ionized water containing 10 mmol/L ammonium formate and 0.1 vol% of formic acid as additives. Acetonitrile (ACN)/isopropanol 5:2 (v/v) with the same concentration of additives is mobile phase B. Equilibration of the column precedes each experiment. At a flow rate of 150 μL/min, gradient elution starts with 50 % each, mobile phase A and B, the latter rises to 100 % B in 40 min and stays there for 10 min. Then re-equilibration of the column with 50 % each, mobile phase A and B follows for 8 min, and samples (kept at 8 °C) are injected in 2 μL volumes per experiment.

Linked to the UHPLC system, the Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher) is operated in Data Dependent Acquisition mode based on an inclusion list for all theoretically possible TG species as $[M + NH_4]^+$ molecules. Prior to experiments, lens settings are tuned once and source parameters are optimized to the TG 51:0 signal. All samples are measured in positive polarity using a HESI II ion source with the following ion source parameters: Source voltage, 4.5 kV; Source temp. 300 °C; Sheath gas, 25 arbitrary units; Aux gas, 8 arbitrary units; Sweep gas, 0 arbitrary units; Capillary temp. 300 °C. Target value for automatic gain control is set to 10^6 ions for entering mass analyzer, maximum ion accumulation time is 500 ms. Full scan profile spectra from *m/z* 400 to 1,150 in positive ion mode are acquired in the Orbitrap mass analyzer at resolution setting of 100,000 at *m/z* 400.

For MS/MS experiments, the 10 most abundant ions in the full scan spectrum are sequentially fragmented in the ion trap collision-induced dissociation, again with the help of the inclusion list alluded to above. Helium serves as collision gas (normalized collision energy, 50; Isolation width, 1.5 Da; Activation Q, 0.2; Activation time, 10 ms). Centroided product spectra are collected at normal scan rate (33 kDa/s), exclusion time is set to 11 s.

LC-MS/MS data are processed with Lipid Data Analyzer (LDA 2) allowing to decipher MS/MS spectra on the characteristics of head group and fatty acyl fragments (Hartler et al., 2017) and annotated acc. to (Liebisch et al., 2020; Liebisch et al., 2013). In this publication, we present an algorithm to predict relative quantities of co-eluting species from multiplexed spectra of TG by fitting linear equations utilizing the Levenberg–Marquardt algorithm (see Supplementary material C for details and proof of principle). We provide this software extension with updated LDA version 2.8.1 (http://genome.tugraz.at/lda2/lda_download.shtml) which can handle virtually any metabolic class that runs into the same issue as TG by producing isomeric fragments from different molecular species.

For further data processing and statistics R (3.5.2) and Rstudio (1.1.463) are applied to abundance values calculated by LDA 2.8.1. In addition to these basic functions, dplyr package enabled swift data handling and mutation. Finally, ggplot2 package is employed to create figures (Wickham, 2016).

2.1.5.4. Analysis of triacylglycerol species by UHPLC-qTOF MS. Combined organic phases of lipid extract (see *2.1.5.1*) are dried under a stream of nitrogen, resolved in 1 mL 2-propanol/chloroform/methanol (7:2:1, v/v/v). A 1:1000 dilution of lipid extracts is used for UPLC-MS analysis. Chromatographic separation is carried out by a procedure reported earlier (Knittelfelder, Weberhofer, Eichmann, Kohlwein, & Rechberger, 2014), with two modifications: First, an AQUITY-UPLC system (Waters Corporation, Wilford, MA, USA) equipped with a Luna ω-C_{18} column (2.1x50 mm, 1.6 μm; Phenomenex) is employed and second, the 15 min-linear gradient starts with 100 % solvent A MeOH/H_2O (1:1 v/v); 10 mM ammonium acetate, 0.1 % formic acid, 8 μM phosphoric acid). A SYNAPT™G1 qTOF HD mass spectrometer (Waters Cooperation) equipped with an electrospray ionization (ESI) source is used for untargeted analysis of TG species. Data are acquired by MassLynx 4.1 software (Waters Corporation). TG species are quantitatively analyzed with "Lipid Data Analyzer 1.6.2" (LDA) software (J. Hartler, Trötzmuller, Chitraju, Spener, Köfeler, & Thallinger, 2011) and expressed as TG species in % of total TG.

2.2. Oxylipin analysis

2.2.1. Preparation of fresh pumpkin seed samples

PhytoP and PhytoF are extracted in triplicate from approximately 0.1 g of each sample. The fresh pumpkin seed is mixed with 2 mL MeOH containing 1 % butylated hydroxytoluene, then 2 ng IS are added to the mixture. After mix-up, 1.5 mL phosphate buffer (pH 2.0) saturated in NaCl are added. The solution is stirred for 1 h at 100 rpm at RT and then centrifuged at 4,000 rpm for 5 min. The supernatant is collected and 4 mL cold chloroform is added. The mixture is vortexed for 30 s and then centrifuged at 2,000 rpm for 5 min at 4 °C. The organic phase is separated and dried under nitrogen flow at 40 °C. Subsequent hydrolysis of the dry residue is attained by incubation with 950 μL KOH (1 M) for 30 min at 40 °C. At the end 1 mL formic acid (40 mM, pH 4.6) is added before the solid phase extraction (SPE) purification step. Mixed-mode ion-exchange SPE cartridges (Oasis MAX; 3 mL, 60 mg; from Waters Corporation) are conditioned with 2 mL MeOH and equilibrated with 2 mL formic acid (20 mM, pH 4.6). After loading the sample, cartridges are washed with i) 2 mL NH_3 2% (v/v), 2 mL formic acid (20 mM, pH 4.6) / MeOH 70:30 (v/v); ii) 2 mL hexane; iii) 2 mL hexane/ethyl acetate 70:30 (v/v). The elution step is carried out with 2 mL hexane/ethanol/acetic

acid 70:29.4:0.6 (v/v/v). Finally, all samples are dried under nitrogen flow at 40 °C. The targeted metabolites are dissolved in 100 µL H_2O/ACN 83:17 (v/v) before injection to the micro-LC-MS/MS system.

2.2.2. Preparation of oil samples

The preparation method applied to oils is slightly different. First, to 100 mg oil 5 mL hexane with 2 mL MeOH, 2 ng IS are added and vortexed for 15 s. The mixed material is supplemented with 2 mL formic acid (40 mM, pH 4.6) before centrifugation at 2,000 rpm for 5 min at RT. The supernatant is separated and lower phase loaded onto the SPE cartridges. From this point on, the rest of the process is carried out as described above.

2.2.3. Chemicals and reagents for octadecanoid analysis

Homemade IS C19-15-F_{2t}-IsoP and all the other standards are synthesized as reported earlier (Cuyamendous et al., 2015; Galano et al., 2017; Oger, Brinkmann, Bouazzaoui, Durand, & Galano, 2008). This pertains to 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-B_1-PhytoP, *ent*-9-L_1-PhytoP, 16(*RS*)-16-A_1-PhytoP as PhytoPs, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF and *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF as PhytoFs. Stock solutions of standards are prepared in MeOH to a concentration of 100 ng/µL, and are stored at − 20 °C. Appropriate dilutions from stock solutions are needed for calibration purposes. LC-MS grade methanol, ACN and HPLC-grade chloroform are obtained from Fisher Scientific (Loughborough, UK). Hexane (CHROMASOLV, HPLC-grade), absolute ethanol, formic and acetic acids, ammonia and KOH (Fluka MS-grade) are from Sigma-Aldrich (Saint Quentin Fallavier, France). Ethyl acetate of HPLC grade was obtained from VWR International (Radnor, PA, USA). Water used in this study was purified on a milliQ system (Millipore). SPE cartridges are a mixed-mode ion-exchange sorbent (Oasis MAX; 3 mL, 60 mg; from Waters).

2.2.4. Analysis of octadecanoids by Micro-LC-MS/MS

Micro-LC-MS analyses are carried out using an Eksigent® MicroLC 200 plus (Eksigent Technologies, Dublin, CA, USA) on a HALO C_{18} analytical column (100*0.5 mm, 2.7 µm; Eksigent Technologies) kept at 40 °C. The mobile phase consists of a binary gradient of solvent A (water with 0.1 % formic acid) and solvent B (ACN/MeOH 8:2 (v/v) with 0.1 % formic acid). The elution at a flow rate of 0.03 mL/min uses the following gradient profile: 17 % solvent B at start, 22 % solvent B at 9.5 min, 30 % solvent B at 11.5 to 15 min and 95 % solvent B at 16 min held for 2.3 min, and then return to initial conditions. This elution profile avoids sample contamination or sample-to-sample carry-over.

MS analyses are done on an SCIEX QTRAP 5500 (Sciex Applied Biosystems, Framingham, MA, USA) equipped with electrospray ionization (ESI) source and operated in the negative mode. The source voltage is kept at − 4.5 kV, N_2 used as curtain gas. Detection of fragmentation ion products from deprotonated molecule $[M - H]^-$ of each PhytoP or PhytoF is provided in multiple reaction monitoring mode and MS parameters are individually optimized for each compound. Analyte quantification is based on analyte to IS ratio using the calibration curves elaborated. The MultiQuant 3.0 software (Sciex Applied Biosystems) is used for data processing. All lipidomic profiling is carried out in triplicate.

2.2.5. Method for octadecanoid characterization

Parameters of extraction yield and of matrix effect follow the methodology published in a previous study (Vigor et al., 2018); specifications for the equipment such as accuracy, precision, resolution and sensitivity applied to oxidized metabolites are validated as in this study.

2.3. Sample preparation for imaging and microscopy

Transversal hand-made sections of fresh unlabeled Cucurbita pepo subsp. pepo var. styriaca seeds and Cucurbita pepo seeds are prepared using conventional razor blades. Sections are mounted in a drop of aqua dest. on high-grade coverslips (Menzel #1.5, Fisher Scientific Inc., Leicestershire, UK) for microscopy. Imaging is performed using an inverted Leica SP5 confocal and multi-photon laser scanning system with spectral detection (Leica, Inc., Mannheim, Germany) and a HCX IRAPO L 25x/0.95 water immersion objective. Coherent anti-Stokes Raman Scattering (CARS) microscopy of neutral lipids and lipid droplets is carried out using a commercial setup consisting of a picosecond laser source and an optical parametric oscillator (OPO; picoEmerald; APE, Berlin, Germany) integrated into the Leica SP5 microscope. For label-free imaging of neutral lipids/lipid droplets (LDs) the laser is tuned to 2845 cm^{-1}, thus enabling imaging of CH_2 symmetric stretching vibrations. CARS signal is detected using a 650/210 emission filter. Two-photon excitation (TPE) of autofluorescence signal of chloroplasts is detected using appropriate emission filters. Both, CARS and TPE signal are recorded simultaneously using a non-descanned photon detector in *Epi*-mode.

3. Results

Data obtained from down-stream processing of pumpkin seeds are presented first for seeds dried at 40 and 60 °C, i.e. respective A- and B-samples, and second for conditioned oil production versus native oil production of A- and B-samples. C-samples originate from rape seeds and serve as controls due to their high ALA content, respective oil produced is a native oil.

3.1. Total fatty acid contents in seed, paste and oil samples

As described above total lipids were extracted from weighted seed, paste and oil samples, transmethylated to FAMEs and subjected to quantitative analysis by GC-FID. Supplementary (suppl.) Fig. A1 clearly shows that oil samples 3A, 3B, 6A, 6B as well as control 6C have, as expected, the highest FA contents, around 3,200 nmol/mg sample. These fatty acids are almost exclusively constituents of TGs, whereas such exclusiveness does not apply to remaining samples. With the exception of sample 4B, the press cake from the paste from pumpkin seeds dried at 60 °C, FA contents are around 500 nmol/mg sample, certainly representing mostly TG-derived fatty acids. This means that still around 1/7 of the oil present in seeds and pastes remain in press cakes. Yet, FAs from seed and paste samples could be 'contaminated' by those stemming from membrane phospho- and galactolipids. The pressing step from samples 2 to 3 and from 5 to 6 is generally impressive for oil yields, but not quantitative as revealed by residual sample 4B upon pressing of sample 2B as shown in suppl. Fig. A1.

3.2. Fatty acid profiling reveals α-linolenic acid in seed, paste and oil samples

Quantitative analyses of fatty acid profiles are based on two different instrumental set-ups. GC-FID detection of FAME species was used with data processing by Xcalibur 2.0 software, to obtain the contribution of each FA species in % of total FAs (Fig. 1A-C, for values see suppl. Tab. A.1). Alternatively, Orbitrap MS/MS profiling of TG molecular species with subsequent calculation by expanded LDA2 software allowed calculation of total amount for each constituent FA species contained in every TG molecular species (Fig. 2, for values see suppl. Tab. A.2). To simplify illustration and explanation of data, we present C_{16} and C_{18} species only, which are the bulk constituents.

Comparison of data for A- and B-samples shown in Figs. 1 and 2 reveals that GC/FID data, expressed in percentages FA species of total FAs, are somewhat lower than that from Orbitrap measurements. This is understandable from the fact that calculation of the former is based on seed, paste and oil samples, whereas the latter are based for each sample on percentage FA species relative to total TG molecular species only. This is specifically evident in the case of minor ALA (FA 18:3) and palmitoleic acid (FA 16:1). As shown in Fig. 1A, these species are barely

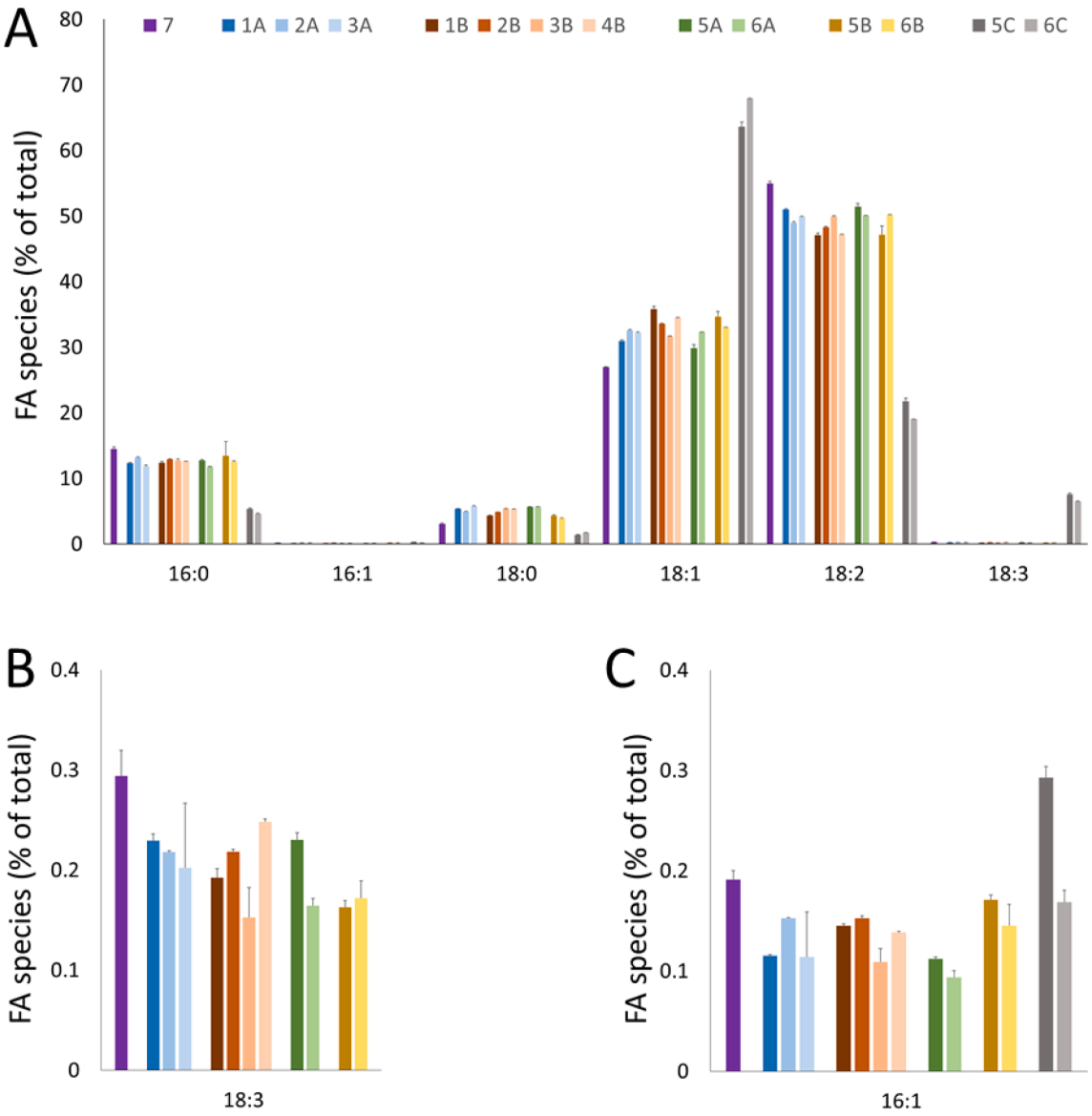

Fig. 1. C16 to C18 fatty acids in dried seeds, pastes and oils from pumpkin and rapeseed plants. Respective samples are from seeds dried at 40 °C (1A, 5A, 5C), at 60 °C (1B, 5B), from pastes (2A, 2B), from press cake (4B), from Styrian pumpkin seed oils (3A, 3B), cold-pressed Styrian pumpkin seed oils (6A, 6B), and cold-pressed from rapeseed (6C). In addition, sample 7 (purple bars) is included, representing fresh seeds from fresh Styrian oil pumpkin. (**A**) Selected fatty acids, (**B**) minor α-linolenic acid (FA 18:3), and (**C**) minor hexadecenoic acid (FA 16:1). Total lipids extracted from samples were transmethylated, FAMEs obtained were quantitatively determined by GC/FID and are shown as percentage of total FAMEs in each sample (n = 5 ± SD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detectible, yet the blow-ups shown in Fig. 1B and C indicate for FA 18:3 values between 0.15 and 0.3 %, for FA 16:1 between 0.08 and 0.2 %. This is to be seen in relation to those depicted in Fig. 3, respective values for FA 18:3 are between 0.4 and 0.8 %, for FA 16:1 between 0.1 and 1.0 %.

Up to here we can summarize the following points: (i) From the bioinformatics point of view, a sophisticated MS/MS platform equipped with LDA2 in combination with the newly elaborated algorithm enabled the calculation of amounts of individual constituents that belong to a class of lipid molecular species. (ii) From the analytical and biochemical points of view, metabolically precious ALA definitely occurs in Styrian pumpkin seed oil, though in amounts down from 0.8 % relative to total TGs. FA 16:1 cannot be ascribed by MS to be pure palmitoleic acid, i.e. FA 16:1(9Z), in all samples. In plant chloroplasts FA 16:1(3E) is a constituent of membrane phosphatidylglycerol (Harwood & James, 1975) that could be freed and thus contaminate the samples from Styrian pumpkin seeds in the course of down-stream processing. But the histograms for FA 16:1 in Fig. 2 clearly show a substantial increase of this FA species in pure oil, i.e. TG molecular species, including those in the oil from non-chloroplast containing rape seed samples. This would indicate that we were dealing with palmitoleic acid in oil samples proper. (iii) From the down-stream processing point of view for the production of oils, conditioning by drying, soaking in salt water, toasting, and pressing, or pressing alone in the case of native oil production, had an effect, whatever the first step was. The comparison of fresh Styrian oil pumpkin seed (sample 7, purple color in Fig. 1A-C) to all other pumpkin seed samples reveals a relative gain for FA 18:1, and losses for FA species 16:0, 18:0, 18:2, and also FA 18:3. Interestingly, this is also seen for control rapeseed samples, with the exception of FA 18:0 showing a small increase (grey bars in Fig. 1A). A look at Fig. 2 indicates that down-stream processing of pumpkin seeds from the drying step on had, with the exception of minor FA 16:1, only moderate impact on FA composition in TGs, in fact none on ALA (FA 18:3), the focus in this study.

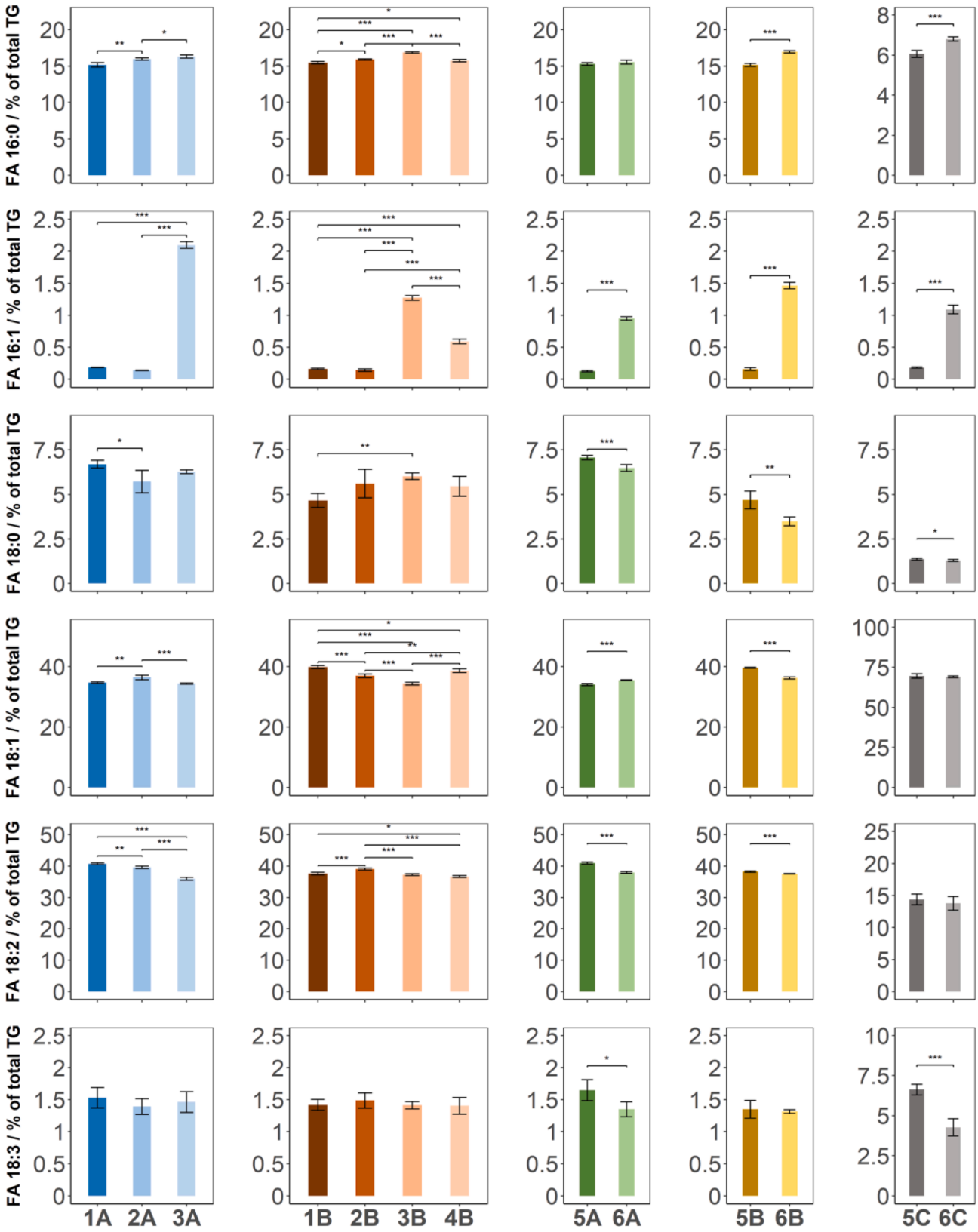

Fig. 2. Main FAs in triacylglycerols as percent of total TG in each sample, determined by LC-MS/MS (Orbitrap Velos Pro) in the course of seed oil production. Sample description as in legend to Fig. 1. Data are shown as mean ± SD (n = 5). P values were determined by ANOVA and Tukey HSD post hoc test (* = 0.05, ** = 0.01, *** 0.001).

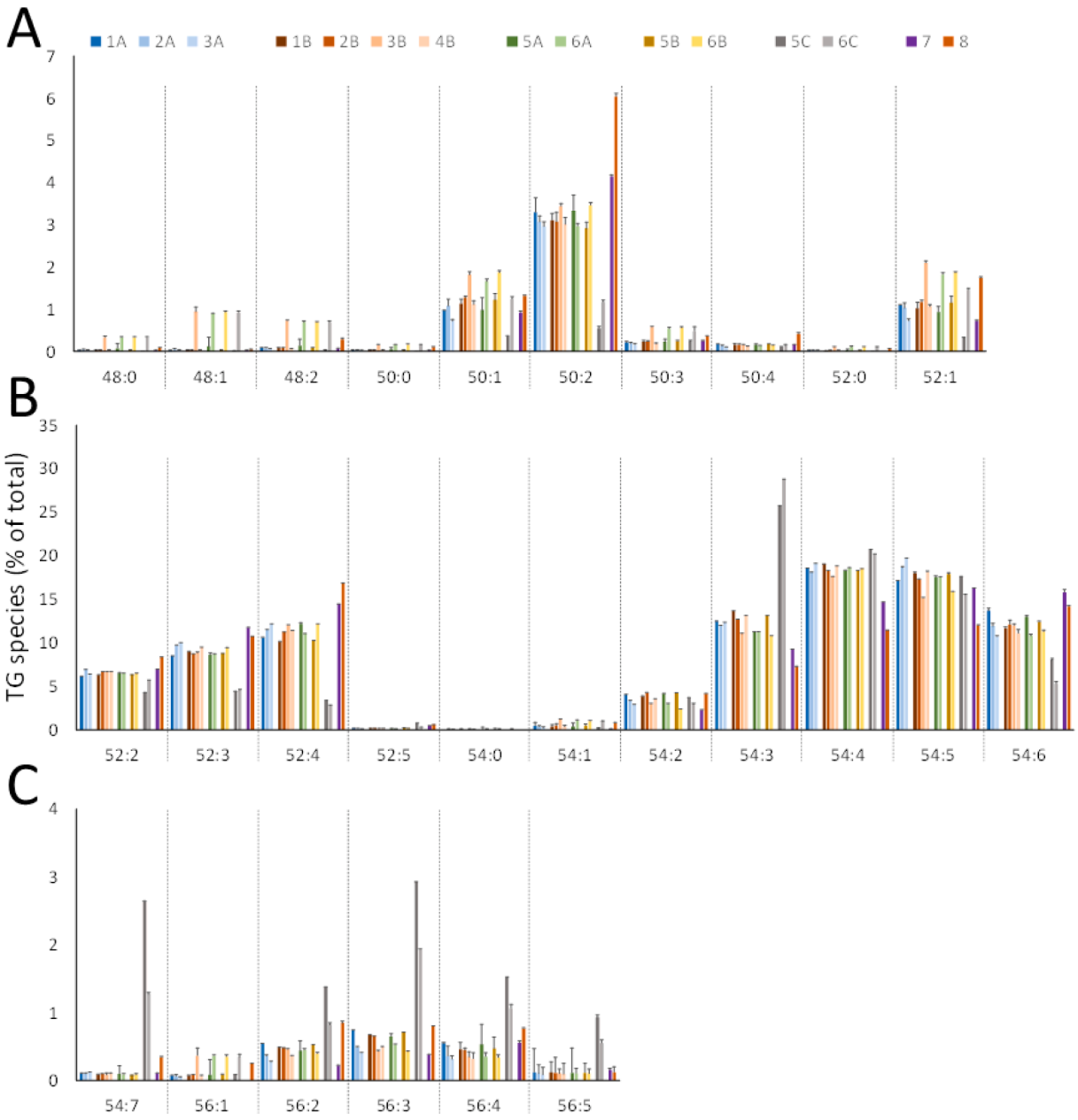

Fig. 3. TG species composition in dried seeds, pastes, and oils from pumpkin and rapeseed plants. Sample descriptions as in legend to Fig. 1. Samples 7 and 8 represent *fresh* Styrian pumpkin seeds and *fresh* 'normal' pumpkin seeds, respectively. **(A)** TG species mainly $\leq$ 5 %, **(B)** mainly $\geq$ 5 %, and **(C)** mainly $\leq$ 1 % (see scale on Y-axis). Total lipids extracted from samples were analyzed by UPLC/qTOF. TG data were processed by "Lipid Analyzer Software" and are shown as percentage composition of each sample (mean $\pm$ SD, n = 5).

Next, we raised the question, how did TG composition change in the course of down-stream processing and what was the impact on constituent ALA in TG species and molecular species?

3.3. Impact of down-stream processing on TG species in seed, paste and oil samples

We profiled all samples with the UPLC/qTOF set-up, resulting TG species data are shown in Fig. 3A-C (note the different scales for % species of total TG species, respective values are presented in suppl. Tab. A.3). From Fig. 1B we knew that in rapeseed samples ALA contents are around 8 % of total FAs, therefore we turned first to these controls (in Fig. 3 seed 5C, dark gray; cold-pressed oil 6C, light gray). Within the minor species shown in Fig. 3A, TG 48:1, 48:2, 50:1, 50:2 and 52:1 in native cold-pressed rapeseed oil clearly gained in percentages. Fig. 3B demonstrates that TG 54:3 was the dominant species in rapeseed samples 5C and 6C, the latter even more upon cold-pressing. For all further species with higher number of double bonds and C-atoms (Fig. 3B and C) the content of these species is lower in native cold-pressed rapeseed oil (sample 6C) than in fresh rapeseeds (sample 5C). Further control samples were seeds from fresh Styrian oil pumpkin (sample 7, purple) and from 'normal' oil pumpkin (sample 8, red). Fig. 3B reveals that the former promoted enhanced levels of TG 54:3, 54:4, 54:5 and 54:6 species compared to those from the latter. These four species belong to the most prominent contributors to total TG species. The opposite is true for all other TG species as can be seen in whole Fig. 3A-C.

The control data tell us (i) that processing of seeds with high ALA content such as rapeseeds effected TG species composition, and (ii) that mutation and plant breeding of pumpkin seeds had an impact on TG species composition having low ALA content in respective seeds.

Judging solely on oil pumpkin samples 1A to 6B, we define in % of total TG species those having ≤ 1 % as minor and ≤ 5 % as low abundant TG species (Fig. 3A and C) and major TG species having ≥ 5 % (Fig. 3B). In the overall Figure blue shades mark samples 1A to 3A, representing seeds dried at 40 °C and processed to Styrian pumpkin seed oil: Only marginal differences in contents of minor and low abundant TG species are seen and differences are major TG species were not consistent. The same is true for seeds dried at 60 °C and processed to Styrian pumpkin seed oil as demonstrated by the brown shades marking samples 1B to 3B. In addition, no consistent and significant difference *between* respective TG species from A- and B-samples are seen.

Green shades marking samples 5A and 6A indicate changes in TG species contents, when native cold-pressed Styrian pumpkin seed oil was produced from seeds dried at 40 °C and yellow shades marking samples 5B and 6B for native cold-pressed Styrian pumpkin seed oil from seeds dried at 60 °C. Fig. 3A and C reveal significant differences as a result of processing only for some minor TG species. This had certainly no significant impact, and interestingly, major TG species as depicted in Fig. 3B were not affected at all, regardless of temperature and cold-pressing.

To sum it up, there was almost no effect on relative amounts of individual TG species in the course of down-stream processing of dried Styrian pumpkin seeds to respective oils. The top question was now, what happened to constituent ALA in TG molecular species.

3.4. *Distribution of α-linolenic acid in TG molecular species in seed, paste and oil samples*

The UHPLC/Orbitrap/LDA2 platform enabled quantitative profiling of TGs at species and molecular species levels. The respective TG profile is shown in supplementary suppl. Tab. B.1 which accords with that of UPLC/qTOF analysis (Fig. 3). Yet it reports more minor molecular species, particularly at high end carbon numbers due to improved detection limits (profile spectrum ranges from TG 32:0 to TG 62:3, in total 97 species under the conditions of n ≥ 3 and total TGs ≥ 0.1 ‰). It also reports minor species with odd carbon numbers; it remains to be determined, whether this was due to straight-chain or methyl branched-chain fatty acids. For all samples, suppl. Tab. B.1 presents for each TG species respective constituent fatty acyls.

Within the 97 TG species occurring in Styrian pumpkin seed oil (samples 1A to 6B in suppl. Tab. B.1), 18 of them contained ALA. If we define criteria such as amount in % of total TG *and* having molecular

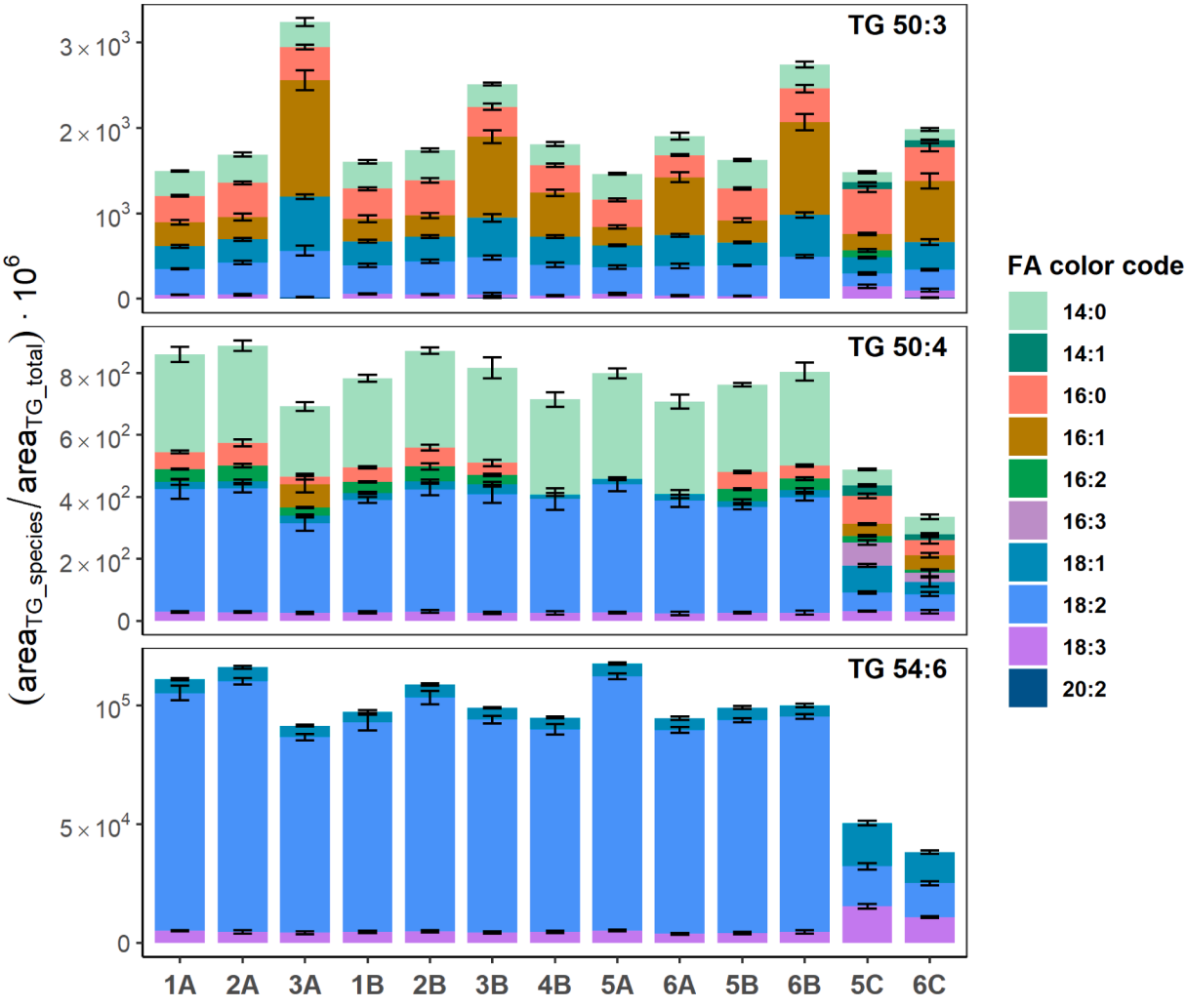

Fig. 4. α-Linolenic acid and palmitoleic acid are constituents in molecular species of selected TG species. **(Upper)** Minor species TG 50:3, the first species to contain ALA, palmitoleic acid is a main constituent with particular enrichment in final oil samples (3A, 3B, 6A, 6B). **(Middle)** Minor species TG 50:4, clear presence of ALA is observed, main constituent is linoleic acid and palmitoleic acid is still present in some samples. **(Lower)** Major species TG 54:6 in samples of Styrian oil pumpkin seeds and rapeseed, composed of oleic acid, linoleic acid and ALA only. MS and MS/MS spectra obtained with and evaluated by the UHPLC/Orbitrap/LDA 2.8.1 platform. TG species *and* their major FA 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 were normalized to total TG to balance the different TG contents found in samples 1A to 6C. Total height of each bar was derived from MS spectra (n ≥ 3), proportions of each constituent FA in respective TG species obtained by simultaneous MS/MS (mean ± SD, n ≥ 3). For graphical demonstration dimension-less values at Y-axis were multiplied by 10^6.

species with ALA, those with 1 % and lower are minor species, then TG species 50:3, 50:4, 51:3, 51:4, 52:5, 52:6, 53:4, 53:5, 54:7, 56:4, 56:5, 56:6, 58:4 and 60:4 fit. These seven minor species each contain several molecular species, some with constituent ALA, but are quantitatively negligible. In the four major species 52:4, 54:4, 54:5 and 54:6 (above 1 %) the percentage of constituent ALA-containing molecular species still is low, yet higher in terms of contribution to total TGs, TG 54:6 being the highest carrier species for ALA.

Three of the 13 TG species are singled out in Fig. 4 depicting graphically the structural contribution of the six main constituent FAs. Accompanying suppl. Tab. A.4 presents all statistically relevant molecular species pertaining to respective TG species mentioned in this paragraph. Fig. 4 upper provides evidence that ALA is constituent FA already in minor TG 50:3. It is composed of seven molecular species, one of them, TG 16:0_16:0_18:3, contains ALA in some samples as suppl. Tab. A.4 demonstrates. Another feature is the presence of constituent FA 16:1, with highest concentrations found in molecular species present in final oils, i.e., Styrian pumpkin seed oils (samples 3A and 3B) and in native Styrian pumpkin seed oils (samples 6A and 6B); this is clearly visible in Fig. 4 and strengthens the argument that FA 16:1 is palmitoleic acid. Another minor species is TG 50:4 (Fig. 4, middle), its main constituent FA is linoleic acid, none-the-less suppl. Tab. A.4 reveals that in all samples ALA is present in TG 14:0_18:1_18:3, whereas in rapeseed oil this FA is constituent in TG 16:0_16:1_18:3 only. But still some palmitoleic acid is present. ALA is more prominent in molecular species of major species TG 54:6 (Fig. 4 lower). Accordingly, suppl. Tab. A.4 proves that TG 18:2_18:2_18:2 is the prominent molecular species, accompanied by TG 18:1_18:2_18:3 in both Styrian oil pumpkin seed and rapeseed samples.

Taken together, inspection by UHPLC/Orbitrap/LDA2 technology allowed (i) to characterize TG molecular species containing ALA and (ii) to state that the down-stream process for Styrian pumpkin sed oils had no significant impact on the structures of these TG molecular species.

3.5. Profiling of octadecanoid metabolites in fresh pumpkin seed samples

The two varieties of fresh pumpkin seeds, one from Styrian oil pumpkin and another from 'normal' oil pumpkin were analyzed from their oxidized metabolites point of view. A suitable sample preparation (see methods part) was a mandatory prerequisite in the challenge to profile and characterize with high sensitivity octadecanoids in natural matrices. The proper tool for this analysis was micro-LC-MS/MS.

First, qualitative and quantitative analyses revealed that the two cultivars share the same major metabolites. Indeed, as seen in Fig. 5 left, diversity was not observed, i.e., in either cultivar six ALA-derived metabolites are seen, namely *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-9-L_1-PhytoP, *ent*-16-B_1-PhytoP, 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP. For respective values see Tab. A.6. The sum of these derivatives represented an average value of 21 ng/g found in fresh Styrian pumpkin seed (sample 7) and of 1622 ng/g in fresh 'normal' pumpkin seed (sample 8). The dynamic range for sample 7 was from 2.77 to 4.01 ng/g, viz. from *ent*-16-F_{1t}-PhytoP to *ent*-9-L_1-PhytoP, for sample 8 from 88.11 to 676.24 ng/g again from *ent*-16-F_{1t}-PhytoP, but to *ent*-16-B_1-PhytoP. Of note, the octadecanoids responsible for the two dynamic ranges were different in the two cultivars.

Second, quantitative analysis showed large differences in contents between samples 7 and 8, in fact representing a characteristic feature for each cultivar (Fig. 5). This is clearly demonstrated by the quantities measured, which in sample 8 (fresh normal oil pumpkin) were at least thirty times higher for 9-*epi*-9-F_{1t}-PhytoP and up to two hundred times higher for *ent*-16-B_1-PhytoP, in comparison to respective values in sample 7 (fresh Styrian oil pumpkin).

3.5.1. Profiling of octadecanoids in oil samples

The determination of octadecanoids in oily matrices was just as challenging as in fresh seed materials. Consequently, the extraction protocol was slightly adapted (see above) and micro-LC-MS/MS served again in the analytical step. Data obtained for samples 3A and 3B, representing conditioned Styrian pumpkin seed oils, and samples 6A and 6B, representing native cold-pressed Styrian pumpkin seed oils, demonstrate that always 9 different ALA-derivatives, i.e., seven PhytoPs and two PhytoFs were present in these samples (Fig. 5 right). In fact, six of the PhytoPs were the same as found in fresh samples 7 and 8, new in the oils were 16(RS)-16-A_{1t}-PhytoP and the two PhytoFs. Interestingly, this qualitative data was independent from whatever process was applied, that is from production technologies and from temperatures of seed drying.

In contrast, quantification revealed differences in total octadecanoid contents (for values see Tab. A.5), ranging from 0.23 to top 5.96 ng/g for 16(*RS*)-16-A_1-PhytoP in sample 6B and *ent*-16-B_1-PhytoP in sample 3A,

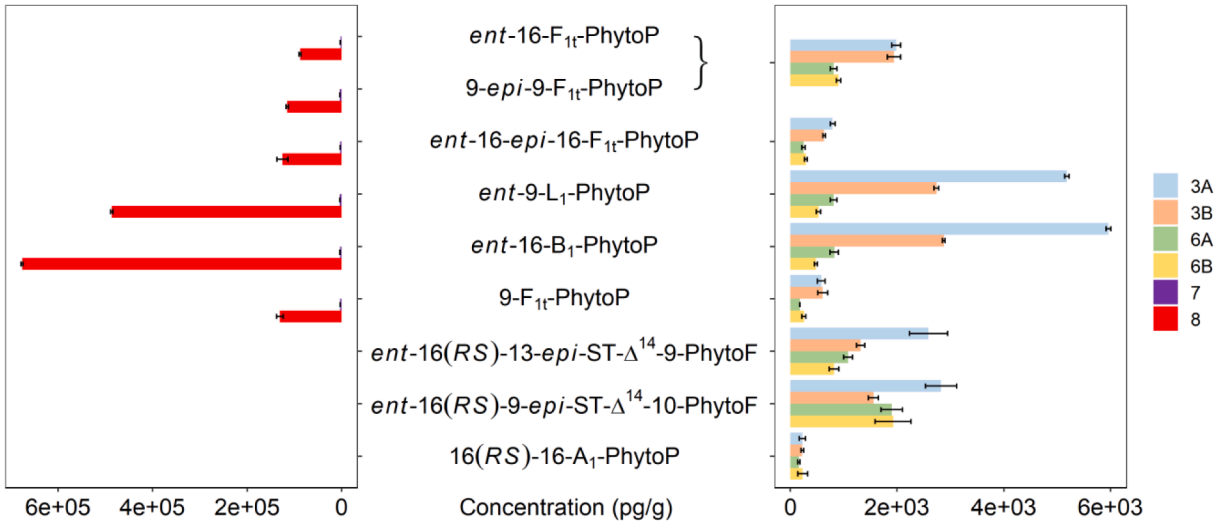

Fig. 5. Micro-LC-MS/MS analyses of octadecanoids. **(Left)** PhytoPs occurring in fresh Styrian oil pumpkin seeds (sample 7, purple) and in fresh 'normal' pumpkin seeds (sample 8, red). PhytoFs were not found. **(Right)** PhytoPs and PhytoFs occurring in Styrian pumpkin seed oils *processed* from seeds at 40 °C (sample 3A, blue) and from seeds dried at 60 °C (sample 3B, orange). PhytoPs and PhytoFs occurring in Styrian pumpkin seed oils *cold-pressed* from seeds dried at 40 °C (sample 6A, green) and from seeds dried at 60 °C (sample 6B, yellow). Concentrations given at the Y-axis are ng octadecanoid species per g seed fresh-weight at left, per g seed dry-weight at right (mean ± SD, n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively, a considerable dynamic range. Similarly, with the same compounds at both ends of the dynamic range in sample 3B, the values span from 0.23 to 2.88 ng/g. Then, in cold-pressed oils (samples 6A and B) PhytoP levels were mostly dramatically lower than in conditioned oils, whereas such data was not found for PhytoFs. This was borne out by octadecanoids such as 16(*RS*)-16-A_1-PhytoP and *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF ranging from 0.58 to 1.90 ng/g (6A) and 0.23 to 1.93 ng/g (6B), respectively. We noted that the 3 compounds occupying the last 3 positions in terms of contents were the same for the four oils. These were the compounds 16(*RS*)-16-A_1-PhytoP, 9-F_{1t}-PhytoP and *ent*-16-*epi*-16-F_{1t}-PhytoP. Conversely, octadecanoids at the top of the ranking in terms of quantity are different, since in cold-press oils *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF is followed by *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF in samples 6A and "*ent*-16-F_{1t}-PhytoP + 9-*epi*-9-F_{1t}-PhytoP" in samples 6B. Taken together, PhytoFs are specific in terms of occurrence in pumpkin seed oils.

4. Discussion

The varieties of Cucurbita pepo L., originating in North and Middle America, belong to genus Cucurbitaceae. They consist of squash and zucchinis, pumpkins and gourds, and have the greatest nutritional and monetary value within the Cucurbitaceae (Paris, 2008). This is particularly true for Cucurbita pepo subsp. pepo plants due to domestication by breeding for either fruit-flesh or seed oil production. From a point mutation in the 19th century the crop Cucurbita pepo subsp. pepo var. styriaca evolved and further on was cultivated for high oil production (Teppner, 2000). To confirm lipid accumulation in C. pepo subsp. pepo var. styriaca by imaging we applied CARS. It is based on the detection of specific molecular vibrations enabling detection of neutral lipids stored in LDs in unfixed seeds without any labeling. Accordingly, Fig. 6 shows increased neutral lipid amounts in pumpkin seeds of C. pepo subsp. pepo var. styriaca (sample 7) compared to those of C. pepo sp. (sample 8). In addition, the images shown in Fig. 6 conform with a recent report demonstrating that naked/thin-coated pumpkin seeds showed a high oil content; this correlates with low protein content and higher seed size in comparison with semi-hulled and hulled pumpkin seeds (Meru, Fu, Leyva, Sarnoski, & Yagiz, 2018). Moreover, genetic relationships based on sequence repeat polymorphism in 104 accessions from the three subspecies of Cucurbita pepo L. identified Cucurbita pepo subsp. pepo var. styriaca as a brand on its own (Gong et al., 2012). It is important to state that the presence of minute amounts of ALA (FA 18:3) in TGs of Styrian pumpkin seed oil was reported earlier and independently of us (Jafari, Goli, & Rahimmalek, 2012; Nakić, Rade, Skevin, Strucelj, Mokrovcak, & Bartolic, 2006).

The production of Styrian pumpkin seed oil afforded in a first step heat delivery to fresh seeds to remove water, viz. at 40° or at 60 °C. In the toasting step temperature delivered to pastes was raised to 125 °C. This was certainly a critical and decisive procedure, as it may affect both, by-products and the oil. Pertaining to the former, formation of polyaromatic hydrocarbons started only at temperatures close 150 °C (Potočnik & Košir, 2017). In the usual temperature regimes between 90 and 130 °C applied to pastes, changes in contents of phytosterols, phenolic compounds and vitamins were observed with uncertain effects on oxidative stability of the final oil (Fruhwirth & Hermetter, 2007, 2008; Murkovic, Piironen, Lampi, Kraushofer, & Sontag, 2004; Vujasinovic, Djilas, Dimic, Basic, & Radocaj, 2012). The desired by-products formed in this temperature range were odorants of mainly pyrazine structures, responsible for the nutty smell and taste of Styrian pumpkin seed oil (Matsui, Guth, & Grosch, 1998; Siegmund & Murkovic, 2004).

Application of pressure to conditioned pastes at 38 °C or of pressure without external heat to dried native seeds produced dark green oils in either case, in the latter case, however, without the nutty smell and taste. The dark green color of the oil, showing orange to red

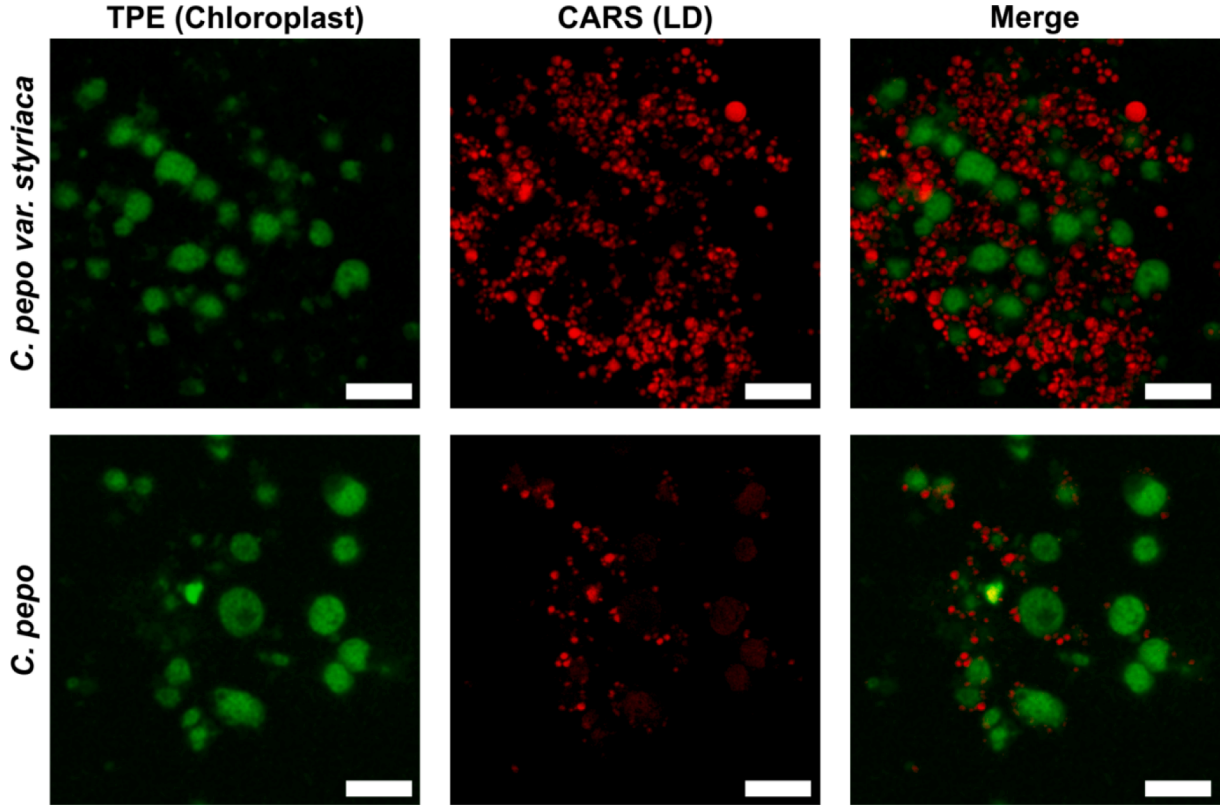

Fig. 6. Spatial distribution and amount of LD in sections of fresh Styrian and 'normal' oil pumpkin seeds. Autofluorescence of chlorophylls in chloroplasts and CH-stretch vibrations of neutral lipids in LD imaged using TPE and label-free CARS microscopy. Significantly increased amount of LD in Styrian oil pumpkin seeds is observed. TPE and CARS signals were imaged simultaneously. Bar = 10 μm.

fluorescence, is a further special feature of these oils from Styrian oil pumpkin. The molecules responsible for this property are protochlorophylls a and b as well as protopheophytin a and b mostly bound to phytol or geranylgeraniol. These substances are bound to the innermost seed coat whatever the thickness be, but are released into the oil only in naked/thin-coated pumpkins carrying the mutation (Ellsworth, 1971; Schoefs, 2001).

Concerning octadecanoids, the current study to our knowledge is the first to report for this variety the production of phytoPs and phytoFs derived from precursor ALA. If we now look, in the case of equal water content, at the non-enzymatic oxylipin profile of Styrian oil pumpkin and 'normal' oil pumpkin, we observe a very clear difference in the content of oxidized ALA-metabolites. This finding highlights the impact of variety on the oxidized derivative profile it may contain. Strictly speaking, this accords with general data on secondary metabolites contained by the plant species known to be impacted by the genotype.

If we know refer to the influence of down-stream processing of the raw materials to respective oils on oxylipin production, our data reveal a strong impact on quantitative but not qualitative values. Conditioned oils 3A and 3B differed markedly from cold-pressed oils 6A and 6B in terms of amount, allowing to put forward the "protective" character of the latter process. Indeed, under such extractive conditions, less oxidized products were formed. Because the octadecanoid profiles and contents of cold-pressed 6A and 6B oils were very close, we can advance the hypothesis of a very moderate impact of heating on seeds within temperatures up to 60 °C. This conclusion does not apply to pastes exposed to different heating temperatures. We observed a huge discrepancy in oxylipin contents from samples 3A and 3B, the one produced from a 40 °C paste contained more oxidized derivatives than the one produced from a 60 °C paste. The higher temperature may be a cause for deterioration of the oxylipins produced. Of course, we can't affirm this interpretation, additional work is necessary for better understanding of this phenomenon.

How can ALA be precursor for C18 oxylipins in Styrian oil pumpkin and in its seeds? ALA content in fresh seeds from Styrian oil pumpkin (sample 7) was only 0.3 % of total FAs; this amount would be available for octadecanoid synthesis and was more than found in all other processed samples as shown in Fig. 1. Taking this criterion, availability of ALA in fresh 'normal' pumpkin seeds (sample 8, not measured) would have been much higher as judged from octadecanoid synthesis shown in Fig. 5 left. Analyses shown in Fig. 5 right reveal much lower amounts of oxylipins detected in processed samples. But interestingly, production of PhytoPs and PhytoFs was higher in conditioned (samples 3A, 3B) than in cold-pressed (samples 6A, 6B) of Styrian pumpkin seed oils. 'Free' fatty acids per se in samples have not been analyzed, yet the data are a quality label for Styrian pumpkin seed oils, as they contain only minimum concentrations of precursor ALA. One can assume that ALA is released by lipolytic processes during seed drying already. Prominent donors would be TGs having constituent ALA (FA 18:3) as demonstrated in Figs. 2 and 4 as well as in Tab. A.1 and A.2. It may be mentioned that prolonged seed drying at 60 °C kills metabolism, whereas drying seeds at 40 °C preserves the potential for germination, a process in fat storing cells preceded by a lipolytic process degrading TGs (Huang, 2018). Soaking these seeds in water may promote such process to some extent.

5. Conclusions

Seeds from Styrian oil pumpkin contain α-linolenic acid in low amounts, the precious ω3 fatty acid of higher plants. By various lipidomic analyses we demonstrate that, during down-stream processing of the seeds to Styrian pumpkin seed oils, α-linoleic acid is preserved in TG molecular structures in each step, be it roasting and/or pressing. The final conditioned oil is appreciated for its green color and nutty taste and smell due to roasting, whereas the final native cold-pressed oil featured green color only and less ALA than in conditioned oils. Moreover, in view of potential nutritional benefits of oxidized products of α-linolenic acid, lipidomic analysis allowed to identify phytoprostanes and phytofurans for the first time in a pumpkin variety. The phytofurans were not found in seeds, but upon processing in respective seed oils, quantitatively more in conditioned oils than in cold-pressed native oils.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131194.

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