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Characterization of the Oxylipin Pattern and Other Fatty Acid Oxidation Products in Freshly Pressed and Stored Plant Oils

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ABSTRACT: Enzymatic and nonenzymatic oxidation of linoleic (LA) and α-linolenic acid (ALA) during pressing and storage of plant oils leads to a variety of oxylipins. We pressed oils from flaxseeds, rapeseeds, and sunflower seeds and analyzed the oxylipin pattern in freshly pressed oils. 9-/13-Hydro(pero)xy-LA/-ALA occurred in high concentration resulting probably from lipoxygenase-catalyzed reactions as well as autoxidation and photooxidation. However, in flaxseed and rapeseed oil, the highest concentrations were found for the terminal epoxy-ALA (15(16)-EpODE) and the hardly known 15-hydroxy-LA (15-HODE, 80 mg/100 g in flaxseed oil). Oils were stored for 6 months and the peroxide value (PV) as well as oxylipin and secondary volatile aldehyde concentrations were determined. While lipid peroxidation in flaxseed oil was surprisingly low, the oxylipin concentration and PV massively increased in rapeseed oil dependent on oxygen availability. Oxylipin concentrations correlated well with the PV, while secondary volatile aldehydes did not reflect the changes of oxylipins and PVs. The comprehensive analysis of hydroxy-, epoxy-, and dihydroxy-LA/-ALA reveals new and unique insights into the composition of plant oils and ongoing oxidation processes.

KEYWORDS: *lipoxygenase, autoxidation, liquid chromatography-mass spectrometry, fatty acid, volatile aldehyde*

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■ INTRODUCTION

Plant oils play an important role in human nutrition, as they are a relevant source of fat, including the essential fatty acids linoleic acid (LA) and α-linolenic acid (ALA),[1−3] as well as secondary plant metabolites such as tocopherols.[4] Especially flaxseed oil but also rapeseed oil are important as one of the few oils containing relevant amounts of n3-polyunsaturated fatty acids (PUFAs).[3] Consumption of plant oils increased substantially during the 20th century, in the United States, for example, mainly due to the growing use of soybean oil and rapeseed oil.[1,2] In 2020−21, 207 million tons of plant oil were produced worldwide[5] of which sunflower oil accounts for 19 million tons and rapeseed oil for 29 million tons.[5] Consumption in Germany reached approximately 1.5 million tons of plant oil in 2020.[6] Plant oils are divided into virgin and processed, i.e., refined oils.[7] Virgin oils are considered to be of high quality, as they are obtained only by (cold) mechanical pressing, still having the characteristic flavor and taste.[7] Mechanical pressing of oil seeds ruptures them;[8] thus, the fatty acids are accessible to oxygen and can be oxidized nonenzymatically by atmospheric oxygen[9] or by enzyme-catalyzed reactions of which lipoxygenases (LOXs) are the best described.[10,11]

Nonenzymatical oxidation comprises autoxidation by radical $^3O_2$ and photooxidation by singlet oxygen $^1O_2$ in a nonradical manner (Figure 1).[12] Autoxidation is a radical chain reaction and begins for LA with the abstraction of the bisallylic hydrogen atom at position 11. The resulting radical is stabilized by delocalization of electrons over five C atoms (positions 9 to 13) and forms a hydroperoxide via the intermediate stage of the peroxy radical and the abstraction of a (bisallylic) hydrogen atom of another (LA) molecule.[13,14] Autoxidation leads almost exclusively to equally formed 9- and 13-hydroperoxides with a conjugated diene system (Figure 1A). The double bond configuration (Z/E isomerization) depends largely on the reaction conditions such as temperature and properties of the surrounded H-donors.[9,13] Accordingly, for ALA, hydroperoxides resulting from autoxidation are predominantly formed at outer positions 9 and 16 and to a lower extent at internal C atoms 12 and 13 (Figure 1B).[15] This is mainly due to the cyclization tendency, i.e., formation of hydroperoxy epidioxides or bicyclic endoperoxides of the inner peroxy radicals resulting from a double bond in the β,γ-position.[9,15] Type 1 photooxidation resembles radical autoxidation,[16] whereas in type 2 activated singlet oxygen reacts directly with the double bond in a manner of 2 + 2 cycloaddition.[17] Thus, the resulting hydroperoxides of photooxidized LA differ from those formed by autoxidation in positions 10 and 12 that are also affected.[12] However, the possible cyclization of the inner peroxy radicals leads to the higher formation of the outer hydroperoxides, depending on the reaction conditions.[18] In summary, nonenzymatic oxidation of PUFAs is complex and leads to a broad pattern of oxylipins.

LOXs are nonheme iron-containing dioxygenases catalyzing the hydroperoxide formation of fatty acids with a 1(Z),4(Z)-pentadiene system. Plant LOXs are classified in LOX-1 and

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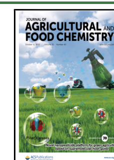

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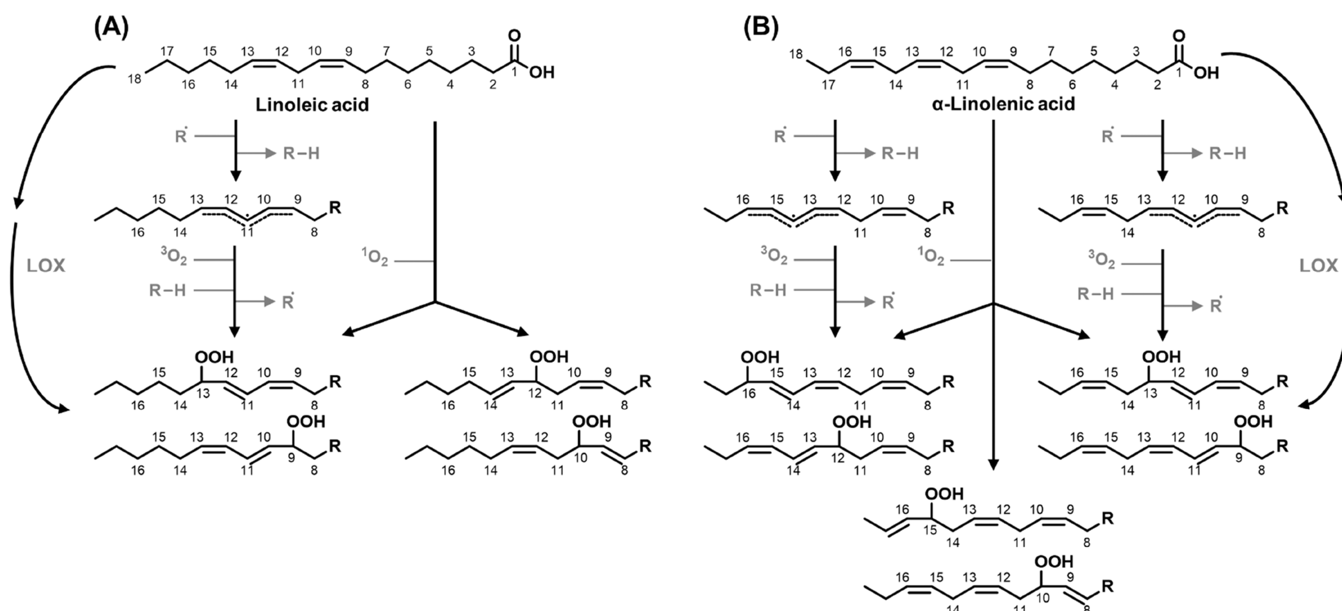

Figure 1. Simplified scheme of the reactions occurring during autoxidation and photooxidation of LA and ALA as well as formation of oxylipins catalyzed by lipoxygenases.

LOX-2 by their subcellular location or based on reaction specificity in the oxidation of LA into 9-LOX and 13-LOX. Additionally, there are also LOXs having a dual or no regiospecificity.[10] They all have in common that with C18 substrates they only abstract the bisallylic hydrogen at C11 with subsequent introduction of molecular oxygen at C9 or C13.[11] The mechanisms responsible for the different regiospecificities are still a matter of intensive research; however, two hypotheses are likely: (i) the substrate orientation model, i.e., fatty acid penetrates the active side tailfirst (13-OOH) or headfirst (9-OOH) and (ii) the space-related model, i.e., regiospecificity depends on how space filling amino acid residues limit the space in the active side.[10,11,19] LOXs occur ubiquitously in plants and were also described in seeds[20,21] such as soybeans (14 g/kg) or flaxseeds (3 g/kg).[22] In oils, LOX-catalyzed PUFA oxidation can be an important source of hydroperoxides which are formed during pressing.[23] A previous study reported concentration of 9- and 13-HODE, which are the hydroxy-analogues of hydroperoxides, e.g., 68 μM and 24 μM in flaxseed oil.[3] However, the number of analyzed C18 oxylipins was limited and the authors used commercially available flaxseed oil obtained with an unknown pressing procedure and which was probably stored in the supermarket already for several months.

Oxidative changes in plant oils during storage are commonly assessed by the peroxide value (PV) and the anisidine value,[7] of which the PV accounts for the entirety of hydroperoxides and thus for primary oxidation,[24,25] while the anisidine value is a marker for α,β-unsaturated aldehydes which are secondary oxidation products.[26,27] These parameters are used to describe the quality of oils; however, being sum parameters, both do not allow insights into oxidation processes actually taking place in the oils during storage. Modern liquid chromatography-mass spectrometry (LC−MS)-based analytical methods as established in our lab enable the simultaneous determination of a broad spectrum of oxidation products[28−31] resulting from a variety of enzymatic and nonenzymatic formation processes. The comprehensive quantification of oxylipins in plant oils is highly relevant because (i) many oxylipins are physiologically potent lipid mediators,[32] (ii) oxylipins provide information on activity of enzymes of fatty acid metabolism in seeds, and (iii) the position-specific analysis of hydro(pero)xy fatty acids allows us to monitor lipid peroxidation during storage. We therefore investigated occurrence and concentration of oxidation products in PUFA-rich plant oils which can be grown in northern Europe. Freshly pressed oils from flaxseeds, rapeseeds, and sunflower seeds were prepared using a screw press. We analyzed the total and nonesterified oxylipins directly in the freshly pressed oils to understand the interaction of enzymatic and nonenzymatic fatty acid oxidation during oil pressing. Additionally, oils were stored for 6 months and analyzed at different time points regarding the PV as well as oxylipin and secondary volatile aldehyde concentrations to link commonly used markers for lipid peroxidation to the specific oxylipin concentrations to gain new information on lipid peroxidation actually taking place during oil storage.

■ MATERIALS AND METHODS

Pressing and Storage of Edible Oils. Flaxseeds (*Linum usitatissimum*), sunflower seeds (*Helianthus annuus*), and rapeseeds (*Brassica napus*) used for pressing were provided by local oil mills. Oil was extracted with a KOMET screw press type CA59G (IBG Monforts GmbH and Co., Mönchengladbach, Germany) operated at 32 rpm with a nozzle of 6 mm inner diameter. After sedimentation of the resulting raw oil, two steps of vacuum filtration (cellulose filter MN 615, Macherey-Nagel, Düren, Germany) were performed.

Freshly pressed and filtered oils were filled in 50 mL amber glass bottles until the bottle was completely full. In addition, 250 mL amber glass bottles were half-filled with the freshly pressed oil. The samples were stored in the dark in a cupboard for 24 weeks at room temperature (20 ° C ± 2 ° C). An originally sealed fully filled 50 mL bottle was opened for each analysis time point. The PV and concentration of volatile secondary oxidation products were analyzed after 0/2/6/8/10/12/14/20/24 weeks. Total as well as free oxylipin concentrations were analyzed after 0/6/10/12/16/20/24 weeks. At time points of 12 weeks and 24 weeks, the half-filled bottles were analyzed as well.

Chemicals and Purchased Plant Oils. 2-Propanol, diethyl ether, methanol (MeOH), and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile (ACN), chloro-

form, and ethanol (EtOH) were obtained from VWR (Darmstadt, Germany). Tin(II) chloride (anhydrous, >98%) was purchased from Fluka/Honeywell (Offenbach, Germany). Ultra-pure water was generated using a Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). All other chemicals were from Merck (Darmstadt, Germany). Internal standards for fatty acid and oxylipin analysis were used as described.[28,33]

Sunflower oil (refined), rapeseed oil (refined), peanut oil (refined), soybean oil (refined), flaxseed oil (virgin), and olive oil (extra virgin) were purchased from an oil mill (Gustav Heess Oleochemische Erzeugnisse GmbH, Leonberg, Germany). For validation of the results, a second batch of oils was bought form a local supermarket: sunflower oil (refined; Thomy, Nestlé Deutschland AG, Neuss, Germany); rapeseed oil (refined; Brölio, Brökelmann + Co Oelmühle GmbH + Co, Hamm, Germany); peanut oil (refined; Mazola, Peter Kölln GmbH & Co. KGaA, Elmshorn, Germany); soybean oil (refined; Sojola, Vandemoortele Lipids NV, Gent, Belgium); flaxseed oil (virgin; REWE Bio, Huilerie Bio Occitane SASU, Bram, France); and olive oil (extra virgin; REWE Bio, HMF Food Production GmbH & Co. KG, Dortmund, Germany).

Quantification of Total Oxylipin and Fatty Acid Concentration. Total (esterified and nonesterified) oxylipin concentrations and fatty acid concentrations were determined as described.[28,30,33] In brief, 5 mg of oil was diluted with 2 mL of 2-propanol. To 50 μL of this solution, 10 μL of deuterated oxylipin internal standards (IS1, 100 nM), 350 μL of 2-propanol, 10 μL of butylated hydroxytoluene (BHT, 0.2 mg/mL in methanol) as an antioxidant, 50 μL of water, and 100 μL of 0.6 M potassium hydroxide in $MeOH/H_2O$ (75/25, v/v) were added and samples were hydrolyzed for 30 min at 60 °C. After neutralization, samples were loaded onto preconditioned solid phase extraction (SPE) cartridges (C8/anion exchange, bed weight 300 mg, volume 3 mL, Bond Elut Certify II, Agilent, Waldbronn, Germany). For reduction of hydroperoxides, 30 μL of tin(II) chloride ($SnCl_2$, 10 mg/mL in MeOH) was added after neutralization prior to SPE. After washing with H_2O and $MeOH/H_2O$ 50/50 (v/v) as well as drying of the cartridges, oxylipins were eluted with 2 mL of acidified ethyl acetate/*n*-hexane (75/25, v/v) and the solvent was removed using a vacuum concentrator (Christ, Osterode am Harz, Germany). Samples were reconstituted in 50 μL of MeOH containing a mix four substances used as second IS (IS 2) for calculation of IS 1 recovery rates.[28] Oxylipins were analyzed by liquid chromatography (1290 Infinity II LC System, Agilent, Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer (QTRAP 5500, AB Sciex, Darmstadt, Germany). Detection was carried out in scheduled selected reaction monitoring mode (SRM) following negative electrospray ionization (ESI(−)). External calibration with internal standards was used for quantification.[28,30] The recovery of the internal standards in each sample was monitored as quality control using IS 2. Only samples with a recovery above 50% (usually around 60−90%) were further evaluated. Moreover, a quality control oil was prepared in triplicate within every batch to assure reliable and reproducible sample preparation.

Fatty acyl concentrations were analyzed by LC−MS (1260 Infinity II LC System, Agilent, Waldbronn, Germany; QqQ 3200 mass spectrometer, AB Sciex, Darmstadt, Germany) following dilution in EtOH from the hydrolysate. Detection was carried out in *pseudo*-SRM following ESI(−). External calibration with deuterated internal standards was used for quantification.[33] To calculate the percentage of fatty acid oxidation, the total/nonesterified oxylipin concentration was divided by the total/nonesterified concentration of the precursor fatty acid.

Quantification of Nonesterified Fatty Acid and Oxylipin Concentration. Nonesterified fatty acid (NEFA) concentrations were determined as described.[33] In brief, 10 mg of oil was dissolved in 1 mL of $CHCl_3$/2-propanol and 10 μL of BHT was added. Samples were loaded on preconditioned aminopropyl SPE cartridges (Supelclean LC-NH_2, bed weight 100 mg, volume 1 mL, Merck, Darmstadt, Germany). Triacylglycerols were removed with 2 mL of $CHCl_3$/2-propanol and NEFAs were eluted with 2 mL of diethyl ether/HAc (98/2, v/v). The eluates were neutralized with 1 M $NaHCO_3$, the upper phase was collected and evaporated to dryness in a vacuum concentrator. The residue was dissolved in EtOH, diluted, and analyzed by LC−MS (1260 Infinity II LC System, Agilent, Waldbronn, Germany; QqQ 3200 mass spectrometer, AB Sciex, Darmstadt, Germany).[33] Nonesterified oxylipins were prepared in the same way using 5 mg of oils and elution was done with 2 mL of MeOH +1% HAc. For reduction of hydroperoxides, 30 μL of tin(II) chloride (10 mg/mL in MeOH) was added prior to sample loading. The eluate was evaporated in a vacuum concentrator, reconstituted in 50 μL of IS 2 dissolved in MeOH, and analyzed by LC−MS. Only samples with a recovery above 50% (usually around 70−90%) were further evaluated. Moreover, a quality control oil was prepared in triplicate within every batch to assure reliable and reproducible sample preparation.

Determination of the PV. The PV was determined according to the DGF method C-VI 6a Part 1 (05)[34] (according to Wheeler). In short, 5 g of oil was dissolved in 50 mL of a mixture of isooctane (Merck, Darmstadt, Germany) and glacial acetic acid (Chem-Solut, Th. Geyer GmbH & Co. KG, Renningen, Germany) 40/60 (v/v). After addition of 500 μL of saturated aqueous potassium iodide (Merck, Darmstadt, Germany) solution, the formed iodine was automatically titrated with sodium thiosulfate (0.01 mol/L, Merck, Darmstadt, Germany) and the endpoint was determined electrochemically (Metrohm, Filderstadt, Germany).

Quantification of Volatile Secondary Oxidation Products. Analysis of the volatile compounds hexanal, *E*-2-heptenal, *E,E*-2,4-heptadienal, nonanal, and *E*-2-decenal was performed by dynamic headspace gas chromatography with a flame ionization detector (GC-FID) as previously described.[35] Quantification was carried out by external calibration of the GC-FID signal of standards. For calibration, the standard compounds were sequentially diluted in refined oil. The absence of the aldehydes of interest in the used refined oil was checked before preparation of the calibration solutions.

RESULTS

Concentration of Fatty Acids in Different Oils. The flaxseed oil used in our study consisted mainly of ALA (46 g/100 g, 1.6 mmol/g) followed by oleic acid (24 g/100 g, 0.86 mmol/g), LA (14 g/100 g, 0.48 mmol/g), and palmitic acid (7.9 g/100 g, 0.31 mmol/g; Table 1). In rapeseed oil, the main fatty acids were oleic acid (61 g/100 g, 2.2 mmol/g), LA (14 g/100 g, 0.51 mmol/g), ALA (10 g/100 g, 0.34 mmol/g), and palmitic acid (5.8 g/100 g, 0.23 mmol/g; Table 1). The sunflower oil consisted mainly of oleic acid (86 g/100 g, 3.1 mmol/g) in addition to palmitic acid (4.7 g/100 g, 0.19 mmol/g), stearic acid (2.9 g/100 g, 0.10 mmol/g), and LA (2.4 g/100

Table 1. Total Fatty Acid Patterns of the Pressed Oils[a]

[g/100 g]	flaxseed oil	rapeseed oil	sunflower oil
C16:1 n7	0.08 ± 0.01	0.21 ± 0.01	0.11 ± 0.01
C16:0	7.9 ± 0.9	5.8 ± 0.7	4.7 ± 0.3
C18:3 n3	45.6 ± 0.5	9.5 ± 0.9	0.066 ± 0.004
C18:2 n6	13.5 ± 0.1	14.3 ± 1.0	2.4 ± 0.3
C18:1 n9 + n7	24 ± 2	61 ± 6	86 ± 6
C18:0	6.8 ± 0.5	1.76 ± 0.09	2.9 ± 0.2
C20:3 n6 + n3	0.019 ± 0.001	<0.0001	<0.0001
C20:2 n6	0.0202 ± 0.0007	0.070 ± 0.007	<0.0001
C20:1 n9	0.171 ± 0.002	1.45 ± 0.07	0.35 ± 0.04
C20:0	0.24 ± 0.01	0.53 ± 0.06	0.25 ± 0.02
C22:1 n9	0.015 ± 0.002	0.52 ± 0.05	0.014 ± 0.003
C22:0	0.18 ± 0.05	0.26 ± 0.06	0.85 ± 0.07

[a]Analysis of total fatty acid concentrations was carried out by LC−MS after hydrolysis of the oils diluted in 2-propanol (mean ± SD, *n* = 3).

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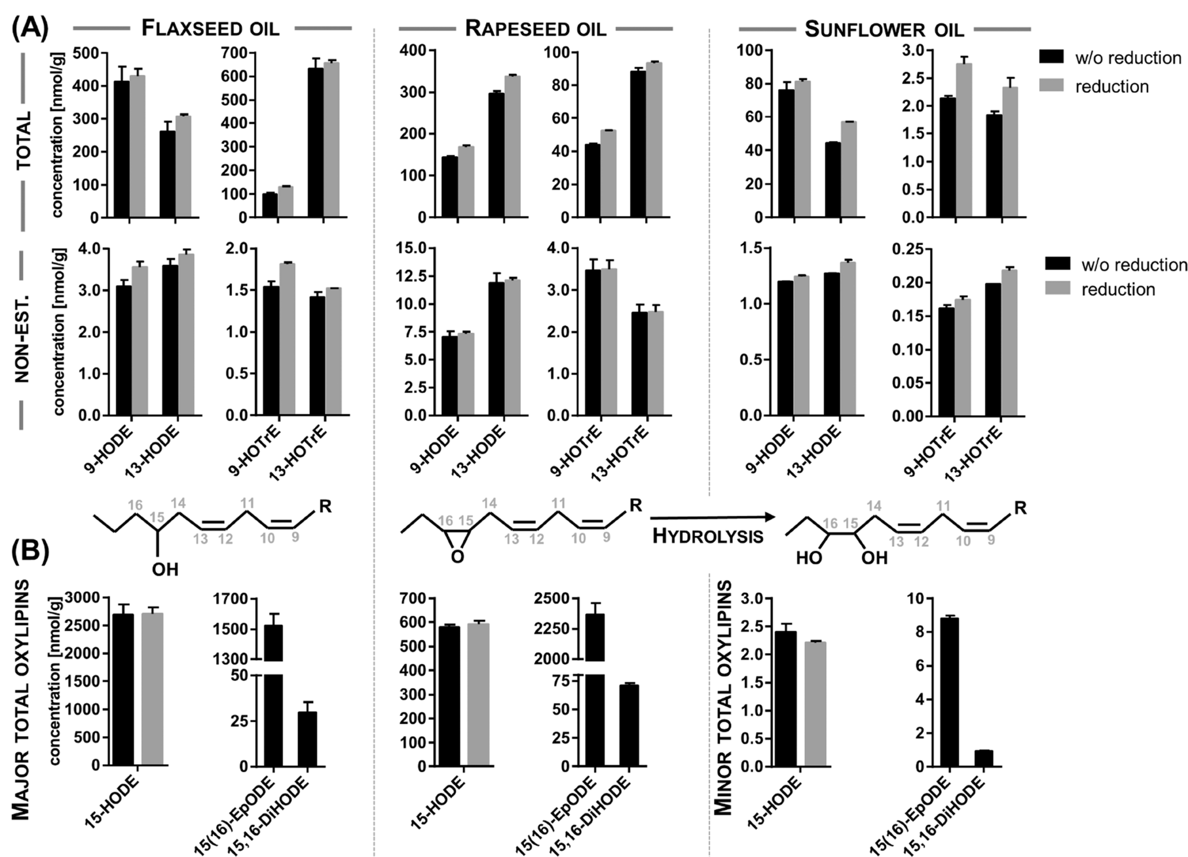

Figure 2. Nonesterified and total oxylipin concentration in freshly pressed oils. (A) Concentration of the well described 9-/13-OH linoleic and α-linolenic acid derived from the radical-mediated hydroperoxide formation. (B) Concentrations of major oxylipins detected in rapeseed and flaxseed oil. Total (nonesterified and esterified oxylipins) as well as nonesterified oxylipins were analyzed by LC−MS (mean ± SD, n = 3) with and without reduction (w/o reduction) of hydroperoxides by $SnCl_2$. HODE: hydroxy linoleic acid; HOTrE: hydroxy α-linolenic acid; EpODE: epoxy α-linolenic acid; DiHODE: dihydroxy α-linolenic acid; R = $-(CH_2)_7-COOH$.

g; Table 1, 0.087 mmol/g). ALA occurred only in minor concentration (0.07 g/100 g, 0.0024 mmol/g).

Concentration of Oxylipins in Freshly Pressed Oils. In freshly pressed flaxseed oil, a slightly higher concentration for total 9-HODE compared to 13-HODE was determined (410 nmol/g and 260 nmol/g; Figure 2A, Table S1). For total hydroxy-ALA, concentrations were considerably higher for 13-HOTrE (630 nmol/g) compared to 9-HOTrE (99 nmol/g). Similar results were found in a second freshly pressed flaxseed oil as well as in two commercially available flaxseed oils (Table S2). On the contrary, for nonesterified oxylipins the 9-/13-regioisomers showed equal concentration (9-HODE: 3.1 nmol/g; 13-HODE; 3.6 nmol/g; 9-HOTrE: 1.5 nmol/g; 13-HOTrE: 1.4 nmol/g, Figure 2A). Reduction of hydroperoxides led only to slightly increased concentration for total and nonesterified 9-/13-OH-PUFA, while for total 10-HODE, a considerably higher total concentration (13.4 nmol/g) was determined after reduction compared to no reduction (6.9 nmol/g; Figure S1). The highest total oxylipin concentrations were found for 15-HODE (2700 nmol/g, Figure 2B), whose concentration did not change after reduction of hydroperoxides, as well as for 15(16)-EpODE (1520 nmol/g). Its hydrolysis product 15,16-DiHODE had a rather low concentration (30 nmol/g) with a ratio of epoxy/diol of 50.

In rapeseed oil, among the hydroxy-LA, higher concentrations were found for the 13-regioisomer (13-HODE 297 nmol/g) compared to 9-HODE (143 nmol/g; Figure 2A, Table S1), only for the nonesterified hydroxy-ALA, i.e., HOTrE, the concentration of the 9-regioisomer was slightly higher (9-HOTrE: 3.5 nmol/g and 13-HOTrE 2.5 nmol/g). According to the fatty acid pattern, the concentrations of HODEs are higher than the HOTrE concentration. Again, reduction of hydroperoxides resulted only in higher levels of total 10- and 12-HODE (e.g., 10-HODE 13.7 nmol/g vs 20.3 nmol/g) while concentration of nonesterified ones did not change (10-HODE 1.84 nmol/g vs 1.87 nmol/g; Figure S1). Similar to flaxseed oil, 15-HODE (580 nmol/g) and 15(16)-EpODE (2370 nmol/g) were the most abundant oxylipins in rapeseed oil (Figure 2B). The ratio of 15(16)-EpODE to its vicinal dihydroxy-PUFA was 34 (15,16-DiHODE 71 nmol/g).

In sunflower oil, higher total concentrations were found for HODEs with 9-HODE (76 nmol/g) being more abundant than 13-HODE (44 nmol/g) compared to ALA-derived HOTrEs with a concentration of about 2 nmol/g (Figure 2A, Table S1). For the nonesterified oxylipins the regioisomers of both fatty acids were detected at equal concentrations (HODE 1.2−1.3 nmol/g; HOTrE 0.16−0.20 nmol/g). Reduction of hydroperoxides led to higher levels of total 10- and 12-HODE by a factor of 3.5 (e.g., 10-HODE 0.53 nmol/g vs 1.88 nmol/g), while the nonesterified oxylipins were detected at comparable concentrations with and without hydroperoxide reduction (0.07 nmol/g vs 0.08 nmol/g; Figure S1). In contrast to flaxseed and rapeseed oil, 9- and 13-HODE are the dominant oxylipins in sunflower oil. Total 15-HODE

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J. Agric. Food Chem. 2022, 70, 12935−12945

(2.4 nmol/g) and 15(16)-EpODE (8.8 nmol/g) had rather low concentration (Figure 2B).

The total 15-HODE levels found in the oils pressed in our lab are consistent with the concentration in commercially available flaxseed, rapeseed, or sunflower oils from the supermarket as well as in the respective authentic oils which were purchased from a local oil mill (Figure 3). Based on the oxylipin patterns, the investigated plant oils can be divided into three groups: Flaxseed oil containing high amounts of total 15-HODE (around 4500 nmol/g), oils with medium levels comprising rapeseed and soybean oil (around 400 nmol/g and 110 nmol/g, respectively) as well as oils with low levels of 15-HODE. The latter group includes peanut oil (around 5 nmol/g), sunflower oil (1.8−5.9 nmol/g), and olive oil (3.9−6.6 nmol/g). No apparent effect of processing on the 15-HODE concentration was found (e.g., virgin rapeseed oil 580 nmol/g; refined: 350−450 nmol/g). Calculation of the relative oxidation level (conc. oxylipin/conc. precursor fatty acid) reveals no difference for total or nonesterified 15-HODE. In both cases, the relative proportion of 15-HODE in the fatty acid concentration is the same (flaxseed oil: around 0.6%; Table 2) or slightly higher for total 15-HODE as it is the case in rapeseed oil (nonesterified 0.04%, total: 0.11%). All other oxylipins showed higher relative oxidation levels for the nonesterified oxylipins by up to a factor of 30 (9-HOTrE rapeseed oil total 0.02% and nonesterified 0.48%). The highest relative oxidation level could be determined for 15(16)-EpODE especially in rapeseed oil (6.6%). Flaxseed oil showed the highest relative oxidation level for LA resulting in a high relative concentration of 9- and 13-HODE. Sunflower oil showed highest relative oxidation for ALA caused by 9- and 13-HOTrE despite the low ALA amounts.

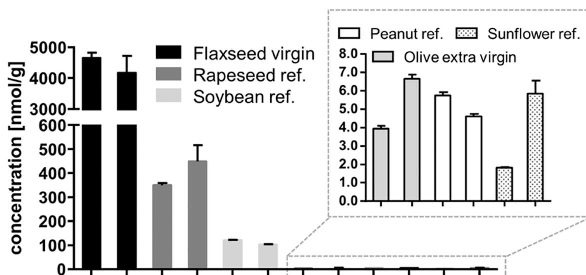

Figure 3. Total concentration of 15-HODE in different commercially available edible oils. Total oxylipins were analyzed in commercially available virgin as well as refined (ref.) edible oils (two different brands per type of oil, obtained from local supermarkets or oil manufacturers) by LC−MS (mean ± SD, n = 3).

Changes in Oxidation Products and the PV during Storage. The PV of stored flaxseed oil was about 1 meqO_2/kg from week 0 to week 14 and increased to 13 meqO_2/kg in week 24 (Figure 4). The PV of flaxseed oil stored in 50% filled bottle was considerably higher in week 24 (26 meqO_2/kg). Concentration of 9-/13-HODE and 13-HOTrE did not change during storage and reduction of hydroperoxides did not result in higher concentrations. 9-HOTrE and 10-HODE showed an increase in concentration and higher levels after reduction of hydroperoxides (Figure S2). The most abundant secondary volatile aldehyde was hexanal (190 nmol/g), of which the concentration did not change over time. A slight increase in concentration of *E*-2-heptenal was observable (week 0: 2.3 nmol/g; week 24: 3.4 nmol/g; Figure S3). Comparable to the total oxylipins, the concentration of nonesterified oxylipins did not change during storage time (Figure S4).

Stored rapeseed oil showed a strong increase in the PV from 0.8 meqO_2/kg at the beginning to 52 meqO_2/kg after 24 weeks (Figure 4). The PV in rapeseed oil stored in half-full bottles was 580 meqO_2/kg, which was more than 10 times higher compared to full bottles. Concentration of 9-/13-HODE/HOTrE as well as 10-/12-HODE directly analyzed without reduction of hydroperoxides did not change over time, while reduction of hydroperoxides strongly increased their concentration (e.g., 13-HODE week 0: 338 nmol/g; week 24: 930 nmol/g). According to the PV, in 50% filled bottles, the increase was more pronounced (week 24: 2200 nmol/g). No effect of the filling level on 10-/12-HODE concentration was observable (Figure S2). In contrast to the oxylipin concentration, the concentration of the most abundant secondary volatile aldehyde hexanal (160 nmol/g) did not change over time and its concentration was only slightly higher in the 50% filled bottles (week 24: 180 nmol/g). Similarly, no change was detectable for *E*-2-heptenal (around 1.8 nmol/g) and *E*,*E*-2,4-heptadienal (around 2.9 nmol/g). Only in half-filled bottles after 24 weeks of storage, higher concentration could be detected (*E*-2-heptenal: 3.0 nmol/g; *E*,*E*-2,4-heptadienal: 4.2 nmol/g; Figure S3). Comparable to the total oxylipins, in oils stored in 50% filled bottles, slightly higher concentration of nonesterified 9-/13-HODE/HOTrE after reduction of hydroperoxides could be detected (e.g., week 24 13-HODE full bottle: 14 nmol/g; 50% filled: 19 nmol/g; Figure S4). The effect of the available air above the oil was less pronounced for nonesterified oxylipins compared to total oxylipins.

Table 2. Percentage of Oxylipin Concentration to Precursor Fatty Acid[a]

% oxidation		flaxseed oil		rapeseed oil		sunflower oil	
		free	total	free	total	free	total
LA	9-HODE (red.)	1.08	0.09	0.50	0.03	0.54	0.09
	13-HODE (red.)	1.17	0.06	0.82	0.07	0.59	0.07
	15-HODE (w/o red.)	0.67	0.56	0.04	0.11	0.01	0.003
ALA	9-HOTrE (red.)	0.20	0.01	0.48	0.02	0.77	0.12
	13-HOTrE (red.)	0.17	0.04	0.34	0.03	0.96	0.10
	15(16)-EpODE (w/o red.)	1.42	0.09	6.58	0.69	2.22	0.37

< 0.01	0.01-0.03	0.03-0.1	0.1-0.32	0.32-1	> 1

[a]Shown is the ratio of oxylipin concentration/precursor PUFA concentration (mean, n = 3). The concentration of nonesterified oxylipins was divided by the concentration of nonesterified fatty acids, or the total oxylipin concentration by the total fatty acid. In order to include the 9- and 13-hydroperoxides, the concentrations after reduction with $SnCl_2$ were used for calculation.

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J. Agric. Food Chem. 2022, 70, 12935−12945

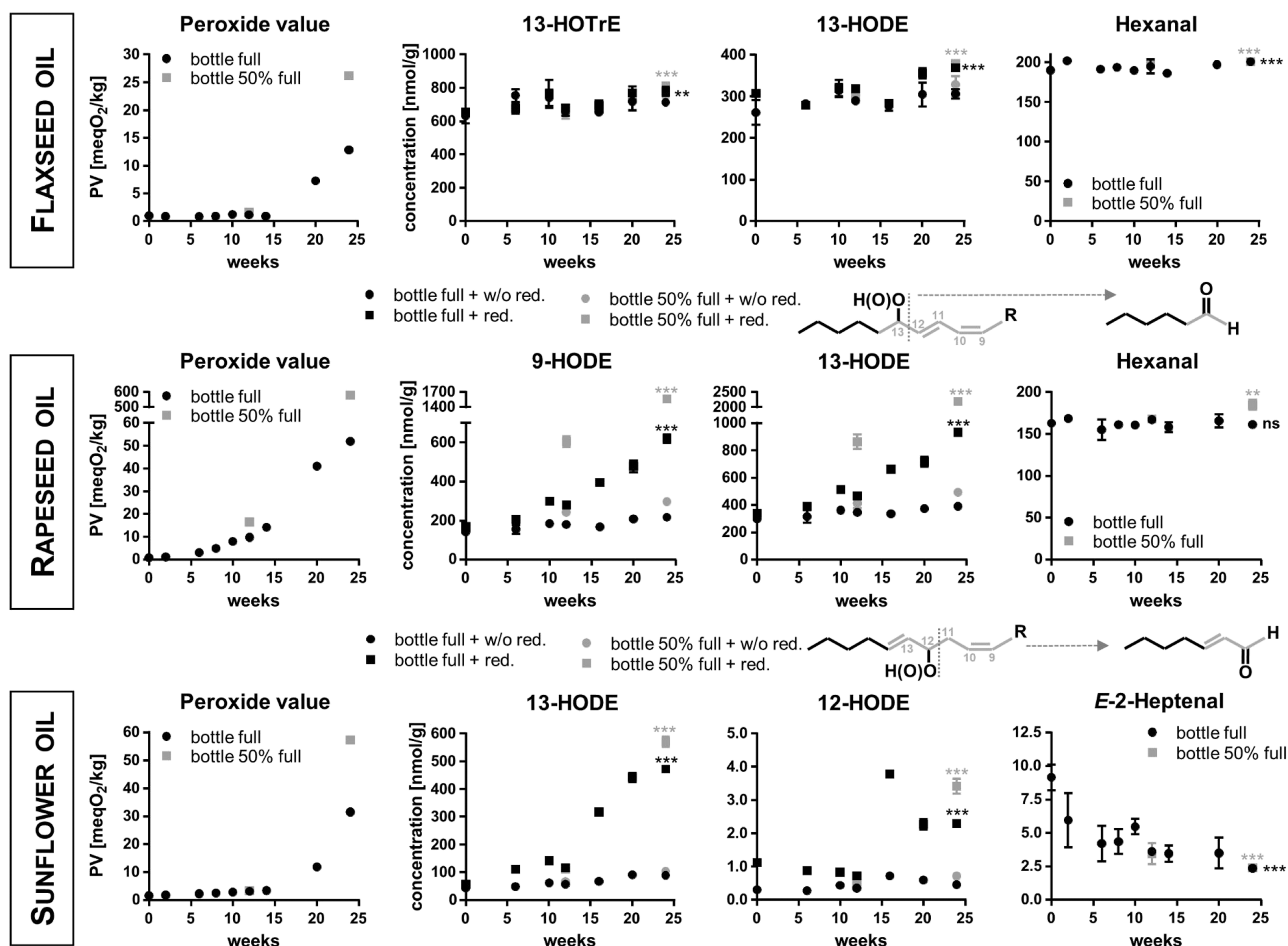

Figure 4. PV, total oxylipin as well as volatile secondary oxidation product concentrations in flaxseed oil (top), rapeseed oil (middle), and sunflower oil (bottom) at different time points of storage. The most abundant oxylipins detected are shown together with their secondary volatile aldehydes. Freshly pressed oils were stored for 24 weeks at 20 ± 2 ° C in the dark and the PV (mean, $n = 2$), total oxylipin concentrations (with/without reduction of hydroperoxides; mean ± SD, $n = 3$), and volatile aldehyde concentrations (mean ± SD, $n = 3$) were analyzed. At each time point, the parameters were determined for the oils stored in full bottles. After 12 weeks and 24 weeks, an analysis of the oil stored in half-full bottles was additionally carried out. A new bottle was opened for all analyses. Statistic evaluation of the concentration at week 24 (reduction; full and half-filled) versus concentration at week 0 (reduction) was performed using multiple *t*-tests with Holm-Sidak correction for multiple comparison ($\alpha = 0.05$; p value <0.05 (*), $p < 0.01$ (**) or $p < 0.001$ (***), ns = not significant). R = $-(CH_2)_7-COOH$.

The PV of stored sunflower oil slightly increased from week 0 (1.5 $meqO_2$/kg) to 3.5 $meqO_2$/kg in week 14 and showed a rapid increase between week 20 and week 24 from 12 $meqO_2$/kg to a final value of 32 $meqO_2$/kg (Figure 4). The PV of sunflower oil stored in a 50% filled bottle was considerably higher in week 24 (57 $meqO_2$/kg). Concentration of total 9-/13-HODE/HOTrE without reduction of hydroperoxides did not change over time (e.g., 13-HODE week 0: 44 nmol/g; week 24: 89 nmol/g), while reduction of hydroperoxides led to considerably higher levels after 16 weeks, 20 weeks, and 24 weeks (13-HODE week 24: 470 nmol/g). The filling level had only a minor impact (13-HODE week 24: 570 nmol/g). The concentration of 12-HODE after reduction decreased in the first 12 weeks from 1.1 nmol/g to 0.7 nmol/g and was increased after 16 weeks, 20 weeks, and 24 weeks (week 24: 2.3 nmol/g). A similar trend was found for 10-HODE (Figure S2). The concentration of *E*-2-heptenal decreased from 9.1 nmol/g to 2.4 nmol/g after 24 weeks of storage which was independent from the filling level. The main secondary oxidation product was hexanal showing slightly elevated concentrations over storage time (week 0: 27 nmol/g; week 24: 39 nmol/g; Figure S3). In contrast to the concentration of total oxylipins, the concentration of nonesterified 9-/13-HODE/HOTrE after reduction of hydroperoxides increased constantly (e.g., 13-H(p)ODE: from 1.4 nmol/g to 3.3 nmol/g; Figure S4).

■ DISCUSSION

Plant oils consist of characteristic patterns of mono- and polyunsaturated fatty acids which are prone to (aut)oxidation and enzymatic-catalyzed oxidation during pressing and storage of oils, affecting sensory properties and quality of edible oils. However, to date only limited data regarding oxidation product patterns and concentrations in oils are available, and the oxidative changes during storage are often analyzed by the sum parameter PV and secondary volatile aldehydes resulting from hydroperoxides. In the present study, the occurrence and concentrations of oxylipins in freshly pressed oils and their formation during 6 months of storage were quantitatively

evaluated and compared to the established parameters, the PV and secondary volatile aldehydes.

Oxidation of unsaturated fatty acids comprises enzymatic and nonenzymatic reactions leading to a variety of oxylipins of which hydroperoxy-PUFA bearing the hydroperoxy group between C9 and C13 for LA and C9 to C16 for ALA are best known.[9,11,14,19] Their formation is characterized by specific ratios of the individual regioisomers, which result from product and substrate specificity of the enzymes on the one hand[19,36] and the kinetically and thermodynamically controlled nonenzymatic oxidation reactions on the other.[9,14] Thus, conclusions about the formation mechanisms can be drawn from the hydro(pero)xy-PUFA pattern in edible oils. Autoxidation and photooxidation of LA leads to equal formation of 9-/13-HODE,[9,13,14] and approximately equal concentration of total and nonesterified 9-/13-HODE was found in flaxseed oil (Figure 2, Table S1). However, autoxidation of ALA would result in threefold higher concentration of 9-HOTrE compared to 13-HOTrE.[15] The sixfold higher concentration of 13-HOTrE found in flaxseed oil (Figure 2) thus might be caused by LOX activity. Previous studies describe the occurrence of LOXs in *Linum usitatissimum*,[22,37,38] which is the cultivated species for flaxseed oil production, with an average content of 3 g/kg[22] and a preferred formation of 13-H(*p*)ODE[38] and 13-H(*p*)OTrE.[38,39] Our data also suggest an active LOX with a specific formation of 13-hydroperoxide. Interestingly, we found similar concentrations of 9-HODE and 13-HODE. The almost equal concentrations of LA-derived HODE and ALA-derived HOTrE, despite a threefold higher concentration of ALA compared to LA, suggests that the flaxseed LOX also forms LA products. In case of autoxidation, the HOTrE concentration would be much higher than the HODE levels. It should be noted that Richardson et al. reported in a previous study about the profile of several oxylipins in different plant oils approximately 2.5-fold higher concentrations for total 9-HOTrE compared to 13-HOTrE.[3] However, we found the described ratio of 13-HOTrE > 9-HOTrE (Figure 2A) also in flaxseed oil obtained from an independent pressing of another flaxseed charge and in two different commercially available flaxseed oils (Table S2).

For nonesterified HOTrE in flaxseed oil and rapeseed oil, equal concentrations of the regioisomers were found (Figure 2). Plant LOXs are classified in 9-LOX and 13-LOX corresponding to the carbon atom in LA that is oxidized.[10,19] While substrates presumably penetrate headfirst into the active site of 9-LOX, substrates for 13-LOX can enter the active side tailfirst,[11,19] restricting the oxidation of fatty acids present as esters in lipids such as triacylglycerols to 13-LOX. Accordingly, concentrations of total 13-HOTrE and 13-HODE in rapeseed oil were higher than those of the 9-regioisomers (Figure 2). The occurrence of LOXs in rapeseeds is well described[40−42] and also 13-H(*p*)ODE being the main product of nonesterified LA with purified LOXs.[43] However, no data are available about the conversion of LA containing lipids as well as about oxidation of ALA by rapeseed LOXs. Data are similarly scarce for LOXs in sunflower seeds and previous studies regarding LOX activity reported conflicting results: While Fauconnier et al. describe no LOX activity[37] or activity appearing only during germination,[44] another study reports LOX activity in sunflower seeds.[45] The equal concentrations we found for 9- and 13-HOTrE could allow the speculation about LOX activity, as based on autoxidation, the concentration of 9-HOTrE should predominate (Figure 2).

The products of autooxidation and the LOX-catalyzed reaction have been investigated for centuries in edible oils. However, in the present study we found that other oxylipins are most abundant in flaxseed and rapeseed oil: 15-HODE bearing a homoallylic hydroxy group and 15(16)-EpODE which is the terminal epoxy-PUFA of ALA (Figure 2B). Different formation pathways for epoxy-PUFA are known in plants comprising (i) peroxygenase-catalyzed transfer of oxygen from a hydroperoxide to the double bond of another fatty acid,[46−49] (ii) activity of cytochrome P450 monooxygenases,[50,51] and (iii) intra- or intermolecular attack of hydroperoxides from one fatty acid on the double bound of the same or another fatty acid molecule.[14,52] However, none of these mechanisms is known to be as strongly regiospecific as would be required to form the ratio of EpODEs present in rapeseed oil (9(10)/12(13)/15(16) of 0.7/0.5/98.7, Table S1), suggesting the existence of a so far unknown (enzymatic) pathway leading to terminal epoxy-ALA. Epoxy-PUFA can be readily hydrolyzed to its corresponding vicinal diols by (soluble) epoxide hydrolases which are described in different plants.[53−56] The ratio of epoxy/diol of ALA was found to be 34 in rapeseed and 50 in flaxseed oil (Figure 2B), implying a low hydrolysis rate.

We identified 15-HODE with a concentration of 2700 μmol/g (80 mg/100 g) as the major oxygenated PUFA in several edible oils. 15-HODE is a so far hardly known oxylipin being reported only in oat seeds where it occurred also in high concentrations of 60−70 mg/100 g.[57,58] For the first time we show that 15-HODE is also a major (hydroxy) fatty acid in plant oils, not only in the oils used in our study, but also in commercially available flaxseed (4200−4700 nmol/g), rapeseed, and soybean oil (Figure 3). Interestingly, 15-HODE has comparable concentrations in oils obtained from the same source, e.g., two different rapeseed oils, suggesting that its occurrence is characteristic for the oils. Although the rapeseed and sunflower oil used in our study were virgin, cold-pressed oils and the rapeseed and sunflower oil bought in the supermarket and from an oil mill were refined, the 15-HODE concentrations are within the same range (rapeseed oil virgin 580 nmol/g and refined 350−450 nmol/g, Figures 2 and 3). It seems that 15-HODE is not removed during the refining process. In addition, unlike other oxylipins, the relative concentration (conc. oxylipin/conc. precursor PUFA) is similar for total and nonesterified 15-HODE/LA ratios (Table 2). This leads to the conclusion that 15-HODE is predominantly bound in lipids. Consistently, Hamberg et al. reported that in oat seeds nonesterified 15-HODE was <1% of total 15-HODE[57] and that 15-HODE occurred mainly in the glycolipid fraction where specifically a 15-HODE-rich galactolipid was identified.[59] To date, the formation pathway(s) of 15-HODE in the seeds are unknown. Due to the homoallylic alcohol structure and no detectable hydroperoxides, nonenzymatic oxidation of LA is chemically unlikely. A ($\omega-n$) hydroxylating activity of CYP as it is known in mammals[60−62] and similar enzyme activity in plant could lead to 15-HODE. In addition to CYP monooxygenase—among others—catalyzing hydroxylation reactions, nonheme iron-containing hydroxylases are known in plants. The mechanism of hydroxylation catalyzed by these enzymes is closely related to the mechanism of desaturation as catalyzed by the fatty acid desaturase 3 (FAD3).[63,64] Hydroxylation and

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J. Agric. Food Chem. 2022, 70, 12935−12945

desaturation mechanisms differ only in the last step, where the intermediary formed carbon radical either binds an oxygen from the oxo-diiron complex[63,65] or disproportionates.[66] The factors controlling both pathways are still a matter of question. Thus, it is likely that a bifunctional FAD3 exists which is capable of both desaturating and hydroxylating fatty acids at the ω-3 position. This hypothesis is supported by Broun et al. who showed that only four changes in the amino acid residues abutting the active side histidines are required to convert a desaturase without hydroxylase activity into a bifunctional hydroxylase−desaturase.[67] Additionally, we found that 15-HODE occurred in high concentrations in all oils which are rich in ALA (Figure 3), i.e., in oils obtained from seeds expressing an active FAD3. Despite the remaining questions about their formation in the seeds, we identified two main oxygenated PUFA with 15-HODE and 15(16)-EpODE which have not been described in edible oils so far, having the potential to be used as markers for authenticity.

Plant oils are stored by the consumer for several months. During this period of time a multitude of nonenzymatic oxidation reactions takes place leading to complex patterns of primary and secondary oxidation products.[9,14] To date, the primary oxidation level is usually determined by assessing the entirety of hydroperoxides (PV)[24,25] and the secondary oxidation products by either using a sum parameter (anisidine value)[26,27] or analysis of the individual volatile products.[68,69] However, sum parameters do not allow us to monitor changes of individual oxidation products, and the volatile products are advanced oxidation products. The detailed analysis of the different hydro(pero)xy-PUFA based on modern LC−MS analysis allows gaining insights into the oxidation processes of stored oils especially by correlating the results to the PV and the concentration of volatile aldehydes.

Flaxseed oil is considered to have poor storage properties with a high tendency to rancidity due to its content of oxidation-prone ALA. However, the PV exceeded the threshold for beginning oxidative changes of 10 meqO_2/kg[7] only after 5 months and was relatively low even in bottles with a large volume of air and higher oxygen availability (Figure 4). Similarly, the primary formed oxylipins (9-/13-H(p)ODE/H(p)OTrE) also showed only a slight increase over the storage time being most pronounced for 9-HOTrE. Interestingly, formation of the photooxidation product 10-HODE hydroperoxide could be detected, although the oils were stored in the dark (Figures 4 and S2). It seems that flaxseed oil is relatively stable against radical chain reaction autoxidation, but not against the reaction with (activated) singlet $^1O_2$ as it is the case during photooxidation type 2. Because virgin, freshly pressed oils were analyzed, antioxidants could be responsible for the low tendency of autoxidation. However, flaxseed oil does not contain high amounts of tocopherols compared to other oils (e.g., flaxseed oil total tocopherol 79 mg/100 g,[70] rapeseed oil 57 mg/100 g,[71] and sunflower oil 65 mg/100 g[72]), and previous studies showed no clear relationship between tocopherol content and oxidative stability.[70,73] Thus, contrary to the high chemical reactivity of ALA, we found that fresh flaxseed oil is less prone to lipid peroxidation and that rancidity was low after a storage time of 6 month. Rancidity is a sensory impairment caused by the formation of volatile secondary products from hydroperoxides.[74,75] Only E-2-heptenal showed a minimal increase during storage, all other detectable volatile aldehydes did not change (Figures 4 and S3). After 6 months, a sensory evaluation revealed a rancid impression which was interestingly predominated by a bitter taste caused probably by oxidized methionine from cyclolinopeptides found in flaxseed oil,[76−78] which may also contribute to the oxidative stability.[73]

The PV of rapeseed oil exceeded the threshold for beginning oxidative changes of 10 meqO_2/kg already after 3 months and was 10-fold higher after 6 months when the oil was stored in half-filled bottles (Figure 4). Thus, the PV directly correlates with the oxylipin concentrations of, e.g., the most abundant oxylipins 9- and 13-H(p)ODE/H(p)OTrE predominantly formed as hydroperoxides. Interestingly, while for the 9- and 13-regioisomers, higher concentrations in 50% filled bottles were detectable, no effect of the filling level was observable for the photooxidation products 10- and 12-HODE (Figure S2). Despite a massive increase in the precursor hydroperoxide 13-HpODE, the concentrations of hexanal did not change (Figure 4).[12] The same result was found for nonanal, only E-2-heptenal and E,E-2,4-heptadienal showed slightly higher concentrations in the half-full bottles (Figure S2). Thus, the secondary volatile aldehydes do not reflect the oxidation status of the tested rapeseed oil.

Sunflower oil was stabile during the first months of storage (Figure 4). After 5 months, the PV rapidly increased and considerable hydroperoxide formation was detectable for all oxylipins. The process exhibits an induction period typical for radical autoxidation in food during storage, after which the reaction rate increases exponentially.[74,75,79] The concentration of E-2-heptenal decreased in the first month, thus correlating to the concentration of 12-H(p)ODE (Figure 4) which is its precursor oxylipin.[9] Interestingly, its concentration further decreased in the following months although the 12-H(p)ODE concentration was higher after 4, 5, and 6 months. Thus, similar to rapeseed oil, concentrations of secondary volatile aldehydes do not reflect the ongoing oxidation processes in sunflower oil well.

NEFAs are considered to negatively influence the oxidative stability of oils through facilitating autoxidation.[80−82] Calculation of the relative total or nonesterified oxylipin concentration (conc. oxylipin/conc. precursor PUFA) revealed for all oxylipins except 15-HODE a higher relative concentration for nonesterified oxylipins compared to total oxylipins in freshly pressed oils, supporting a higher oxidation ratio of NEFA compared to TG during pressing (Table 2). However, it should be noted that it is not possible to distinguish between oxidation of NEFA and liberation of oxidized fatty acids from lipids by hydrolysis. During storage, only in sunflower oil, increasing concentrations of nonesterified oxylipins were observable (Figure S4), while in rapeseed oil, only minimal changes in the nonesterified oxylipins were detected despite a massive increase in the total oxylipin concentration (Figures 4 and S2). The peroxy radicals formed during the autoxidative chain reaction are highly reactive and attack nearly all (bisallylic) hydrogen atoms.[52] Thus, from a kinetics perspective, oxidation of highly abundant fatty acids esterified in triacylglycerols is much more likely than specific oxidation of low concentrated NEFAs. The preferred oxidation of triacylglycerols over NEFAs was also described by Shen et al. in heated soybean oil.[83] Interestingly, they also reported a rapid hydrolysis of triacylglycerols, while in our experiment the concentration of NEFAs did not change during storage (Table S3). Thus, we found two different effects regarding oxidation of NEFAs vs bound fatty acids: On the one hand, in freshly pressed oils, presumably oxidation of NEFAs predominates, on the other hand during storage, triacylglycerols are preferentially

oxidized. It might be concluded that the higher oxidation of NEFAs in freshly pressed oil is enzymatically based, while oxidized triacyclglycerols are derived from nonenzymatic oxidation.

Flaxseeds, rapeseeds, and sunflower seeds were pressed and the obtained oils were directly analyzed regarding their patterns of oxylipins, giving hints regarding the oxidation reaction during pressing. Oils were stored for 6 months to throughout investigate the changes during storage. A variety of oxylipins were detectable in freshly pressed oils probably resulting from a complex interaction of autoxidative, photo-oxidative, and LOX-catalyzed reactions. The characteristic oxylipin pattern depended on seeds, precursor PUFA distribution as well as LOX activity, and substrate/product specificity. In contrast to expectations, it was not the 9- and 13-hydroxy-regioisomers having the highest concentrations in flaxseed and rapeseed oil but the so far less known 15-HODE and 15(16)-EpODE. These products are most likely enzymatically formed. 15-HODE probably results as a byproduct from LA desaturation to ALA by FAD3 and is mainly bound to triacylglycerols, meaning that it remains in the oil after refining. Thus, on the one hand, it is a robust parameter and could be used as an authenticity marker of oils. On the other hand, its effect on human health warrants to be further investigated. The combined analysis of the PV, oxylipin concentrations, and secondary volatile aldehydes showed that freshly pressed rapeseed oil is much more prone to oxidative processes than flaxseed oil. We could show that the oxylipin concentrations correlate well with the PV, while the secondary volatile aldehydes did not reflect the ongoing oxidative changes and thus their use as oxidation markers is limited. Our study provides new and unique insights into the oxidation processes of fatty acids during pressing and storage and is another important step toward a comprehensive quantitative characterization of our food.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c04987.

> Concentration of nonesterified and total 10-/12-HODE, concentration of total oxylipins in flaxseed oil, rapeseed oil, and sunflower oil at different time points of storage, concentration of volatile aldehydes in flaxseed oil, rapeseed oil, and sunflower oil at different time points of storage, concentration of free oxylipins in flaxseed oil, rapeseed oil, and sunflower oil at different time points of storage, concentration of oxylipins in freshly pressed oils, concentration of hydroxy-PUFA in freshly pressed and commercially available flaxseed oils and concentration of NEFA in freshly pressed oils as well as after 12 weeks and 24 weeks of storage (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACN, acetonitrile; ALA, α-linolenic acid; BHT, butylated hydroxy toluene; DiHODE, dihydroxy α-linolenic acid; EpODE, epoxy α-linolenic acid; EtOH, ethanol; HAc, acetic acid; HODE, hydroxy linoleic acid; HOTrE, hydroxy α-linolenic acid; IS, internal standard; LA, linoleic acid; LC−MS, liquid chromatography-mass spectrometry; LOX, lipoxygenase; MeOH, methanol; NEFA, nonesterified fatty acids; PUFA, polyunsaturated fatty acid; PV, peroxide value; SPE, solid phase extraction; SRM, scheduled selected reaction monitoring

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