OCCURRENCE AND FORMATION OF OXIDIZED FATTY ACIDS IN EDIBLE OILS AND THEIR USE AS NOVEL PARAMETERS TO EVALUATE OIL QUALITY AND AUTHENTICITY

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Für meine Familie, Freunde und mich

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Chapter 1

Introduction and Scope

Today's Western diet is characterized by a high consumption of the vegetable oils soybean oil and rapeseed oil [1-2]. In 1961, the Central Committee for Medical and Community Program of the American Heart Association published a statement, which advised Americans to replace saturated fats in the diet by polyunsaturated fatty acids (PUFA) as it was assumed that this would reduce the risk for coronary heart diseases. Consequently, it was recommended to reduce the consumption of animal fat as contained in milk, cream, butter or meat and replace it by vegetable oils and, to a lesser extent, by margarine and shortenings [3]. Over the last 50 years, the *per capita* consumption of edible oils in Germany has risen from around 4.4 kg to 17.5 kg per year [4]. 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, C18:2 n6) and α-linolenic acid (ALA, C18:3 n3) [1-2, 5] as well as secondary plant metabolites such as tocopherols [6]. Especially flaxseed oil but also rapeseed oil are important because they are one of the few oils containing relevant amounts of n3-PUFA [5].

Most plant oils consist of a large amount of C18-PUFA [5] which are precursors for a multitude of fatty acid oxidation products [7-9]. In order to evaluate their patterns in oils, their complex formation routes in plants and seeds as well as during processing and storage have to be considered. Generally, the first step of lipid peroxidation is the formation of fatty acid hydroperoxides by either the catalytic activity of lipoxygenases (LOX) or non-enzymatic oxidation (Fig. 1.1) [7].

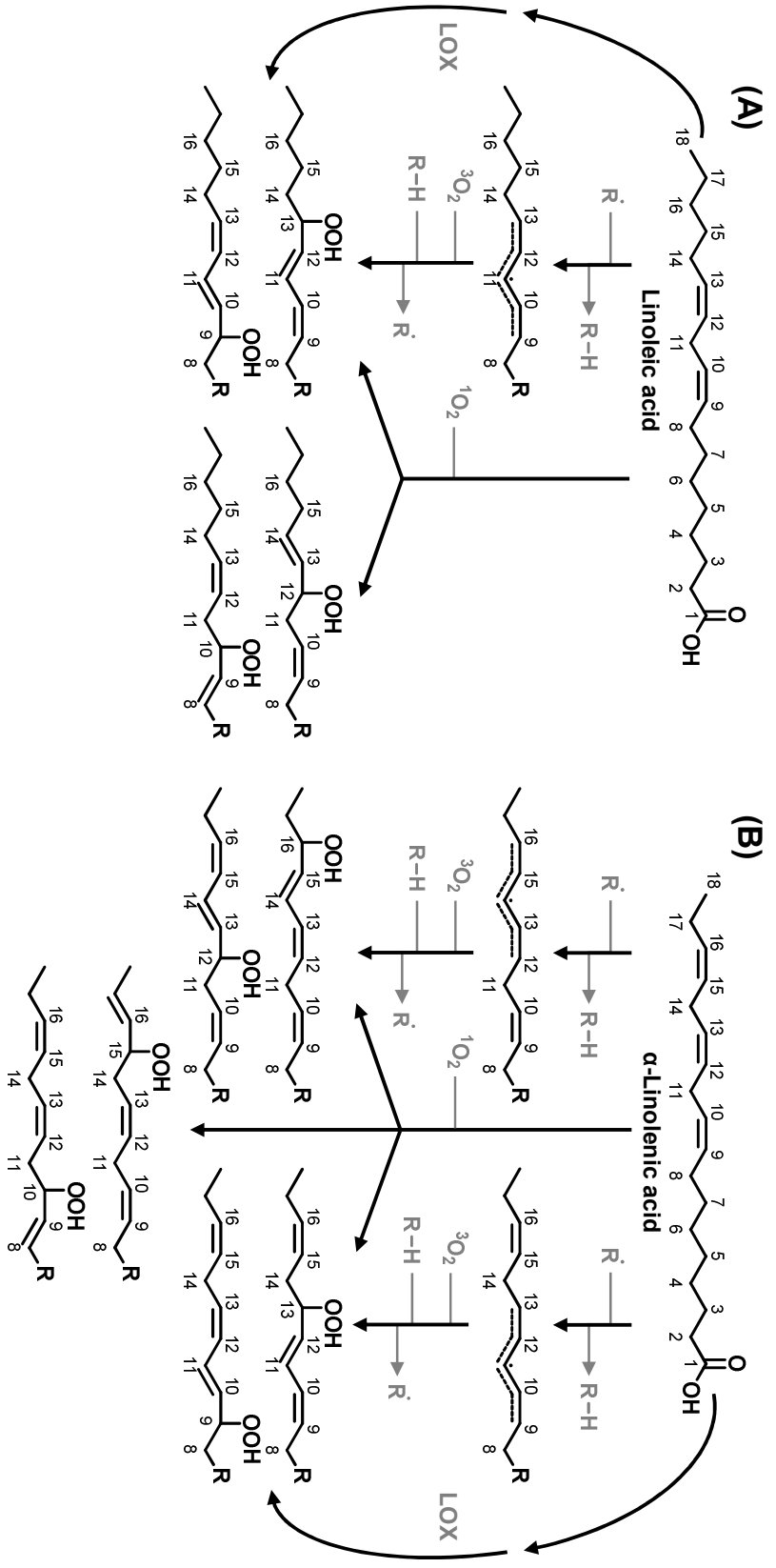

Fig. 1.1: Simplified scheme of the reactions occurring during autoxidation and photooxidation of (A) LA and (B) ALA as well as formation of oxylipins catalyzed by lipoxygenases.

Non-enzymatic oxidation comprises autoxidation by radical $^3O_2$ and photooxidation by singlet oxygen $^1O_2$ in a non-radical manner [8]. The steps of the radical chain reaction-based autoxidation of LA are i) abstraction of the bisallylic hydrogen atom at position 11 of the 1(*Z*),4(*Z*) pentadiene system, ii) addition of $^3O_2$ at one of the outer positions of the double bound system, giving rise to a peroxy radical and iii) abstraction of a hydrogen atom from another fatty acid leading to the formation of 9- and 13- hydroperoxides. For ALA, the 16-hydroperoxide is additionally formed [10-11]. The configuration of the newly formed conjugated double bond system depends mainly on the temperature and the availability of hydrogen donors [12]. In contrast, photooxidation follows an “ene”-mechanism comprising direct addition of activated $^1O_2$ to double bonds. This results in addition to outer hydroperoxides also in the formation of inner hydroperoxides at positions 10, 12 and 15 with isolated double bonds, albeit at a lower percentage due to the cyclization tendency, i.e. formation of hydroperoxy epidioxides or bicyclic endoperoxides, resulting from a double bond in β,γ-position [8].

9- and 13-hydroperoxides are also formed by the catalytic activity of LOX. LOX are iron-containing non-heme dioxygenases catalyzing the hydroperoxide formation of fatty acids with an 1(*Z*),4(*Z*)-pentadiene system [13]. LOX occurring in plants are either classified by their subcellular location in LOX-1 and LOX-2 or into 9-LOX and 13-LOX based on their reaction specificity in the oxidation of LA. Of note, LOX with no or dual regiospecificity also exist [14]. Plant LOX abstract exclusively the bisallylic hydrogen at C11 with subsequent introduction of molecular oxygen at C9 or C13 [13]. Two hypotheses are likely to explain the different regiospecificities. The first is the substrate orientation model: if a fatty acid enters the active side with its carboxy end ahead, i.e. head-first, 9-OOH results; if it penetrates the active site tail-first, 13-OOH is formed [15]. The second model is related to the space within the active side: while plant 9-LOX contain a small valine residue at the bottom of the substrate binding pocket, in nearly all plant 13-LOX a space-filling histidine or phenylalanine residue is located at this amino acid position. Replacing of this histidine residue by valine was shown to turn a 13-LOX into a 9-LOX [16].

Fatty acid hydroperoxides are metabolized in plants by different enzymatic pathways. A prominent class are atypical cytochrome P450 monooxygenases (CYP), which are subgrouped as CYP74 family. They require neither molecular oxygen nor NAD(P)H dependent CYP450-reductase as cofactors. Instead, they use the acylhydroperoxides generated by LOX or autoxidation as both oxygen donor and substrate [7]. To this class belongs beside hydroperoxide lyase, epoxy alcohol synthase and divinylether synthase, the allene oxide synthase which initiates the most investigated oxylipin biosynthesis pathway in plants: the octadecanoid pathway resulting in the formation of jasmonic acid [7, 17]. Jasmonates, to which jasmonic acid, the receptor-active jasmonoyl-L-isoleucine and other derivatives belong, play a key role in immunity, growth, development as well as biotic and abiotic stress response of plants [18]. Another enzyme acting on hydroperoxides are peroxygenases. They catalyze the intra- or intermolecular transfer of an oxygen atom from a hydroperoxide to a double bond yielding epoxy alcohols or epoxy fatty acids, respectively, as well as the reduced hydroperoxide, i.e. hydroxy-PUFA [19].

In addition to hydroperoxides formed by LOX or chemical oxidation and their subsequent enzymatic conversion to a variety of downstream oxidation products, some other epoxy- and hydroxy-PUFA were described in different plant seeds. Epoxy-PUFA have raised interest due to their relevance for industrial purposes, e.g. as plasticizers in polyvinyl chloride [20]. The seed oils of several *Asteraceae* genera such as *Vernonia* and *Euphorbiaceae* contain high percentages of the LA-derived 12(13)-EpOME (vernolic acid). Its formation route seems to differ between species and is either based on the activity of CYP and/or a Δ12-desaturase-like epoxidase [20-21] and/or peroxygenase [22]. The other epoxy-LA derivative 9(10)-EpOME (coronaric acid) was also identified e.g. in seeds of *Chrysanthemum coronarium* [23]. Castor oil made from *Ricinus communis* seeds consists of 85-90% 12-hydroxy-oleic acid (9*Z*-12-OH-C18:1), so-called ricinoleic acid [24], which finds wide application in the industry, e.g. for manufacturing of cosmetics, nylon, vanishes, paints or lubricants [25]. Ricinoleic acid is formed by direct hydroxylation of oleic acid by the oleate 12-hydroxylase [26]. Oleate 12-hydroxylases

from different plants show a high sequence identity to Δ12-desaturases, e.g. 67% for the oleate 12-hydroxylase from *Ricinus communis* and the Δ12-desaturases from *Arabidopsis* [26]. Seven amino acid residues were identified to distinguish between a hydroxylating or desaturating activity [27]. In the Δ12-desaturase from *Arabidopsis*, four of these amino acids are adjacent to the histidine residues which are essential to catalysis; replacing these four amino acids by their equivalents from a *Lesquerella* hydroxylase turned a strict desaturase into bifunctional desaturase-hydroxylase [27]. In addition, the mechanism of both hydroxylation and desaturation of oleic acid to ricinoleic and LA, respectively, starts with the initial abstraction of a hydrogen atom at C12 [28]. Then, the pathways separate: elimination of a second hydrogen atom at C13 leads to the formation of LA [29], while for the formation of ricinoleic acid the hydroxy group bound to the di-iron complex in the active site is transferred to the C12 radical [28, 30]. Like LA, ricinoleic acid can be desaturated to densipolic acid (12-OH-9*Z*,15*Z*-C18:2) [31] occurring in some *Paysonia* species in concentrations of 40-50% [32] and one study also reports a signal with similar chromatographic properties as densipolic acid in flaxseeds [31] as well as the ability of developing flaxseeds to desaturate ricinoleic acid at C15 to densipolic acid [33].

Oxylipins in plant oils originate from i) enzymatic fatty acid oxidation in the plants and seeds as described above, ii) enzymatic and non-enzymatic oxidation during oil pressing as well as iii) autoxidation and photooxidation during storage. During oil pressing, cells are disrupted and oxygen as well as LOX come into contact with the PUFA. LOX were described in seeds [34-35] where they occur in high concentrations, e.g. in soybeans (14 g/kg) or flaxseeds (3 g/kg) [36]. Once being produced and filled in bottles, oils are stored by consumers for weeks or months. Over time, a multitude of non-enzymatic oxidation reactions takes place: The primarily formed hydroperoxides can degrade by β-cleavage to odor-active carbonyl compounds which are associated with a rancid perception of the product and reduce the sensory value of an oil [8]. Thus, the oxidative status of oils is crucial for their quality. In the context of official food control, the lipid peroxidation of oils is determined by the peroxide value (PV), the anisidine value and the Totox value

(anisidine value + 2 x PV) [37]. The PV is a parameter for peroxide-bound oxygen and thus for primary oxidation, while the anisidine value accounts for the formation of α-, β-unsaturated aldehydes, i.e. secondary oxidation [37]. However, both are controversially discussed parameters: The determined PV of an oil frequently has a high measurement uncertainty and depends on the standardized method used for the determination [38-39]. The significance of the anisidine value as a criterion for the rancidity of an oil is markedly limited by the fact that not only the odor-active free aldehydes, but also those bound to triacylglycerols are measured [38]. Additionally, being non-specific sum parameters, they do not provide any information about which oxidation processes actually take place in the oil and which products are formed.

Modern liquid chromatography-mass spectrometry (LC-MS) based analytical methods enable the simultaneous determination of a broad spectrum of oxidation products resulting from a variety of enzymatic and non-enzymatic formation processes [40-41]. By analysis of hydro(pero)xy-, epoxy-, di- and multihydroxy-PUFA as well as phytoprostanes a comprehensive picture about i) the amount of physiologically potent lipid mediators, ii) activity of enzymes of the fatty acid metabolism in oilseeds and iii) lipid peroxidation during processing and storage can be drawn. In a first step, well-characterized sample preparation strategies are needed to get reliable, reproducible results. Some approaches for determination of total oxylipins exist, mostly comprising hydrolysis for cleaving bound oxylipins with or without preceding lipid extraction [42-45]; however, only limited information on the impact of the different experimental procedures on the oxylipin concentrations is available. Thus, it is impossible to deduce a sample preparation strategy for reproducible and precise analysis from the literature. In **chapter two**, a detailed sample preparation strategy for quantification of total oxylipins in protein- and water-rich human plasma is described. Liquid-liquid extraction and protein precipitation for efficient lipid and oxylipin extraction are compared and the best hydrolysis conditions for total oxylipin determination are deduced. The importance of thoroughly evaluated protocols is shown by the finding that improper sample handling can lead to artificial formation of oxylipins during solid

phase extraction. The developed sample preparation procedure is characterized by good reproducibility and is the reliable basis for the analysis of total oxylipins in biological samples, foods and plant oils.

Oxylipins are formed from PUFA by a multitude of oxidation mechanisms [9]. However, the importance of PUFA is not limited to their oxidation products. The fatty acids themselves exhibit a variety of physiological functions such as influencing properties of biomembranes, storing and providing of energy or being involved in cell signaling [46-48]. The gold standard of fatty acid analysis is gas chromatography coupled to flame ionization detection (GC-FID) due to the high separation efficiency and quantification based on response factors which does not require external calibration [49-51]. However, for this technique, transesterification or derivatization is necessary, which is laborious as well as time-consuming and can lead to discrimination of analytes [52]. Of note, this need makes the quantification of non-esterified fatty acids (NEFA) challenging due to the high amount of esterified fatty acids occurring in different lipid classes. LC-MS analysis of fatty acids offers the opportunity of analyzing fatty acids directly. In addition, the analysis of the entire fatty acid distribution can be linked to the determination of total oxylipins, meaning that both parameters can be quantified from one sample without additional sample preparation as described in **chapter three**. This chapter deals with the method development for quantification of fatty acids in different matrices, how to rapidly separate isomers differing only in the position of the double bond and to make analytes with low fragmentation tendency due to the linear hydrocarbon backbone accessible to LC-MS analysis. The performance and accuracy of the method is cross-validated by using a previously established GC-FID approach and the broad applicability of the method is demonstrated by showing the fast, combined analysis of total fatty acids and oxylipins as well as NEFA in biological and oil samples. Together with the developed sample preparation strategy in chapter two, these are the fundamental analytical methods which are used for a comprehensive, accurate and precise analysis in the following chapters.

A sufficient supply with long-chain n3-PUFA such as EPA and DHA is associated with a variety of positive health effects such as reduced risk of coronary heart diseases [46]. However, the intake of EPA and DHA is inadequate for large parts of the Western population [46, 53] and conversion rates of ALA to EPA and DHA by elongases and desaturases are low [54] Besides the physiological function of the PUFA themselves, consumption of higher concentrations of n3- compared to n6-PUFA reduces pro-inflammatory lipid mediators derived from arachidonic acid (ARA, C20:4 n6) on the one hand [55-57] and increases the formation of cardio-protective and anti-inflammatory lipid mediators derived from n3-PUFA on the other hand [55, 57-58]. The German Nutrition Society (DGE) and the American Heart Association among others recommend two servings of (oily) fish per week or a daily intake of at least 250 mg EPA and DHA per day for the prevention of coronary heart diseases [59-60]. There are several n3-PUFA supplements available on the market to help consumers who do not eat (enough) fish to ensure a sufficient supply with EPA and DHA. They are based on fish oil, krill oil or micro-algae oil. Production of n3-PUFA-rich concentrates from fish is a complex procedure including pressing of crude oil from cooked fish, refinement, transesterification to ethyl esters and concentration of n3-PUFA [61-62]. Micro-algae oil is obtained from simple lipid extraction of cells from *Schizochytrium* grown in fermenters and subsequent refinement [63]. On the contrary, krill oil is a non-refined solvent or supercritical carbon dioxide extract from the Antarctic krill (*Euphausia superba*) [64]. The diversity of sources and production techniques for the n3-PUFA-rich oils results in varying EPA and DHA concentrations, lipid compositions, PUFA binding forms and degree of oxidation [65-66]. In previous studies, the accuracy of EPA and DHA declaration and the PV were determined, showing large difference between the supplements [65, 67-69]. However, no data is available about the total and non-esterified oxylipin patterns as well as the NEFA concentrations. This is not only interesting with respect to get information about the production of the n3-rich oils and about their authenticity, but also about the oxidation level and thus the quality of the oils. Moreover, several of the oxylipins derived from EPA and DHA are highly potent lipid mediators [9] and are usually not declared on the supplementation products. **Chapter four** shows the results of the

analysis of total and non-esterified oxylipins and fatty acids in 11 different n3-PUFA supplements by state-of-the-art LC-MS analysis. It is investigated whether the measured EPA and DHA content matches the manufacturer's declaration and what can be learned from the overall fatty acid patterns. The total degree of PUFA oxidation and the NEFA concentrations are evaluated as possible new parameters for oil quality and the individual oxylipin concentrations might be an interesting marker in terms of authenticity. This study provides an in-depth description of the composition of n3-rich oils and shows new possibilities to describe the quality and authenticity of these important dietary supplements.

Despite the high importance of EPA and DHA for human health, the main fatty acids in human nutrition are linoleic acid (LA) and α-linolenic acid (ALA) which are provided by consumption of plant oils [1-2, 5]. While oils used by the food industry are mostly refined, consumers appreciate virgin, cold-pressed oils for their characteristic flavor and taste and secondary plant metabolites being present. Mechanical pressing ruptures the seeds, so that the oil leaks out [70]. At the same time, the fatty acids are exposed to oxygen and can be oxidized non-enzymatically by atmospheric oxygen [8] or by the catalytic activity of enzymes such as LOX [13]. 9- and 13-hydroperoxy-LA/-ALA resulting from autoxidation and LOX activity (Fig. 1.1) [13, 71] have been considered for decades to be the main PUFA oxidation products in oils. To date, only very limited data is available about a broader pattern of oxylipins in oil and thus about enzymatic activity in oilseeds and during oil pressing. In addition, the oxidative changes are commonly assessed by the PV and anisidine value [37] making insights in ongoing oxidative processes difficult. Using the state-of-the-art LC-MS method developed in chapter two, simultaneous determination of a broad spectrum of oxidation products resulting from a variety of enzymatic and non-enzymatic formation routes was performed in **chapter five**. Freshly pressed oils from flaxseeds, rapeseeds and sunflower seeds were prepared using a screw press. Total and non-esterified oxylipins were analyzed directly in the freshly pressed oils to understand the interaction of enzymatic and non-enzymatic fatty acid oxidation during oil pressing. Additionally, oils were stored for six months and analyzed at different

time points regarding 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.

Oils are not only consumed in their pure form, but mainly in complex and processed foods. Their production includes e.g. heat which is a strong promoter of lipid peroxidation [8]. Application of high temperature on several foods often leads to desired taste and smell; the best example for this is frying [72]. The oxidative deterioration of the oil during frying is so far assessed by sensory evaluation and analysis of the smoking point as well as the amount of polar compounds and polymerized triacylglycerols [73]. They result from complex decomposition reactions of hydroperoxides at advanced stages of lipid peroxidation because hydroperoxides are described to be unstable at the high temperatures used during frying [71, 74]. On the contrary, previous studies showed a specific formation of *trans*-epoxy fatty acids during frying [75-77]. Though these data suggest that oxylipins can be formed during frying, detailed data about the patterns of individual oxidation products in frying oils and how they are formed and degraded during frying are scarce. Therefore, in **chapter six**, potato chips were fried in high-oleic sunflower oil for 4 x 5 cycles on two days and the oil was comprehensively analyzed regarding the formation of hydro(pero)xy-FA, epoxy-FA and dihydroxy-FA using the sensitive LC-MS approach. Based on these data, it is possible to quantitatively characterize the ongoing changes in oxylipin concentrations during the frying process and new potential markers for the evaluation of thermally treated plant oils are suggested.

Our nutrition nowadays comprises a variety of processed foods and meals, for whose production many different oils are used. Oxidative processes occur during all steps of food preparation leading to complex patterns of primary and secondary oxidation products. Several studies show the absorption of hydroxy- and epoxy-PUFA from the diet in the gastrointestinal tract [78-81]. Thus, our diet is a relevant source of oxylipins circulating in the plasma. It is therefore essential to gain information about the oxylipin concentrations in our foods and how their

concentrations changes during processing of oils, e.g. refinement. In **chapter seven**, at first different refined and virgin oils were analyzed to collect data about the oxylipin pattern in the oils which are used to produce foods and meals, and how the refining process changes the oxylipin concentrations. Secondly, in 12 different (fatty) foods and meals from restaurants and the supermarket the fatty acid and oxylipin concentrations were investigated. The samples were selected to cover a wide range of different type of food, e.g. different fatty acid distributions, processed meat, fish and vegetarian replacements as well as convenient, frequently consumed meals in Germany. This allowed to gain information about the amount of oxylipins in the product and to deduce the degree of oxidation by calculating the ratio of oxylipin to its precursor PUFA. Additionally, the amount of oxylipins per portion was estimated. Thus, this chapter provides for the first time data about ingested oxylipin patterns and concentrations by common foods.

The mechanisms of autoxidation and photooxidation as well as lipoxygenases are well-known for centuries [8]. Hence, from the food chemistry perspective, the view on oxylipins in plant oils focuses on the main products of these reactions: 9-, 10-, 12- and 13-HODE as well as 9- and 13-HOTrE. As it has become obvious during the thesis that this is only a limited view on the patterns of fatty acid oxidation products. Several so far less noticed oxylipins – for which an authentic standard substance is commercially available – turned out to be highly abundant and/or could be specific markers for quality evaluation of oils. Thus, it seems that plant oils contain a broader spectrum of oxylipins than has been found and even expected so far. In **chapter eight**, it was aimed to perform the first comprehensive characterization of oxylipins in flaxseed, rapeseed and sunflower oil beyond the limited view on autoxidation products as it has been the state of the art for decades. Oils were screened by liquid chromatography-high resolution mass spectrometry (LC-HRMS) to collect data about retention time, exact mass and fragmentation patterns allowing to draw conclusions about oxylipin structures. Results were confirmed using GC-MS as an orthogonal separation technique. The tentatively identified structures together with reports from the literature showed evidence for an involvement of fatty acid desaturase 3 (FAD3) in oxylipin

formation. This was investigated by analysis of oxylipins in flaxseeds and *Arabidopsis thaliana* seeds with high and low FAD3 activity as well as in seeds of *Paysonia densipila* and *Physaria gordonii*. Using a semi-quantitative approach, it was possible to determine the levels of the new oxylipins in cold-pressed flaxseed, rapeseed and sunflower oil and also in sunflower oil from preheated seeds mimicking hot-pressing of oils. This chapter provides unique qualitative and (semi-)quantitative data about the patterns of PUFA oxidation products in edible oils and how they are formed, allowing to draw a more complete picture about their occurrence which is of fundamental importance for food chemists.

Overall, this thesis improves our understanding of the composition and ongoing oxidative changes in edible oils and oily products by using comprehensive state-of-the-art LC-MS-based methods to analyze the oxylipin pattern. It gives deeper insights in how oxylipins are formed and degraded in oils during pressing, storage and heating and suggests new parameters to evaluate the authenticity and quality of edible oils.

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Chapter 2

Targeting esterified oxylipins by LC-MS – Effect of sample preparation on oxylipin pattern *

A major part of oxygenated metabolites of polyunsaturated fatty acids – i.e. eicosanoids and other oxylipins – in biological samples is found in the esterified form. Yet, their biological role is only poorly understood. For quantification of esterified oxylipins in biological samples current protocols mostly apply alkaline hydrolysis with or without prior lipid extraction to release oxylipins into their free form which can be subsequently quantified via liquid chromatography-mass spectrometry. Herein, a detailed protocol for precise and reproducible quantification of esterified oxylipins in plasma is presented comprising i) extraction of lipids and removal of proteins with iso-propanol, ii) alkaline hydrolysis with potassium hydroxide to saponify lipids and iii) solid phase extraction of the liberated oxylipins on C8/anion exchange mixed mode material. Unequal extraction of internal standards and lipid classes during lipid extraction before hydrolysis led to distorted concentrations, emphasizing that the choice of solvent used in this step is important to minimize discrimination. Regarding the hydrolysis conditions, at least 30 min incubation at 60 °C is required with 0.1 M KOH in sample. Drying of the SPE cartridges is a critical parameter since autoxidation processes of PUFA, which are present in high concentrations after cleavage, lead to artificial formation of epoxy fatty acids. With the developed protocol, the inter-day, intra-day and inter-operator variance was < 21% for most oxylipins including hydroxy-, dihydroxy- and epoxy-PUFA. The applicability of the developed methodology is demonstrated by investigating the changes in the oxylipin pattern following omega-3 fatty acid feeding to rats.

* modified from Ostermann, A. I.[#]; Koch, E.[#]; Rund, K. M.; Kutzner, L.; Mainka, M.; Schebb, N. H. Targeting esterified oxylipins by LC-MS – Effect of sample preparation on oxylipin pattern. Prostag Oth Lipid M 2020, 146, 106384.

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2.1 Introduction

Eicosanoids and other oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFA) which are formed endogenously in a network of enzymatic and autoxidative processes termed arachidonic acid (ARA) cascade. The biology of unesterified – i.e. free – oxylipins has been investigated in numerous studies over the past decades [1-3]. However, only a small portion of oxylipins in human plasma is found in the free form while the major part of oxylipins, especially epoxy- and hydroxy-PUFA as well as isoprostanes and -furanes, are bound in lipids [4-5], e.g. phospholipids and triglycerides [5-6], and lipoproteins [3, 7-8]. Although physiological effects of esterified oxylipins have been described [9] and some reports exist investigating the profile of free vs. bound oxylipins [4], the pattern of oxylipins in lipoprotein fractions [7-8] or changes in esterified oxylipins during dietary interventions [10-11], their biological role still remains largely unknown [2, 6].

Although liquid chromatography-tandem mass spectrometry (LC-MS/MS) based methods exist to quantify selected lipids with oxylipin moieties [12-13], esterified oxylipins are most commonly quantified indirectly using LC–MS/MS following cleavage from the lipids as a sum of free and esterified oxylipins [4, 14-22]. While other approaches have been described, e.g. treatment with phospholipase A2 [23], alkaline hydrolysis is mostly used to cleave the ester bond. However, sample treatment before hydrolysis, hydrolysis conditions and extraction procedures differ hugely [24]. Different procedures are described directly hydrolyzing the sample following addition of organic solvent [4, 11, 14, 16, 21-22]. In other protocols liquid-liquid extraction (LLE) is used to extract lipids from plasma before hydrolysis [17-18, 20] – or liquid-solid extraction in case of tissue [19] – or proteins are at first precipitated and lipids subsequently extracted by LLE [15]. In some though not in all methods the extraction solvent or the sample is acidified prior extraction [15, 18-19].

Alkaline hydrolysis is conducted using 0.2-0.5 M potassium hydroxide and 0.5-5 M sodium hydroxide or 0.1 M sodium carbonate in the sample [4, 11, 14-16, 19-22] and hydrolysis conditions range from 4 °C [19-20] to 90 °C [15] for 20 to 60 min [4, 14-

[16, 18, 21] or overnight (18 h) [19-20, 22]. Also, two step procedures are described in which free oxylipins are generated by the addition of water following trans-esterification to methyl esters using sodium methoxide [17]. For the extraction of the resulting free oxylipins, solid phase extractions [4, 14, 16-17, 21-22] or LLE [15, 18, 20] are used.

While protocols are widely applied [3, 24], only limited information on the impact of the different experimental procedures for the cleavage of lipids on the determined oxylipin concentration is available. Thus, it is impossible to deduce the best procedure for reproducible and precise analysis from the literature. Therefore, we herein describe a sample preparation strategy for the quantification of total (i.e. sum of free and esterified) oxylipins following alkaline hydrolysis and provide detailed data on sample preparation steps, extraction efficiency and variability. The final standard operation procedure (SOP) was applied to investigate the effects of feeding an omega-3 PUFA (n3-PUFA) rich diet on the profile of total oxylipins in rat plasma.

2.2 Experimental

2.2.1 Materials

Oxylipin standards and deuterated oxylipin internal standards were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany). For the sample preparation 15 different internal standards (IS) covering all relevant oxylipin classes were used. The purity of the standards was checked according to Hartung et al. [25]. LC-MS-grade methanol (MeOH), acetonitrile (ACN), *iso*-propanol and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). *n*-Hexane (*n*Hex, HPLC grade) was obtained from Carl Roth (Karlsruhe, Germany) and ethanol (absolute, p.a.) from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany) or VWR (Darmstadt, Germany). Nitrogen (5.0) was supplied from a nitrogen tank (Westfalen Gas, Münster Germany) of similar quality generated by a Nitrogen Generator (Fey Druckluft, Laatzen, Germany). Pooled human EDTA-plasma was

obtained following centrifugation (15 min, 4 °C, 1200 x *g*) of EDTA-blood and mixing of the plasma supernatants from 4-6 healthy male and female volunteers aged between 25-38 years. Plasma was immediately stored at -80 °C until analysis.

2.2.2 LC-MS analysis of total oxylipins

In the following section, the final protocol developed in this study is reported. Details of all investigated conditions are reported in the appendix. Internal standards and additives (10 µl 0.2 mg/ml butylated hydroxytoluene (BHT) in MeOH, 100 µM indomethacin and 100 µM *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) [26] in methanol) were added to 100 µl freshly thawed plasma and samples were vortexed briefly. 400 µl ice-cold *iso*-propanol (-30 °C) were added, vortexed and samples were frozen at -80 °C for at least 30 min in a pre-cooled sample rack. Samples were thawed briefly at room temperature, vortexed and centrifuged (4 °C, 10 min, 20000 x *g*). The supernatant was hydrolyzed at 60 °C for 30 min using 100 µl 0.6 M KOH in MeOH/water (75/25, *v/v*). Following hydrolysis, samples were put on ice and neutralized with 20 µl 25% aqueous HAc.

Extraction of oxylipins was conducted as described [27] with modifications. Bond Elut Certify II cartridges (Agilent, Waldbronn, Germany) were prepared with one column volume (~ 3 ml) of each, ethyl acetate (EA)/*n*Hex (75/25, *v/v*) with 1% HAc, MeOH and 0.1 M Na_2HPO_4 buffer (in H_2O/MeOH (95/5, *v/v*), adjusted to pH 6.0 with HAc). The neutralized sample was diluted on the cartridge with 2 ml 0.1 M aqueous Na_2HPO_4 buffer (adjusted to pH 6.0 with HAc). The pH was checked in a representative sample per batch using pH stripes (5.1-7.2 scale, Macherey-Nagel, Düren, Germany) and adjusted to pH 6.0, if necessary, with diluted HAc. In our experience, the same amount of acid can be used for neutralization of similar samples (e.g. plasma from different individuals). Samples were washed with one column volume ultrapure water (18.2 MΩ) as well as MeOH/H_2O (50/50, *v/v*) and dried with vacuum (-200 mbar, 30 sec). Oxylipins were eluted using 2 ml of EA/*n*Hex (75/25, *v/v*) with 1% HAc in prepared glass tubes containing 6 µl of 30% methanolic glycerol.

Samples were evaporated to dryness using a vacuum concentrator (1 mbar, 30 °C, ~70 min; Christ, Osterode am Harz, Germany) and the residue was reconstituted in 50 µl MeOH containing 40 nM of 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea used as IS2 to calculate recovery rates of IS as a measure for extraction efficiency. Samples were centrifuged sharply (4 °C, 10 min, 20000 x *g*). 5 µl of the supernatant were analyzed by means of LC-MS/MS (QTRAP, Sciex, Darmstadt, Germany) as described [27, 28]. The oxylipins are detected in scheduled selected reaction monitoring mode following fragmentation using commonly applied transitions [29].

2.2.3 Effect of n3-PUFA feeding on the oxylipin pattern in rats

Male, 6-week-old Sprague-Dawley rats (n=6; 200-210 g) obtained from Charles River Laboratories International Inc. (Sulzfeld, Germany) were fed for 23 days with either a standard diet containing sunflower oil (Control) or an n3-PUFA rich diet (EPA+DHA) containing sunflower oil with 1% EPA and 1% DHA ethyl esters. Both diets contained 10% total fat and were described in detail in [30]. Animals had access to food and water *ad libitum*. Rats were sacrificed by cardiac puncture following anesthesia with xylazine/ketamine (66/5 mg/kg BW). Blood was collected and plasma was generated by centrifugation (10 min at 855 x *g*, 4 °C). Plasma was directly frozen in liquid nitrogen and stored at -80 °C until analysis. All animal experiments were approved by the animal welfare service of the state of Lower Saxony (Oldenburg, Germany; 33.9-42502-04-13/1134, 28.05.2013).

2.2.4 Data analysis

Statistical analysis was performed using Graphpad Prism software (version 8.0, San Diego, CA; USA). All other calculations were done in Microsoft Excel (version 2010 and 365, Redmond, WA, USA).

2.3 Results

LLE ($CHCl_3$/MeOH, methyl tert*-butyl ether (MTBE)/MeOH and EA/nHex) with acidified solvents* yielded high recovery rates for all oxylipins (> 65%, slightly lower with MTBE/MeOH) and lipid classes (≥ 85%, except PC C21:0 and PS

C16:0 with EA/*n*Hex (58% and 68% respectively)) from BSA (Fig. 2.1) while *direct hydrolysis* yielded low recovery rates for different analytes e.g. resolvins, leukotrienes and hydroxy-PUFA (Fig. 11.1).

Decreasing the content of acid in the LLE extraction solvent resulted in insufficient extraction of oxylipins with all extraction protocols and of saturated PC and PS using EA/*n*Hex (Fig. 11.2). Increased recovery of IS with acidified solvents was also observed in plasma (Fig. 11.3). Using *protein precipitation*, all tested solvents led to acceptable and similar recovery rates of deuterated oxylipin standards (Fig. 2.1 II A). For all lipid classes (except FFA), a general trend was observable: lipids containing saturated fatty acids were insufficiently extracted while better extraction was achieved for lipids with unsaturated moieties (Fig. 2.1 II B). The only solvent yielding good recovery rates (i.e. ≥ 85 %) for all lipids containing unsaturated fatty acids was *iso*-propanol. Accordingly, calculated plasma concentrations of hydroxy- and epoxy- PUFA were higher using *iso*-propanol (Fig. 11.4).

A *direct comparison of LLE with acidified solvents versus protein precipitation* for extraction of lipids from plasma revealed differences in calculated concentrations of hydroxy-PUFA, with protein precipitation resulting in higher concentrations compared to LLE. In contrast, epoxy-PUFA and 5(*R,S*)-5-F_{2t}-IsoP were higher with LLE (Fig. 2.3). Only concentrations of dihydroxy-PUFA were similar with all investigated extraction procedures. It should be noted that inter-day variance (two days) with LLE was higher (up to ~30% for some hydroxy- and epoxy-PUFA) compared to protein precipitation (< 15%).

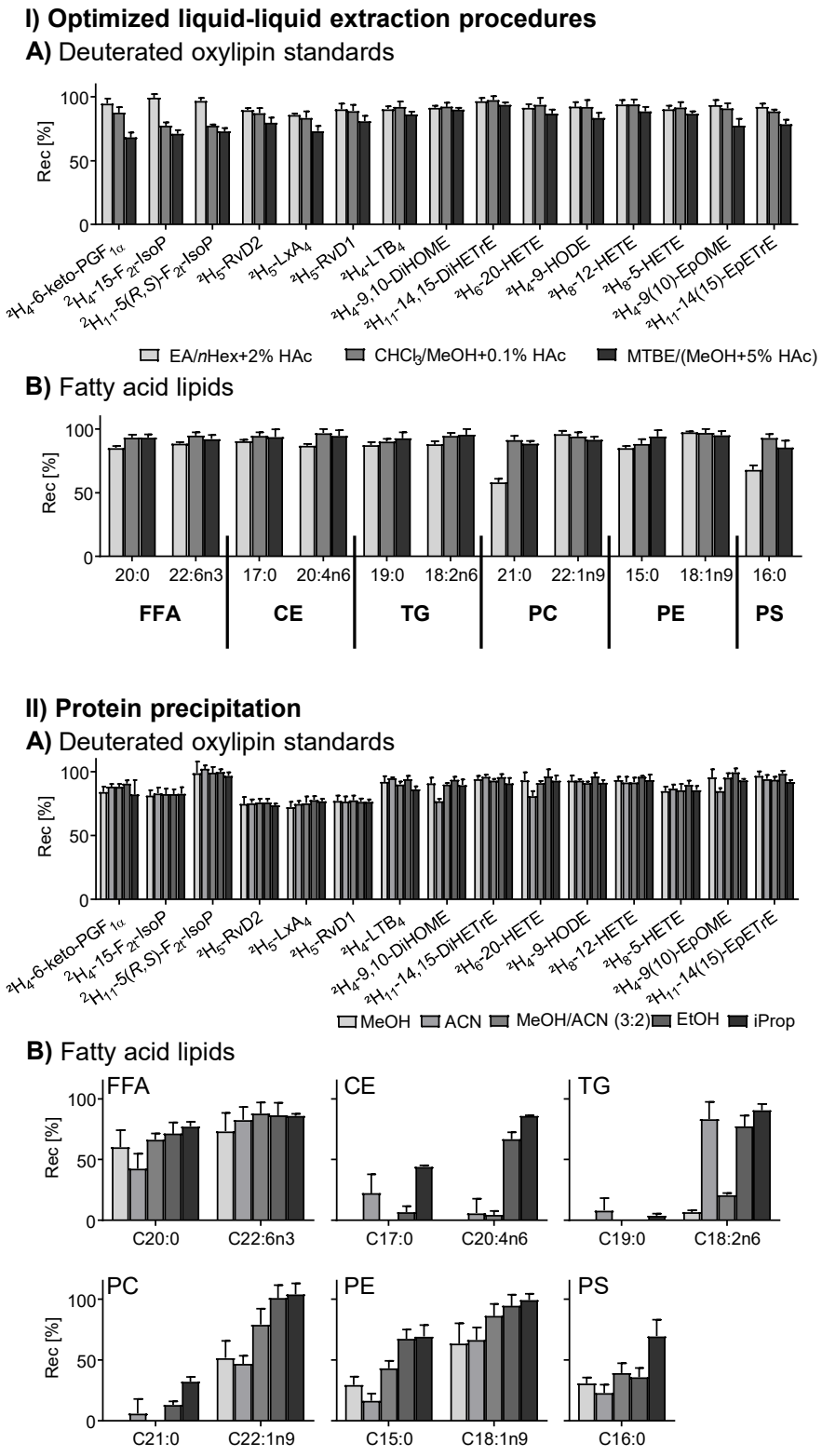

Fig. 2.1: Extraction efficiency of (I) optimized LLE and (II) protein precipitation. Shown are recoveries of (A) deuterated oxylipin standards to mimic extraction of free oxylipins and (B) lipids as surrogate for esterified oxylipins from BSA (mean ± SD; n = 4 (LLE) and n = 3 (protein precipitation)). LLE were performed as described in the appendix in Tab. 11.1 and protein precipitation was performed with 400 µl organic solvent. Oxylipins were directly analyzed with LC-MS following LLE and extracted by SPE on Bond Elut Certify II material after protein precipitation.

Lower sample volume or higher base concentration during hydrolysis resulted in higher concentrations of epoxy-PUFA and several hydroxy-PUFA (especially 4-HDHA, up to ~30%, Fig. 2.4). *Hydrolysis times* of 30-45 min yielded comparable oxylipin concentrations while hydrolysis for 15 min resulted in lower concentrations of hydroxy-PUFA (Fig. 2.4). After 30 min hydrolysis, 70% of PGE_2 was converted to PGB_2, while no residual PGE_2 could be found (Fig. 2.2). Interestingly, following hydrolysis of a PGD_2 standard spiked to plasma, no PGJ_2 formation was observed.

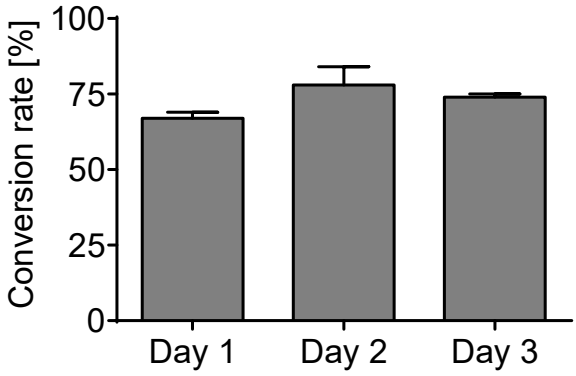

Fig. 2.2: Conversion of PGE_2 to PGB_2 during hydrolysis. PGE_2 was spiked to 100 µl plasma. Samples were prepared according to the final protocol. Recoveries were calculated against a diluted PGE_2 standard and equimolar conversion of PGE_2 to PGB_2 was assumed. Shown is mean conversion ± SD (n = 3) on three days.

During *SPE* on silica-based cartridge material (Bond Elut Certify II), drying of the stationary phase prior elution of the sample (either with nitrogen positive pressure or vacuum) led to increased concentrations of epoxy-PUFA (Fig. 2.5 I, Fig. 11.5) at longer drying times. Use of a neat fatty acid standard (ARA) revealed that specifically *cis*-epoxy-PUFA were formed while only a slight increase of the corresponding *trans*-epoxy-PUFA was observed (Fig. 2.5 II). Comparing *cis*- and *trans*-epoxy-PUFA plasma concentrations also revealed a higher increase in *cis*- compared to *trans*-epoxy-PUFA (Fig. 11.5 D). In contrast, oxylipin concentrations were not altered with longer drying times when using a polymer-based cartridge material (Oasis MAX, Fig. 2.5 I). Using controlled drying times, SPE on Oasis MAX (2 min) and Bond Elut Certify II (30 sec) led to comparable concentrations for oxylipins (Fig. 2.5 I and Fig. 2.6). However, intra- and interday variances – especially for epoxy-PUFA – were higher for Oasis MAX cartridges (Fig. 2.6 and Fig. 11.6).

Modifications of the procedure affect oxylipin concentration: Increasing the sample volume from 100 µl to 200 µl while maintaining the organic content of the

sample on the cartridge ≤ 25% and a sample volume on column of 3 ml (one column volume) resulted either in losses of hydroxy- or epoxy-PUFA (Fig. 11.7).

Feeding of rats for 23 days with a diet containing 1% of each DHA and EPA ethyl esters increased the plasma concentration of n3-PUFA oxylipins compared to the control group receiving sunflower oil (Tab. 2.1). EPA metabolites were increased 38-82-fold (absolute increase from 0.6-3 nM to 35-190 nM), while increase in DHA metabolites was 4.5-7.5-fold (from 3-18 nM to 25-110 nM). ARA derived oxylipins (except 20-HETE) were reduced 0.4-0.3-fold (absolute decrease from 65-190 nM to 19-130 nM) compared to the control group.

2.4 Discussion

2.4.1 Extraction of lipids prior to hydrolysis

Lipids are commonly directly hydrolyzed in the biological matrix without prior extraction steps [4, 11, 14, 16, 21-22]. To increase solubility of the analytes in the sample, organic solvent – mostly MeOH (25-56% of the sample volume [4, 14, 16, 21]) – is added. In our hands, this approach led to inacceptable recovery rates of internal standards, e.g. < 60% for hydroxy-PUFA (using 75% MeOH during hydrolysis at a concentration of 1.5 M sodium hydroxide; Fig. 11.1).

Three LLE protocols for the extraction of lipids prior to hydrolysis were tested: i) extraction with ethyl acetate and *n*-hexane which is used as elution solvent in the SPE procedure used routinely in the lab for the extraction of free oxylipins (EA/*n*Hex, [27]), ii) extraction according to Folch et al. with chloroform and methanol ($CHCl_3$/MeOH), a standard protocol for lipid extraction [31] and iii) methyl *tert*-butyl ether (MTBE/MeOH) which is an established alternative lipid extraction procedure allowing to omit the use of halogenated organic solvents [32]. It is not surprising that both, $CHCl_3$/MeOH and MTBE/MeOH were well suited for the extraction of lipids and nonpolar oxylipins. Of note, with EA/*n*Hex, extraction of phosphatidylcholine (PC) and phosphatidylserine (PS) with saturated fatty acid moieties was insufficient. With increasing polarity of the analytes extraction efficiency decreased with all protocols, presumably due to increasing amounts of the analytes

being present in plasma in the deprotonated form. Consequently, acidification of the extraction solvent resulted in enhanced extraction power.

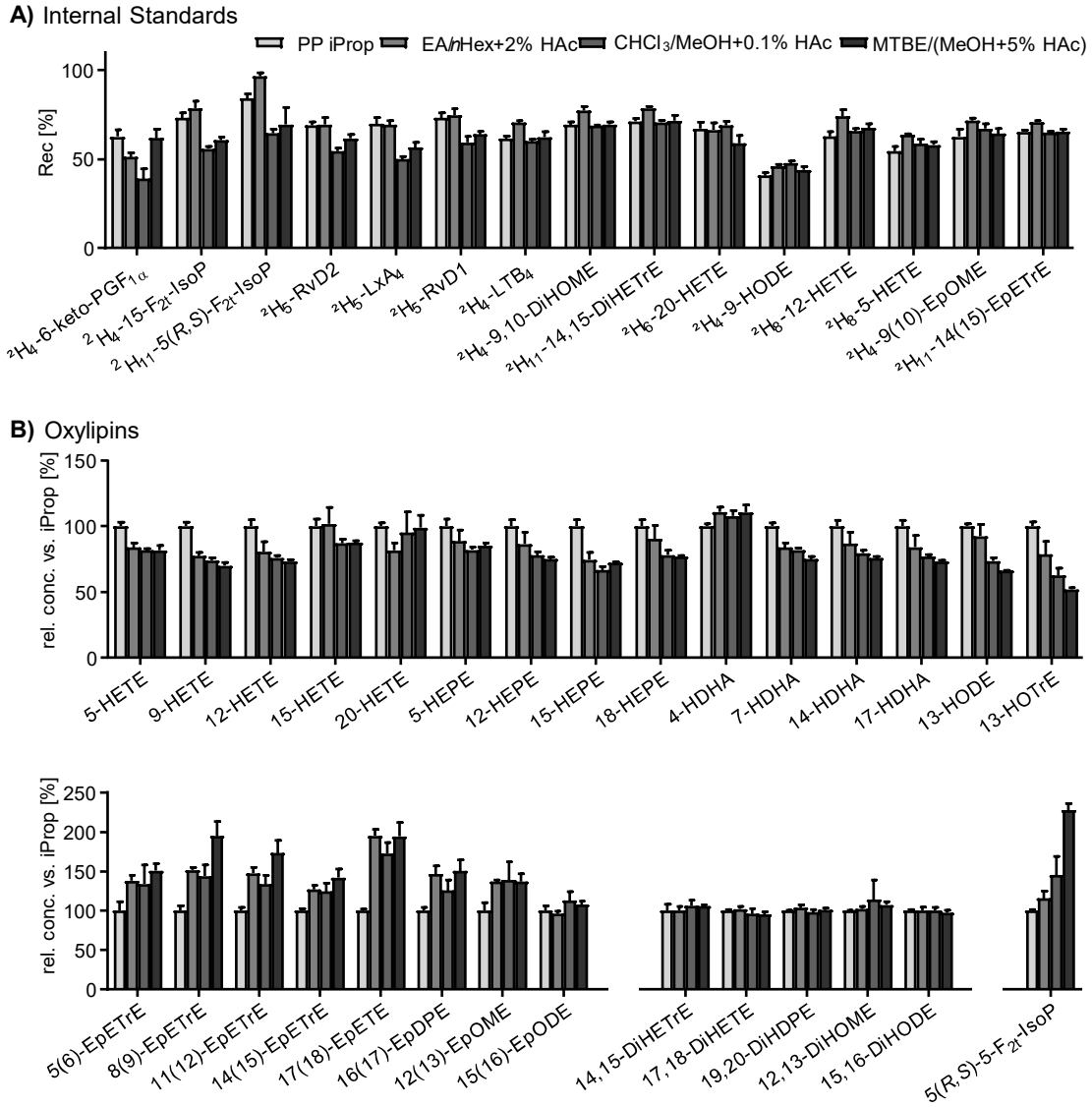

Fig. 2.3: Extraction of lipids from plasma using optimized LLE procedures with acidified solvents or protein precipitation with *iso*-propanol. Shown are (A) recoveries of internal standards as well as (B) relative concentrations of representative oxylipins in 100 µl plasma following protein precipitation with *iso*-propanol (PP iProp, 400 µl) or LLE (mean ± SD, n = 3).

Pronounced differences in calculated concentrations of hydroxy- and epoxy-PUFA as well as IsoP were observed when comparing acidified and non-acidified extraction solvents. This can be explained by the huge differences in recovery rates of lipids (the predominant form of oxylipins in plasma [7]) and the non-esterified oxylipin IS. Accordingly, absolute areas which only reflect the extraction of lipids were more comparable (Fig. 11.3). Since no esterified isotope labeled

oxylipins are commercially available, these results emphasize the importance of equal extraction efficiency among all lipid and oxylipin classes to ensure reliable and reproducible quantification of esterified oxylipins. This is not only crucial in the context of IS availability, but also considering differences in distribution of esterified oxylipins among lipid classes in biological samples of different origin (e.g. plasma vs. tissue).

Protein precipitation is a less labor-intensive alternative to remove proteins and extract lipids from biological matrices. Organic solvents have been widely used for this purpose [33-36]. All investigated approaches (*iso*-propanol, acetonitrile, methanol, methanol/acetonitrile (3/2, *v/v*) and ethanol) were well-suited to extract free oxylipin IS from the biological matrix (Fig. 2.1 II A). However, there were pronounced differences for the different lipid classes (Fig. 2.1 II B) with *iso*-propanol yielding overall the best extraction efficiency. Comparing the most effective protein precipitant – *iso*-propanol – and optimized LLEs with acidified solvents, oxylipin concentrations in plasma were in the same range (Fig. 2.3). However, in terms of simplicity and sample handling time, protein precipitation is the preferred method.

2.4.2 Hydrolysis conditions

Due to limited solubility in the predominantly organic sample after protein precipitation (*iso*-propanol/water (4/1, *v*/*v*)), base (sodium hydroxide, potassium hydroxide and sodium carbonate), base concentration as well as dilution solvent were critical parameters, since non-optimal setups resulted in two phases during hydrolysis or precipitation of the alkali salt. In this context, methanol in the solvent for the base (75/25 MeOH/H_2O, *v*/*v*) works as a bridging agent and enables a better solubility of potassium hydroxide in the aqueous, *iso*-propanol containing sample.

Different sample volumes of ~600 µl with ~0.1 M KOH and ~800 µl with ~0.56 M KOH led to comparable concentrations of oxylipins. Because of the elution power of higher portion of organic solvent on the SPE cartridge the low sample volume setup (i.e. hydrolysis following addition of 100 µl of 0.6 M KOH resulting in ~600 µl sample with ~0.1 M KOH) was chosen for the final protocol. Hydrolysis

time was set to 30 min and longer times (45 min) led to similar oxylipin concentrations suggesting that the analytes are stable and do not degrade during the chosen hydrolysis conditions. Interestingly, in a recent report it was discussed, that longer hydrolysis time and higher temperature (37 °C for 60 min or 60 °C for 30 min; 0.2 M KOH, ~43% MeOH during hydrolysis) led to degradation of 7-HDHA in the sample [21], while this analyte is more stable in our hands (60 °C for 30 min, ~0.1-0.56 M KOH, 63% *iso*-propanol and 17% MeOH during hydrolysis, Fig. 2.4).

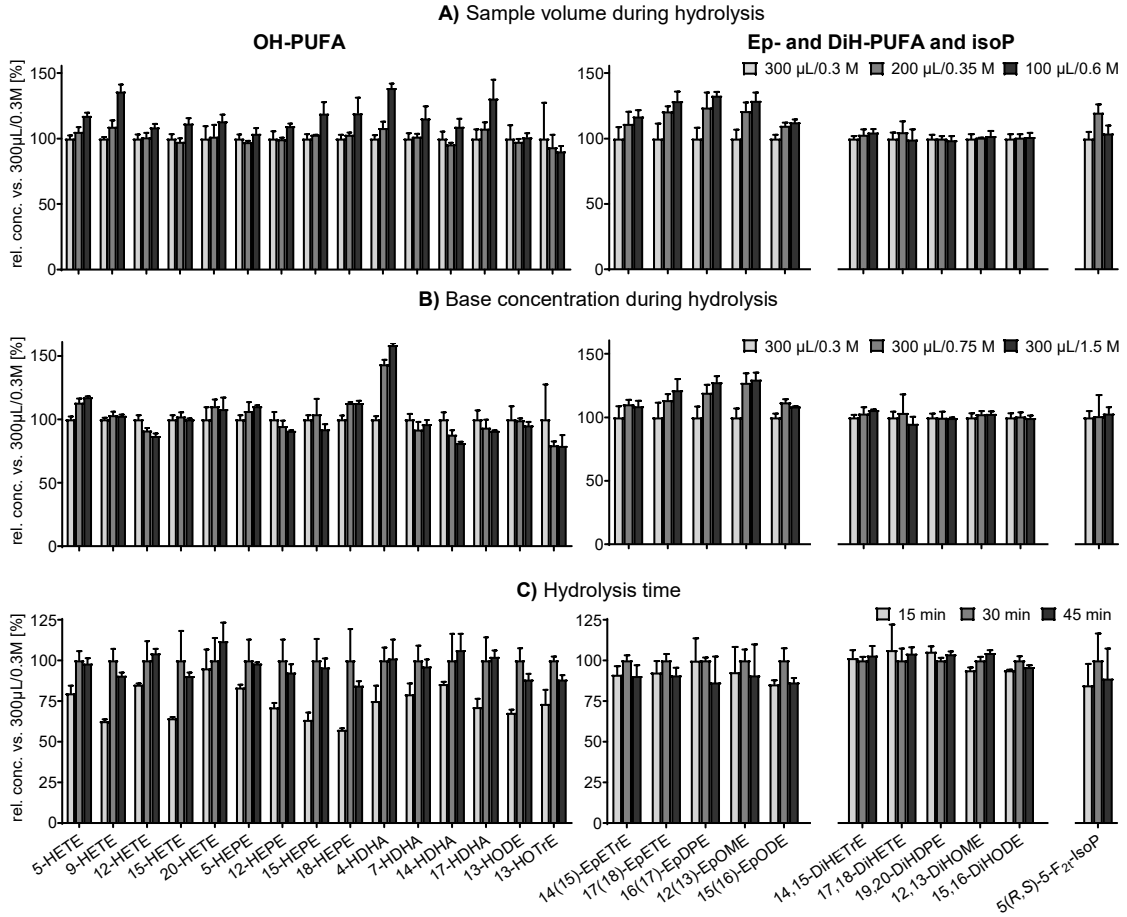

Fig. 2.4: Optimization of hydrolysis conditions. Shown are relative concentrations of a representative set of oxylipins in 100 µl plasma following protein precipitation with *iso*-propanol (400 µl) and hydrolysis (mean ± SD, n = 3). (A) Sample volume was varied keeping ~0.1 M KOH during hydrolysis (30 min); (B) Base concentration was varied with constant sample volume (800 µl, 30 min); (C) Hydrolysis time was varied using 0.1 M KOH in sample.

For thromboxanes, degradation under alkaline conditions has been described [17] which was also observed with our protocol (recovery of the $^{2}H_4$-TxB$_2$ < 5%, data not shown). Also, some classes of prostanoids, e.g. *β*-hydroxy-keto-prostanoids

such as PGE_2 and PGD_2 degrade under alkaline conditions [17, 37]. In this process, PGE_2 dehydrates to PGA_2 which subsequently isomerizes to the more stable PGB_2 [37]. Using the presented hydrolysis conditions, the conversion rate of PGE_2 to PGB_2 was about 70% (Fig. 2.2) and no residual PGE_2 was observed in the chromatograms. Thus, the present protocol is suitable to use PGB_2 as a surrogate for estimating the concentration of PGE_2 in plasma.

2.4.3 Solid phase extraction of cleaved oxylipins

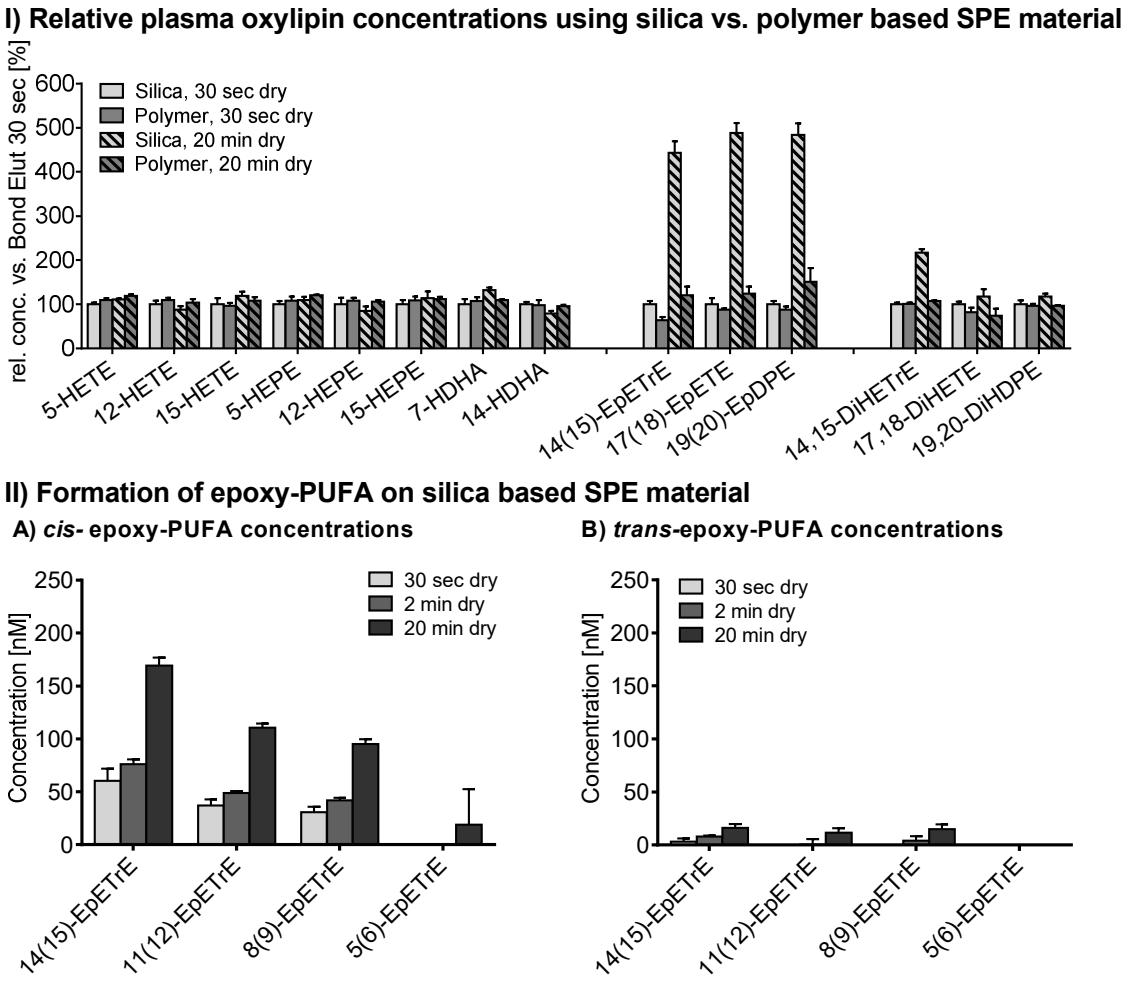

Fig. 2.5: Impact of sample drying on (I) plasma oxylipin concentrations using different cartridge materials and (II) the formation of epoxy-PUFA on silica-based cartridge material. (I) Samples were extracted either with silica (Bond Elut Certify II) or polymer (Oasis MAX) based cartridge material and dried with vacuum (mean ± SD, n=3). Relative concentrations were calculated against the mean of the concentration following 30 s of drying on silica-based cartridges. (II) Effect of sample drying on epoxy-PUFA concentrations formed on silica-based cartridge material (Bond Elut Certify II) using a neat ARA standard (0.7 mM) (mean ± SD, n=3).

Oxylipins are extracted from the hydrolyzed samples using SPE on silica modified with C8 and anion exchange moieties (Bond Elut Certify II [14, 27, 38-39]). While for free oxylipins, this SPE mode results in good removal of potential matrix interferences [38] and reproducible oxylipin concentrations [40], drying of the hydrolyzed sample (both, by positive and negative pressure) was a critical parameter and unsuitable drying resulted in the artificial formation of *cis*-epoxy-PUFA (Fig. 2.5, Fig. 11.5 D). It has been described that unsaturated fatty acids adsorbed to a monolayer of silica are oxidized by atmospheric oxygen to – mainly – epoxy-PUFA (while unsaturated fatty acids adsorbed in a bulk phase are predominantly converted to hydroperoxides) [41-42]. Due to their orientation to the monolayer, *cis*-epoxy-PUFA are the main product of this reaction [41-42]. These results indicate that autoxidation following the reaction described happens during the drying process of the hydrolyzed sample [43], presumably on non-endcapped residues of the cartridge material. In contrast to the extraction of free oxylipins – where drying is not such a crucial parameter – the high concentration of free PUFA in the hydrolyzed sample (e.g. 0.7 mM ARA in the plasma used in this study determined by GC-FID) can be readily oxidized on the silica material. Consistently, SPE on a silica-free cartridge material with a polymer backbone and similar hydrophilic binding sites as well as strong anion exchange moieties (Oasis MAX) did not result in an increase of epoxy-PUFA, even at long drying times (Fig. 2.5 I). Interestingly, drying of samples on silica-based SPE material has not been discussed before in the context of artifact formation, although it has been used to extract free oxylipins from cleaved samples [14, 39].

For reliable quantification of oxylipins in large sample sets such as cohort studies, the analytical protocol needs to be highly reproducible. A direct comparison of SPE on silica- and polymer-based C8/anion exchange mixed mode material showed that the SPE on silica-based material (with exact 30 sec of sample drying) resulted in notably lower inter-day, intra-day and inter-operator variations in the reported oxylipin concentrations compared to the polymer-based material (Fig. 2.6).

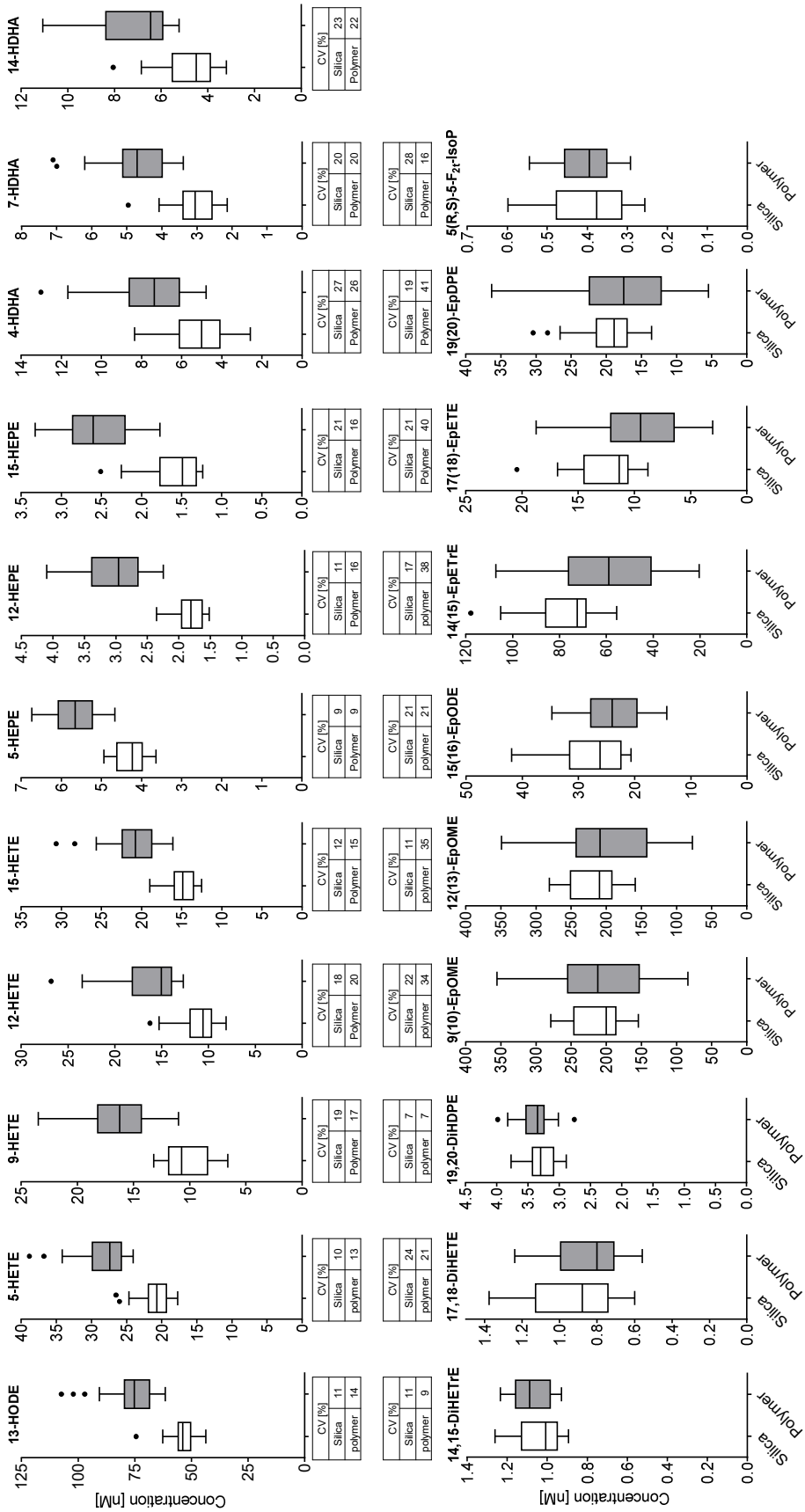
13-HODE
5-HETE
9-HETE
12-HETE
15-HETE
5-HEPE
12-HEPE
15-HEPE
4-HDHA
7-HDHA
14-HDHA
14,15-DiHETrE
17,18-DiHETE
19,20-DiHDPE
9(10)-EpOME
12(13)-EpOME
15(16)-EpODE
14(15)-EpETrE
17(18)-EpETE
19(20)-EpDPE
5(R,S)-5-F2t-IsoP
Concentration [nM]
Silica
Polymer
CV [%]
Silica
Polymer
polymer

Fig. 2.6 (previous page): Box-Whisker-plots and coefficients of variation of plasma oxylipin concentrations following SPE of the hydrolyzed sample on Bond Elut Certify II (left) and Oasis MAX (right) cartridges. Four different operators prepared and analyzed a triplicate set of samples on three consecutive days. Individual sample concentrations are shown in the Fig. 11.6.

Thus, silica-based cartridge material is in our hands most suitable for the extraction of oxylipins from the hydrolyzed sample if the operator tightly abides by the optimized drying time. Using the overall optimized procedure, variations of most oxylipins were ≤ 21%. Taking international guidelines on method validation (e.g. European Medicines Agency [44] or Food and Drug Administration [45]) into account which define a precision of < 15% for analytes well above the LLOQ, variations of most analytes pass these criteria or are only slightly higher (≤ 21%) with our method. Also, this is in the same range as for free oxylipins [3]. Coefficients of variation described by Quehenberger et al. are overall lower (direct hydrolysis, SPE on Strata-X polymeric RP-columns) [21], however, they are based on the analysis of standards and no inter-operator variance is considered. In combination with the validation of the calibration, including definition of linear range, limit of detection etc. described in [27-28], alkaline hydrolysis using the presented procedure followed by LC–MS analysis of the free oxylipins is with this precision well suited for the analysis of total oxylipins in biological samples. However, because no reference material of esterified oxylipins in lipids is available it is actually impossible to validate the accuracy of the method.

2.4.4 Impact of n3-PUFA supplementation on total oxylipin concentrations in rat plasma

The optimized method was finally applied to investigate the effects of n3-PUFA feeding on the oxylipin pattern. Rat plasma was analyzed following feeding with 1% of EPA and 1% of DHA in the chow for three weeks. The recoveries of internal standards were above 55% and the variation of the determined concentration per group (n = 5-6 animals) were for all analytes acceptable with a standard deviation < 30% (except for 12-LOX metabolites) which supports the broad applicability of the method developed here for the quantification of total oxylipins (Tab. 2.1). In line with previous reports for free oxylipins in rodents [14, 30] and in humans [46-47], concentrations of EPA and DHA derived oxylipins were massively increased after

feeding the n3-PUFA rich diet compared to the standard diet. While absolute changes of EPA and DHA metabolites were in the same range, relative changes in EPA metabolites were unequally higher. This is probably caused by lower baseline EPA compared to DHA plasma level in rats [14, 48] and has been discussed earlier in a murine supplementation study [30].

Tab. 2.1: Plasma concentrations of oxylipins following feeding of an EPA+DHA enriched diet to rats. The EPA+DHA group received a diet enriched with 1% EPA and DHA ethyl esters, while the control group was feed with a diet containing sunflower oil. Shown are concentrations of a representative set of analytes derived from EPA, DHA and ARA. [a] Shown are mean ± SEM (n = 4-6). [b] t-test, non-paired, α = 0.05; [c] t-test, Welch correction, α = 0.05. Outlier excluded based on ROUT outlier test, Q = 1%.

Oxylipin	Control group[a]	EPA + DHA group[a]	Fold change	Significance
		Alcohols		
5-HETE	111 ± 5	39 ± 3	0.3	$p < 0.0001$[b]
9-HETE	65 ± 4	19 ± 2	0.3	$p < 0.0001$[b]
12-HETE	189 ± 4	130 ± 40	0.7	n.s.[c]
15-HETE	90 ± 4	27 ± 2	0.3	$p < 0.0001$[b]
20-HETE	6.8 ± 0.6	28 ± 5	4.1	$p < 0.05$[c]
5-HEPE	1.2 ± 0.1	47 ± 6	38	$p < 0.01$[c]
12-HEPE	1.3 ± 0.2	190 ± 80	146	n.s.[c]
15-HEPE	< LLOQ	35 ± 4	-	-
18-HEPE	0.61 ± 0.09	44 ± 6	72	$p < 0.01$[c]
20-HEPE	3.1 ± 0.2	110 ± 40	34	n.s.[c]
4-HDHA	5.5 ± 0.4	35 ± 5	6.3	$p < 0.01$[c]
7-HDHA	3.2 ± 0.2	24 ± 4	7.5	$p < 0.01$[c]
14-HDHA	9 ± 3	60 ± 10	6.8	$p < 0.05$[c]
17-HDHA	18 ± 1	80 ± 10	4.5	$p < 0.05$[c]
22-HDHA	3.2 ± 0.3	110 ± 20	34	$p < 0.05$[c]
		Epoxides		
14(15)-EpETrE	260 ± 20	100 ± 8	0.4	$p < 0.0001$[b]
17(18)-EpETE	1.3 ± 0.1	105 ± 9	82	$p < 0.001$[c]
19(20)-EpDPE	12 ± 1	80 ± 10	6.6	$p < 0.01$[c]
		Vicinal diols		
14,15-DiHETrE	3.7 ± 0.4	2.7 ± 0.6	0.7	n.s.[b]
17,18-DiHETE	0.15 ± 0.03	9.4 ± 1.0	63	$p < 0.001$[c]
19,20-DiHDPE	1.5 ± 0.1	9.7 ± 0.5	6.4	$p < 0.001$[c]

2.5 Conclusion

A detailed procedure covering sample preparation steps for the quantification of esterified oxylipins in biological samples is presented. It is highlighted that different steps during sample preparation have a direct impact on the oxylipin concentration. Both, LLE and protein precipitation yielded oxylipin concentrations in the same range; however, protein precipitation is the preferred method because of its simplicity. Best conditions for hydrolysis are 30 min at 60 °C after addition of 100 µl 0.6 M KOH (in 75/25 MeOH/water). Our results indicate that these conditions lead to efficient liberation of oxylipins from the lipids and no degradation during hydrolysis. The SPE procedure, however, has to be strictly controlled if extraction of cleaved oxylipins is carried out on silica-based material since artificial formation of epoxy-PUFA can occur. With the methodology described herein, precise quantification of oxylipins in biological matrices is achieved. Based on this the biological role of esterified oxylipins can be further investigated and the analytical strategy could pave the route for their use as biomarkers for diseases.

2.6 References

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Chapter 3

Rapid quantification of fatty acids in plant oils and biological samples by LC-MS *

Analysis of fatty acids in food and biological samples such as blood is indispensable in modern life sciences. We developed a rapid, sensitive and comprehensive method for the quantification of 41 saturated and unsaturated fatty acids by means of LC-MS. Optimized chromatographic separation of isobaric analytes was carried out on a C8 reversed phase analytical column (100 x 2.1 mm, 2.6 µm core-shell particle) with a total run time of 15 min with back pressure lower than 300 bar. On an old triple quadrupole instrument (3200, AB Sciex), pseudo *selected reaction monitoring mode was used for quantification of the poorly fragmenting fatty acids, yielding limits of detection of 5-100 nM. Sample preparation was carried out by removal of phospholipids and triacylglycerols by solid phase extraction (non-esterified fatty acids in oils) or saponification in* iso*-propanol (total fatty acids). This is not only a rapid strategy for quantification of total fatty acids, but allows the direct combination with the LC-MS-based analysis of fatty acid oxidation products (eicosanoids and other oxylipins) from the same sample. The concentrations of total fatty acids determined by means of LC-MS were consistent with those from GC-FID analysis demonstrating the accuracy of the developed method. Moreover, the method shows high precisions with a low intra-day (≤ 10% for almost all fatty acids in plasma and ≤ 15% in oils) and inter-day as well as inter-operator variability (< 20%). The method was successfully applied on human plasma and edible oils. The possibility to quantify non-esterified fatty acids in samples containing an excess of triacylglycerols and phospholipids is a major strength of the described approach allowing to gain new insights in the composition of biological samples.*

* modified from Koch, E.; Wiebel, M.; Hopmann, C.; Kampschulte, N.; Schebb, N. H. Rapid quantification of fatty acids in plant oils and biological samples by LC-MS. Anal Bioanal Chem 2021, 413, 5439−5451.

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3.1 Introduction

Fatty acids play a fundamental role in the biology of living organisms e.g. by influencing properties of biomembranes, storing and providing of energy or being involved in cell signaling [1-3]. Especially long-chain polyunsaturated fatty acids (PUFA) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are involved in many (patho)physiological processes i.a. through their oxidation products. These eicosanoids and other oxylipins are highly potent lipid mediators regulating for example inflammation, vasoconstriction or pain [4-5]. Dietary supplementation of n3-PUFAs or reducing the intake of n6-PUFA is a promising way to modulate endogenous fatty acid distribution which has been demonstrated in numerous intervention studies [6-8].

The basic structure of fatty acids is a linear hydrocarbon chain with a varying number of double bonds. A large number of structurally similar molecules, e.g. n3- vs. n6-PUFA, leads to challenges in analytics. Fatty acids are often analyzed by means of gas chromatography coupled to flame ionization detection (GC-FID) or mass spectrometry (GC-MS) due to the high separation efficiency and good sensitivity of GC [9-11]. However, transesterification or derivatization is necessary for this analytical procedure, which is on the one hand laborious and time-consuming and on the other hand can lead to discrimination of analytes [12]. Similarly, the need for derivatization makes quantification of non-esterified fatty acids (NEFA) in biological samples by GC challenging due to the high amount of fatty acids occurring in different lipid classes such as triacylglycerols or phosphorlipids. Fractionation of these lipid classes by solid phase extraction (SPE) is a powerful tool to separate NEFA from other lipid species [13]: However, already 1-2% of unremoved triacylglycerols or phospholipids disturbs the quantification of low abundant NEFA in biological samples and plant oils.

The use of liquid chromatography-mass spectrometry (LC-MS) has raised strong interest in fatty acid analysis in recent years. Even though some published methods include derivatization of the fatty acids e.g. to improve ionization efficiency [14-15], determination by LC-MS offers the possibility of analyzing fatty

acids directly [16-17]. Reversed phase columns based on modified silica gel are commonly used as stationary phase usually in combination with more nonpolar solvents such as *iso*-propanol/acetonitrile mixtures [16, 18].

However, none of the published LC-MS approaches [15-16, 19-25] fulfills our needs regarding comprehensiveness, chromatographic separation of isobaric fatty acids, high sample throughput and applicability for a wide range of matrices. Therefore, we developed herein a new method which (i) covers a comprehensive set of biologically occurring fatty acids, (ii) allows rapid analysis (< 15 min) but separation of positional isomers and (iii) requires only simple sample preparation by saponification following dilution in organic solvent. The latter allows us to analyze the oxidation products of fatty acids, eicosanoids and other oxylipins, from the same sample preparation using an established targeted oxylipin metabolomics method [26]. The method optimization and performance were characterized and compared to earlier published methods and the accuracy was demonstrated by cross-validation with a standard GC-FID approach. Finally, the method was successfully applied to analyze human plasma and refined as well as virgin plant oils.

3.2 Experimental

3.2.1 Chemicals and biological materials

Fatty acid standards (C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C15:0, C17:0, C18:2 n6, C18:1 n9, C18:0, C20:0, C21:0, C8:0-d15, C12:0-d23 and C20:5 n3-d5) were purchased from Merck (Darmstadt, Germany). The internal standards C16:0-d4 and C18:0-d5 were bought from Eurisotop (Saarbrücken, Germany). All other fatty acid standards were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Fatty acid methyl esters (FAME) standards for the FAME reference mix were from Restek (marine fish oil mix; Bad Homburg vor der Höhe, Germany), Merck (FAME C22:4 n6, FAME C22:5 n3, FAME C25:0, Supelco 37 Component FAME Mix; Darmstadt, Germany), Fluka/Honeywell (FAME C19:0; Offenbach, Germany) and Cayman Chemicals

(FAME C18:4 n3, FAME C20:3 n9, FAME C20:4 n3; Ann Arbor, Michigan, USA). The used edible oils (refined and virgin sunflower oil as well as virgin flaxseed oil) samples were bought in local supermarkets in Wuppertal, Germany. Pooled human EDTA plasma was generated from healthy individuals as described [26] in accordance with the guidelines of the Declaration of Helsinki and approved by the ethic committee of the University of Wuppertal. Acetonitrile (ACN) and ethanol (EtOH) were obtained from VWR (Darmstadt, Germany) and methanol (MeOH), *iso*-propanol as well as acetic acid (HAc) from Fisher Scientific (Schwerte, Germany). Ultra-pure water was generated using the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). All other chemicals were from Merck (Darmstadt, Germany).

3.2.2 LC-ESI(-)-MS analysis

Analysis was carried out on a 1260 Infinity LC System (Agilent, Waldbronn, Germany) coupled to an API 3200 instrument (AB Sciex, Darmstadt, Germany). Ionization was carried out in negative electrospray ionization (ESI(-)) mode with the following source settings: ion spray voltage -4500 V, curtain gas (nitrogen, N_2-generator NGM 33, cmc Instruments, Eschborn, Germany) 35 psi, nebulizer gas (gas 1, purified compressed air; “zero air”) 70 psi generated with a RAMS 05ZA (cmc Instruments, Eschborn, Germany), drying gas (gas 2, purified compressed air) 55 psi, temperature 500 °C. The sprayer offset was 0.511 cm for the vertical and 0.519 cm for the horizontal axis. The electrode protrusion was approximately 1 mm. 10 µl of samples were injected by an HTC PAL autosampler (CTC Analytics, Switzerland, local distributor: Axel Semrau, Sprockhövel, Germany) equipped with a 25 µl syringe and a 20 µl sample loop. Samples were cooled at 4 °C. Separation of fatty acids was carried out on a Kinetex C8 core-shell reversed phase column (100 x 2.1 mm, particle size 2.6 µm, pore size 10 nm; Phenomenex, Aschaffenburg, Germany) kept at 40 °C. The analytical column was equipped with an inline filter (0.3 µm, 1290 infinity II inline filter, Agilent, Waldbronn, Germany) and a SecurityGuard Ultra C8 cartridge as precolumn (2.1 mm ID, Phenomenex, Aschaffenburg, Germany). Solvent B of the mobile phase consisted of ACN/MeOH/HAc (80/15/0.1, *v/v/v*) and solvent A was

0.1% HAc mixed with 5% of solvent B. The following linear gradient was used: 0.0-1.0 min isocratic 20% B, 1.0-1.5 min linear from 20% B to 66% B, 1.5-8.0 min isocratic 66% B, 8.0-11.0 min linear from 66% B to 100% B, 11.0-14.0 min isocratic 100% B, 14.0-14.5 min linear from 100% B to 20% B followed by equilibration for 0.5 min. This resulted in a total run time of 15 min. Analyst software (version 1.6.2, Sciex) was used for instrument control as well as data acquisition and Multiquant (version 2.1.1, Sciex) for peak integration and quantification.

3.2.3 Calibration and quantification

Stock solutions of the individual fatty acids were mixed and diluted in EtOH using glass volumetric flasks to concentration levels of 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0 and 2.5 µM. For fatty acids which often occur in high concentrations in biological samples (C16:0, C16:1 n7, C18:0, C18:1 n9, C18:2 n6, C20:4 n6) the final concentration levels were 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 µM. Concentrations of fatty acids in stock solution (200 µM) were verified by GC-FID (Fig. 11.8) following HCl catalyzed transmethylation to fatty acid methyl esters (FAME) according to Ostermann et al. [12]. If the determined concentration of a fatty acid in stock solution was not within ± 15%, a correction factor was used. Additionally, the concentrations of PUFA in the calibration solution are monitored using a FAME reference mix to compensate for autoxidative degradation. The FAME reference mix was directly analyzed by GC-FID and prepared as sample for LC-MS determination. If the PUFA concentration determined by GC-FID and LC-MS was not within ± 10%, a second correction factor was used. C8:0-d15, C12:0-d23, C16:0-d4, C18:0-d5, C18:1 n9-d17, C18:2 n6-d4, C20:0-d3, C20:3 n6-d6, C20:4 n6-d8, C20:5 n3-d5 and C22:6 n3-d5 were used as internal standards at a concentration of 0.2 µM.

Tab. 3.1 (following two pages): LC-ESI(-)-MS/MS parameters and performance for the quantification of fatty acids. Shown are all fatty acids covered by the method, their mass transitions for quantification in scheduled SRM mode, specific electronical MS parameters (declustering potential (DP), collision energy (CE)), their internal standards (IS), retention time (t_R), full peak width at half maximum (FWHM), the calibration range as well as the limit of detection (LOD) and the lower limit of quantification (LLOQ). Scheduled selected reaction monitoring mode (SRM) using nitrogen as collision gas (12 psi) with a detection window of ± 35 s around the expected retention time was used for analyte detection.

Fatty Acid	Mass transition Q1	Q3	Electronic parameters DP [V]	CE	Internal standard	Retention time[1] [min]	FWHM[2] [s]	Calibration range [µM]	LOD [µM]	LOD ng on column	LLOQ [µM]
C21:0	325.2	325.2	-48	-16	C20:0-d3	12.25 ± 0.01	3.3 ± 0.4	0.075 - 2.5	0.05	0.2	0.075
C22:6 n3	327.4	327.4	-46	-16	C22:6 n3-d5	9.09 ± 0.06	10.2 ± 0.6	0.05 - 2.5	0.025	0.08	0.05
C22:6 n3 CO_2	327.4	283.4	-46	-16	C22:6 n3 CO_2-d5	9.09 ± 0.07	9.3 ± 0.8	0.075 - 2.5	0.05	0.2	0.075
C22:5 n3	329.2	329.2	-48	-20	C22:6 n3-d5	10.02 ± 0.03	6.3 ± 0.2	0.05 - 2.5	0.01	0.03	0.025
C22:5 n3 CO_2	329.2	285.2	-48	-20	C22:6 n3 CO_2-d5	10.02 ± 0.04	6.3 ± 0.6	0.075 - 2.5	0.05	0.2	0.075
C22:5 n6	329.2	329.2	-48	-20	C22:6 n3-d5	10.49 ± 0.02	4.9 ± 0.2	0.05 - 2.5	0.01	0.03	0.025
C22:5 n6 CO_2	329.5	285.2	-48	-20	C22:6 n3 CO_2-d5	10.49 ± 0.02	4.8 ± 0.4	0.05 - 2.5	0.025	0.08	0.05
C22:4 n6	331.3	331.3	-40	-20	C22:6 n3-d5	10.88 ± 0.02	4.1 ± 0.1	0.05 - 2.5	0.005	0.02	0.01
C22:4 n6 CO_2	331.3	287.3	-40	-20	C22:6 n3 CO_2-d5	10.88 ± 0.02	4.1 ± 0.3	0.075 - 2.5	0.05	0.2	0.075
C22:2 n6	335.3	335.3	-46	-20	C22:6 n3-d5	11.76 ± 0.01	3.2 ± 0.3	0.05 - 1.0	0.01	0.03	0.025
C22:1 n9	337.5	337.5	-46	-26	C20:0-d3	12.11 ± 0.01	3.1 ± 0.8	0.05 - 2.5	0.025	0.08	0.05
C22:0	339.2	339.2	-46	-20	C20:0-d3	12.45 ± 0.01	3.3 ± 0.6	0.075 - 2.5	0.05	0.2	0.075
C23:0	353.2	353.2	-48	-16	C20:0-d3	12.65 ± 0.01	4.4 ± 0.8	0.075 - 1.0	0.05	0.2	0.075
C24:1 n9	365.4	365.4	-48	-20	C20:0-d3	12.51 ± 0.01	4.0 ± 0.5	0.075 - 1.0	0.05	0.2	0.075
C24:0	367.2	367.2	-46	-20	C20:0-d3	12.84 ± 0.01	4.8 ± 0.9	0.25 - 2.5	0.1	0.4	0.25
Internal standards											
d15-C8:0	158.2	158.2	-26	-9		3.28 ± 0.01	3.14 ± 0.1				
d23-C12:0	222.2	222.2	-44	-18		5.08 ± 0.02	4.4 ± 0.2				
d17-C18:1 n9	298.2	298.2	-52	-20		10.72 ± 0.02	4.2 ± 0.2				
d4-C18:2 n6	283.2	283.2	-52	-20		9.45 ± 0.06	10.4 ± 0.6				
d8-C20:4 n6	311.2	311.2	-48	-20		9.29 ± 0.06	9.3 ± 0.4				
d8-C20:4 n6 CO_2	311.2	267.2	-48	-22		9.28 ± 0.06	8.2 ± 1.7				
d5-C20:5 n3	306.2	306.2	-44	-20		7.38 ± 0.04	7.4 ± 0.6				
d5-C20:5 n3 CO_2	306.2	262.2	-44	-20		7.38 ± 0.04	6.6 ± 0.9				
d5-C22:6 n3	332.5	332.5	-42	-20		9.01 ± 0.06	10.0 ± 0.8				
d5-C22:6 n3 CO_2	332.5	288.2	-42	-24		9.01 ± 0.06	9.5 ± 1.3				
d4-C16:0	259.2	259.2	-46	-21		10.35 ± 0.03	6.4 ± 0.4				
d5-C18:0	288.2	288.2	-48	-21		11.44 ± 0.01	3.4 ± 0.3				
d6-C20:3 n6	311.5	311.5	-46	-17		10.32 ± 0.03	5.3 ± 0.3				
d3-C20:0	314.2	314.2	-50	-21		12.03 ± 0.01	3.6 ± 0.3				

[1] Mean ± SD of the retention times in different batches

[2] Mean ± SD of the full width at half maximum (FWHM) in different batches

[3] No LOD can be determined because analyte is also detectable in blank injection

[4] quadratic regression

[5] Determined by at least 2x peak height of blank injection and accuracy of 80-120%

[6] No calibration possible due to high background levels

Fatty Acid	Mass transition Q1	Q3	Electronic parameters DP [V]	CE [V]	Internal standard	Retention time[1] [min]	FWHM[2] [s]	Calibration range [µM]	LOD [µM]	LOD ng on column	LLOQ [µM]
C6:0	115.2	115.2	-24	-14	C8:0-d15	2.81 ± 0.01	3.4 ± 0.1	0.1 - 2.5	-[3]	-[3]	0.1[5]
C7:0	129.2	129.2	-42	-10	C8:0-d15	3.05 ± 0.01	3.2 ± 0.1	0.075 - 2.5	-[3]	-[3]	0.075[5]
C8:0	143.2	143.2	-32	-10	C8:0-d15	3.30 ± 0.01	3.2 ± 0.2	0.1 - 2.5	-[3]	-[3]	0.1[5]
C9:0	157.1	157.1	-42	-14	C8:0-d15	3.61 ± 0.01	3.3 ± 0.1	-[6] -	-[3]	-[3]	-[6]
C10:0	171.2	171.2	-44	-14	C8:0-d15	4.01 ± 0.01	3.5 ± 0.2	0.05 - 2.5	-[3]	-[3]	0.05[5]
C11:0	185.1	185.1	-36	-16	C12:0-d23	4.52 ± 0.01	3.8 ± 0.1	0.05 - 2.5	-[3]	-[3]	0.05[5]
C12:0	199.1	199.1	-44	-14	C12:0-d23	5.19 ± 0.02	4.5 ± 0.2	0.1 - 2.5	-[3]	-[3]	0.1[5]
C13:0	213.2	213.2	-46	-16	C12:0-d23	6.10 ± 0.03	5.8 ± 0.3	0.05 - 2.5	0.005	0.01	0.01
C14:1 n5	225.2	225.2	-46	-16	C12:0-d23	5.74 ± 0.03	5.3 ± 0.2	0.05 - 2.5	0.005	0.01	0.01
C14:0	227.1	227.1	-46	-14	C12:0-d23	7.32 ± 0.04	7.7 ± 0.5	0.075 - 2.5	-[3]	-[3]	0.075[5]
C15:1 n5	239.2	239.2	-36	-14	C12:0-d23	6.79 ± 0.04	7.0 ± 0.3	0.05 - 2.5	0.006	0.01	0.012
C15:0	241.3	241.3	-44	-14	C12:0-d23	9.00 ± 0.06	11.4 ± 0.6	0.05 - 2.5	0.01	0.02	0.025
C16:1 n7	253.3	253.3	-48	-20	C18:1 n9-d17	8.28 ± 0.05	9.4 ± 0.4	0.1 - 20	0.005	0.01	0.01
C16:0	255.2	255.2	-44	-20	C16:0-d4	10.38 ± 0.03	6.8 ± 0.4	0.5 - 20	-[3]	-[3]	0.5[5]
C17:0	269.3	269.3	-48	-20	C20:0-d3	11.03 ± 0.02	4.1 ± 0.3	0.05 - 2.5	0.005	0.01	0.01
C18:4 n3	275.3	275.3	-36	-16	C20:5 n3-d5	6.25 ± 0.03	6.0 ± 0.2	0.05 - 2.5	0.01	0.03	0.025
C18:4 n3 CO_2	275.3	231.3	-36	-16	C20:5 n3-d5	6.25 ± 0.03	5.9 ± 0.5	0.075 - 2.5	0.05	0.1	0.075
C18:3 n6	277.2	277.2	-46	-22	C20:5 n3-d5	7.75 ± 0.05	8.3 ± 0.6	0.05 - 2.5	0.025	0.07	0.05
C18:3 n3	277.2	277.2	-44	-24	C20:5 n3-d5	7.46 ± 0.04	7.9 ± 0.4	0.05 - 2.5	0.025	0.07	0.05
C18:2 n6	279.3	279.3	-46	-16	C18:2 n6-d4	9.52 ± 0.06	9.6 ± 0.3	0.1 - 15	-[3]	-[3]	0.01[5]
C18:1 n9	281.4	281.4	-46	-18	C18:1 n9-d17	10.80 ± 0.02	4.7 ± 0.2	0.1 - 15[4]	-[3]	-[3]	0.05[5]
C18:0	283.2	283.2	-46	-20	C18:0-d5	11.47 ± 0.01	5.5 ± 0.5	1 - 20	-[3]	-[3]	1.0[5]
C19:0	297.4	297.4	-46	-20	C20:0-d3	11.79 ± 0.01	3.4 ± 0.3	0.05 - 2.5	0.005	0.01	0.01
C20:5 n3	301.2	301.2	-46	-16	C20:5 n3-d5	7.43 ± 0.05	7.8 ± 0.4	0.05 - 2.5	0.025	0.08	0.05
C20:5 n3 CO_2	301.2	257.2	-46	-16	C20:5 n3 CO_2-d5	7.43 ± 0.05	7.6 ± 0.6	0.075 - 2.5	0.05	0.2	0.075
C20:4 n6	303.2	303.2	-46	-18	C20:4 n6-d8	9.43 ± 0.06	9.8 ± 0.5	0.1 - 20	0.02	0.06	0.05
C20:4 n6 CO_2	303.2	259.2	-46	-18	C20:4 n6 CO_2-d8	9.43 ± 0.06	9.1 ± 0.9	0.1 - 20	0.05	0.2	0.1
C20:4 n3	303.3	303.3	-46	-18	C20:5 n3-d5	8.74 ± 0.06	10.0 ± 0.5	0.05 - 2.5	0.01	0.03	0.025
C20:4 n3 CO_2	303.3	259.2	-46	-18	C20:5 n3 CO_2-d5	8.75 ± 0.05	9.4 ± 1.0	0.075 - 2.5	0.05	0.2	0.075
C20:3 n9	305.4	305.4	-46	-14	C22:6 n3-d5	10.69 ± 0.02	4.5 ± 0.1	0.05 - 2.5	0.006	0.02	0.013
C20:3 n6	305.4	305.4	-46	-14	C20:3 n6-d6	10.37 ± 0.03	5.5 ± 0.1	0.05 - 2.5	0.005	0.02	0.01
C20:2 n6	307.3	307.3	-48	-24	C22:6 n3-d5	11.09 ± 0.02	4.0 ± 0.2	0.05 - 2.5	0.005	0.02	0.01
C20:1 n9	309.4	309.4	-48	-16	C20:0-d3	11.62 ± 0.01	3.4 ± 0.2	0.05 - 2.5	0.01	0.03	0.025
C20:0	311.2	311.2	-44	-20	C20:0-d3	12.04 ± 0.01	3.5 ± 0.2	0.05 - 2.5	-[3]	-[3]	0.05[5]

For calibration, the peak area ratios (analyte/IS) were plotted against the concentration ratios (analyte/IS). Calibration curves were calculated using linear or quadratic least square regression (weighting: $1/x^2$, Tab. 3.1). The limit of detection (LOD) was determined by a signal-to-noise ratio of ≥ 3 and the lower limit of quantification (LLOQ) by signal-to-noise ratio of ≥ 5 and accuracy of ± 20% within the calibration curve. For fatty acids which are ubiquitously detectable in blank injection i.e. injection of EtOH, the LLOQ was set to the concentration yielding a peak height of at least twofold of the peak height in blank injections and accuracy within the calibration curve of ± 20%.

3.2.4 Sample preparation

For quantification of total fatty acid concentrations in oils, 4-5 mg oil were diluted with 1.5 ml of *iso*-propanol. 100 µl of this solution were mixed with 300 µl of *iso*-propanol, 10 µl of antioxidant mixture (0.2 mg/mL butylated hydroxy toluene (BHT), 100 µM indomethacin, 100 µM trans-4-(-4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*-AUCB) in MeOH), 50 µl water and 100 µl 0.6 M KOH in MeOH/H_2O (75/25, *v*/*v*). Samples were hydrolyzed for 30 min at 60 °C. Following neutralization (20 µl of 25% HAc), samples were diluted (20 µl/500 µl) in EtOH and additionally 10 µl/100 µl for high concentrated fatty acids and 50 µl/100 µl for low concentrated fatty acids.

For quantification of total fatty acids in plasma, 100 µl plasma were mixed with 10 µl of antioxidant mixture and 400 µl of ice-cold *iso*-propanol. Following centrifugation (4 °C, 20,000 x *g*, 10 min), 450 µl of the supernatant were collected. For hydrolysis (30 min, 60 °C), 100 µl of 0.6 M KOH in MeOH/H_2O (75/25, *v*/*v*) were added. After neutralization by addition of 20 µl of 25% HAc samples were diluted (20 µl/500 µl and subsequently 50 µl/100 µl) in EtOH. NEFA in plasma were analyzed in the same way without hydrolysis and the following dilution: 10 µl/100 µl for high concentrated fatty acids and 40 µl/100 µl for low concentrated fatty acids.

For quantification of NEFA in plant oils, solid phase extraction on aminopropyl columns (1 ml volume, 100 mg bed weight, Supelco/Merck, Darmstadt, Germany) was used to remove triacylglycerols [13]. Approximately 10 mg of oils were diluted

in 1 ml chloroform/*iso*-propanol 2/1 (*v*/*v*). 10 µl of BHT (0.02 mg/ml in MeOH) as antioxidant and 10 µl of C20:4 n6 (160 µM) as internal standard was added. The columns were washed with two cartridge volumes of diethyl ether/HAc 98/2 (*v*/*v*) and two cartridge volumes of chloroform/*iso*-propanol 2/1 (*v*/*v*). Samples were loaded onto the cartridges and triacylglycerols were removed with two cartridge volumes of chloroform/*iso*-propanol 2/1 (*v*/*v*). NEFA were eluted with two cartridge volumes of diethyl ether/HAc 98/2 (*v*/*v*). The eluate was neutralized with 1 ml 1 M $NaHCO_3$, the upper layer was collected and evaporated to dryness (vacuum concentrator, 30 °C, 1 mbar; Christ, Osterode, Germany). The residue was reconstituted in 200 µl EtOH, diluted 50 µl/100 µl for low concentrated fatty acids as well as 10 µl/100 µl and subsequently 40 µl/100 µl for high concentrated fatty acids.

3.3 Results and discussion

A sensitive and selective quantification of fatty acids by means of LC-MS was developed: Mass-spectrometric detection was optimized and a rapid, efficient chromatographic separation was developed. The method performance was characterized and the results were compared with those from GC-FID analysis. Finally, the method was applied to the analysis of total fatty acids as well as NEFA in plasma and in plant oils.

3.3.1 Optimization of mass spectrometric detection

Fatty acids contain a carboxy group; thus, ionization was carried out in ESI(-) mode. This leads to the formation of $[M-H]^-$ ions which were the dominating ions detected in MS full-scan experiments. The declustering potential was optimized in single ion monitoring mode of the $[M-H]^-$ ions for each fatty acid (Tab. 3.1). Monitoring of collision induced dissociation (CID) fragment spectra revealed no fragmentation for fatty acids with ≤ 3 double bonds, while for PUFA with ≥ 4 double bonds the formation of $[M-H-44]^-$ ions was observed, resulting from a decarboxylation (Fig. 3.1). The lack of detection of fragments of the linear hydrocarbon backbone is consistent with earlier reports [20]. Interestingly, for n6-PUFA

the intensity of the fragment resulted from CO_2 loss was higher compared to n3-PUFA which might be due to the spatial proximity of the double bonds to the carboxyl group in n6-PUFA. This may lead to a higher fragmentation rate through stabilization of the fragment ion charge in the unsaturated carbon chain.

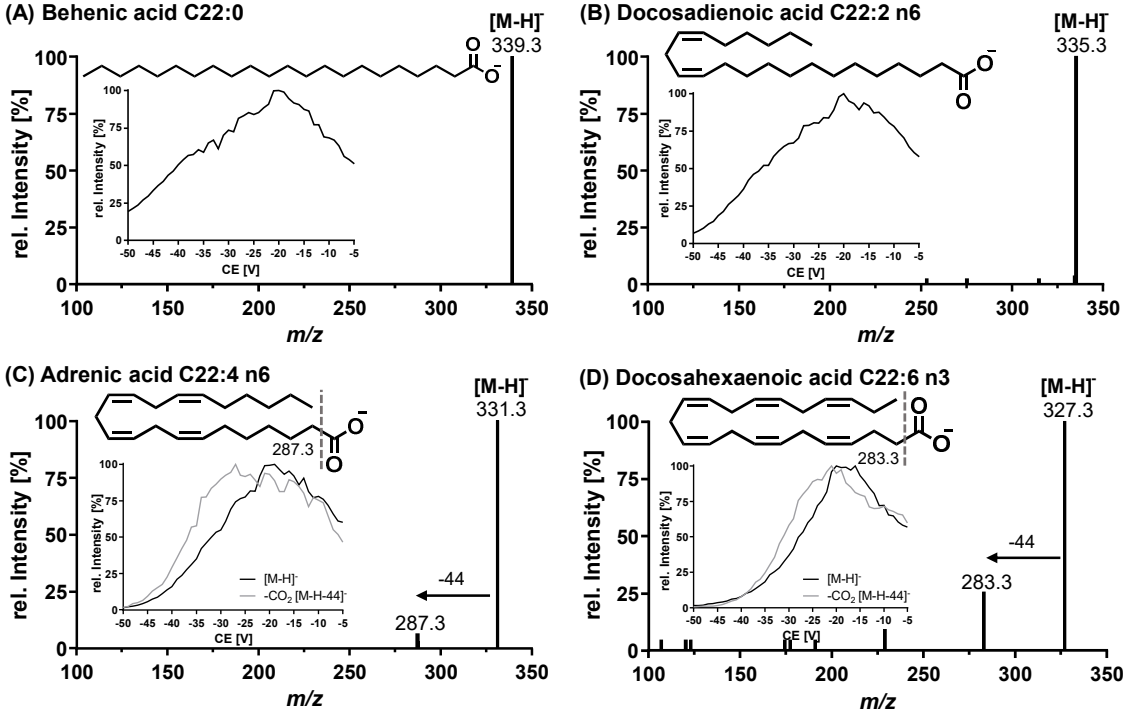

Fig. 3.1 Collision induced dissociation (CID) product spectra of $[M-H]^-$ ions of selected fatty acids with 22 carbon atoms and increasing number of double bounds. (A) Behenic acid; (B) Docosadienoic acid; (C) Adrenic acid; (D) Docosahexaenoic acid. Insert: Optimization of collision energy (CE) for *pseudo*-SRM and decarboxylation. Ionization was carried out in negative electrospray ionization mode.

Pseudo scheduled selected reaction monitoring mode (*pseudo*-SRM), i.e. isolating the *m/z* of $[M-H]^-$ ions in Q1 and Q3, was used for quantification of fatty acids which do not show fragment ions in CID. Using the highest collision energy which did not lead to a decrease of the intensity of the $[M-H]^-$ ion in *pseudo*-SRM mode, co-eluting isobaric matrix is likely to be fragmented which increases the specificity of the detection. PUFA bearing ≥ 4 double bonds were detected in *pseudo*-SRM and additionally in regular SRM mode by using transition of the loss of CO_2. However, because *pseudo*-SRM and decarboxylation are rather unspecific, chromatographic separation is crucial for isomeric fatty acids. The following critical isobaric separation pairs were identified among the biological occurring PUFA: C18:3 n3 (ALA) and n6 (GLA), C20:3 n6 (DGLA) and n9, C20:4 n3 (n3-ARA) and n6 (ARA) as well as C22:5 n3 (n3-DPA) and n6 (n6-DPA).

3.3.2 Optimization of chromatographic separation

Liquid chromatographic separation of low to moderately polar substances, including fatty acids, is commonly achieved using a C18 column [16, 20-21, 27]. Using a state-of-the-art sub-2 µm particle filled C18 reversed phase column (column 1; Zorbax Eclipse Plus, 150 x 2.1 mm, Agilent, Waldbronn, Germany) and a linear H_2O/MeOH/ACN gradient (Tab. 3.2), retention times of fatty acids were unacceptably long. Even using an optimized gradient with a long isocratic step (11 min) at 100% B, long chain fatty acids eluted at late retention times (C24:0 20.28 min). Moreover, ALA and GLA were not separated (R = 0.87). Different columns with lower hydrophobicity were tested aiming to increase the selectivity to separate isomers. ALA and GLA were the most critical separation pair, thus a gradient was chosen yielding an optimal retention factor (k) and an isocratic step at their retention time (Tab 3.2): In order to adjust the appropriate elution power (percentage B) for the isocratic step, it was calculated from the linear starting gradient at which %B ALA elutes and the isocratic step was set to this calculated percentage B minus 5%.

With a biphenyl stationary phase (column 2; Nucleoshell, 150 x 2.0 mm, 2.7 µm particle size (core-shell), Macherey-Nagel, Düren, Germany) sufficient separation of ALA and GLA (R = 0.88) could not be achieved. It seems that the π-π-interactions between the isolated double bonds of the fatty acids and the aromatic double bonds of the biphenyl phase do not provide sufficient selectivity. This is supported by the results from a second biphenyl phase (Raptor Biphenyl, 100 x 2.1 mm, 2.7 µm core-shell particle), Restek, Bad Homburg vor der Höhe, Germany). On this column with a considerably lower hydrophobicity, a separation of ALA and GLA was not possible (R = 0.95); nevertheless, despite the shorter length, the separation was comparable to that of column 2. Modification of the aromatic ring structure to pentafluorophenyl residues (column 3; PFP Kinetex, 100 x 2.1 mm, 2.6 µm core-shell particle) or linkage of a phenyl moiety to the silica gel via an ether bridge (column 4; Synergi Polar-RP, 100 x 2.0 mm, 2.5 µm particle size, both Phenomenex, Aschaffenburg, Germany) led to poor separation of ALA and GLA (R = 0.65 and 0.46, respectively).

Stationary phase	Brand Manufacturer	Column dimension: Lenght [mm]	Internal diameter [mm]	Particle size[1] [µm]	Pore size[1] [nm]	Carbon load[1] [%]	Isocratic step [%B]	$k_{C6:0}$	$FWHM_{C6:0}$ [s]	$RT_{C24:0}$ [min]	$R_{GLA/ALA}$	$R_{C20:3n9/n6}$	$R_{n6/n3\text{-}ARA}$	$R_{n6/n3\text{-}DPA}$
linear gradient[+]														
C18, doubly endcapped	ZORBAX Eclipse Plus Agilent	150	2.1	1.8	9.5	9.0	linear	5.63	7.6	-	1.18	2.63	2.21	3.39
Biphenylpropyl, multi-endcapping	Nucleoshell Macherey-Nagel	150	2.0	2.7 (core-shell)	9.0	5.2	linear	3.41	10.6	28.30	0.57	3.07	2.25	3.63
Pentaflourophenyl, TMS endcapping	Kinetex Phenomenex	100	2.1	2.6 (core-shell)	10	9.0	linear	3.19	16.7	23.50	0.51	2.29	1.75	2.73
Phenyl (ether linked), polar endcapping	Synergi Polar RP Phenomenex	100	2.0	2.5	10	11	linear	3.30	14.4	23.28	0.33	1.14	0.89	1.32
C8, hybrid silica, endcapping	Triart YMC	100	2.0	1.9	12	17	linear	6.55	9.9	28.83	1.36	3.17	2.55	3.76
C8, TMS endcapping	Kinetex Phenomenex	100	2.1	2.6 (core-shell)	10	8.0	linear	4.77	13.1	28.28	1.68	3.56	2.91	4.42
optimized gradient (isocratic steps at different percentage of B)[‡]														
C18, doubly endcapped	ZORBAX Eclipse Plus Agilent	150	2.1	1.8	9.5	9.0	90[#]	0.90	6.6	20.28	0.87	2.83	1.72	3.02
Biphenylpropyl, multi-endcapping	Nucleoshell Macherey-Nagel	150	2.0	2.7 (core-shell)	9.0	5.2	68	1.44	2.4	12.98	0.88	2.75	2.43	3.17
Pentaflourophenyl, TMS endcapping	Kinetex Phenomenex	100	2.1	2.6 (core-shell)	10	9.0	56*	2.56	2.4	11.74	0.65	2.30	1.94	2.67
Phenyl (ether linked), polar endcapping	Synergi Polar-RP Phenomenex	100	2.0	2.5	10	11	58*	2.10	3.9	11.77	0.46	0.92	1.11	1.14
C8, hybrid silica, endcapping	Triart YMC	100	2.0	1.9	12	17	71	2.36	2.4	13.26	1.25	2.76	3.35	3.69
C8, TMS endcapping	Kinetex Phenomenex	100	2.1	2.6 (core-shell)	10	8.0	66	2.36	2.4	12.96	1.88	2.89	3.21	3.68

[+]gradient: 20% B, 2 min; linear to 90% B in 24 min; to 100% B in 1 min; 100% B 4 min; back to 20% B and equilibration in 5 min

[#]gradient: 50% B, 1 min; linear to 90% B in 2 min; 90% B 6 min; to 100% B in 2 min; 100% B for 11 min; back to 50% B and equilibration in 3 min

[‡]gradient: 20% B, 1 min; in 0.5 min to the respective %B of the isocratic step; hold it for 6.5 min; linear to 100% B in 3 min; 100% B for 3 min; back to 20% B an equilibration in 1.5 min

*initial gradient condition was A/B 90/10

[1] according to manufacturer

Tab. 3.2 (previous page): Selection of a LC-column for the chromatographic separation of fatty acids. Summarized are the stationary phases of the tested analytical columns and the column dimensions. The quality of the separation and the suitability of the method was characterized by the retention factor (k) and the full peak width at half maximum (FWHM) of the first eluting fatty acid to adjust the initial gradient conditions, the retention time of the last eluting fatty acid to define the total run time and the chromatographic resolution of the isobaric analytes. ACN/MeOH/HAc (80/15/0.1; v/v/v) was used as organic eluent (B) and the aqueous eluent (A) was 0.1% acetic acid with 5% B. The flow rate was 0.3 ml/min.

A baseline separation of ALA and GLA ($R > 1.5$) could be achieved on a C8 reversed phase column with dimensions of 100 x 2.1 mm, 2.6 µm core-shell particle (Kinetex, Phenomenex, Aschaffenburg, Germany). Increasing the hydrophobicity by ethyl-bridged hybrid silica (column 5, Triart, 100 x 2.0 mm, 1.9 µm particle size, YMC, Dinslaken, Germany) failed to further improve the separation ($R_{ALA/GLA} = 1.25$).

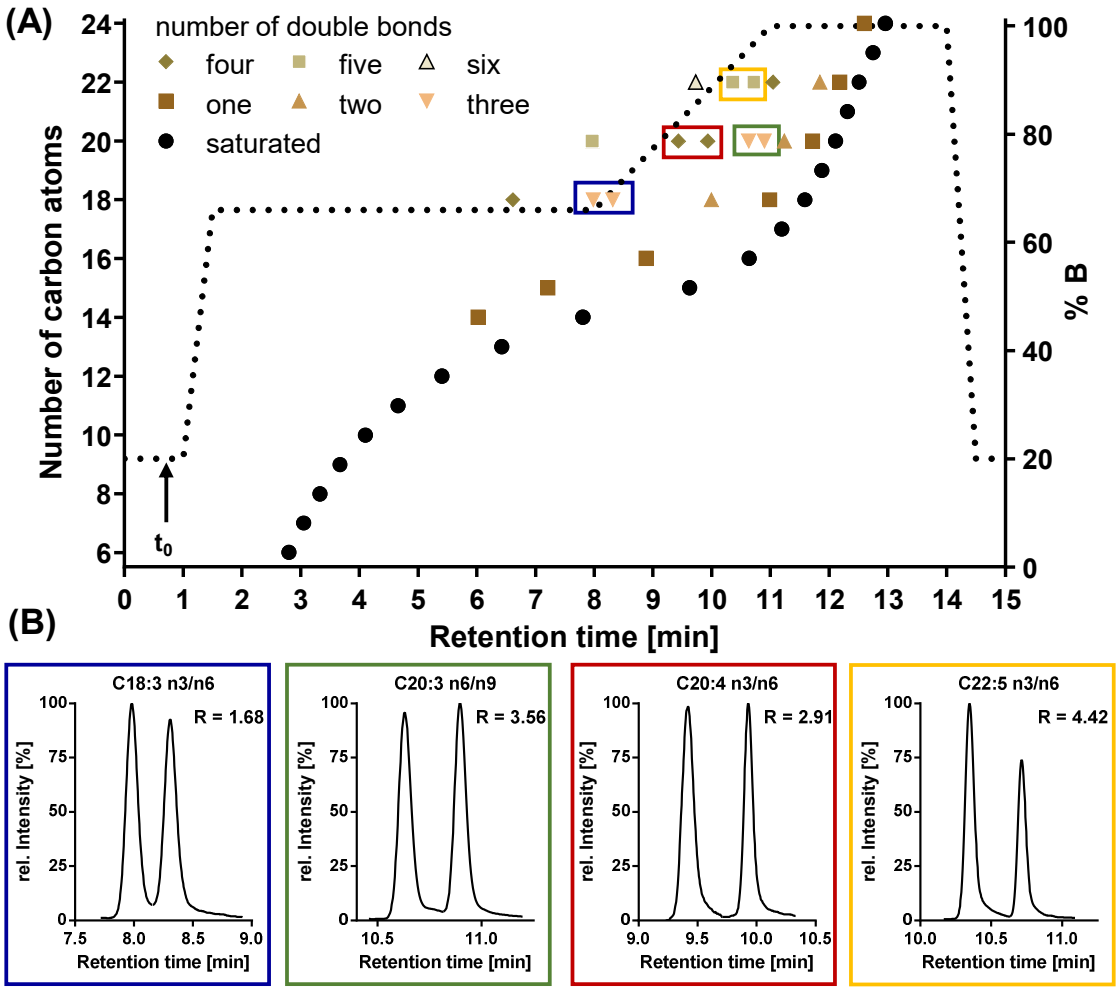

Fig. 3.2: (A) Relationship between retention time of the fatty acids and the number of carbon atoms as well as double bounds. (B) Chromatographic separation of isomeric fatty acids. Separation was carried out on an RP-8 column (2.1 × 100 mm, 2.6 µm core-shell particle, pore size 10 nm) with (**A**) a H_2O/ACN/MeOH/HAc gradient with a flow rate of 0.3 ml/min. The void volume was approx. 0.24 ml (0.8 min).

With the optimized chromatographic conditions on the Kinetex C8 column 41 fatty acids and 11 internal standards could be separated within 13.5 min (Fig. 3.2, Fig. 11.9). The saturated fatty acids eluted over the entire run time, while the retention times for unsaturated fatty acids depended on the number of double bonds (Fig. 3.2). Hu et al. and Bromke et al. also described a pronounced relationship between retention time, number of carbon atoms and number of double bonds [20-21]. C24:0 eluted last with a retention time of 12.96 min. In order to remove potentially retained non-polar matrix, the isocratic step at 100% B was held for one void volume (0.24 ml, 0.8 min). Including re-equilibration, the final run time was 15 min with highly stable retention times showing a variation (relative standard deviation, RSD) of < 0.20% or < 0.02 min for intra-batch (n = 24) and < 0.75% or < 0.07 min for inter-batch (three batches, n = 30; Tab. 3.1).

Regarding the starting conditions of the gradient, it turned out that a pre-concentration step [28] with low elution power (20% B) is required for a good separation of early eluting fatty acids. The lipophilic nature of fatty acids makes the use of the more non-polar injections solvent EtOH necessary, otherwise long-chain fatty acids are discriminated (Fig. 11.10). Without the pre-concentration step the strong elution power of the injection solvent deteriorates the peak shape of the early eluting analytes (Fig. 3.3). With a retention factor k >1 the analytes are well separated from void volume (k = 2.36 for C6, Tab. 3.2) using 20% B for the initial step. ALA and GLA are separated by a long isocratic step at 66% B (6.5 min, R = 1.88), other critical separation pairs such as n3- and n6-DPA were separated within a linear solvent gradient. Interestingly, while C20:3 n9 and n6 were easily separable (R = 2.89), C20:3 n6 and n3 as well as C18:1 n9 and n7 could not be separated on any of the tested columns. It is consistent with literature that RP-LC does not allow to separate C20:3 n6 and n3 [16, 22]. However, in our experience, C20:3 n3 and C18:1 n7 do not occur or only occur at low concentrations in biological samples [7, 10, 29]. Thus, the quantification of C20:3 n6 and C18:1 n9 in sum with the respective isomer does not seem problematic for a correct determination

of the quantitative fatty acid pattern in cells, blood, tissues and the most edible oils.

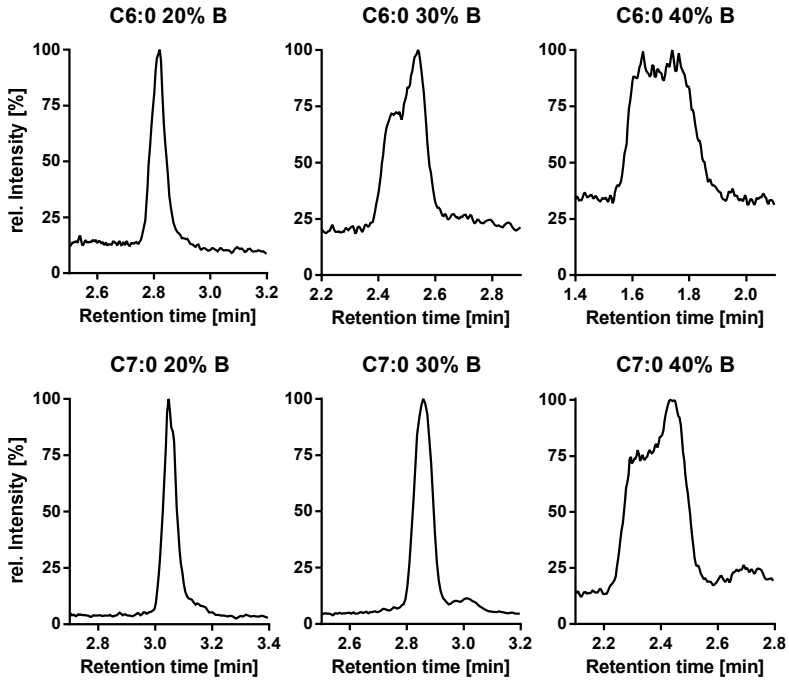

Fig. 3.3: Effect of the preconcentration step in the gradient on peak shapes of the first eluting fatty acids. Shown are 10 µl injections of a fatty acid standard (0.5 µM) in ethanol at different initial gradient conditions. The initial conditions were held for 1 min, then the % B was increased to 66% B in 0.5 min.

The method described herein is superior compared to previous LC-MS approaches. It requires no derivatization as used by several other groups [14-15, 23-24], which shortens sample preparation considerably. The total run time of 15 min allows rapid analysis. Even though a run time of around 15 min is also achieved in other methods, these methods quantify a considerably lower number of analytes (23 fatty acids [25]; 14 fatty acids [14]; 30 fatty acids [22]).

3.3.3 Sensitivity

The limit of detection (LOD) and lower limit of quantification (LLOQ) was determined according to the Guideline on Bioanalytical Method Validation of the European Medicines Agency (EMA) [30]. The LOD was set to the lowest injected standard yielding a signal-to-noise ratio (S/N) of ≥ 3 and the LLOQ was set to the lowest standard yielding a signal-to-noise ratio of ≥ 5 and an accuracy of 80-120% within the calibration curve. The LODs of the fatty acids detected by *pseudo*-SRM ranged mainly between 5-25 nM (0.01-0.08 ng on column, Tab. 3.1), whereas the LODs for the transition based on decarboxylation were higher (25-50 nM; 0.08-0.2 ng on column) due to the low intensity of the $[M-H-44]^-$ fragment. Therefore,

fatty acids bearing ≥ 4 double bounds were also quantified in *pseudo*-SRM mode. The sensitivity is consistent with earlier reported LODs, e.g. 0.02-0.1 µM (0.05-0.32 ng on column) [25] and 0.03-0.3 µM (0.1-1 ng on column) [22]. It should be noted that we used a 20-year-old middle class mass spectrometer. With state-of-the-art high resolution MS instruments such as LTQ Orbitrap Elite [16] or highly sensitive QqQ MS QTRAP 5500 [20] LODs of 1-2 orders of magnitude lower can be achieved.

The group of Hu et al. found a low LOD also for C16:0 and C18:0 (0.05 ng/ml; 0.20 nM and 0.18 nM, respectively) [20]. In our hands, blank injections and even LC-MS measurements without injection also showed peaks for C16:0 and C18:0 (Fig. 11.11, 11.12) which could not be completely reduced by using pure solvents, glassware instead of plastic and methanol as well as *iso*-propanol for washing the injection system between runs. High background signals of these fatty acids – used ubiquitously in consumer products such as plastic ware – were also described by other groups [18, 23]. For these analytes, we set the LLOQ to the concentration yielding a peak height of at least twofold of the peak height in blank injections and an accuracy within the calibration curve of 80-120% (Tab. 3.1) which was 0.5 µM for C16:0 and 1 µM for C18:0. The upper limit of quantification was set to 20 µM. By using a deuterated internal standard for each of these compounds (C16:0-d4 and C18:0-d5) ion suppression occurring at this high concentration could be compensated allowing a linear regression. (Fig. 11.11, 11.13). In order to enable simultaneous quantification of C18:1 n9, which is a main FA in biological samples, quadratic least square regression (weighting: $1/x^2$) was used. Only low carry-over was observed in the preceding injection of a high concentrated standard (Fig. 11.12). For quantification of all other fatty acids linear calibration up to 2.5 µM was used. This strategy using fatty acid specific concentration ranges of the calibration series (Tab. 3.1) allows the rapid quantification of fatty acids in biological samples with only one set of calibrators. Due to the use of a large number of isotopically labeled IS the analysis is also robust and shows high accuracy and precision.

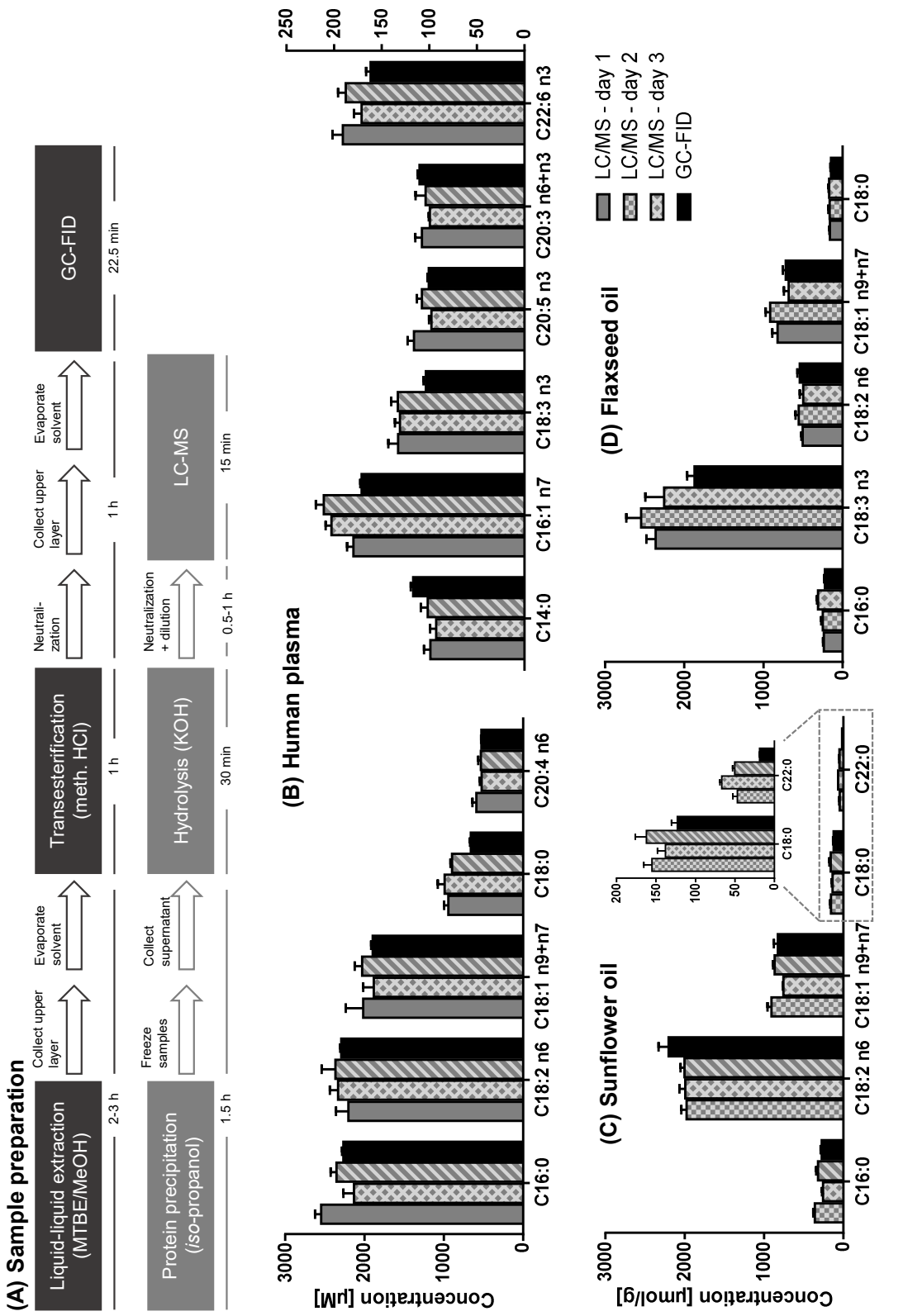

Fig. 3.4: Accuracy and precision of the method: The intra- and inter-day variability of total fatty acid concentrations as well as comparison of the LC-MS method to quantification by GC-FID in human plasma and edible oils are shown. (A) Scheme of sample preparation. Total fatty acid concentrations in (B) human plasma, (C) sunflower oil and (D) flaxseed oil determined on three days by means of LC-MS (mean ± SD, n = 3) and compared to the concentrations determined by means of GC-FID (mean ± SD, n = 3).

3.3.4 Accuracy and precision

The accuracy of the developed analytical LC-MS method was assessed by comparing the fatty acid concentrations in plasma and plant oils with those obtained by a validated GC-FID analysis (Fig. 3.4), which can be considered the gold standard of fatty acid analysis. The use of GC-FID provides an orthogonal quantification which is not dependent on standard concentrations due to the mass-sensitive detector allowing to deduce absolute concentration based on one reference compound. Sample preparation for gas chromatographic determination included lipid extraction with methanol/methyl *tert*-butyl ether (MTBE) and trans-esterification to FAME [12], whereas for LC analysis, the samples were diluted with *iso*-propanol and the supernatant after centrifugation was directly hydrolyzed (Fig. 3.4 A) [31]. The major fatty acids quantified in the plasma were C16:0, C18:2 n6 and C18:1 n9+n7, followed by C18:0 and C20:4 n6 as previously described for healthy subjects [7, 32]. The main n3-PUFA were ALA, EPA and DHA having concentrations of around 150 µM (Fig. 3.4 B). Given the difference in sample preparation, both methods showed an excellent match of the determined concentrations (agreement 80-120%, Fig. 3.4 B). Only for C18:0 slightly higher concentrations are obtained by means of LC-MS, presumably because of its high background signal. Differently diluted hydrolyzed plasma samples show that the matrix leads only to low or no ion suppression allowing robust quantification of the fatty acyls in biological samples (Fig. 11.14).

In plant oils similar levels of total fatty acids were found following quantification by means of GC-FID or LC-MS (Fig. 3.4 C, D). For the main fatty acids in flaxseed oil or sunflower oil, the concentration agreement was also good (70-130%, Fig. 3.4 C-D). Therefore, we conclude that the simple sample preparation by dilution with *iso*-propanol and saponification is suitable for the quantification of fatty acids in protein-rich matrices as well as in fatty matrices. It should be noted that more fatty acids could be quantified by LC-MS than by GC-FID due to the higher sensitivity of the LC-MS method. The LLOQ of the LC-MS measurement is ≤ 75 nM for almost all fatty acids, whereas it is more than one order of magnitude higher for the GC analysis. This made it possible to quantify for example

C22:5 n6 in plasma or C22:1 n9 in flaxseed oil allowing to gain a more comprehensive picture of the fatty acid pattern of biological samples and plant oils. It should be noted that GC-(MS) following transesterification on the one hand allows the simultaneous detection of a more comprehensive set of fatty acids compared to LC-MS and on the other hand electron ionization MS provides more structural information based on fragments. For example, Lisa et al found 81 fatty acyls in animal fats including also branched and *trans*-isomers [33]. However, if only major fatty acids are of interests as it is the case in numerous studies of modern life science, LC-MS is in our hands the method of choice because of the rapid sample preparation and analysis.

In order to evaluate the precision of the analytical procedure, human plasma samples as well as two edible oils with different fatty acid pattern were analyzed on three separate days (inter-day variance Fig. 3.4, n = 3). The intra-day variability was assessed by calculating the RSD on each single day (n =3, Tab. 11.2). Both parameters were lower than 15% for almost all fatty acids in plasma and plant oils and thus meet the criteria required by the EMA guideline [30] demonstrating a high precision of the developed method. Only long chain saturated and monounsaturated fatty acids such as C20:0 and C20:1 n9 showed in part higher variations. Interestingly, the results using transitions based on decarboxylation resulted in higher RSD, e.g. 15% for C22:5 n3 vs. 5% in *pseudo*-SRM, which might be explained by low intensity of the $[M-H-44]^-$ fragment and thus low peak heights. Therefore, quantification should be carried out by *pseudo*-SRM mode and the second transition can additionally be used for confirmation.

The determination of fatty acids by LC-MS offers a much faster and more sensitive method than GC-FID analysis. We could show that the method leads to consistent and precise results. The easy and rapid sample preparation via direct saponification in *iso*-propanol is not only fast but allows the simultaneous total oxylipin determination (Tab. 11.3) [31]. Thus, our approach makes it possible to analyze both oxidized fatty acids and their precursors from a single sample preparation.

3.3.5 Non-esterified fatty acids in plasma and edible oils

Concentrations of NEFA were determined in plasma (Tab. 11.4) and virgin, cold pressed sunflower oil as well as refined sunflower oil (Fig. 3.5) using the described LC-MS method. In plasma, NEFA could be directly analyzed after dilution of the sample with *iso*-propanol. The obtained concentrations (Tab. 11.4) were in good agreement with the results described by other groups [16-17]. The accuracy of the measurement was supported by the standard addition procedure using ARA resulting in a concentration of 2.0 µM in plasma, compared to 2.1 ± 0.1 µM by external calibration (Fig. 11.15). This underlines the robustness of the method against matrix effects and demonstrates that the method allows the reliable quantification of three parameters from a single sample preparation: Quantification of total fatty acids, NEFA and total oxylipins.

For quantification of NEFA in plant oils the excess of triacylglycerols was reduced by SPE using aminopropyl cartridges as described [13, 34]. As expected, the concentrations of NEFA were considerably lower in refined sunflower oil than in virgin sunflower oil, since the NEFA are removed in the deacidification step of the refining process [35]. C18:2 n6 was the fatty acid with the highest concentration, followed by C18:1 n9+n7, C16:0 and C18:0. Thus, the concentrations of NEFA represent the total fatty acid distribution (Fig. 3.4 C). Because of the sensitivity of the method, we could detect low-concentrated fatty acids such as C22:1 n9, which often could not be reported [36-37]. Due to the direct analysis of the NEFA by LC-MS, our method does not require derivatization for GC analysis, where different derivatization strategies such as esterification [38], silylation [36] or dimethyl-amidation [39] are used. The targeted approach allows quantification of NEFA even in the presence of triacylglycerols that may not have been completely removed by the SPE. Thus, the presented LC-MS method herein allows the reliable quantification of NEFA in biological samples, which is of pivotal importance for the characterization of both edible oils [35, 40] as well as biological samples such as plasma [2, 41].

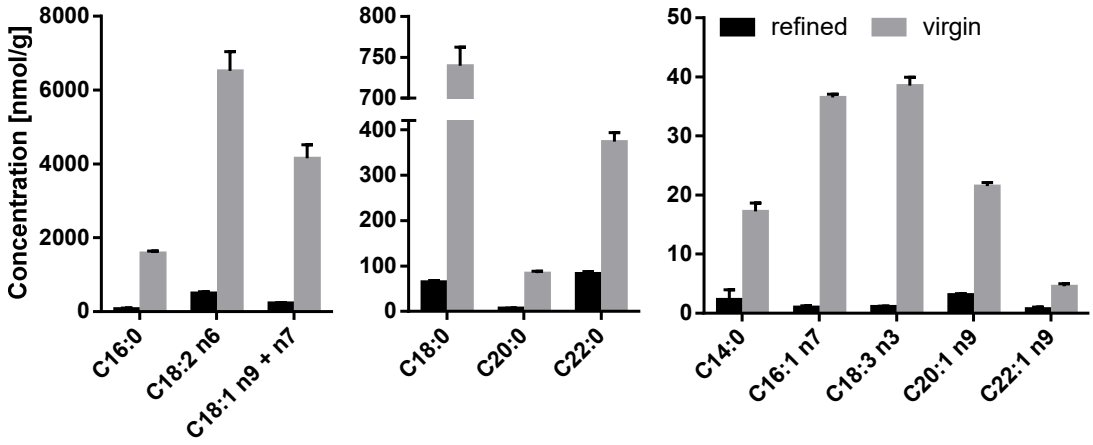

Fig. 3.5 Concentration of non-esterified fatty acids in virgin and refined sunflower oil determined by LC-MS. 10 mg sunflower oil were dissolved in chloroform/*iso*-propanol (2/1, v/v) and triacylglycerols were removed by solid phase extraction on aminopropyl cartridges. Refined and virgin oils were obtained from a local supermarket and analyzed in triplicate (mean ± SD).

3.4 Conclusion

A new LC-MS method for the quantification of fatty acids in biological samples was developed. Using an optimized C8 reversed phase column, 41 fatty acids and 11 isotopically labeled fatty acids as internal standards could be separated within a total run time of only 15 min. Despite using a rather old, middle class QqQ MS, the method is sensitive with a LLOQ of 10-75 nM for most fatty acids. The low inter-day and inter-operator variability of < 20% indicates a high precision of the method. The concentrations of fatty acids determined by LC-MS in plasma and plant oils are consistent with those of a GC-FID analysis ensuring accurate and comparable results by the developed method. Major strength of the approach is the rapid sample preparation by hydrolysis and dilution allowing high sample throughput. Moreover, the analysis can be combined with the analysis of PUFA oxidation products (eicosanoids and other oxylipins) [31]. Finally, LC-MS analysis allows to quantify NEFA in presence of triacylglycerols which is of pivotal importance for the analysis of biological samples and plant oils.

3.5 References

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Chapter 4

Comprehensive analysis of fatty acid and oxylipin patterns in n3-PUFA supplements *

*Supplementing long-chain omega-3 polyunsaturated fatty acids (n3-PUFA) improves health. We characterized the pattern of total and non-esterified oxylipins and fatty acids in n3-PUFA supplements made of fish, krill or micro-algae oil by LC-MS. All supplements contained the declared amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); however, their content per capsule and the concentration of other fatty acids varied strongly. Krill oil contained the highest total n3-oxylipin concentration (6000 nmol/g) and the highest degree of oxidation (EPA 0.7%; DHA 1.3%), while micro-algae oil (*Schizochytrium *sp.) showed the lowest oxidation (< 0.09%). These oils contain specifically high amounts of the terminal hydroxylation product of EPA (20-HEPE, 300 nmol/g) and DHA (22-HDHA, 200 nmol/g) which can serve as an authenticity marker for micro-algae oil. Refined micro-algae and fish oil were characterized by NEFA levels of ≤ 0.1%. Overall, the oxylipin and fatty acid pattern allows gaining new insights into the origin and quality of n3-PUFA-rich oils in supplements.*

* modified from Koch, E.; Kampschulte, N.; Schebb, N. H. Comprehensive Analysis of Fatty Acid and Oxylipin Patterns in n3-PUFA Supplements. J Agric Food Chem 2022, 70 (13), 3979-3988.

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4.1 Introduction

The western diet is characterized by a high ratio of n6-polyunsaturated fatty acids (PUFA) to n3-PUFA of up to 15:1 [1]. This is mainly due to the high consumption of linoleic acid (LA) from vegetable oils such as soybean, sunflower or corn oil [2], while the intake from oil containing the n-3 PUFA α-linolenic acid (ALA, 18:3 n3) such as flaxseed oil is low [3]. Particularly, the supply with long-chain n3-PUFA, namely eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) derived from marine sources, is inadequate for large parts of the western population [1, 4]. EPA and DHA are essential for human physiology and DHA is for example a main fatty acid in the human brain [5]. An appropriate supply has thus been shown to reduce the risk of diseases being best investigated but not limited to coronary heart disease [4-5] as a recent study demonstrates a reduction in all causes of premature death by efficient n3-PUFA supply [6].

PUFA can be oxidized in the human body by the enzymes of the arachidonic acid (ARA) cascade to a complex pattern of products yielding eicosanoids and other oxylipins [7-8]. The catalytic activity of cyclooxygenase leads to the formation of prostanoids such as prostaglandins – e.g. prostaglandin E2, a potent mediator of pain, fever and inflammation – and thromboxanes which play a key role in the blood coagulation cascade [7, 9]. Lipoxygenases convert PUFA to regio- and stereospecific hydro(pero)xy PUFA [10]. For several of EPA and DHA derived hydroxy- as well as multiple hydroxylated PUFA, anti-inflammatory/pro-resolving properties are described [11]. Cardioprotective epoxy-PUFA are formed in the third branch of the ARA cascade by cytochrome P450 monooxygenases (CYP) [12] which also catalyze ω/(ω-n)-hydroxylation leading e.g. to the vasoconstrictory oxylipin 20-hydroxyeicosatetraenoic acid (HETE) [13]. There is a growing body of evidence that epoxy-PUFA are involved in e.g. angiogenesis, vasodilation and hypertension [13-15] and the EPA-derived 17(18)-epoxyeicosatetraenoic acid (EpETE) is a potent anti-arrhythmic mediator which served as a lead for the development of drugs against atrial fibrillation [16]. Epoxy-PUFA are rapidly metabolized by soluble epoxide hydrolases to the corresponding vicinal

dihydroxy-PUFA which are less bioactive [17-18]. Autoxidation not only gives rise to a variety of oxidized PUFA and via initial formation of hydroperoxides various hydroxy-PUFA [19-20], but also epoxy-PUFA [21] as well as prostaglandin-like isoprostanes and isofuranes are formed [20]. Consumption of higher concentrations of n3- compared to n6-PUFA in the diet results in replacement of ARA by EPA and DHA in cell membrane phospholipids [22-25]. This reduces pro-inflammatory lipid mediator precursors on the one hand [23, 26-27] and increases the formation of cardioprotective and anti-inflammatory/pro-resolving lipid mediators derived from n3-PUFA on the other hand [23, 27-28]. EPA and DHA can also exhibit direct biological activity by modulating membrane fluidity or directly interacting with receptors [29].

Biochemically, humans can convert ALA to long-chain PUFA by two elongases and action of desaturases; however, the synthesis of EPA and DHA for subjects on a western diet is very low [30-31]. For example, using [^{13}C]ALA, Hussein et al. showed that the conversion of ALA to EPA was only about 0.3%, while [^{13}C]DHA was detectable only in traces [32]. For an adequate supply of n3-PUFA, sufficient dietary intake of DHA and EPA is thus indispensable. Besides consuming at least 1-2 servings of oily fish per week such as salmon or mackerel [33-34], there are numerous n3-PUFA supplements available on the market. The n3-PUFA-rich oils in the capsules are obtained from marine sources being rich in EPA and DHA: micro-algae, krill or fish. The raw material for fish oil is obtained from fish e.g. caught for fishmeal, bycatch or cutting from fishes for food use [35]. Cooking of the fish material denaturates the proteins and allows the oil to be pressed off. After separation from water, the crude fish oil is obtained which is subjected to an oil refining process. To concentrate the n3-PUFA, the bound fatty acids are converted into ethyl esters (EE) which are fractionated using urea complexation, molecular distillation and chromatography [35-37]. For medical products, EE are often used directly, while the EE are re-esterified to triacylglycerols (rTG) for food supplements [38]. Several studies have shown that the bioavailability of EE is lower compared to fatty acid bound in (r)TG or phospholipids (PL) [39-40]; however, this effect is minor compared to whether the products are consumed together with a

fat-rich meal or not [40-42]. Micro-algae oil is considered to be a more sustainable alternative for n3-PUFA supplementation due to overfished stocks and global peak production of fish oil [43-44]. For EPA- and DHA-rich oil, micro-algae cells from the genera *Schizochytrium* grown in fermenters are disrupted and the extracted oil is subjected to an oil refining process as used for other plant oils [43-44]. Due to its high content of EPA and DHA of at least 10% and 22% [45], respectively, further enrichment of the PUFA is not necessary. Accordingly, in micro-algae oil triacylglycerols occur in their natural fatty acid distribution [38]. There might be evidence that the bioavailability of rTG after hydrolysis to monoacylglycerols and non-esterified fatty acids in the human gastrointestinal tract is higher compared to naturally occurring TG [40-41]. Data is similarly ambiguous regarding the bioavailability of krill oil which is a non-refined oil being rich in PL and thus considered particularly efficient [39-41]. Krill oil is the less processed product among n3-supplements. It is obtained from Antarctic krill (*Euphausia superba*) by solvent or supercritical carbon dioxide extraction without additional refining containing a high content of PL and astaxanthin [46].

The quality and lipid composition of n3-PUFA supplements have been investigated in several studies [38, 47-50]. However, in previous studies, it was only investigated, whether the amount of EPA and DHA corresponds to the declaration of the manufacturer, in which lipids the n3-PUFA are bound or if the oxidation level determined by peroxide value and anisidine value indicates rancidity of the oils. With some exceptions, the measured EPA and DHA content matches the declared or legally demanded content [48-49]. For the peroxide value, studies report varying results. While De Boer et al. and Bannenberg et al. report about 14% of the supplements studied had PV greater than the maximum limit of 5 meq O_2/kg [48, 50-51], in the study by Albert et al. this was the case in 83% of the samples [47]. However, the peroxide value is a sum parameter for the actual reactive species and provides no information about the composition of the oxidized and non-oxidized PUFA. In a few studies, oxylipins have already been analyzed in algae oils and supplements, e.g. ALA- and LA-derived oxylipins in “high-stability” as well as “omega-9” algae oil [52] and oxylipins in four fish oil- and one algae oil-

based supplements [53]. However, the number of studied supplements, covered oxylipins and n3-sources of supplements was limited and no reports are available about the levels and pattern of both non-esterified fatty acids and oxylipins in n3-PUFA supplementation products.

The content of EPA and DHA, their oxidation products, the presence of other fatty acids and the concentrations of non-esterified fatty acids i) are relevant parameters in the assessment of quality, ii) provide information on the production of n3-rich oils and iii) allow to evaluate authenticity. Therefore, we investigated in the present study 11 different supplements based on fish, micro-algae and krill oil regarding their total and non-esterified fatty acid (NEFA) and oxylipin pattern using state-of-the-art liquid chromatography-mass spectrometry (LC-MS) methods [54-56] and comprehensively describe their composition.

4.2 Experimental

4.2.1 n3-PUFA supplementation products

Eleven different n3-PUFA supplements comprising two different krill oils, three micro-algae oils and six fish oil-based products were investigated in this study. Supplements were selected to fulfill the following criteria: i) from different marine sources, ii) fatty acids bound in different lipid classes, iii) different levels of declared EPA and DHA concentration, iv) different prices and v) sold by companies which are well established on the German market. Samples were obtained from local drug stores or pharmacies in Wuppertal, Germany: Mivolis Premium Omega-3 krill oil (KO1; dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany), Doppelherz System Antarktis krill oil (KO2; Queisser Pharma, Flensburg, Germany), Altapharma Omega-3 micro-algae oil (AO1; Dirk Rossmann GmbH, Burgwedel, Germany), Omega3-Loges micro-algae oil (AO2; Dr. Loges + Co. GmbH, Winsen (Luhe), Germany), Doppelherz System Omega-3 micro-algae oil (AO3; Queisser Pharma, Flensburg, Germany), Mivolis Omega-3 1000 fish oil (FO1; dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany), Doppelherz aktiv Omega-3 1400 fish oil (FO2; Queisser Pharma, Flensburg, Germany),

tetesept Omega-3 1000 fish oil (FO3; tetesept pharma GmbH, Frankfurt am Main, Germany), Doppelherz System Omega-3 Konzentrat fish oil (FO4; Queisser Pharma, Flensburg, Germany), Doppelherz aktiv Mama plus+ fish oil-based product (M+; Queisser Pharma, Flensburg, Germany), SPM Active (fractionated marine lipid concentrate; SPM; Metagenics, Aliso Viejo, CA, USA; bought in a local drug store in California). The capsules were cut with a scalpel and the oils were transferred to glass vials. The analysis was carried out in triplicate. For each replicate a new capsule was used. The fat content of one capsule according to the manufacturer's declaration is shown in Tab. 4.1. In KO2 and M+ the total fatty acid content was determined by GC-FID.

4.2.2 Chemicals

Iso-propanol, diethyl ether, methanol (MeOH) and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile (ACN), chloroform and ethanol (EtOH) were obtained from VWR (Darmstadt, Germany). Ultra-pure water was generated using the 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 concentrations were used as described [54-55].

4.2.3 Quantification of total oxylipin and total fatty acid concentrations

Total oxylipin concentrations and total fatty acid concentrations were determined as described [54-56]. In brief, 5 mg of the capsule content was diluted with 2 ml *iso*-propanol. To 50 µl of this solution, 350 µl of *iso*-propanol, 10 µl of butylated hydroxytoluene (BHT) as antioxidant, 50 µl of water and 10 µl of deuterated oxylipin internal standard (IS) solution containing $^{2}H_4$-6-keto-$PGF_{1\alpha}$, $^{2}H_4$-8-*iso*-$PGF_{2\alpha}$, $^{2}H_4$-$PGF_{2\alpha}$, $^{2}H_5$-RvD2, $^{2}H_{11}$-8,12-*iso*-i$PF_{2\alpha}$-VI, $^{2}H_5$-LxA_4, $^{2}H_5$-RvD1, $^{2}H_4$-PGB_2, $^{2}H_4$-LTB_4, $^{2}H_4$-9,10-DiHOME, $^{2}H_{11}$-11,12-DiHETrE, $^{2}H_6$-20-HETE, $^{2}H_4$-13-HODE, $^{2}H_4$-9-HODE, $^{2}H_8$-15-HETE, $^{2}H_3$-13-oxo-ODE, $^{2}H_8$-12-HETE, $^{2}H_8$-5-HETE, $^{2}H_4$-12(13)-EpOME, $^{2}H_{11}$-14(15)-EpETrE, $^{2}H_7$-5-oxo-ETE and $^{2}H_{11}$-8(9)-EpETrE were added. 100 µl of potassium hydroxide in MeOH/H_2O were added and samples were hydrolyzed (60 °C for 30 min). After neutralization with 20 µl

of 25% HAc in water, samples were loaded onto pre-conditioned solid phase extraction (SPE) cartridges (C8/anion exchange, bed weight 300 mg, volume 3 ml, Bond Elut Certify II, Agilent, Waldbronn, Germany). Samples were washed with one column volume of water and one column volume of MeOH/H_2O (50/50, *v*/*v*). Cartridges were dried with vacuum (-200 mbar) for 30 sec. Oxylipins were eluted with 2 ml of acidified ethyl acetate/*n*-hexane and the solvent was removed using a vacuum concentrator (Christ, Osterode am Harz, Germany). Samples were reconstituted in IS 2 in MeOH and 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). A Zorbax Eclipse plus C18 reversed-phase column (150 x 2.1 mm ID, particle size 1.8 µm; Agilent, Waldbronn, Germany) and a binary gradient was used for chromatographic separation. Solvent A was 0.1% HAc with 5% B and solvent B was ACN/MeOH/HAc (80/15/0.1, *v*/*v*/*v*). The flow rate was 0.3 ml/min. The gradient was as follows: 0 min 21% B, 1 min 21% B, 1.5 min 26% B, 10 min 51% B, 19 min 66% B, 25.1 min 98% B, 27.6 min 98% B, 27.7 min 21% B, 31.1 min 21% B. Detection was carried out in scheduled selected reaction monitoring mode (SRM) following negative electrospray ionization (ESI(-)). External calibration with IS was used for quantification [54, 56]. For quality control, the recovery of the IS in each sample was monitored using a set of IS 2 as described in detail here [54]. The acceptance criterion for all IS was a recovery above 50%. Moreover, a quality control oil was prepared in triplicate to assure efficient and reproducible sample preparation.

Total fatty acid 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. A Kinetex C8 core-shell reversed-phase column (100 x 2.1 mm ID, particle size 2.6 µm; Phenomenex, Aschaffenburg, Germany) and a binary gradient was used for chromatographic separation. Solvent A was 0.1% HAc with 5% B and solvent B was ACN/MeOH/HAc (80/15/0.1, *v*/*v*/*v*). The flow rate was 0.3 ml/min. The gradient was as follows: 0 min 20% B, 1 min 20% B, 1.5 min 66% B, 8 min 66%

B, 11 min 100% B, 14 min 100% B, 14.5 min 20% B, 15 min 20% B. Detection was carried out in *pseudo*-SRM following ESI(-). External calibration with IS was used for quantification [55].To calculate the % of total fatty acid oxidation, the sum of all total oxylipins covered by the method from one precursor fatty acid was divided by the total concentration of the precursor fatty acid.

4.2.4 Quantification of non-esterified oxylipin and NEFA concentrations

NEFA concentrations were determined as described [55]. In brief, 10 mg of the capsule content were dissolved in 1 ml $CHCl_3$/*iso*-propanol and BHT was added. Samples were loaded on pre-conditioned normal phase SPE cartridges (Supelclean LC-NH_2, bed weight 100 mg, volume 1 ml, Merck, Darmstadt, Germany). Triacylglycerols were removed with 2 ml $CHCl_3$/*iso*-propanol and NEFAs were eluted with diethyl ether/HAc (98/2, *v*/*v*). The eluates were neutralized with 1 M $NaHCO_3$ and the upper phase was evaporated to dryness in the vacuum concentrator. The residue was dissolved in EtOH, diluted according to the expected NEFA concentration and analyzed by LC-MS as described above. Non-esterified oxylipins were prepared in the same way using only 5 mg of the oils and MeOH + 1% HAc for elution. The eluate was directly evaporated in the vacuum concentrator, reconstituted with IS 2 in MeOH and analyzed by LC-MS. For the quantification of non-esterified oxylipins in the krill oil samples, the SPE protocol with the Bond Elut Certify II cartridges was used as described above to remove phospholipids. For quality control, the recovery of the IS in each sample was monitored using a set of IS 2 as described in detail here [54]. The acceptance criterion for all IS was a recovery above 50%. Moreover, a quality control oil was prepared in triplicate to assure efficient and reproducible sample preparation.

To calculate the % NEFA of total fatty acids, the NEFA concentration was divided by the total fatty acid concentration. For calculation of non-esterified fatty acid oxidation ratio in %, the sum of all non-esterified oxylipins covered by the method from one precursor fatty acid was divided by the precursor NEFA concentration. The value was expressed in percentage.

	Total fat per capsule[a] [mg]	Recommended number of capsules per day[a]	EPA determined [mg/capsule]	EPA declared[a] [mg/capsule]	EPA per day[c] [mg]	DHA determined [mg/capsule]	DHA declared[a] [mg/capsule]	DHA per day[c] [mg]
AO1	500	1	96 ± 8	*250*[b]	96	155 ± 8	*250*[b]	155
AO2	640	2	129 ± 4	96	258	187 ± 2	192	374
AO3	575	2	110 ± 20	100	220	210 ± 40	200	420
FO1	1000	**1**-2	167 ± 6	160	167	103 ± 5	100	103
FO2	1400	1	269 ± 9	285	269	167 ± 8	190	167
FO3	1000	1	170 ± 20	180	170	110 ± 10	120	110
FO4	900	1	360 ± 30	300	360	260 ± 30	200	260
KO1	525	2	70 ± 4	63	140	34 ± 1	31	68
KO2	420[d]	**2**-3	56 ± 3	60	112	26.0 ± 0.4	28	52
SPM	250	2	49 ± 7	not specified	98	76 ± 9	not specified	152
M+	340[d]	2	20 ± 1	not specified	40	103 ± 9	100	206

[a] according to manufacturer's information
[b] only sum of EPA + DHA is indicated by the manufacturer
[c] calculated by the number of capsules per day as recommended by the manufacturer (see column 3)
[d] total fatty acid concentration in one capsule was determined following extraction of the capsule content with toluene and analyzed by GC-FID due to the presence of non-lipid ingredients in the capsule

Tab. 4.1: Amount of EPA and DHA in the n3-PUFA supplements and specifications according to the manufacturer. Shown are the total fatty acid concentrations of EPA and DHA in the liquid content of the different capsules determined by LC-MS following hydrolysis (mean ± SD, n = 3). The amount of EPA and DHA per capsule was calculated based on manufacturer's information on total amount of oil in one capsule.

4.3 Results and discussion

The concentrations of total and non-esterified oxylipins as well as fatty acids were determined in different n3-PUFA supplements produced from fish oil, micro-algae oil or krill oil. The obtained comprehensive data set allows drawing conclusions about the manufacturing practices and the quality of the products.

4.3.1 Fatty acid pattern of fish, micro-algae and krill oil-based supplements

The micro-algae oil products show a simple fatty acid profile consisting mainly of C16:0, C18:0, C18:1 n9+n7, n3-docosapentaenoic acid (n3-DPA) and EPA as well as DHA (Fig. 4.1) with a ratio of DHA/EPA of around 1.6. In minor concentrations (10-20 mg/g), C14:0, LA, ARA and n6-DPA were detectable (Tab. 11.5). All suppliers state that they use oils from the micro-algae *Schizochytrium* sp. which is an approved novel food in the EU [57]. (Micro-)Algae and phytoplankton are the main biological sources of long-chain n3-PUFA and the different species differ considerably regarding their PUFA content as a few strains predominantly produce EPA, while in others, DHA is the dominating fatty acid [58-59]. The large genus of *Schizochytrium* sp. are predominantly DHA-forming species that contain only about 1% EPA, besides 27-50% DHA and 11-20% n6-DPA [60-63]. Using non-genetic modification strategies, different *Schizochytrium* sp. strains have been developed and registered as novel food [44, 57]. Among those, Martek Biosciences Corporation, which is today a part of DSM Nutritional Products, developed one strain that produces EPA at a concentration about half that of DHA [44]. This is the actual pattern of all three micro-algae products found in this study (Fig. 4.1, Tab. 11.5). Based on this and the almost identical PUFA concentrations, it can be concluded that all companies use the same *Schizochytrium* sp. oil as ingredient for their micro-algae products. Alternatively, the identical fatty acid pattern might be explained by the strictly controlled conditions under which the micro-algae grow in large-scale fermentation vessels [43, 64-65]. This would indicate that on the one hand, the product is independent of environmental or geographical factors

leading to constant specifications of the obtained oils and on the other hand that the fatty acid profile can be precisely controlled [65].

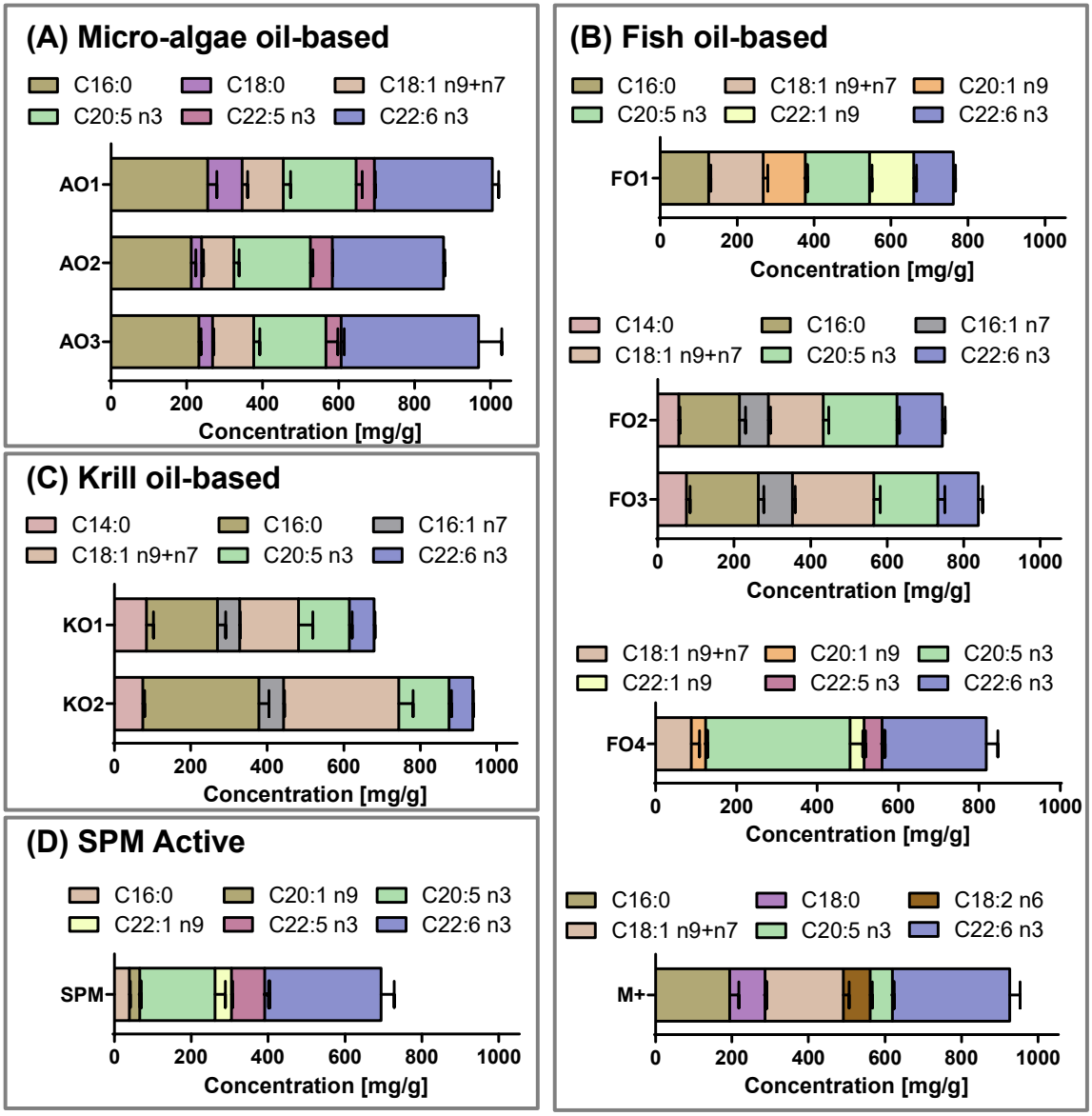

Fig. 4.1: Total fatty acid distribution in the supplements. Shown are the concentrations of the main fatty acids in the tested supplements. Fatty acids were analyzed by LC-MS following hydrolysis (mean ± SD, n= 3).

The fish oil-containing capsules show distinct differences in the fatty acid pattern. While FO2 and FO3 had a comparable pattern with the highest concentration for C14:0, C16:0, C16:1 n7, C18:1 n7+n9, EPA and DHA, for FO1, high amounts of C20:1 n9 and C22:1 n9 were determined (Fig. 4.1, Tab. 11.5). FO4 contained high concentrations of EPA and DHA and low levels of other fatty acids such as C18:1 n7+n9, C20:1 n9, C22:1 n9 and n3-DPA. The marketing as "concentrate"

can therefore be considered appropriate. The DHA/EPA ratio is similar for all fish oil products at around 0.6. The differences in the fatty acid compositions of fish oils can be explained by the different species of fish used as well as by e.g., the geographical location or the climate [66-67]. Moreover, fish oil production is technologically more complex than that of micro-algae oil products which are used directly after refinement. For the production of n3-PUFA-rich fish oils, the n3-PUFA are concentrated including transesterification to EE, urea complexation, short-path distillation, chromatography and re-esterification to TG [35, 37]. While short-path distillation separates short-chain from long-chain EE based on different volatility, urea complexation allows to remove saturated and mono-unsaturated fatty acids from PUFA [36-37, 68]. It can therefore be speculated that FO2 and FO3 were subjected to urea complexation, while FO1 was dominantly concentrated by short-path distillation. However, it should be noted that combinations of processes are also applied, as is presumably the case with FO4. Although the production of fish oil capsules is a multi-step process that ends with the concentration of specific fatty acids, the overall fatty acid pattern is surprisingly little standardized. This is consistent with an earlier study showing a broad spectrum of fatty acid distribution [53]. However, the same groups of fish oil (Fig. 4.1, Tab. 11.5) can be found in the previous study as well: fish oil containing i) almost exclusively EPA and DHA, ii) relevant amounts of C14:0 (8%) and C16:0 (19% [53]; in our study: 6% and 17%, respectively in FO2/FO3) or iii) comparable concentration of EPA (12% [53] vs. 17% in our study), DHA (13% [53] vs. 11%), C20:1 n9 (11% [53] vs. 11%) and C22:1 n9 (7% [53] vs. 12%). It seems that this reflects the different production technologies for the concentration of n3-PUFA from fish oil.

The main fatty acids in krill oil were C14:0, C16:0, C16:1 n7, C18:1 n7+n9, EPA and DHA with a DHA/EPA ratio of around 0.5 (Fig. 4.1, Tab. 11.5). Compared to other supplements, the concentration of EPA and DHA in krill oil was much lower. In addition to the main fatty acids, krill oil contains a wide range of other fatty acids. This can be explained by the diverse lipid fractions being present in krill oil compared to the refined natural TG or re-esterified TG in plant and fish oil: the

lipid fraction of krill oil supplements consists of PL (33-46%), TG (46-63%) and NEFA (3-7%) [38]. The fatty acid pattern of the krill oil is very comparable between the two manufacturers suggesting that both companies use the same krill oil as ingredient.

Based on the n3-PUFA content, all products contribute to the supply of these essential food ingredients. However, taking into account the consumption recommendation, different daily PUFA intakes result for the different products. The German Nutrition Society (DGE) recommends a daily intake of 250 mg EPA and DHA for the prevention of coronary heart disease [69]. Similarly, the American Heart Association and the *Dietary Guidelines for Americans 2020-2025* recommend two servings of (oily) fish per week, resulting in at least 250 mg EPA and DHA per day [70-72]. Four n3-PUFA supplements clearly exceed this value, five are in the range of 250 mg and the two krill oils are below these recommendations. One could argue that the krill oil products might be more efficient due to the potentially higher bioavailability of PL [40]. However, it should be noted that for efficient absorption of EPA and DHA, the ingestion together with a (high-fat) meal has a much stronger effect than the n3-PUFA binding form [40-42]. Thus, the krill oil products might not contain enough n3-PUFA for an efficient supplementation. During pregnancy, the need for DHA is particularly high, as DHA is important for the development of the brain as well as the neural and visual system of the fetus [73-74]. The European Food Safety Authority (EFSA) recommends that pregnant and lactating women consume 350-450 mg of DHA per day [75]. However, following the recommended intake of the M+ product, pregnant and lactating women consume a maximum of 200 mg of DHA per day. This is already too low for the general population and could lead to a massive undersupply of n3-PUFA during pregnancy and lactation if not enough fatty cold-water fish is consumed.

Overall, all products contain a relevant amount of n3-PUFA consistent with manufacturer's declaration. The content and the DHA to EPA ratio differ considerably between the different sources, i.e. micro-algae, krill and fish. While all analyzed micro-algae and krill oil products seem to contain an oil from one commercial source due to the very similar fatty acid pattern, the fish oil products

have a diverse composition. As no information regarding other fatty acids besides EPA and DHA is available, the consumer only has the option of making a purchase decision based on the amount of these two n3-PUFA. In addition, one capsule of the fish oil-based products contains 900-1400 mg fat and thus much more fat than the capsules based on micro-algae or krill oil (420-640 mg fat/capsule, Tab. 4.1). We therefore conclude that information regarding total fat content, amount of saturated, monounsaturated and polyunsaturated fatty acids according to the common nutrition facts labels would therefore be important for the consumer.

4.3.2 Oxylipin pattern of fish, micro-algae and krill oil-based supplements

EPA and DHA as well as other PUFA are converted by plants and animals to eicosanoids and other oxylipins [7-8]. Moreover, PUFA are prone to (aut)oxidation due to their high degree of unsaturation resulting in a complex pattern of oxidation products including oxylipins, e.g. (multi)hydroxylated and epoxidized fatty acids [19-20]. Krill oil and FO4 showed the highest total oxylipin concentration. The sum of all ALA, EPA and DHA-derived oxylipins was 5700 nmol/g in KO1 and 2800 nmol/g in FO4 (Fig. 4.2 A, Tab. 11.6). Consistent with the fatty acid pattern, the concentrations of oxylipins formed from n3-PUFA massively exceeds those derived from n6-PUFA. For example, the concentrations of oxylipins from LA and ARA in KO1 and FO4 are 280 nmol/g and 220 nmol/g, respectively. Only in M+ nearly equal concentrations were found for n3- and n6-PUFA-derived oxylipins (2100 nmol/g vs. 1500 nmol/g) mainly due to a high linoleic acid content in this product which is 6% of the total fatty acids, while it is only 1.1-1.7% in fish oil. The three micro-algae oils tested have identical absolute n3-PUFA oxylipin concentrations of around 1200 nmol/g. Together with the identical fatty acid pattern, this again underlines the hypothesis that the distributors of the capsules AO1-3 purchased the micro-algae oil from the same manufacturer. However, the price range of the three micro-algae oil-based products was 0.17-0.42 € per capsule.

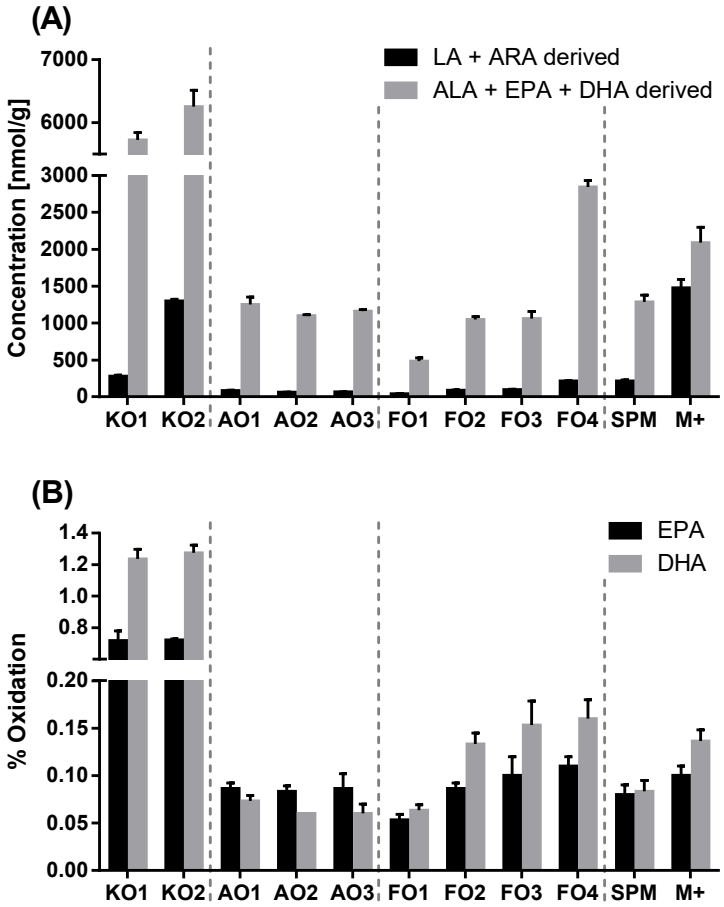

Fig. 4.2: (A) Concentration of total n3- and n6-PUFA-derived oxylipins as well as (B) level of PUFA oxidation. In (A) shown are the sums of all detectable LA/ARA as well as ALA/EPA/DHA-derived oxylipins analyzed by LC-MS following hydrolysis and SPE (mean ± SD, n = 3).
(B) Concentrations of all detectable oxylipins from EPA or DHA were divided by the respective total fatty concentration (mean ± SD, n= 3).

Krill oil products had a high percentage of oxidation products with 1.3% for DHA and 0.7% of EPA. On the contrary, the micro-algae oils, together with FO1 and SPM, showed the lowest oxidation (0.06-0.09% for DHA and 0.05-0.09% for EPA) based on the ratio of the concentration of all detectable oxylipins of EPA and DHA to the concentration of the precursor fatty acid (Fig. 4.2 B). It therefore seems to be technically possible to achieve a maximum degree of oxidation of < 0.1%, which could be used as a marker for an efficient removal of oxidation products during the refinement procedure. The acute oxidation of an oil is commonly assessed by the peroxide value and the anisidine value and results show that usually around 20-30% of the tested supplements exceed the international quality standards [51] set e.g. by the Global Organization for EPA and DHA Omega-3s (GOED) [47-49]. However, several of the products tested in previous studies had a low peroxide value demonstrating that it is possible to achieve high oxidative stability e.g., by improving the encapsulation procedure or choosing the adequate type and concentration of antioxidants [48]. These results are well in line with the

low percentage of oxidation products we found in our study for refined micro-algae and fish oil-based products.

We analyzed all supplementation products with specific detection of the individual oxylipins derived from PUFA by means of state-of-the-art analytical methods. In krill oil, high concentrations of hydroxy-PUFA (Tab. 4.2, Tab. 11.6) such as 5-/9-/12-/15-/18-hydroxyeicosapentaenoic acid (e.g. in KO1 12-HEPE 500 nmol/g and 18-HEPE 680 nmol/g) or for DHA 4-/11-/17-/20-hydroxydocosahexaenoic acid (e.g. in KO1 4-HDHA 320 nmol/g and 20-HDHA 330 nmol/g) were detected. On the contrary, the ω-hydroxylated PUFA 20-HEPE (KO1 10 nmol/g) and 22-HDHA (KO1 7 nmol/g) as well as epoxy- and dihydroxy-PUFA (e.g., 17(18)-EpETE) in KO1 53 nmol/g and 17,18-dihydroxyeicosatetraenoic acid (DiHETE 2.6 nmol/g) showed rather low concentration. Similar trends can be found for the n6-PUFA ARA (Tab. 11.6).

In fish oil, hydroxy-, epoxy- and dihydroxy-EPA and -DHA were approximately within the same range (Tab. 4.2, Tab. 11.6). Consistent with the fatty acid distribution, the n3-oxylipin concentrations were FO1 < FO2 ~ FO3 < FO4. In each fish oil, the highest concentrations of EPA-derived oxylipins were found for 17(18)-EpETE and 18-HEPE. A high concentration of 17(18)-EpETE is consistent with earlier reports which found it as main oxylipin in fish oil-based supplements [53]. We found 19(20)-epoxydocosapentaenoic acid (EpDPE) to be twofold higher compared to other epoxy-DHA regioisomers what is also in line with previous findings [53]. However, our method also includes numerous hydroxy-DHA metabolites which occur at similar a concentration as 19(20)-EpDPE.

Tab. 4.2 (following page): Concentrations of selected oxylipins derived from EPA and DHA in the tested supplements. Concentrations were determined by LC-MS analysis following hydrolysis and SPE (mean ± SD, n= 3). The values are colored according to their concentration (logarithmic scale: > 300 nmol/g (dark blue), 300-100 nmol/g, 100-30 nmol/g, 30-10 nmol/g, 10-3 nmol/g, < 3 nmol/g (light blue)).

	Concentration [nmol/g]	KO1	KO2	AO1	AO2	AO3	FO1	FO2	FO3	FO4	SPM	M+
EPA	5-HEPE	339 ± 4	300 ± 20	11 ± 2	9.1 ± 0.3	6.7 ± 0.4	9.3 ± 0.5	25.6 ± 0.7	26 ± 3	84 ± 2	14 ± 2	11 ± 1
	9-HEPE	440 ± 10	450 ± 30	18 ± 3	13.6 ± 0.6	9.6 ± 0.2	21.8 ± 1.1	52.5 ± 0.8	56 ± 6	140 ± 2	20 ± 2	21 ± 2
	12-HEPE	500 ± 10	490 ± 30	15 ± 3	10.0 ± 0.2	6.2 ± 0.2	16.2 ± 1.1	44.3 ± 1.2	45 ± 5	129 ± 2	20 ± 2	21 ± 3
	15-HEPE	430 ± 3	440 ± 30	13 ± 2	8.6 ± 0.3	4.9 ± 0.3	14.8 ± 1.4	39.1 ± 2.2	40 ± 4	121.9 ± 0.4	17 ± 2	19 ± 2
	18-HEPE	678 ± 9	740 ± 40	23 ± 4	15.6 ± 0.6	10.2 ± 0.4	22.8 ± 1.8	65.0 ± 1.5	66 ± 7	214 ± 4	32 ± 3	25 ± 2
	20-HEPE	10.3 ± 0.9	12 ± 1	270 ± 30	300 ± 10	281 ± 8	< 0.96	7.1 ± 0.5	7.7 ± 0.6	7.6 ± 0.3	21 ± 3	9 ± 1
	5,18-diHEPE	12.5 ± 0.9	10.1 ± 0.4	< 0.96	< 0.96	< 0.96	< 0.96	< 0.96	< 0.96	< 0.96	< 0.96	< 0.96
	17(18)-EpETE	53 ± 9	42 ± 2	76 ± 3	72 ± 8	77.0 ± 0.9	70 ± 7	78 ± 6	77.5 ± 9.6	180 ± 20	58 ± 4	22 ± 5
	5,6-DiHETE	15.6 ± 1.0	16.1 ± 0.5	6.7 ± 0.6	5.5 ± 0.1	5.3 ± 0.2	4.18 ± 0.09	28 ± 2	22.7 ± 3.0	49 ± 3	87 ± 7	< 0.14
	17,18-DiHETE	2.63 ± 0.08	2.9 ± 0.2	2.8 ± 0.3	2.86 ± 0.04	3.7 ± 0.2	3.6 ± 0.3	18 ± 1	11.7 ± 1.4	19 ± 1	49 ± 4	4.5 ± 0.5
	PGB_3	2.94 ± 0.14	3.5 ± 0.4	< 0.36	< 0.36	< 0.36	< 0.36	< 0.36	< 0.36	< 0.36	< 0.36	< 0.36
DHA	4-HDHA	317 ± 8	342 ± 3	33 ± 5	31.7 ± 0.9	31.7 ± 0.3	10.12 ± 0.06	35 ± 2	35.6 ± 4.0	122 ± 6	59 ± 2	120 ± 9
	7-HDHA	156 ± 9	145 ± 6	26 ± 6	20.4 ± 0.8	20 ± 1	8.9 ± 0.7	23.8 ± 0.8	26.8 ± 2.8	92 ± 5	29.6 ± 1.0	91 ± 6
	14-HDHA	190 ± 10	180 ± 10	18 ± 5	11.3 ± 0.5	9.3 ± 0.1	8.2 ± 0.9	22 ± 1	22.6 ± 1.4	80 ± 2	25 ± 4	83 ± 8
	17-HDHA	235 ± 4	210 ± 20	16 ± 2	10.2 ± 0.4	8.4 ± 0.6	6.5 ± 0.7	28 ± 1	28.4 ± 3.0	72.9 ± 0.7	21 ± 3	75 ± 7
	20-HDHA	332 ± 4	350 ± 30	35 ± 5	22.0 ± 0.6	19.7 ± 0.7	13.0 ± 0.8	37.6 ± 1.0	40.4 ± 4.8	150 ± 5	46 ± 4	120 ± 10
	22-HDHA	7.3 ± 0.2	7.1 ± 0.6	170 ± 10	166 ± 4	202 ± 5	< 0.38	1.5 ± 0.2	1.6 ± 0.4	1.4 ± 0.2	2.7 ± 0.4	14 ± 1
	19(20)-EpDPE	71 ± 7	71.3 ± 0.7	72 ± 2	58 ± 5	81 ± 4	23.7 ± 2.1	32 ± 2	31.9 ± 2.9	88 ± 8	56 ± 2	80 ± 20
	4,5-DiHDPE	31 ± 2	34 ± 1	26.0 ± 0.7	18 ± 1	16.4 ± 0.8	9.7 ± 0.4	50 ± 2	46.0 ± 3.0	94 ± 6	180 ± 10	27 ± 2
	19,20-DiHDPE	1.62 ± 0.07	1.8 ± 0.2	5.45 ± 0.04	5.1 ± 0.2	7.8 ± 0.2	2.59 ± 0.03	13.1 ± 0.4	9.6 ± 0.8	10.6 ± 0.2	47 ± 4	20 ± 1

>300 | 300-100 | 100-30 | 30-10 | 10-3 | < 3

Micro-algae oil contains high concentrations of 280 nmol/g for 20-HEPE, which are 10-20 times higher than the concentrations of the other hydroxy-EPA in micro-algae oil as well as 20-HEPE in the other supplements (Tab. 4.2). Also for DHA, the highest concentration was found for the terminal hydroxy metabolite 22-HDHA. Interestingly, 20-HETE, the terminal hydroxy-product of ARA being a minor PUFA in micro-algae oil, could only be detected in low concentrations comparable to other hydroxy-ARA (Tab. 11.6). In mammals, cytochrome P450 monooxygenases are known to catalyze ω-hydroxylation of PUFA [8]. To date, information about corresponding enzymes in micro-algae is missing. In the model plant *Arabidopsis thaliana*, CYP86A1 has been described to catalyze hydroxylation of C10 to C18 fatty acids [76]. This suggests that there are also corresponding enzyme systems in micro-algae, which apparently exhibit a pronounced activity. Being bound in triacylglycerols in high concentrations, 20-HEPE and 22-HDHA remain in the micro-algae oil after the refining process and thus could be used as an authenticity marker for micro-algae oil-based n3 supplements.

The extent to which the degree of oxidation and thus the uptake of physiologically relevant lipid mediators enhances or impairs the positive effect of the n3-supplements has not yet been sufficiently investigated. A large number of EPA and DHA supplementation studies have been carried out. However, the preparations administered to the subjects are often not tested for their oxylipin concentration [77]. Thus, study outcomes might be influenced by the quality and oxidation level of the supplements, as eicosanoids and other oxylipins are believed to be at least partly responsible for the physiological effects of EPA and DHA [4, 8]. Among the great variety of oxylipins, the so-called specialized pro-resolving mediators (SPM) and their precursors 17-HDHA and 18-HEPE are considered to play an important role in the resolution of acute inflammation [11]. Because synthetic SPMs also have potent effects in animal models [11], a SPM-enriched supplement product is already available for sale on the US market. However, the analysis of this product showed rather low concentrations of 18-HEPE and 17-HDHA and no SPMs at all. On the contrary, krill oil contains the highest concentrations of the SPM precursors 18-HEPE and 17-HDHA followed by fish oil. Even 5,18-diHEPE (RvE2 or an

isomer) was detectable in the krill oil-based supplements. However, consumption of supplements does not only provide single oxylipins with a potential positive health effect but these products also always contain oxidation products of all fatty acids with unknown biological activity.

4.3.3 Non-esterified fatty acids and oxylipins

The NEFA concentration is considered an important parameter in the quality assessment of edible oils. Micro-algae oils had a low NEFA percentage (ratio NEFA/total fatty acid) of 0.03-0.06% (Tab. 4.3) whereas for fish oils, large differences could be observed: While FO1 contains very low NEFA concentrations (0.004% NEFA), FO4 shows 30 times higher concentrations (0.1%). Manufacturers may have different processes for refining the crude fish oil; nevertheless, it is apparently technically possible to produce fish oil capsules with low NEFA levels (< 0.1%).

The krill oils had – consistent with their non-refined nature – a NEFA content of around 4% (Tab. 4.3, Tab. 11.7). International guidelines such as the Codex Alimentarius set a maximum NEFA content of 0.3% for refined and 2% for cold-pressed and virgin plant oils as well as up to 1.25% for animal fats [78-79]. The high percentage of NEFA in krill oils presumably results from the quality of the raw material. The lipid composition of *Euphausia superba* contains around 13% NEFA [80], because the enzymatic system of krill comprises lipases which show high activity at 0 °C and even if the krill organism is no longer alive [81]. Thus, the amount of NEFA may serve as a quality marker for fast processing of krill. If the krill is highly chopped up before processing or is not fresh, for example due to long transport routes, increased hydrolysis can be observed. Unlike quality aspects, the high NEFA concentration may have benefits as they are known to have a very good bioavailability. Possibly, not the binding of n3-PUFA to phospholipids, but the high concentration of NEFA is responsible for the often postulated better availability of n3-PUFA from krill oil compared to (r)TG-based fish or micro-algae oil [39-40].

Tab. 4.3: Percentage of NEFA in total fatty acids and their oxidation level. Shown are the relative concentrations of non-esterified fatty acids calculated against the respective total fatty acid concentration as well as the oxidation level calculated by dividing the concentration of the non-esterified oxylipin by the concentration of the non-esterified precursor fatty acid (mean ± SD, n = 3). Non-esterified fatty acids and oxylipins were isolated from oils by solid phase extraction and analyzed by LC-MS.

	% NEFA (NEFA/total FA)	% Oxidation (non-esterified oxylipins/NEFA)				
		LA	ALA	ARA	EPA	DHA
KO1	3.7 ± 0.6	0.69 ± 0.01	0.89 ± 0.01	3.53 ± 0.06	2.45 ± 0.06	1.59 ± 0.04
KO2	3.9 ± 0.9	2.9 ± 0.1	2.5 ± 0.2	2.9 ± 0.2	2.1 ± 0.1	1.49 ± 0.08
AO1	0.030 ± 0.004	0.59 ± 0.03	NEFA < LLOQ	NEFA < LLOQ	0.163 ± 0.004	0.180 ± 0.004
AO2	0.057 ± 0.002	0.79 ± 0.04	NEFA < LLOQ	NEFA < LLOQ	0.15 ± 0.03	0.14 ± 0.02
AO3	0.03 ± 0.01	0.58 ± 0.04	NEFA < LLOQ	NEFA < LLOQ	0.13 ± 0.01	0.070 ± 0.002
FO1	0.0040 ± 0.0002	9.5 ± 0.2	NEFA < LLOQ	NEFA < LLOQ	0.016 ± 0.003	0.07 ± 0.02
FO2	0.08 ± 0.01	0.44 ± 0.04	0.135 ± 0.003	0.80 ± 0.01	0.57 ± 0.02	0.34 ± 0.01
FO3	0.070 ± 0.003	0.34 ± 0.06	0.16 ± 0.03	0.93 ± 0.05	0.67 ± 0.03	0.502 ± 0.003
FO4	0.12 ± 0.01	4.6 ± 0.5	0.28 ± 0.03	1.0 ± 0.1	1.0 ± 0.1	0.46 ± 0.06
SPM	0.15 ± 0.02	1.5 ± 0.5	0.8 ± 0.3	1.14 ± 0.09	0.68 ± 0.03	0.18 ± 0.02
M+	0.31 ± 0.04	5.4 ± 0.5	10.4 ± 0.6	NEFA < LLOQ	1.9 ± 0.3	0.67 ± 0.06

Non-esterified oxylipins can result from NEFA oxidation or hydrolytic processes of oxidized lipids. To investigate the ratio of NEFA to non-esterified oxylipins, the sum of the non-esterified oxylipin concentrations derived from one fatty acid was divided by the respective NEFA concentration (Tab. 4.3, Tab. 11.7, 11.8). Similar to the total oxidation level, krill oil-based supplements showed the highest percentage of oxidation for ARA, EPA and DHA, while in micro-algae oils and FO1 the lowest apparent oxidation was found. Interestingly, the highest percentage of oxidation products to precursor fatty acid could be observed for LA, e.g. 9.5% in FO1 and 5.4% in M+, while the oxidation of DHA in FO1 was only 0.07% and 0.7% in M+. The ratio of oxylipins to precursor PUFA was comparable or slightly higher for non-esterified versus total EPA and DHA. One could speculate that the LA origin is not the n3-rich oil, but the carrier substance of minor additives such as tocopherols. For example, several of the analyzed supplements such as AO1-3, KO2 and M+ contain sunflower or soybean oil according to manufacturer. Overall, the micro-algae and fish oil products contain minor amounts of NEFA while the high NEFA and non-esterified oxylipin concentrations in krill oil can be explained by their unrefined nature. In addition, our results indicate that little autoxidation or hydrolysis takes place in the capsules since the oxidation rates of non-esterified EPA and DHA are rather low.

4.4 Conclusion

Fish, micro-algae and krill oil-based n3-supplements were analyzed regarding the concentration of non-esterified and total oxylipins as well as their fatty acid pattern. For all products tested, the measured total EPA and DHA concentrations were in line with the declaration on the package; however, the concentrations of saturated and monounsaturated fatty acids varied strongly within the fish oil supplements and no information about their content is available for the consumer. The products show a distinct oxylipin pattern and the hydroxy-EPA and hydroxy-DHA patterns could be used as characteristic parameters for micro-algae oil. Being a non-refined product, krill oil contains the highest amounts of oxylipins, including the biologically active lipid mediators 15-HEPE, 18-HEPE and

17-HDHA. Regarding the highly processed fish oil and the refined micro-algae oils, the ratio of oxylipins to the concentration of the precursor PUFA allows to determine the efficient removal of oxidation products and the oxidation status. We could show that < 0.1% oxidation and NEFA content are found in most products and thus are technically feasible and may be used as novel quality markers for a good refining process of oils used in the n3-PUFA supplements.

4.5 References

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Chapter 5

Characterization of the oxylipin pattern and other fatty acid oxidation products in freshly pressed and stored plant oils *

Enzymatic and non-enzymatic 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- and 13-hydro(pero)xy-LA and -ALA occurred in high concentrations 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 six months and the peroxide value (PV) as well as the 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 PV. The comprehensive analysis of hydroxy-, epoxy- and dihydroxy-LA and -ALA reveals new and unique insights into the composition of plant oils and ongoing oxidation processes.

* modified from Koch, E.; Wiebel, M.; Löwen, A.; Willenberg, I.; Schebb, N. H. Characterization of the Oxylipin Pattern and Other Fatty Acid Oxidation Products in Freshly Pressed and Stored Plant Oils. J Agric Food Chem 2022, 70 (40), 12935-12945.

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5.1 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 (PUFA) [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 of plant oils in Germany reached approximately 1.5 million tons 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 their 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 non-enzymatically by atmospheric oxygen [9] or by enzyme catalyzed reactions of which lipoxygenases (LOX) are the best described [10-11].

Non-enzymatical oxidation comprises autoxidation by radical $^3O_2$ and photo-oxidation by singlet oxygen $^1O_2$ in a non-radical manner (Fig. 1.1) [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 (Fig. 1.1 A). 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 position 9 and 16 and to a lower extent at internal C-atoms 12 and 13 (Fig. 1.1 B) [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 an "ene" reaction [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, non-enzymatic oxidation of PUFA is complex and leads to a broad pattern of oxylipins.

LOX are non-heme iron containing dioxygenases catalyzing the hydroperoxide formation of fatty acids with a 1(*Z*),4(*Z*)-pentadiene system. Plant LOX are classified in LOX-1 and 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 LOX 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 hypothesis 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]. LOX 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, of 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 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 non-enzymatic 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 the 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 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 non-esterified oxylipins directly in the freshly pressed oils to understand the interaction of enzymatic and non-enzymatic fatty acid oxidation during oil pressing. Additionally, oils were stored for six months and analyzed at different time points regarding 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.

5.2 Experimental

5.2.1 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 & 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 concentration 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.

5.2.2 Chemicals and purchased plant oils

Iso-propanol, diethyl ether, methanol (MeOH) and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile (ACN), chloroform 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 from 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); olive oil (extra virgin; REWE Bio, HMF Food Production GmbH & Co. KG, Dortmund, Germany).

5.2.3 Quantification of total oxylipin and fatty acid concentrations

Total (esterified and non-esterified) oxylipin concentrations and fatty acid concentrations were determined as described [28, 30, 33]. In brief, 5 mg oil of was diluted with 2 ml of *iso*-propanol. To 50 µl of this solution, 10 µl of deuterated oxylipin internal standards (IS1, 100 nM), 350 µl of *iso*-propanol, 10 µl of butylated hydroxytoluene (BHT, 0.2 mg/ml in methanol) as 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 pre-conditioned 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 acid 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 % of fatty acid oxidation, the total/non-esterified oxylipin concentration was divided by the total/non-esterified concentration of the precursor fatty acid.

5.2.4 Quantification of non-esterified fatty acid and oxylipin concentrations

Non-esterified fatty acid (NEFA) concentrations were determined as described [33]. In brief, 10 mg of oil were dissolved in 1 ml of $CHCl_3$/*iso*-propanol and 10 µl BHT was added. Samples were loaded on pre-conditioned aminopropyl SPE cartridges (Supelclean LC-NH_2, bed weight 100 mg, volume 1 ml, Merck, Darmstadt, Germany). Triacylglycerols were removed with 2 ml of $CHCl_3$/*iso*-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]. Non-esterified oxylipins were prepared in the same way using 5 mg of the 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.

5.2.5 Determination of peroxide value

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).

5.2.6 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.

5.3 Results

5.3.1 Concentration of fatty acids in the 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; Tab. 5.1). 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; Tab. 5.1). 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 g; Tab. 5.1, 0.087 mmol/g). ALA occurred only in minor concentration (0.07 g/100 g, 0.0024 mmol/g).

Tab. 5.1: Total fatty acid patterns of the pressed oils. Analysis of total fatty acid concentrations was carried out by LC-MS after hydrolysis of the oils diluted in *iso*-propanol (mean ± SD, n = 3).

[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

5.3.2 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; Fig. 5.1 A, Tab. 11.9). 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 (Tab. 11.10). On the contrary, for non-esterified 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, Fig. 5.1 A). Reduction of hydroperoxides led only to slightly increased concentrations for total and non-esterified 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; Fig. 11.16). The highest total oxylipin concentrations were found for 15-HODE (2700 nmol/g, Fig. 5.1 B), 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.

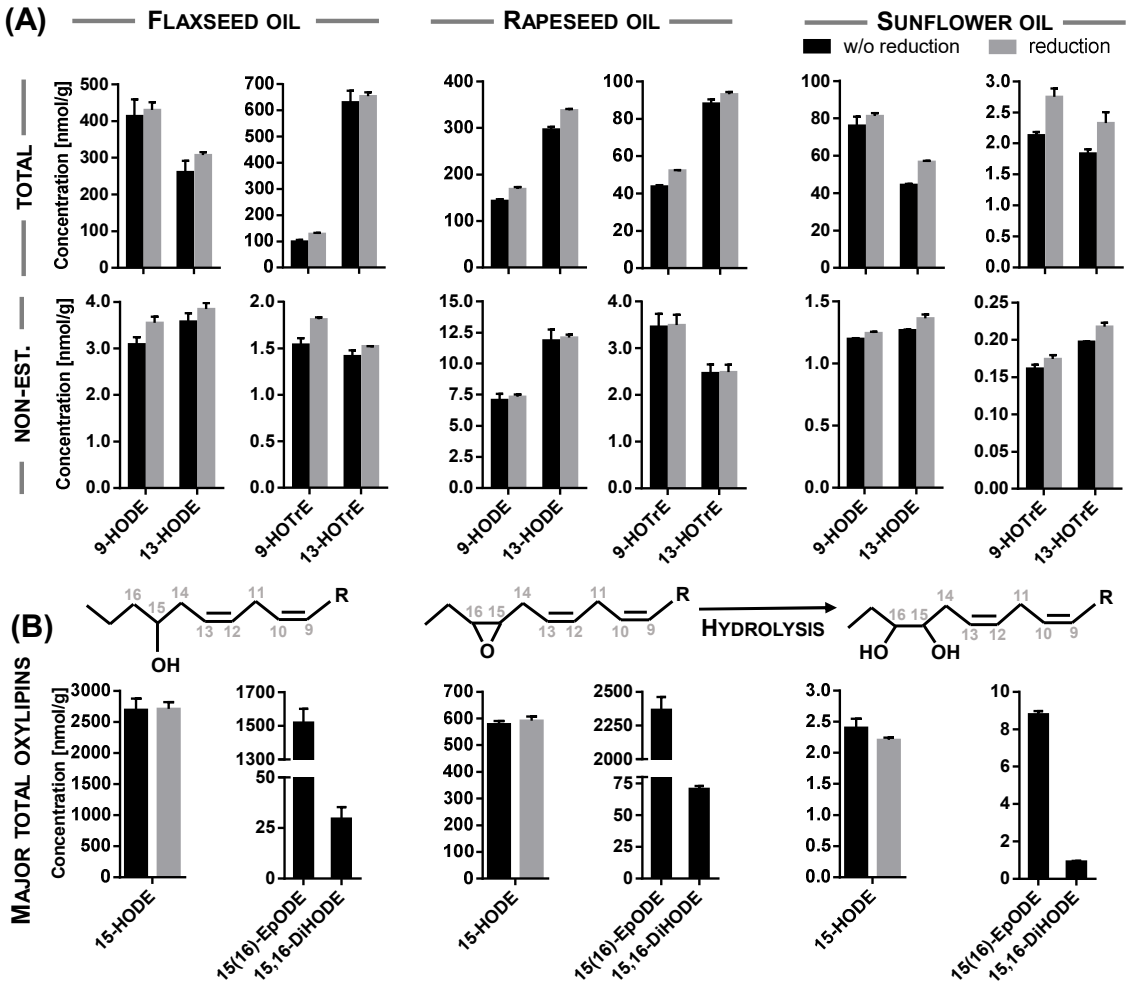


Fig. 5.1: Non-esterified and total oxylipin concentration in freshly pressed oils. (A) Concentration of the well described 9-/13-OH-linoleic and α-linolenic acid derived from the radical mediated hydroperoxide formation. (B) Concentrations of major oxylipins detected in rapeseed and flaxseed oil. Total (non-esterified and esterified oxylipins) as well as non-esterified 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.

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; Fig. 5.1 A, Tab. 11.9), only for the non-esterified 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 HODE are higher than the HOTrE concentrations. 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 concentrations of non-esterified ones did not change (10-HODE 1.84 nmol/g vs. 1.87 nmol/g; Fig. 11.16). Similar to flaxseed oil, 15-HODE (580 nmol/g) and 15(16)-EpODE (2370 nmol/g) were the most

abundant oxylipins in rapeseed oil (Fig. 5.1 B). The ratio of the 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 HODE with 9-HODE (76 nmol/g) being more abundant than 13-HODE (44 nmol/g) compared to ALA derived HOTrE with a concentration of about 2 nmol/g (Fig. 5.1 A, Tab. 11.9). For the non-esterified 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 non-esterified oxylipins were detected at comparable concentrations with and without hydroperoxide reduction (0.07 nmol/g vs. 0.08 nmol/g; Fig. 11.16). In contrast to flaxseed and rapeseed oil, 9- and 13-HODE are the dominant oxylipins in sunflower oil. Total 15-HODE (2.4 nmol/g) and 15(16)-EpODE (8.8 nmol/g) had rather low concentration (Fig. 5.1 B).

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 (Fig. 5.2). 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 non-esterified 15-HODE. In both cases, the relative proportion of 15-HODE in the fatty acid concentration is the same (flaxseed oil: around 0.6%; Tab. 5.2) or slightly higher for total 15-HODE as it is the case in rapeseed oil (non-esterified 0.04%, total: 0.11%). All other oxylipins showed higher relative oxidation level for the non-esterified oxylipins by up to a factor

of 30 (9-HOTrE rapeseed oil total 0.02% and non-esterified 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.

5.3.3 Changes in oxidation products and peroxide value 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 (Fig. 5.3). The PV of flaxseed oil stored in a 50% filled bottle was considerably higher in week 24 (26 meqO_2/kg). Concentrations 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 of concentration and higher levels after reduction of hydroperoxides (Fig. 11.17). 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; Fig. 11.18). Comparable to the total oxylipins, the concentration of non-esterified oxylipins did not change during storage time (Fig. 11.19).

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 (Fig. 5.3). The PV in rapeseed oil stored in half-full bottles was 580 meqO_2/kg, which was more than ten times higher compared to full bottles. Concentration of 9- and 13-HODE and -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 (Fig. 11.17). In contrast to the oxylipin concentrations, the concentrations 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 concentrations could be detected (*E*-2-heptenal: 3.0 nmol/g; *E,E*-2,4-heptadienal: 4.2 nmol/g; Fig. 11.18). Comparable to the total oxylipins, in oils stored in 50% filled bottles, slightly higher concentrations of non-esterified 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; Fig. 11.19). The effect of the available air above the oil was less pronounced for non-esterified oxylipins compared to total oxylipins.

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 (Fig. 5.3). 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- and 13-HODE and -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 (Fig. 11.17). 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; Fig. 11.18). In contrast to the concentration of total oxylipins, the concentration of non-esterified 9- and 13-HODE and -HOTrE after reduction of hydroperoxides increased constantly (e.g. 13-H(p)ODE: 1.4 nmol/g to 3.3 nmol/g; Fig. 11.19).

5.4 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 six months of storage were quantitatively evaluated and compared to the established parameters PV and secondary volatile aldehydes.

Oxidation of unsaturated fatty acids comprises enzymatic and non-enzymatic 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 non-enzymatic oxidation reactions on the other hand [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- and 13-HODE [9, 13-14] and approximately equal concentration of total and non-esterified 9- and 13-HODE was found in flaxseed oil (Fig. 5.1, Tab. 11.9). 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 (Fig. 5.1) thus might be caused by LOX activity. Previous studies describe the occurrence of LOX 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 the 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 (Fig. 5.1 A) also in flaxseed oil obtained from an independent pressing of another flaxseed charge and in two different commercially available flaxseed oils (Tab. 11.10).

For non-esterified HOTrE in flaxseed oil and rapeseed oil, equal concentrations of the regioisomers were found (Fig. 5.1). Plant LOX 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 (Fig. 5.1). The occurrence of LOX in rapeseeds is well described [40-42] and also 13-H(p)ODE being the main product of non-esterified LA with purified LOX [43]. However, no data are available about the conversion of LA containing lipids as well as about oxidation of ALA by rapeseed LOX. Data are similarly scarce for LOX 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 (Fig. 5.1).

The products of autoxidation and LOX catalyzed reactions 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 (Fig. 5.1 B). 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, Tab. 11.9) 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 (Fig. 5.1 B), implying a low hydrolysis rate.

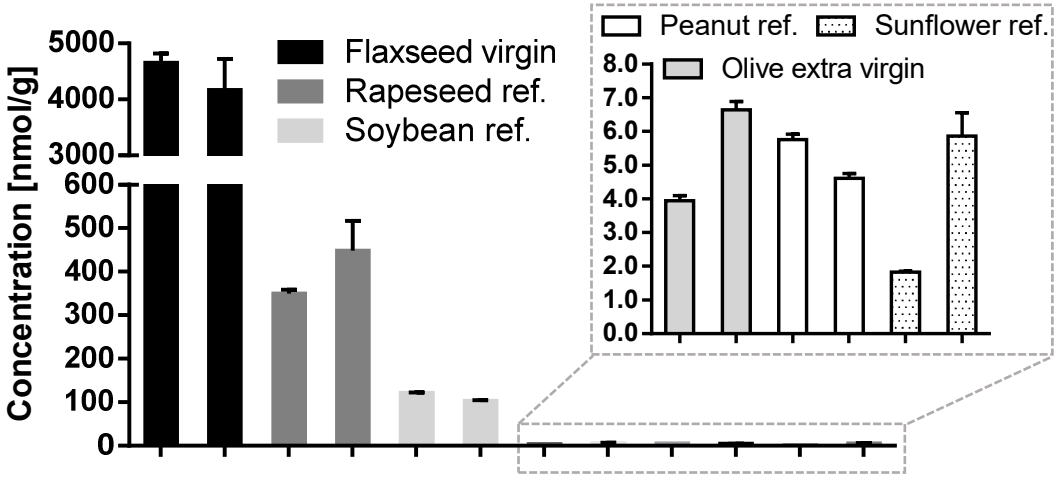


Fig. 5.2: Total concentration of 15-HODE in different commercially available edible oils. Total oxylipins were analyzed in commercially available virgin as well as refined (ref.) edible oils (two different brands per type of oil, obtained from local supermarkets or oil manufacturers) by LC-MS (mean ± SD, n = 3).

We identified 15-HODE with a concentration of 2700 µmol/g (80 mg/100 g) as a major oxygenated PUFA in some 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 (Fig. 5.2). 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 from 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, Fig. 5.1, Fig. 5.2). It seems that 15-HODE is not removed during the refining process. In addition, unlike other oxylipins, the relative concentration (conc. 15-HODE/conc. LA) is similar for total and non-esterified 15-HODE (Tab. 5.2). This leads to the conclusion that 15-HODE is predominantly bound in lipids. Consistently, Hamberg et al. reported that in oat seeds non-esterified 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].

Tab. 5.2: Percentage of oxylipin concentration to precursor fatty acid. Shown is the ratio of oxylipin concentration/precursor PUFA concentration (mean, n = 3). The concentration of the non-esterified oxylipin was divided by the concentration of the non-esterified fatty acid, or the total oxylipin by the total fatty acid concentration. In order to include the 9- and 13-hydroperoxides, the concentrations after reduction with $SnCl_2$ were used for calculation.

% 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

To date, the formation pathway(s) of 15-HODE in the seeds are unknown. Due to the homoallylic alcohol structure and no detectable hydroperoxides, non-enzymatic oxidation of LA is chemically unlikely. A (ω-n) hydroxylating activity of CYP as it is known in mammals [60-62] and similar enzyme activity in plants could lead to 15-HODE. In addition to CYP monooxygenase – among others – catalyzing hydroxylation reactions, non-heme 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

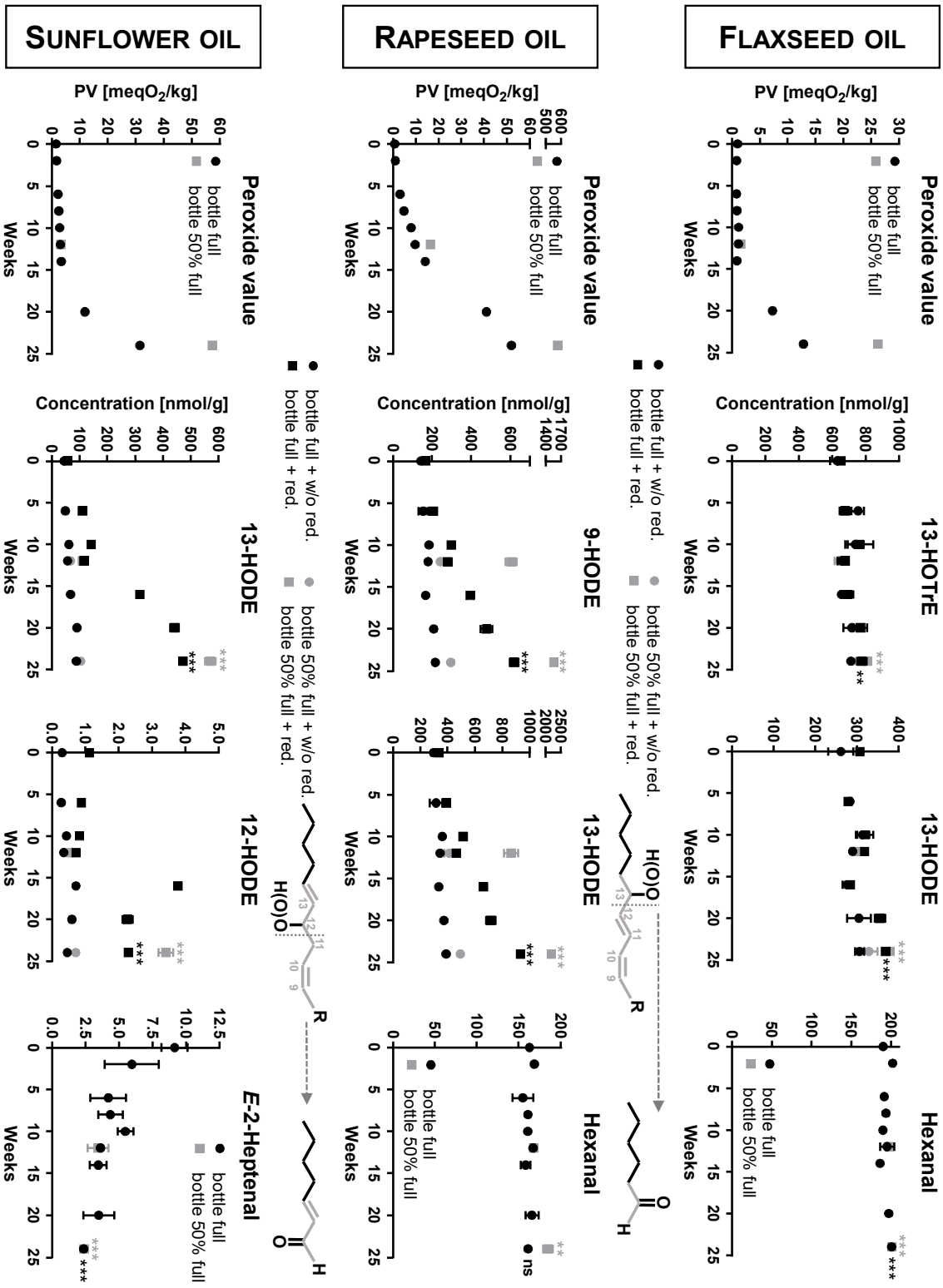
desaturase 3 (FAD3) [63-64]. Hydroxylation and desaturation mechanisms differ only in the last step, where the intermediary formed carbon radical either binds an oxygen from the oxo-diiron complex [63, 65] or disproportionates [66]. The factors controlling both pathways are still a matter of question. 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 (Fig. 5.2), 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 non-enzymatic 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 five months and was relatively low even in bottles with a large volume of air and higher oxygen availability (Fig. 5.3). Similarly, the primary formed oxylipins, namely

9- and 13-H(p)ODE and -H(p)OTrE, also showed only a slight increase over the storage time being most pronounced for 9-HOTrE (Fig. 5.3, Fig. 11.17). Interestingly, formation of the photooxidation product 10-HODE hydroperoxide could be detected, although the oils were stored in the dark (Fig. 11.17). 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 six months. 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 (Fig. 5.3, Fig. 11.18). After six 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].

Fig. 5.3 (following page): Peroxide value, 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 °C ± 2 °C in the dark and the peroxide value (mean, n = 2), the total oxylipin concentrations (with/without reduction of hydroperoxides; mean ± SD, n = 3) and the 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 rapeseed oil exceeded the threshold for beginning oxidative changes of 10 $meqO_2/kg$ already after three months and was tenfold higher after six months when the oil was stored in half-filled bottles (Fig. 5.3). Thus, the PV

directly correlates with the oxylipins concentration of e.g. the most abundant oxylipins 9-/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 (Fig. 11.17). Despite a massive increase in the precursor hydroperoxide [12] 13-HpODE, the concentrations of hexanal did not change (Fig. 5.3). 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 (Fig. 11.18). 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 (Fig. 5.3). After five 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 months, thus correlating to the concentration of 12-H(p)ODE (Fig. 5.3) 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 four, five and six months. Thus, similar to rapeseed oil, concentrations of secondary volatile aldehydes do not reflect the ongoing oxidation processes in sunflower oil well.

NEFA are considered to negatively influence the oxidative stability of oils through facilitating autoxidation [80-82]. Calculation of the relative total or non-esterified oxylipin concentration (conc. oxylipin/conc. precursor PUFA) revealed for all oxylipins except 15-HODE a higher relative concentration for non-esterified oxylipins compared to total oxylipins in freshly pressed oils, supporting a higher oxidation ratio of NEFA compared to TG during pressing (Tab. 5.2). 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 concentration of non-esterified oxylipins were observable (Fig. 11.19), while in rapeseed oil only minimal changes in the non-

esterified oxylipins were detected despite a massive increase in the total oxylipin concentrations (Fig. 5.3, Fig. 11.17). The peroxy radicals formed during autoxidative chain reactions 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 NEFA. The preferred oxidation of triacylglycerols over NEFA 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 NEFA did not change during storage (Tab. 11.11). Thus, we found two different effects regarding oxidation of NEFA *vs*. bound fatty acids: On the one hand, in freshly pressed oils presumably oxidation of NEFA predominates, on the other hand, during storage triacylglycerols are preferentially oxidized. It might be concluded that the higher oxidation of NEFA in freshly pressed oil is enzymatically based, while oxidized triacyclglycerols are derived from non-enzymatic oxidation.

5.5 Conclusion

Flaxseeds, rapeseeds and sunflower seeds were pressed and the obtained oils were directly analyzed regarding their patterns of oxylipins giving hints regarding the oxidation reactions during pressing. Oils were stored for six 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, photooxidative 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 by-product 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 as well as oxylipin and secondary volatile aldehydes concentrations showed that freshly pressed rapeseed oil is much more prone to oxidative processes than flaxseed oil. We could show that the oxylipin concentrations correlated 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.

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Chapter 6

Trans-hydroxy, *trans*-epoxy and *erythro*-dihydroxy fatty acids increase during deep-frying *

Deep-frying of food is a common cooking technique causing thermal oxidation of fatty acids (FA). Here, we investigated for the first time the formation of hydroxy-, epoxy- and dihydroxy-FA derived from oleic, linoleic (LA) and α-linolenic acid (ALA) during frying. Potato chips were fried in high-oleic sunflower oil for 4 x 5 cycles on two days and the oil was comprehensively analyzed by liquid chromatography-tandem mass spectrometry. During frying, the E,*Z-9- and* E,*Z-13-hydroperoxy-LA and -ALA concentrations decrease while their corresponding hydroxy-FA remain constant. The concentrations of both* E,E-*9-/-13-hydroperoxy-LA and* E,E-*9-/13-hydroxy-LA increase with the frying cycles, which is also found for the concentration of* trans-*epoxy-FA. The increase in* trans-*epoxy-FA is more pronounced than that of the corresponding* cis-*epoxy-FA, exceeding their concentrations on the second day of frying. This selective change in the* cis-/trans-*epoxy-FA ratio is also observed for their hydrolysis products: Concentrations of* erythro-*dihydroxy-FA, derived from* trans-*epoxy-FA increase during frying stronger than* threo-*dihydroxy-FA derived from* cis-*epoxy-FA. Based on these data we suggest that the ratio of* E,E-/E,*Z-hydroxy-FA, in combination with the* cis-/trans-*epoxy-FA ratio as well as the* threo-/erythro-*dihydroxy-FA ratio are promising new parameters to evaluate the heating of edible oils and to characterize the status of frying oils.*

* modified from Koch, E.; Löwen, A.; Nikolay, S.; Willenberg, I.; Schebb, N. H. *Trans*-Hydroxy, *Trans*-Epoxy and *Erythro*-Dihydroxy Fatty Acids Increase during Deep-Frying. J Agric Food Chem 2023, 71 (19), 7508-7513.

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6.1 Introduction

Deep-frying is a common processing of food because it is fast and delivers crispy food products with desired roasted, fried flavor, taste and golden-brown color. During deep-frying, the fried food such as French fries is completely immersed in heated oil which is partly absorbed by the product when it cools down [1]. The thermal treatment of the oil causes oxidation and degradation leading to complex patterns of oxidized fatty acids (FA), secondary oxidation products such as aldehydes as well as dimers and polymers [2].

The understanding of the lipid oxidation occurring during frying is of fundamental importance, in order to allow controlling of the frying oil quality during the frying process. It is indispensable to define the maximum time/number of frying cycles for which an oil can be used before it has to be completely replaced with fresh oil [2-3]. Thus, reliable parameters to determine the frying oil quality are needed [3]. So far, the quality of oils used for frying is primarily assessed by sensory evaluation [4]. If an impairment is observed (e.g. scratchy taste), different parameters can be analyzed in the laboratory to prove the deterioration of the oil: smoking point (≤ 170 °C), di- and oligomeric triacylglycerols (≥ 12%) and the polar compounds (≥ 24%) [4]. Autoxidatively formed hydroperoxides, such as *E*,*Z*-9- or *E*,*Z*-13-hydroperoxy linoleic acid (LA) have been found to be unstable and degrade to secondary oxidation products due to the high temperatures during frying [1, 5]. However, the formation of different oxidized FA during frying such as epoxy-FA has been described [1-2, 6]. It is important to distinguish between continuous heating and frying conditions as the latter causes more severe oil deterioration, e.g. due to loss of antioxidants [2-3]. Several studies describe the formation of epoxidized oleic acid (epoxy stearic acid) and epoxidized LA (EpOME) [7-9]. Velasco et al. reported levels of 3.4-14 mg/g of 9(10)-epoxy stearic acid and 9(10)-EpOME as well as 12(13)-EpOME in frying oils from Spanish restaurants [9]. Kalogeropoulos et al. found 0.2-0.9 mg/g epoxy- and keto-FA in different vegetable oils after 8 session of deep-frying [8] and Brühl et al. determined 0.05-16.6 mg/g of 9(10)-epoxy stearic acid and 9(10)-EpOME as well as 12(13)-EpOME [7].

Additionally, Zhang et al. reported a decrease in the concentrations of several hydroxy-PUFA and an increase in dihydroxy-LA concentrations for one deep-frying cycle, while after three deep-frying cycles (30 min in total), hydroxy- and epoxy-PUFA were increased [6].

Though these data suggest that oxylipins, particularly epoxy-FA can be formed, detailed data about the patterns of individual oxidation products in frying oils and how they are formed and degraded during frying are scarce. Therefore, in the present study, we performed a frying experiment with high-oleic sunflower oil and comprehensively analyzed the formation of hydro(pero)xy-FA, epoxy-FA and dihydroxy-FA during frying at 175 °C of potato chips, using a sensitive liquid chromatography-tandem mass spectrometry-based (LC-MS) approach. Based on these data, we are able to quantitatively characterize the ongoing changes in oxylipin concentrations during the frying process and suggest new potential markers for evaluation of heating of vegetable oils.

6.2 Experimental

6.2.1 Chemicals

Iso-propanol, methanol (MeOH) and acetic acid were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile and ethanol were obtained from VWR (Darmstadt, Germany). Tin(II) chloride (anhydrous, > 98%) was purchased from Fluka/Honeywell (Offenbach, Germany). Ultra-pure water was generated using the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). Oxylipin and fatty acid internal standards as well as fatty acids and oxylipins were used as described [10-11]. 10*E*,12*E*-9-HODE, 9*E*,11*E*-13-HODE, *erythro*-9,10-dihydroxy stearic acid, *threo*-9,10-dihydroxy stearic acid, *trans*-9(10)-epoxy stearic acid and *trans*-12(13)-EpOME were purchased from Larodan (Stockholm, Sweden).

6.2.2 Deep-frying experiments

Potatoes of the variety Verdi with a tuber size > 40 mm and refined high oleic sunflower oil were used for the frying trial. The fatty acid pattern of the oil was determined by LC-MS [12]: oleic acid 77 g/100 g, LA 10 g/100 g, C16:0 4.3 g/100 g, C18:0 2.5 g/100 g and ALA, C20:0, C20:1 n9 as well as C22:0 were < 1 g/100 g. The potatoes were washed on a semi-technical scale, abrasively peeled with a carborundum peeling system (16K, Flottwerk GmbH & Co. KG, Rotenburg a. d. Fulda, Germany), cut into 1.2-1.3 mm thick slices (Urschel Laboratories Inc., Chesterton, Indiana, United States), washed with water and blotted dry by hand. Then, 200 g of raw potato slices were deep-fried for 3 min each at 175 °C in a gastronomy-scale deep-fryer (FriFri Eco 4 + 4 professional, Lincat Limited, Lincoln, United Kingdom), which was filled with 3.3 kg of frying oil. Twenty frying cycles were performed on each of two consecutive days, resulting in a total of 40 frying cycles. Between experimental days, the fryer was switched off so that the frying oil cooled down at the end of the first day and was reheated at the beginning of the second day. Samples were taken i) from the fresh oil, ii) after first heating of the fresh oil, iii) every 5th cycle of frying on day 1 (20 cycles), iv) before and after re-heating on day two and v) after 5 and 20 cycles on day two. Oil samples were hydrolyzed and analyzed by LC-MS. After taking the samples, the loss of oil in the fryer was compensated for by adding 220 g of fresh frying oil.

6.2.3 Quantification of total oxylipin concentrations

Total (sum of esterified and non-esterified) oxylipin concentrations were determined as described [11, 13]. In brief, 5 mg of oil was diluted with 1.9 ml of *iso*-propanol and 50 µl of this solution were used. 350 µl of *iso*-propanol, 10 µl of deuterated oxylipin internal standards (IS1, 100 nM), 10 µl of butylated hydroxy toluene (0.2 mg/ml in methanol) as antioxidant, 50 µl of water and 100 µl of 0.6 M potassium hydroxide in MeOH/H_2O (75/25, *v*/*v*) were added. Samples were hydrolyzed for 30 min at 60 °C to release esterified oxylipins from lipids such as triacylglycerols, and subsequently neutralized with 25% acetic acid in H_2O. Samples were prepared with and without reduction of hydroperoxides to determine the concentrations of hydroperoxides. For reduction of hydroperoxides,

30 µl of tin(II) chloride ($SnCl_2$, 10 mg/ml in MeOH) were added after neutralization prior to SPE. Samples were loaded onto pre-conditioned SPE cartridges (C8/anion exchange, bed weight 300 mg, volume 3 ml, Bond Elut Certify II, Agilent, Waldbronn, Germany). 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. Samples were reconstituted in 50 µl MeOH containing a mix of four substances used as second IS (IS 2) for calculation of IS 1 recovery rate [11]. 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 electrospray ionization in negative mode. The MS parameters were set as follows: ion spray voltage -4500 V; capillary temperature 650 °C; curtain gas (N_2) 50 psi; nebulizer gas (GS1, N_2) 30 psi and drying gas (GS2, N_2), 70 psi. Declustering potentials, entrance potentials, collision cell exit potentials, collision energies as well as MRM transitions were optimized for each oxylipin [10-11]. Quantification was performed by external calibration with deuterated IS from each structural class and different precursor-PUFA in a concentration of 20 nM. The recovery of the internal standards in each sample was monitored using a set of the IS 2 [11]. Only samples with a recovery above 50% were further evaluated. Moreover, a quality control oil (olive oil, stored in small aliquots at -80 °C) was prepared in triplicate within every batch to assure reliable and reproducible sample preparation and LC-MS analysis. The *trans*-epoxy-FA were quantified using the *cis*-epoxy-FA calibration curve and the *E*,*E*-hydroxy-FA using the *E*,*Z*-hydroxy-FA calibration curve. For determination of the *erythro*-dihydroxy-FA, the calibration curve of the *threo*-dihydroxy-FA was used.

6.2.4 Acidic hydrolysis of cis- *and* trans*-epoxy-FA yielding* threo- *and* erythro*-dihydroxy-FA*

Standards of *trans*-9(10)-epoxy stearic acid, *cis*-9(10)-epoxy stearic acid, *trans*-12(13)-EpOME and *cis*-12(13)-EpOME were diluted in 500 µl acetonitrile to a

concentration of 0.5-1 µM. 500 µl of 1 M aqueous H_2SO_4 were added and samples were hydrolyzed for 24 h at room temperature. Following addition of 500 µl of 1 M $NaHCO_3$, the acetonitrile was removed from the solution by evaporation using a vacuum concentrator. Oxylipins were extracted twice with 600 µl of ethyl acetate. Following evaporation, the residue was reconstituted in MeOH and analyzed by LC-MS.

6.3 Results and Discussion

The concentrations of *E*,*Z*- and *E*,*E*-hydroperoxy-FA, *E*,*Z*- and *E*,*E*-hydroxy-FA, *cis*-/*trans*-epoxy-FA and *threo*-/*erythro*-dihydroxy-FA in frying oils after different numbers of frying cycles were analyzed to investigate the ongoing oxidative processes during deep-frying in detail.

6.3.1 Changes in hydroxy-PUFA concentrations during deep-frying

The initial concentrations of *E*,*Z*-9- and *E*,*Z*-13-hydroperoxy-LA (HpODE) were the highest (1600 nmol/g and 2000 nmol/g) and decreased with the number of frying cycles to 280 nmol/g and 380 nmol/g after 20 cycles (Fig. 6.1; determined by calculating the difference of the corresponding HODE concentrations obtained with (gray bar) and without (black bar) reduction of hydroperoxides). Re-heating of the oil on the next day further reduced the concentration to 60 nmol/g and 90 nmol/g, respectively. Thus, after 2 x 20 cycles of frying, the concentrations of *E*,*Z*-9-HODE and *E*,*Z*-13-HODE including hydroperoxides (gray bars) were comparable to the concentrations of the corresponding hydroxy-PUFA (black bars) of around 320 nmol/g for *E*,*Z*-9-HODE and 490 nmol/g for *E*,*Z*-13-HODE (Fig. 6.1). Their concentrations did not change during frying (cold, fresh oil: *E*,*Z*-9-HODE 360 nmol/g; *E*,*Z*-13-HODE 510 nmol/g).

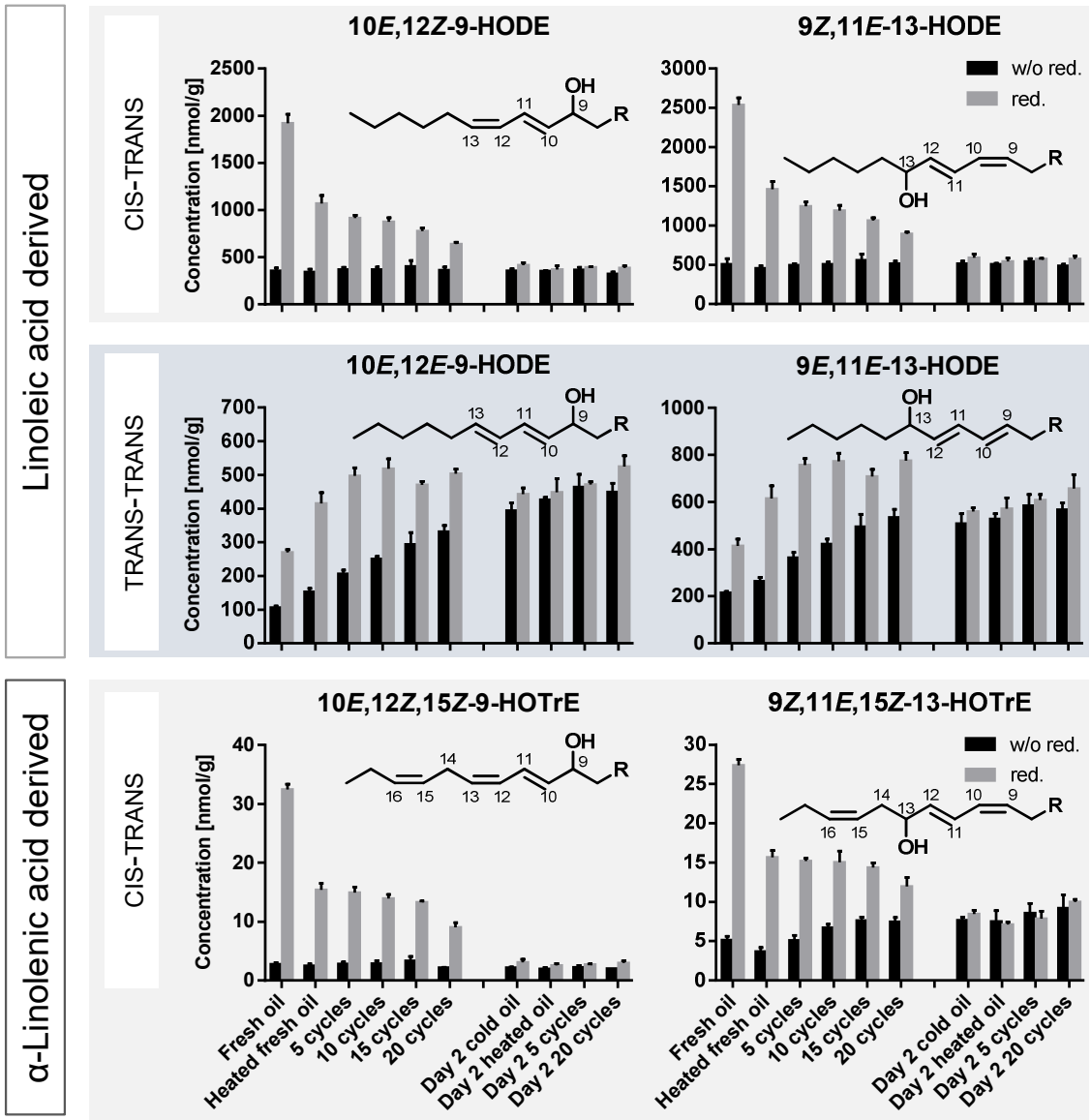


Fig. 6.1: Concentration of hydroxy- and hydroperoxy-linoleic and α-linolenic acids in deep-fried oil. High-oleic sunflower oil was heated to 175 °C and potato slices were fried for 3 min. Samples were taken from the fresh oil before heating, before the first frying cycle and after 5, 10, 15 and 20 cycles of potato frying. Deep-frying oil was cooled down at room temperature and the experiment was repeated the next day with the same oil. Hydroxy fatty acids were analyzed by LC-MS after hydrolysis of lipids with (red.) and without reduction (w/o red.) of hydroperoxides (mean ± SD, n = 3). For HOTrE, only 10*E*,12*Z*,15*Z*-9-HOTrE and 9*Z*,11*E*,15*Z*-13-HOTrE standards were commercially available and could be evaluated (R = -$(CH_2)_6$-COOH).

FA hydroperoxides are not stable at high temperatures and rapidly degrade, giving rise to alkoxy and hydroxy radicals which are highly reactive intermediates of the lipid peroxidation, forming secondary oxidation products [1-2, 5]. Consistently, the decrease of *E*,*Z*-9-/13-HpODE detected by us did not lead to an increase in *E*,*Z*-9-/13-HODE concentrations (Fig. 6.1). Similar to HpODEs, the *E*,*Z*,*Z*-9-hydroperoxy-ALA (HpOTrE) and *E*,*Z*,*Z*-13-HpOTrE concentrations decreased

from 30 nmol/g and 22 nmol/g in the fresh oil to 6.8 nmol/g and 4.5 nmol/g after 20 cycles and 1.0 nmol/g and 0.8 nmol/g after 2 x 20 cycles, supporting the degradation of *E*,*Z*-hydroperoxides during frying (Fig. 6.1).

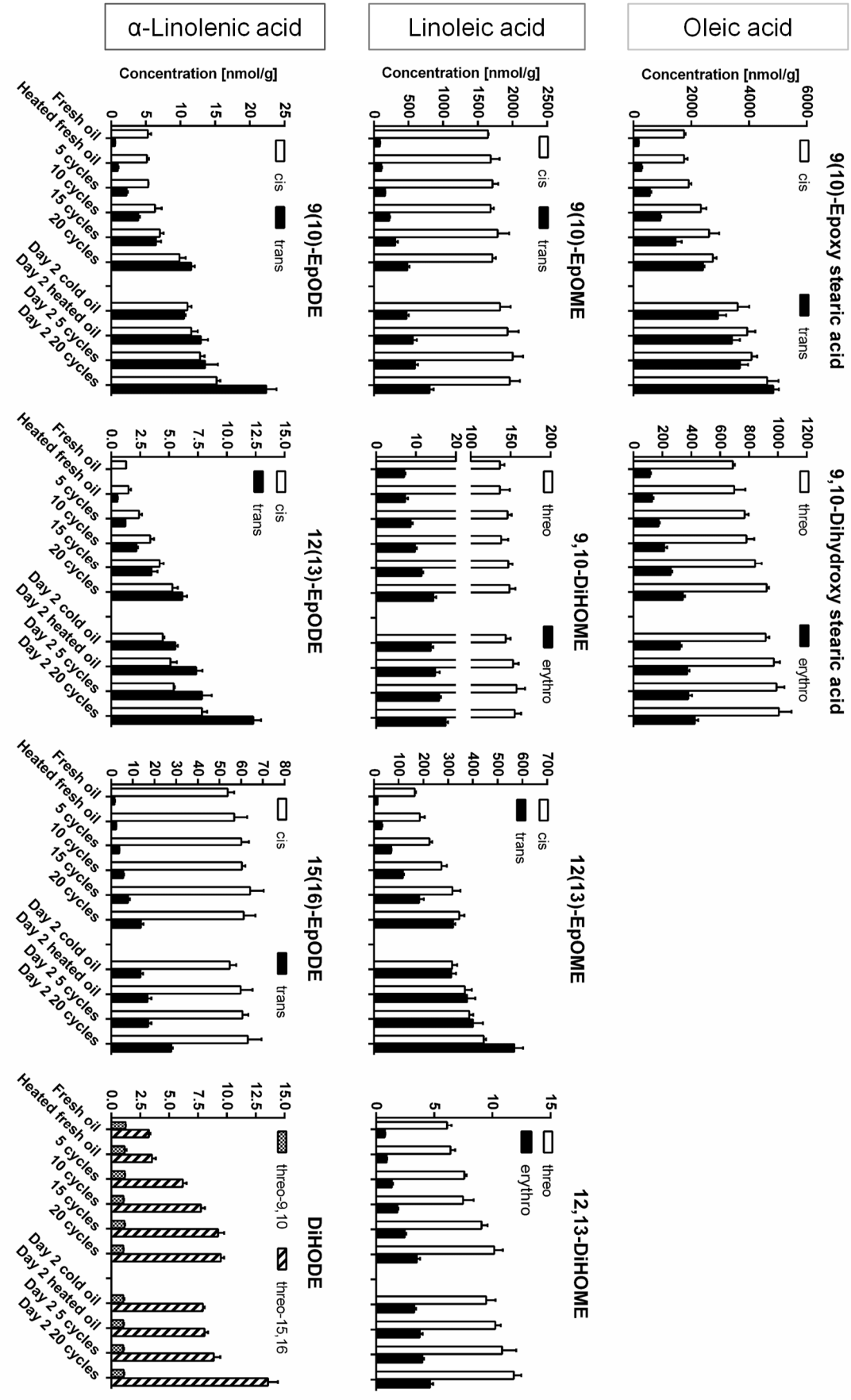
For *E*,*E*-hydroperoxy-LA and *E*,*E*-hydroxy-LA, concentrations after reduction of hydroperoxides specifically increased from 270 nmol/g in the fresh, cold oil to 500 nmol/g after 20 cycles of frying for *E*,*E*-9-HODE and from 410 nmol/g to 780 nmol/g for *E*,*E*-13-HODE (Fig. 6.1). This shows that the *E*,*E*-hydroperoxides are formed at high temperatures at higher rates and/or are more stable than the *E*,*Z*-hydroperoxides. The *E*,*E*-9-HpODE and *E*,*E*-13-HpODE concentrations were similar in the fresh oil (160 nmol/g and 200 nmol/g) and after 20 cycles of frying (170 nmol/g and 240 nmol/g) and even slightly increased during frying (after 5 cycles: 290 nmol/g and 390 nmol/g). Additionally, for the hydroxy-FA *E*,*E*-9-HODE and *E*,*E*-13-HODE, increasing concentrations were observed over 20 cycles of frying: the concentrations for *E*,*E*-9-HODE and *E*,*E*-13-HODE were 110 nmol/g and 210 nmol/g in the fresh oil and raised to 330 nmol/g and 530 nmol/g, respectively. This shows that unlike the *E*,*Z*-hydroperoxides, a part of the formed the *E*,*E*-hydroperoxides are reduced to their corresponding hydroxy-FA. Thus, the ratio of *E*,*Z*-HODE to *E*,*E*-HODE decreases from 3.4 for 9-HODE in the fresh oil to 0.72 after 2 x 20 cycles of frying and from 2.4 to 0.86 for 13-HODE. The preferred formation of *E*,*E*-hydroperoxides over *E*,*Z*-hydroperoxides at high temperatures has been reported in the literature, as the *E*,*E*-configuration is thermodynamically favored [14-15]. Consistently, we showed that *E*,*E*-HpODE and *E*,*E*-HODE isomers are formed in a time-dependent manner with the frying cycles, while the kinetically controlled products *E*,*Z*-HpODE are not. *E*,*Z*-HpODE isomers being present in the oils were rapidly degraded to secondary oxidation products. Thus, high levels of *E*,*E*-HODE and low levels of *E*,*Z*-HODE are an indicator for the heating of oil. Therefore, we suggest the *E*,*Z*-/*E*,*E*-9-HODE and *E*,*Z*-/*E*,*E*-13-HODE ratio as a marker for the oil quality. If the ratio falls below < 1 our data show that this indicates repeated frying. However, further studies are needed to validate this potential new marker for oil quality.

6.3.2 Formation of epoxy-FA and dihydroxy-FA during frying

Deep-frying of high-oleic sunflower oil massively increased the concentrations of *trans*-epoxy-FA of oleic acid, LA and ALA. For example, the concentration of *trans*-12(13)-epoxy-LA (EpOME) raised from 15 nmol/g in the fresh, cold oil to 320 nmol/g after 20 cycles of frying and to a final concentration of 570 nmol/g after re-heating and additional 20 frying cycles (Fig. 6.2). On the contrary, the concentrations of *cis*-epoxy-FA changed only slightly (*cis*-12(13)-EpOME in the fresh oil 170 nmol/g, after 20 frying cycles 350 nmol/g and 440 nmol/g after 20 cycles on day two). Thus, an initial *cis*-/*trans*-ratio of 11 decreased to 1.1 after 20 cycles and turned into 0.78 after 40 cycles in total. A higher concentration of the *trans*-isomer compared to the *cis*-form after 40 cycles of frying was also found for 9(10)-epoxy stearic acid (*cis*: 4600 nmol/g; *trans*: 4800 nmol/g), 9(10)-epoxy-ALA (EpODE) (*cis*: 15 nmol/g; *trans*: 22 nmol/g) as well as 12(13)-EpODE (*cis*: 7.8 nmol/g; *trans*: 12 nmol/g, Fig. 6.2). The concentration of *trans*-9(10)-EpOME and *trans*-15(16)-EpODE increased strongly as well and the *cis*-/*trans*-ratio decreased from 35 to 2.3 for 15(16)-EpODE and from 19 to 2.4 for 9(10)-EpOME. Our results are in line with previous studies reporting considerable amount of epoxy-FA in frying oils and specifically high concentrations for *trans*-epoxy-FA [1, 8-9]. Here, we could show for the first time that the epoxy-FA of all fatty acids including *trans*-EpODE derived from ALA are elevated during frying.

While few studies used *trans*-epoxy-FA concentrations to characterize used frying oils [7-9], we suggest the *cis*-/*trans*-epoxy-FA ratio to be a good marker for heated vegetable oils. Both isomers share the same transition in SRM mode with similar, but different retention times [10]. Thus, the *cis*-/*trans*-ratio can be directly determined by the peak areas from the LC-MS chromatogram and does not require pure standards and determination of concentrations.

Fig. 6.2 (following page): Concentrations of epoxy- and dihydroxy-linoleic and α-linolenic acids in deep-fried oil. High-oleic sunflower oil was heated to 175 °C and potato slices were fried for 3 min. Samples were taken from the fresh oil before heating, before the first frying cycle and after 5, 10, 15 and 20 cycles of potato frying. Deep-frying oil was cooled down at room temperature and the experiment was repeated the next day with the same oil. Total epoxy-FA and dihydroxy-FA were analyzed by LC-MS after hydrolysis of lipids (mean ± SD, n = 3). 12,13-DiHODE could not be evaluated due to matrix interference.

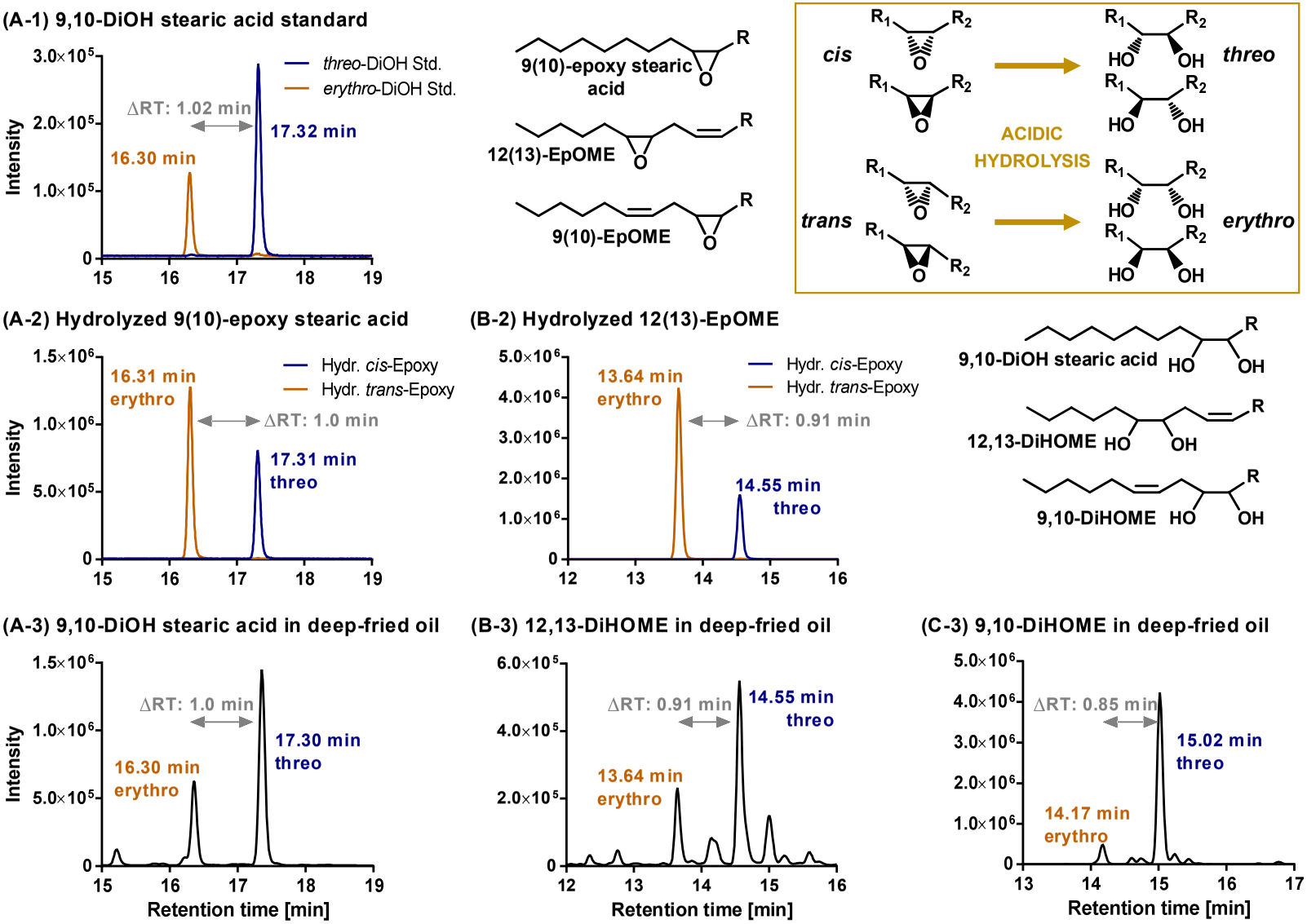

Oleic acid
Linoleic acid
α-Linolenic acid
9(10)-Epoxy stearic acid
9,10-Dihydroxy stearic acid
9(10)-EpOME
9,10-DiHOME
12(13)-EpOME
12,13-DiHOME
9(10)-EpODE
12(13)-EpODE
15(16)-EpODE
DiHODE
Concentration [nmol/g]
cis
trans
threo
erythro
threo-9,10
threo-15,16
Fresh oil
Heated fresh oil
5 cycles
10 cycles
15 cycles
20 cycles
Day 2 cold oil
Day 2 heated oil
Day 2 5 cycles
Day 2 20 cycles

Using the same calibration for *cis*- and *trans*-epoxy-FA, we found that for 9(10)-epoxy stearic acid, 12(13)-EpOME, 9(10)-EpODE and 12(13)-EpODE, the *cis*-/*trans*-ratio turned from > 1 to < 1. Thus, this is an easily accessible marker, because even by directly evaluating the MS signals of *cis*- and the *trans*-epoxy-FA, it is easily evaluable if the oil is fresh (*cis*/*trans* > 1) or used several times (*cis*/*trans* < 1). Higher concentrations of the *trans*- compared to *cis*-epoxy-FA were also previously described for 12(13)-EpOME and 9(10)-epoxy stearic acid in used frying oils from restaurants [9], supporting our idea of using the *cis*-/*trans*-epoxy-PUFA ratio which could be – together with the other markers described in this manuscript – a sensitive and selective marker for heat application on vegetable oils.

Epoxy-FA can be hydrolyzed to their corresponding vicinal diols [16]. The *cis*-epoxy-FA gives rise to the *threo*-dihydroxy-FA and from a *trans*-epoxy-FA the *erythro*-configuration is formed (Fig. 6.3). This can be explained by the underlying nucleophilic type-2 substitution mechanism [17]. We confirmed this by acidic hydrolysis of *cis*- and *trans*-9(10)-epoxy stearic acid standard which led exclusively to the formation of *threo*- and *erythro*-9,10-dihydroxy stearic acid, respectively (Fig. 6.3). Similarly, acidic hydrolysis of *cis*- and *trans*-12(13)-EpOME led to the formation of two peaks with a comparable retention time difference of around 1 min as it was found for *erythro*- and *threo*-9,10-dihydroxy stearic acid. In the present study, we quantified for the first time *erythro*- and *threo*-9,10-dihydroxy stearic acid, *erythro*- and *threo*-12,13-dihydroxy-LA (DiHOME) and – characterrized and quantified based on the retention time shift – also *erythro*- and *threo*-9,10-DiHOME. Interestingly, while the concentrations of the *threo*-dihydroxy-FA changed only slightly, the (*trans*-epoxy-FA derived) *erythro*-dihydroxy-FA concentrations increased strongly with the number of frying cycles (Fig. 6.2). For example, in the fresh oil, *threo*-9,10-dihydroxy stearic acid was with 690 nmol/g the more abundant isomer compared to 120 nmol/g for the *erythro* form. However, the *threo*-concentrations changed only slightly during frying to 1000 nmol/g after 2 x 20 cycles. On the contrary, the levels of *erythro*-9,10-dihydroxy stearic acid increased strongly during frying (fresh oil: 120 nmol/g; after 2 x 20 cycles:

420 nmol/g). The specific increase in the *erythro*-dihydroxy-FA during frying compared to the *threo*-isomers might be explained by higher formation rate of *trans*-epoxy-FA compared to *cis*-epoxy-FA (Fig. 6.2). The epoxy-to-diol ratio remains constant or increases over time for all epoxy-/dihydroxy-FA pairs, except for 15,16-DiHODE. For example, in the fresh oil, the *trans*-9(10)-epoxy-/*erythro*-9,10-dihydroxy stearic acid ratio was found to be 1.5 and was increased to 11 after 2 x 20 cycles. Thus, it seems that formation rate of epoxy-FA during frying is higher than the hydrolysis rate. However, the specific increase in *trans*-epoxy-FA results in higher absolute concentrations for *erythro*-dihydroxy-FA and thus the *threo*-/*erythro*-ratio changes during frying: For example, for *threo*-/*erythro*-9,10-dihydroxy-stearic acid ratio decreased from 6.0 in the fresh oil to 2.4 after 2 x 20 cycles of frying. A decrease by more than 300% was also detectable for *threo*-/*erythro*-12,13-DiHOME (fresh, cold oil 8.1; 2 x 20 cycles: 2.6). Thus, we suggest in addition to the ratio of *E*,*Z*-to-*E*,*E*-HODE and *cis*-/*trans*-epoxy-FA, the ratio of *threo*-/*erythro*-dihydroxy-FA as a new potential marker for the characterization of frying oils.

Fig. 6.3 (following page): LC-MS chromatograms of *threo*- and *erythro*-dihydroxy fatty acids and their occurrence in deep-fried oil. Shown are in (A-1) transitions of the *erythro*- and *threo*-9,10-dihydroxy stearic acid standards; in (A-2) and (B-2) the chromatograms resulting from acidic hydrolysis of *cis*- and *trans*-9(10)-epoxy stearic acid as well as *cis*- and *trans*-12(13)-EpOME. (A-3), (B-3) and (C-3) shows the pattern of the *erythro*- and *threo*-dihydroxy fatty acid in deep-fried high-oleic sunflower oil on day 2 after 20 cycles. In the right upper corner, the structure of *cis*- and *trans*-epoxy fatty acids and *erythro*- and *threo*-dihydroxy fatty acids are shown. (R = $-(CH_2)_7-COOH$).


(A-1) 9,10-DiOH stearic acid standard
threo-DiOH Std.
erythro-DiOH Std.
ΔRT: 1.02 min
16.30 min
17.32 min
Intensity
9(10)-epoxy stearic acid
12(13)-EpOME
9(10)-EpOME
cis
trans
ACIDIC HYDROLYSIS
threo
erythro
(A-2) Hydrolyzed 9(10)-epoxy stearic acid
Hydr. cis-Epoxy
Hydr. trans-Epoxy
16.31 min
erythro
ΔRT: 1.0 min
17.31 min
threo
(B-2) Hydrolyzed 12(13)-EpOME
13.64 min
erythro
ΔRT: 0.91 min
14.55 min
threo
9,10-DiOH stearic acid
12,13-DiHOME
9,10-DiHOME
(A-3) 9,10-DiOH stearic acid in deep-fried oil
ΔRT: 1.0 min
16.30 min
erythro
17.30 min
threo
(B-3) 12,13-DiHOME in deep-fried oil
ΔRT: 0.91 min
14.55 min
threo
13.64 min
erythro
(C-3) 9,10-DiHOME in deep-fried oil
ΔRT: 0.85 min
15.02 min
threo
14.17 min
erythro
Retention time [min]

6.4 Conclusion

In the present study we investigated how the patterns and concentrations of oxidized fatty acids change during repeated frying of freshly cut and washed 1 mm potato slices. Using conditions representing the industrial frying process of potato chips, we described that deep-frying led to the formation of the thermodynamically favored *E*,*E*-HpODE and *E*,*E*-HODE isomers in a time-dependent manner, while the kinetically controlled products *E*,*Z*-HpODE were degraded. Additionally, a strong increase in the concentrations of *trans*-epoxy-FA was detectable, while the elevation of *cis*-epoxy-FA was modest. For 12(13)-EpOME, 9(10)-epoxy stearic acid, 9(10)-EpODE and 12(13)-EpODE the concentrations for the *trans*-isomer exceeded that of the *cis*-isomer after longer frying times. The concentrations of *erythro*-dihydroxy-FA derived from *trans*-epoxy-FA increased strongly with the frying cycles, while the concentrations of *threo*-dihydroxy-FA derived from *cis*-epoxy-FA were only slightly higher. Therefore, we suggest the ratios of i) *E*,*Z*-/*E*,*E*-HODE, ii) *cis*-/*trans*-epoxy-FA and iii) *threo*-/*erythro*-dihydroxy-FA as new markers for heating of plant oils. Especially the combination of all three parameters makes them attractive for characterization of heated oils. Their potential is worth to be evaluated in future studies which could gain more information about the factors influencing the oxylipin formation, e. g. moisture of potato slices or temperature. A deeper insight into the mechanisms of oxylipin formation and degradation during heating of oils might be not only interesting in terms of assessing the quality of frying oils, but also for other questions dealing with the proof of heating, e.g. (illegal) refining of (virgin) oils.

6.5 References

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Chapter 7

Do meals contain a relevant amount of oxylipins? LC-MS-based analysis of oxidized fatty acids in food *

Several oxylipins are potent lipid mediators and are discussed to be absorbed after oral intake. However, information about their concentrations in oils and processed foods are scarce. Here, we analyzed the concentrations of mono-, di- and multihydroxy- as well as epoxy fatty acids in virgin and refined oils as well as in different foods/meals. Oil refining causes hydrolysis of epoxy-PUFA and thus high dihydroxy-PUFA concentrations (e.g. 15,16-DiHODE 290 µg/g in refined vs. 15 µg/g in virgin rapeseed oil), making the epoxy-to-diol ratio a potential marker for refined oils. Low oxylipin levels were found in foods with high amounts of saturated fatty acids such as hamburger patties (around 30 µg/g). High concentrations (up to 1200 µg/g, 80 mg per serving) and high oxylipin/precursor-PUFA ratios were found in fried falafel and processed foods such as vegetarian sausage/fish fingers. Our study provides first insights in the oxylipin concentrations of our daily food, indicating a relevant intake.

* modified from Koch, E.; Löwen, A.; Schebb, N. H. Do meals contain a relevant amount of oxylipins? LC-MS-based analysis of oxidized fatty acids in food. Food Chem 2024, 438, 137941.

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7.1 Introduction

Today's western diet is characterized by a high consumption of vegetable oils such as soybean oil and rapeseed oil. Over the last 50 years, the per capita consumption of vegetable oils in the US as well as in Europe has dramatically increased, e.g. in Germany, it has risen from around 4.4 kg to 17.5 kg per year [1]. The resulting effect – a more than threefold increase in linoleic acid (LA) and doubled intake in α-linolenic acid (ALA) at the end of the 20th century compared to its beginning – is controversially discussed in the context of human health [2].

Plant fats and oils as well as animal and fish fat are the main dietary fatty acid (FA) sources. Beside the direct use of plant oils e.g. in salads, the predominant amount is consumed in processed foods. Fatty acids are susceptible to oxidation including enzymatical and non-enzymatical pathways leading to a large variety of oxidation products [3-4]. For example, for LA and ALA, 9- and 13-hydro(pero)xides [3], 9(10)-/12(13)-/15(16)-epoxy-PUFA [4], keto-PUFA [3] as well as di- and trihydroxylated PUFA [5] can be formed. A recent study from Wheelock and coworkers largely extended the number of C18-oxylipins which can be found in human plasma [5]. Several oxylipins play an important role as lipid mediators regulating for example inflammation, pain, blood pressure or renal function [4].

The oxylipins circulating in human plasma can result from both endogenous formation from PUFA [4] or the diet [6-9]. Several of the daily consumed products are highly processed and thermally stressed which can cause lipid peroxidation [10]. Hydroperoxides which are initially formed during autoxidation are reported to decompose to hydroxy-PUFA and aldehydes in the gastrointestinal tract [11-12]. On the contrary, different studies demonstrate an absorption of hydroxy- and epoxy-PUFA [6-9]. Wilson et al. reported an increase in concentrations of ^{13}C-labeled hydroxy- and epoxy-PUFA in human plasma of healthy women after consumption of fat containing the labeled oxylipins [7-8]. Similarly, d4-13-HODE administered to rats by gavage was detectable in esterified plasma lipids and rat tissue [9]. Finally, Ferreiro-Vera found an increase in 13-HODE concentration in human serum after intake of fried sunflower oil [6].

Despite the high relevance of oxylipins occurring in our diet, data regarding their patterns and concentrations in food are scarce or not available. We and some other groups investigated the amounts of oxylipins in edible oils [13-14], fish [15-17] and milk [18]. Although being often limited regarding the number of analyzed oxylipins, these studies have already reported relevant amounts of oxidized PUFA in food. However, nothing is known about the amount and pattern of oxylipins in processed foods and whole meals.

Here, we analyzed the oxylipin patterns in refined and virgin oils gaining information about this main ingredient in processed foods and meals. Then, we investigated twelve different (fatty) foods and meals from restaurants and supermarkets. The samples were selected to cover a wide range of different type of food: i) foods with different fatty acid distributions, i.e. high amounts of saturated fatty acids, oleic acid, LA and PUFA; ii) processed animal and fish foods; iii) vegetarian replacements of meat/fish which is a rapidly growing market; iv) convenient meals from (fast food) restaurants and v) frequently consumed foods in Germany. We comprehensively analyzed the fatty acid and the total oxylipin concentrations to gain information about the amount of oxylipins in the product and calculated the ratio of oxylipin to the precursor PUFA to deduce the degree of oxidation. Finally, we estimated the amount of oxylipins which is consumed per portion. Owing to that, our study provides for the first time data about oxylipin patterns in real food and the concentrations consumed by common meals.

7.2 Experimental

7.2.1 Chemicals

Iso-propanol, methanol (MeOH), toluene and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile and ethanol (EtOH) were obtained from VWR (Darmstadt, Germany). Ammonium acetate was bought from Roth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Hydrochloric acid was obtained from Acros Organics/Fisher Scientific (Schwerte, Germany) Ultra-pure

water was generated using the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). All other chemicals, except for oxylipins, were from Merck (Darmstadt, Germany). Internal standards for fatty acid and oxylipin analysis as well as fatty acids and oxylipins were used as described [19-20]. 10*E*,12*E*-9-HODE, 9*E*,11*E*-13-HODE, *erythro*-9,10-dihydroxy stearic acid, *threo*-9,10-dihydroxy stearic acid and *trans*-9(10)-epoxy stearic acid were additionally purchased from Larodan (Stockholm, Sweden).

7.2.2 Food Samples

All food samples were obtained from local restaurants and supermarkets in Wuppertal, Germany. The following food samples were analyzed: i) rich in saturated fatty acids: grilled sausage, hamburger patties and pizza type “Margherita; ii) rich in oleic acid: falafel, vegan curry, sauce “Bolognese” and “Schnitzel”; iii) rich in LA: vegetarian meat replacement products (vegetarian sausage alternative and vegetarian chicken filet) as well as vegetarian fish fingers; iv) samples rich in n3-PUFA: traditional and omega-3 fish fingers as well as tuna filets.

The two vegan vegetable curry were bought in two different restaurants. Curry one consisted of around 220 g sauce, 366 g vegetables and 343 g rice. Curry two consisted of around 135 g sauce, 288 g vegetables and 301 g rice. The falafel samples were bought as falafel sandwiches in two different takeaways. Both sandwiches contained three falafels with a weight of 21-24 g. The sauce “Bolognese” were bought in a local supermarket (Barilla, Parma, Italy and La Doria S.p.A., Angri, Italy). The “Schnitzel” were bought prepared (fried) in a local supermarket and were made from pork (111 g in total; approx. 70 g of meat) or chicken (148 g; approx. 100 g of meat). Samples were homogenized with the coating. The vegetarian meat alternatives were bought unprepared in a local supermarket. The vegetarian sausage (41 g) was based on pea protein and contained coconut oil and sunflower oil according to manufacturer (LikeMeat GmbH, Düsseldorf, Germany). The vegetarian grilled chicken filet (75 g) was based on soybean and wheat proteins and contained rapeseed oil and sunflower oil according to manufacturer (Tivall Deutschland GmbH, Frankfurt/Main, Germany). Both samples were prepared using a contact grill prior to homogenization.

The grilling sausages were bought from two different local butchers and were roasted in a pan without oil prior to homogenization (132 g and 165 g). Hamburger patties (both 34 g) were bought as hamburgers from two major fast-food restaurant chains (McDonalds, Burger King). Tuna filet in sunflower oil were bought in the local supermarket (Saupiquet D GmbH, Krefeld, Germany and Frinsa Deutschland GmbH, Düsseldorf, Germany). Tuna sample 1 contained after draining the oil 134 g product (61 g sunflower oil) and Tuna sample 2 contained after draining the oil 130 g (55 g sunflower oil). Pizza Margherita was ordered at two different pizza restaurants. The vegetarian fish fingers were bought in a local supermarket (Heine Delikatessen GmbH & Co. KG, Eckernförde, Germany). They are based on salsify, partially oiled hemp seed protein, sunflower oil and flaxseed oil. The vegetarian fish fingers were analyzed raw (25 g; fish 15 g, breadcrumb coating 10 g) and after preparing in the oven (19 g; fish 9 g, breadcrumb coating 10 g). The omega-3 fish fingers and the normal fish fingers were bought in a local supermarket (Iglo GmbH, Hamburg, Germany). The fish fingers were analyzed raw (omega-3: 32 g; fish 19 g, breadcrumb coating 13 g; normal: 32 g; fish 19 g, breadcrumb coating 13 g) and after preparing in the oven (omega-3: 25 g; fish 12 g, breadcrumb coating 13 g; normal: 25 g; fish 13 g, breadcrumb coating 12 g). Representative pieces of the foods (> 5 g) were homogenized using a hand blender.

7.2.3 Quantification of total fat amount

The total fat content of the processed food samples was determined according to Schulte et al. [21]. In brief, 5 g of the homogenized food samples were weighed into a centrifuge tube and 5 ml of water, 5 ml of 8 M hydrochloric acid and 5 ml of toluene were added. Sample were capped, vortexed and heated in a drying oven at 120°C for 2 h. Samples were vortexed several times during heating. Samples were centrifuged and exactly 1 ml of the toluene phase was transferred into a vial. The toluene was removed using a gentle steam of nitrogen and the amount of fat was weighed.

7.2.4 Lipid extraction for total oxylipin and fatty acid analysis

Lipid extraction from the food samples was performed according to Matyash [22] with slight modifications. 50 mg of the homogenized food samples were weighed in 1.5 ml reaction tubes (Sarstedt, Nürnbrecht, Germany). To the samples were added: 10 µl of butylated hydroxy toluene (BHT, 0.2 mg/ml in MeOH) used as antioxidants, 2 x 3 mm stainless steel balls, 50 µl of ultra-pure water and 300 µl of MeOH acidified with 5% HAc. Samples were homogenized using a ball mill for 2 x 2 min at 25 Hz in pre-cooled racks (Retsch GmbH, Haan, Germany). 600 µl of methyl *tert*-butyl ether (MTBE) were added and the samples were shaken on a Vortex Genie 2 for 1.5 min. 300 µl of 0.15 M ammonium acetate were added and samples were centrifuged for phase separation at 4°C for 5 min at 10 000 x *g*. The upper phase was collected and the remaining phase was washed with 300 µl of MTBE. The upper phase was again collected and combined. Samples were evaporated to dryness using a vacuum concentrator (30°C, 1 mbar; Christ, Osterode, Germany). The lipid residue was reconstituted in 800 µl of *iso*-propanol.

7.2.5 Quantification of total oxylipin and fatty acid concentrations

Total (esterified and non-esterified) oxylipin concentrations and fatty acid concentrations were determined as described [20, 23-24]. In brief, for analysis of oxylipins in food samples, 50-250 µl of the reconstituted lipid extracts (depending on the lipid content) were diluted with *iso*-propanol to a final volume of 400 µl. For analysis of plant oils, 5 mg of oils were diluted in 2 ml of *iso*-propanol. To 50 µl of this solution, 350 µl of *iso*-propanol were added. 10 µl of deuterated oxylipin internal standards (IS1, 100 nM), 10 µl of BHT (0.2 mg/ml in MeOH) as 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 pre-conditioned solid phase extraction (SPE) cartridges (C8/anion exchange, bed weight 300 mg, volume 3 ml, BondElut Certify II, Agilent, Waldbronn, Germany). 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. Samples were reconstituted in 50 µl of MeOH containing a mix of

four substances used as second IS (IS 2) for calculation of IS 1 recovery rate [19]. 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 [19, 25]. The recovery of the internal standards in each sample was monitored as quality control using IS 2. Only samples with a recovery above 50% were further evaluated. Moreover, a quality control oil with known concentrations was prepared in triplicate within every batch to assure reliable and reproducible sample preparation. The concentrations are calculated as total amounts, i.e. per g of the sample.

Total fatty acid 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 mode following ESI(-). External calibration with deuterated internal standards was used for quantification [20]. The concentrations are calculated as total amounts, i.e. per 100 g of the sample. Additionally, the ratio% of oxylipin to precursor fatty acids was calculated by dividing the total oxylipin concentration (in nmol/g) by the total concentration of the precursor fatty acid (in nmol/g) and multiplied with 100 to get %.

7.3 Results and Discussion

Foods contain substantial concentrations of fatty acids – especially C18-(PU)FA – which are precursors of a variety of oxylipins. In this study, the patterns and concentrations of these oxylipins were determined in refined and virgin oils as well as different food samples to estimate the consumption of oxidized fatty acids by the diet.

7.3.1 Oxylipin pattern in virgin and refined plant oils

The concentrations of oxylipins in virgin rapeseed oil were higher for hydroxy- and epoxy-FA compared to refined rapeseed oil (Tab. 7.1). For example, for 13-HODE, the concentration in the virgin rapeseed oil was 237 nmol/g, while it was 17 nmol/g in the refined oil. Similarly, for 15(16)-EpODE, 2060 nmol/g *vs*. 27 nmol/g were found. Similar differences were found for hydroxy-PUFA (e.g. 13-HODE: virgin 2300 nmol/g; refined 370 nmol/g) and epoxy-PUFA (e.g. 15(16)-EpODE 77 nmol/g vs. 17 nmol/g) in refined compared to virgin sunflower oil. Thus, refining of oils reduces the content of hydroxy fatty acids and epoxy fatty acids. However, the concentrations of 15-HODE were similar in refined and virgin oils (430 nmol/g *vs*. 455 nmol/g in virgin and refined rapeseed oil, respectively). Consistent with our previous findings [13], we conclude that 15-HODE levels are not altered by refining, while the other analyzed hydroxy-PUFA are massively reduced. These results were supported by the analysis of oxylipins in rapeseed oil which was collected after different steps during the refining process (Fig. 11.20): in crude rapeseed oil, the hydroxy-PUFA concentration were higher, while they were reduced after the bleaching process. Again, 15-HODE remained unchanged. Interestingly, the deodorization step caused the specific formation of 9- and 13-*E*,*E*-hydroxy-LA concentrations which is consistent with our previous finding that *E*,*E*-hydroxy-PUFA in contrast to *E*,*Z*-hydroxy-PUFA increase during strong heating of oils [26].

Epoxy-FA can be hydrolyzed to their corresponding dihydroxy-PUFA e.g. under acetic conditions [26] . In the virgin oils, the dihydroxy-FA concentrations are low (15,16-DiHODE in rapeseed oil 49 nmol/g) and the epoxy-to-diol ratio showed a clear dominance of the epoxy-FA, e.g. for the pair 15(16)-EpODE/15,16-DiHODE, the epoxy-to-diol ratio was 42 in virgin rapeseed oil. This is dramatically different in the refined oils (Tab. 7.1): here, considerably high dihydroxy-FA concentrations were detected (15,16-DiHODE 940 nmol/g in rapeseed oil), while the epoxy fatty acid concentrations were low. Thus, the epoxy-to-diol ratio was considerably decreased. For example, in refined rapeseed oil, the epoxy-to-diol ratio for 15(16)-EpODE/15,16-DiHODE was 0.03.

[nmol/g]	Virgin rapeseed oil			Refined rapeseed oil			Virgin sunflower oil			Refined sunflower oil			Virgin soybean oil			Refined soybean oil		
	mean	± SD	ep/diol	mean	± SD	ep/diol	mean	± SD	ep/diol	mean	± SD	ep/diol	mean	± SD	ep/diol	mean	± SD	ep/diol
9(10)-epoxy stearic acid	262	± 7	0.56	96	± 9	0.12	1120	± 8	5.4	940	± 50	1.1	910	± 50	2.1	64	± 4	0.068
threo-9,10-dihydroxy stearic acid	467	± 6		770	± 20		207	± 4		880	± 20		440	± 10		936	± 7	
trans-9(10)-epoxy stearic	43	± 3	0.14	35	± 2	0.10	43	± 5	0.92	43	± 3	0.76	650	± 40	1.9	24	± 4	0.046
erythro-9,10-dihydroxy stearic acid	317	± 6		340	± 10		46.6	± 0.7		57	± 3		350	± 20		510	± 20	
9-HODE	176	± 6		12.0	± 0.2		2620	± 40		284	± 3		900	± 70		37	± 1	
E,*E*-9-HODE	19	± 1		3.16	± 0.03		135	± 8		221	± 5		220	± 20		7.7	± 0.5	
10-HODE	31.8	± 0.9		5.1	± 0.2		21	± 1		36.8	± 0.7		49	± 5		19.5	± 0.9	
12-HODE	21.2	± 0.6		3.50	± 0.06		12.0	± 0.4		22.1	± 0.2		31	± 1		13	± 2	
13-HODE	237	± 6		17.2	± 0.1		2300	± 110		370	± 10		1450	± 30		79	± 4	
E,*E*-13-HODE	38	± 1		5.8	± 0.4		156	± 8		370	± 10		171	± 6		14	± 1	
15-HODE	430	± 30		455	± 9		7.3	± 0.2		5.1	± 0.2		79	± 2		129	± 2	
9(10)-EpOME	62	± 1	5.8	34	± 1	2.1	2700	± 60	19	1910	± 70	5.4	1900	± 200	10	87	± 5	0.2
threo-9,10-DiHOME	10.7	± 0.1		16.6	± 0.7		145	± 3		351	± 6		188	± 8		540	± 10	
trans-9(10)EpOME	14.0	± 1.0	24	10.6	± 0.1	4.7	n.d.		-	86	± 6	4.2	105	± 6	29	24.6	± 0.3	1.1
erythro-9,10-DiHOME	0.58	± 0.03		2.3	± 0.1		1.48	± 0.01		20	± 1		3.60	± 0.02		22	± 1	
12(13)-EpOME	113	± 2	38	23	± 2	0.92	239	± 5	60	322	± 10	18	930	± 60	20	62	± 3	0.53
threo-12,13-DiHOME	3.01	± 0.08		25	± 1		4.0	± 0.2		17.7	± 0.2		46	± 2		118	± 1	
trans-12(13)EpOME	11.5	± 0.9	65	8.48	± 0.08	5.8	20	± 1	50	51	± 2	22	63	± 3	36	18.89	± 0.02	3.3
erythro-12,13-DiHOME	0.18	± 0.03		1.45	± 0.06		0.40	± 0.02		2.32	± 0.05		1.76	± 0.04		5.7	± 0.2	
9-HOTrE	52	± 3		10.3	± 0.5		6.3	± 0.3		2.29	± 0.09		68	± 2		9.7	± 1.0	
13-HOTrE	67	± 4		4.0	± 0.2		5.91	± 0.09		< LLOQ			149	± 3		5.2	± 0.4	
9(10)-EpODE	21.1	± 0.2	20	11.62	± 0.02	2.3	8.2	± 0.7	8.0	5.5	± 0.2	4.6	360	± 70	23	12.5	± 0.8	0.089
threo-9,10-DiHODE	1.03	± 0.05		5.0	± 0.1		1.02	± 0.02		1.19	± 0.03		16	± 1		141	± 2	
12(13)-EpODE	15.0	± 0.4	18	5.7	± 0.2	1.5	0.70	± 0.07	-	0.54	± 0.03	-	62	± 5	31	5.3	± 0.2	0.68
threo-12,13-DiHODE	0.82	± 0.06		3.7	± 0.3		< LLOQ			< LLOQ			2.02	± 0.06		7.81	± 0.08	
15(16)-EpODE	2060	± 70	42	27	± 2	0.029	77	± 5	20	16.6	± 0.2	4.3	2100	± 200	23	30	± 1	0.052
threo-15,16-DiHODE	49	± 2		940	± 30		3.8	± 0.1		3.9	± 0.3		92	± 5		580	± 20	

Tab. 7.1 (previous page): Concentrations of total (esterified + non-esterified) oxylipins in different refined and virgin plant oils. Concentrations of oxylipins were determined by LC-MS following hydrolysis and solid phase extraction. Samples were analyzed in triplicate (mean ± SD, n = 3; n.d. = not determinable due to matrix interference).

Similar effects of refining on the epoxy- and dihydroxy-PUFA concentrations were found for virgin and refined sunflower oil and – more pronounced – for soybean oil (Tab. 7.1). Analysis of oxylipins in samples from different steps of rapeseed oil refining revealed that epoxy-PUFA are removed and dihydroxy-PUFA are formed (probably from the epoxy-PUFA) during the bleaching step (Fig. 11.20). While deodorization does not change *cis*-epoxy-PUFA concentrations, this step leads to the formation of *trans*-epoxy-PUFA. This is consistent with our previous finding that together with the increase of *E*,*E*-hydroxy-PUFA concentrations (see above), the concentrations of *trans*-epoxy-PUFA are specifically increased during strong heating of oils [26].

The refinement process causes degradation of *E*,*Z*-hydroxy-PUFA and hydrolysis of *cis*-epoxy-FA to dihydroxy-FA. Thus, dihydroxy-FA might be a potential marker to assess if an edible oil was refined. Even more specific might be the use of the epoxy-to-diol ratio. Future studies are needed to evaluate the applicability of dihydroxy-FA as new parameter for the characterization and identification of refined oils; however, the epoxy-to-diol-ratio seems to be a promising approach.

7.3.2 Concentrations of oxylipins in foods containing high amounts of saturated fatty acids

Three food samples contained high amounts of saturated fatty acids: Grilled sausage, hamburger patties and pizza type “Margherita”. Grilled sausage consists of substantial amount of 15-20 g/100 g fat (Tab. 7.2). The main fatty acids were palmitic acid (3.5-4.4 g/100 g), stearic acid (1.9-2.2 g/100 g), oleic acid (6.3-11 g/100 g) and LA (1.1-1.7 g/100 g, Tab. 7.2). High amounts of palmitic acid (4.9-5.3 g/100 g), stearic acid (2.3-2.5 g/100 g) and oleic acid (8.8-10 g/100 g) were also found in hamburger patties which also contained palmitoleic acid (C16:1 n7, 0.93-1.0 g/100 g). The total amount of fat was very similar with 20-22 g/100 g. In pizza “Margherita”, specifically high concentrations of myristic acid (C14:0, 1.9 g/100 g) were detected. Additionally, palmitic acid (5.3-5.5 g/100 g),

stearic acid (1.9-2.0 g/100 g) and oleic acid (3.2-3.6 g/100 g) occurred at high levels. The total fat amount was determined to be 19-23 g/100 g.

Tab. 7.2: Total fatty acid concentrations in the analyzed meals/foods. Lipids were extracted from food samples by a modified Matyash extraction [22]. Total fatty acid concentrations were determined by LC-MS after hydrolysis of esterified fatty acids in two representative food samples.

[mg/100 g]	Vegan vegetable curry	Falafel	Sauce "Bolognese"	Schnitzel	Veg. chicken	Veg. grilling sausage
C6:0	8.76 - 31.3	< LLOQ	< LLOQ	< LLOQ	< LLOQ	34.5
C8:0	107 - 430	7.08 - 22.4	< LLOQ	3.18 - < LLOQ	< LLOQ	370
C10:0	90.3 - 401	< LLOQ	< LLOQ	5.20 - 3.07	< LLOQ	305
C 11:0	< LLOQ - 2.21	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.31
C 12:0	698 - 3090	6.30 - 12.5	4.16 - 4.82	9.01 - 1.68	15.0	2910
C 13:0	0.462 - 2.24	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.95
C 14:1 n5	< LLOQ	< LLOQ	7.92 - 30.7	2.49 - < LLOQ	< LLOQ	< LLOQ
C 14:0	217 - 1260	25.1 - 49.8	51.2 - 119	22.8 - 13.7	8.44	1130
C 15:1 n5	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 15:0	< LLOQ	5.06 - 8.42	5.10 - 15.7	3.31 - 2.35	1.30	3.12
C 16:1 n7	11.8 - 18.4	52.2 - 61.5	63.2 - 158	82.5 - 57.1	13.2	7.40
C 16:0	336 - 909	1260 - 2870	620 - 1110	1030 - 772	378	971
C17:0	2.47 - 4.92	14.0 - 18.8	11.9 - 29.7	6.48 - 8.04	2.85	4.42
C 18:4 n3	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 18:3 n6	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 18:3 n3	307 - 634	873 - 1070	17.7 - 24.9	445 - 937	265	74.8
C 18:2 n6	692 - 1270	3520 - 4460	304 - 80.5	1290 - 2080	915	2930
C 18:1 n9+7	2430 - 4870	10500 - 12900	2430 - 1580	4470 - 6880	2590	2570
C 18:0	116 - 279	360 - 535	339 - 670	260 - 291	110	395
C 19:0	< LLOQ	< LLOQ	< LLOQ - 4.37	< LLOQ	< LLOQ	< LLOQ
C 20:5 n3	< LLOQ	< LLOQ	< LLOQ - 0.929	< LLOQ	< LLOQ	< LLOQ
C 20:4 n6	< LLOQ	< LLOQ	5.71 - 5.11	26.3 - 27.4	< LLOQ	2.92
C 20:4 n3	< LLOQ	< LLOQ	< LLOQ - 1.12	< LLOQ	< LLOQ	< LLOQ
C 20:3 n9	< LLOQ	< LLOQ	0.443 - 0.497	2.01 - 2.42	< LLOQ	< LLOQ
C 20:3 n6+n3	< LLOQ - 0.283	< LLOQ	2.43 - 2.77	2.50 - 4.45	< LLOQ	< LLOQ
C 20:2 n6	2.08 - 4.01	7.47 - 9.60	4.38 - < LLOQ	5.36 - 8.65	2.41	0.677
C 20:1 n9	46.8 - 87.4	163 - 249	20.5 - 16.7	76.9 - 146	49.6	17.9
C 20:0	24.5 - 39.3	102 - 142	9.24 - 7.59	40.5 - 63.0	26.0	26.2
C 21:0	0.732 - 1.36	4.20 - 4.48	< LLOQ	1.10 - 1.72	< LLOQ	< LLOQ
C 22:6 n3	< LLOQ	< LLOQ	< LLOQ	2.56 - 2.02	< LLOQ	< LLOQ
C 22:5 n3	< LLOQ	< LLOQ	1.40 - 3.13	3.82 - 3.79	< LLOQ	< LLOQ
C 22:5 n6	< LLOQ	< LLOQ	< LLOQ	2.12 - < LLOQ	< LLOQ	< LLOQ
C 22:4 n6	< LLOQ	< LLOQ	1.21 - 0.961	7.29 - 3.37	< LLOQ	< LLOQ
C 22:2 n6	< LLOQ - 0.378	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 22:1 n9	10.6 - 36.7	21.1 - 60.5	0.785 - 0.804	15.2 - 35.2	8.17	0.956
C 22:0	12.4 - 22.6	52.8 - 67.3	16.0 - 1.73	21.8 - 33.2	16.1	38.9
C 23:0	0.742 - 1.72	5.26 - 5.94	< LLOQ	0.884 - 1.31	0.207	1.60
C 24:1 n9	8.73 - 17.3	35.1 - 44.2	< LLOQ	12.3 - 28.8	10.5	1.91
C 24:0	7.04 - 13.9	37.4 - 46.6	5.45 - < LLOQ	14.2 - 17.8	6.40	21.8
Total fat ([a] determ. / [b] accord. to manu-facturer), [g/100 g]:	4.58 [a] - 12.9 [a]	16.6 [a] - 24.1 [a]	4.00 [b] - 4.60 [b]	7.51 [a] - 13.1 [b]	7.00 [b]	12.0 [b]

Tab. 7.2: continued.

[mg/100 g]	Grilling sausage pork	Hamburger patty	Tuna in sunflower oil	Pizza "Margherita"	Veg. fish fingers	Omega-3 fish fingers	Fish fingers
C6:0	< LLOQ	< LLOQ	< LLOQ	262 - 235	< LLOQ	< LLOQ	< LLOQ
C8:0	< LLOQ	< LLOQ	< LLOQ	142 - 127	< LLOQ	< LLOQ	< LLOQ
C10:0	8.80 - 8.67	< LLOQ	< LLOQ	507 - 486	< LLOQ	< LLOQ	< LLOQ
C 11:0	< LLOQ	< LLOQ	< LLOQ	19.1 - 16.4	< LLOQ	< LLOQ	< LLOQ
C 12:0	13.5 - 11.7	16.9 - 17.2	< LLOQ	640 - 685	2.81	2.89	< LLOQ
C 13:0	< LLOQ	< LLOQ	< LLOQ	27.7 - 22.6	< LLOQ	< LLOQ	< LLOQ
C 14:1 n5	< LLOQ	186 - 216	< LLOQ	154 - 154	< LLOQ	< LLOQ	< LLOQ
C 14:0	173 - 172	528 - 568	9.62 - 13.6	1860 - 1910	13.5	24.5	14.6
C 15:1 n5	< LLOQ	3.74 - 3.40	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 15:0	7.55 - 6.17	68.1 - 60.9	2.97 - 4.35	187 - 173	3.67	4.28	4.33
C 16:1 n7	275 - 326	931 - 1030	12.7 - 12.2	219 - 214	13.4	46.4	31.0
C 16:0	3550 - 4430	4850 - 5270	393 - 686	5500 - 5280	870	681	607
C17:0	32.1 - 41.2	125 - 114	7.25 - 8.43	91.0 - 74.7	5.49	7.56	6.62
C 18:4 n3	< LLOQ	< LLOQ	1.67 - 0.806	< LLOQ	< LLOQ	6.59	2.14
C 18:3 n6	5.48 - 4.98	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 18:3 n3	86.6 - 152	86.4 - 78.8	26.7 - 26.7	93.7 - 140	1010	626	570
C 18:2 n6	1090 - 1720	328 - 412	1730 - 4790	284 - 267	4240	1500	1460
C 18:1 n9+7	6260 - 10900	8830 - 10000	855 - 2680	3610 - 3150	2970	5110	4890
C 18:0	1930 - 2220	2500 - 2280	261 - 528	2040 - 1880	471	251	200
C 19:0	< LLOQ	15.0 - 12.5	1.92 - 2.00	11.9 - 9.89	< LLOQ	< LLOQ	< LLOQ
C 20:5 n3	3.55 - < LLOQ	6.49 - 6.58	24.8 - 17.0	7.92 - 12.3	< LLOQ	79.1	72.4
C 20:4 n6	58.3 - 43.7	35.8 - 34.3	17.4 - 7.38	21.5 - 14.7	< LLOQ	5.75	3.38
C 20:4 n3	< LLOQ	6.16 - 5.34	< LLOQ	5.15 - 7.43	< LLOQ	2.55	1.35
C 20:3 n9	5.15 - 3.97	2.94 - 2.76	< LLOQ	< LLOQ - 1.31	< LLOQ	< LLOQ	< LLOQ
C 20:3 n6+n3	21.6 - 22.6	18.0 - 15.6	0.656 - < LLOQ	14.8 - 11.9	< LLOQ	0.707	0.581
C 20:2 n6	37.9 - 45.7	< LLOQ	1.57 - 1.56	< LLOQ	1.42	6.26	6.01
C 20:1 n9	122 - 152	90.8 - 81.7	10.4 - 20.2	52.6 - 42.3	23.1	116	123
C 20:0	37.4 - 71.4	33.4 - 37.1	15.6 - 35.7	34.6 - 33.0	32.5	57.8	55.3
C 21:0	< LLOQ	< LLOQ	< LLOQ	4.04 - 3.58	< LLOQ	1.58	1.52
C 22:6 n3	< LLOQ	< LLOQ	142 - 112	< LLOQ	< LLOQ	106	81.3
C 22:5 n3	12.9 - 13.2	18.6 - 16.5	3.50 - 2.32	13.1 - 15.1	< LLOQ	7.49	4.63
C 22:5 n6	< LLOQ	< LLOQ	16.9 - 5.68	< LLOQ	< LLOQ	1.62	< LLOQ
C 22:4 n6	11.8 - 12.0	6.28 - 5.14	1.05 - 0.423	3.69 - < LLOQ	< LLOQ	< LLOQ	< LLOQ
C 22:2 n6	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 22:1 n9	2.19 - 2.87	3.48 - 2.93	0.608 - 0.813	7.16 - 6.12	< LLOQ	47.7	77.5
C 22:0	< LLOQ - 13.7	9.39 - 13.3	25.0 - 55.0	10.5 - 13.0	63.6	32.1	31.8
C 23:0	< LLOQ	< LLOQ	0.315 - 2.12	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C 24:1 n9	< LLOQ	< LLOQ	3.54 - 3.03	6.19 - 6.55	< LLOQ	24.7	24.9
C 24:0	< LLOQ	< LLOQ	8.16 - 23.9	< LLOQ	28.8	13.8	14.0
Total fat ([a] determ. / [b] accord. to manu-facturer), [g/100 g]:	14.6 [a] - 19.5 [a]	21.8 [a] - 20.4 [a]	4.53 [a] - 10.3 [a]	18.9 [a] - 22.5 [a]	10.1 [b]	8.00 [b]	8.60 [b]

In all samples, high oxylipin concentrations were determined for the oleic acid derived 9(10)-epoxy and 9,10-dihydroxy stearic acid (Fig. 7.1, Tab. 11.12-11.18). In grilled sausage, higher levels of LA-derived oxylipins compared to their levels in hamburger patties and pizza were found (e.g. 13-HODE: grilled sausage 18-22 nmol/g; pizza 8.5-8.7 nmol/g; hamburger patty 3.5-4.7 nmol/g). Both findings

are consistent with the fatty acid pattern with oleic acid being the most abundant fatty acid and grilled sausage also containing considerable amounts of LA, while pizza and hamburger patties did not (Tab. 7.2). Interestingly, in grilled sausage, a high relative concentration (oxylipin conc./precursor fatty acid conc.) was determined for the terminal epoxide of ALA, 15(16)-EpODE (around 0.3-0.5%), while the other ALA-oxylipins had a rather low (relative) concentration of around 0.02-0.03%. For pizza, threefold higher relative concentrations were determined for both, LA and ALA derived oxylipins (up to 0.15%).

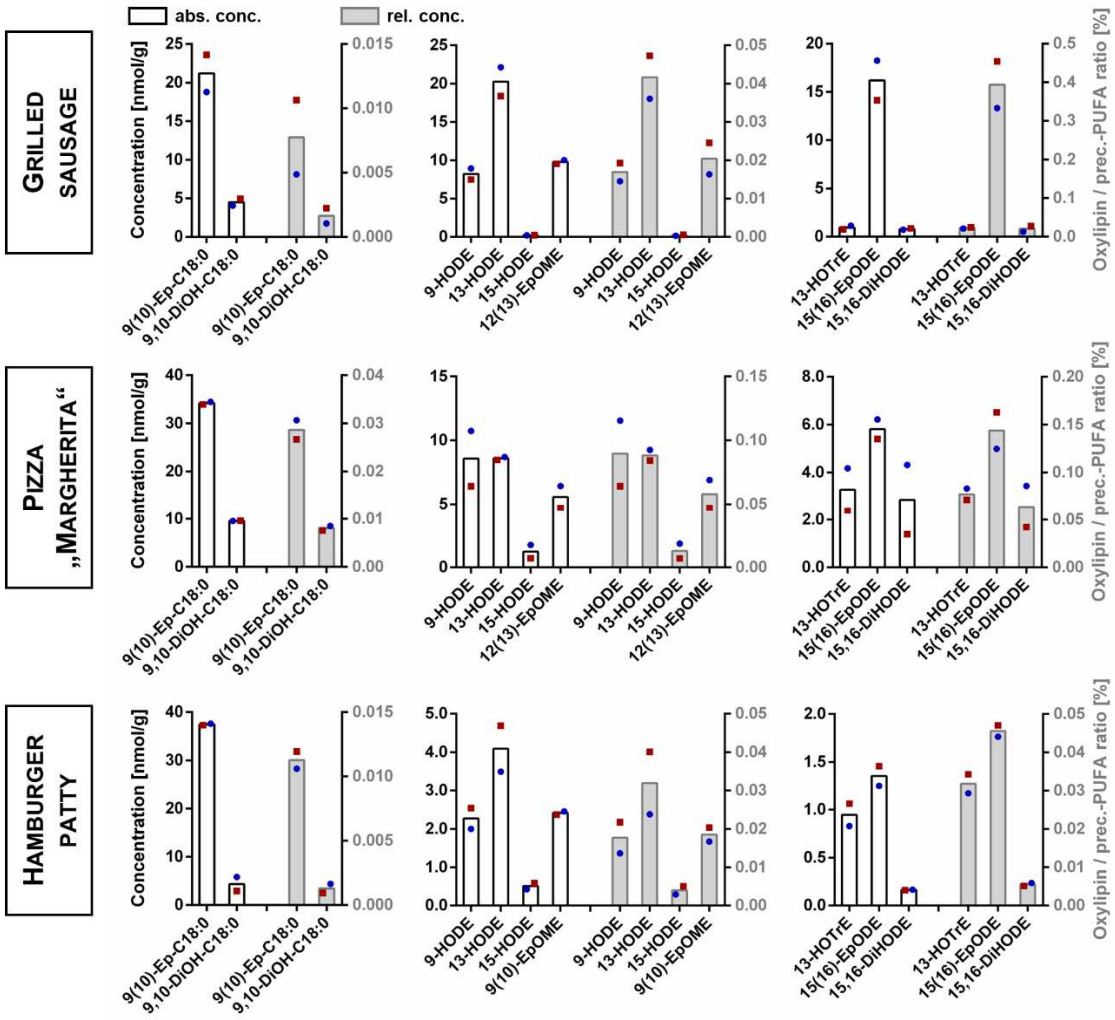

Fig. 7.1: Concentrations of oxylipins in food samples rich in saturated fatty acids. Two different grilled sausages, pizza type “Margherita” and hamburger patties were analyzed in duplicate. Shown are the determined oxylipin concentrations (black bars) as well as the ratio between oxylipin and precursor-FA concentration (grey bars) and the individual oxylipin concentration of the two samples (blue and red dots).

Based on these three food samples containing high amounts of saturated fatty acids it can be concluded that the absolute concentrations of oxylipins are low

due to low precursor fatty acid concentrations. Moreover, the ratio of oxylipins to precursor FA are also low, indicating only a low to moderate oxidation of FA. This can be explained by the stability of saturated fatty acids against autoxidation [27] which hampers the radical chain reaction resulting in lower oxidation of unsaturated FA.

7.3.3 Oxylipin pattern in food samples high in oleic acid

From the twelve food samples analyzed in this study, four contained high amounts of oleic acid: falafel, vegan curry, sauce “Bolognese” and “Schnitzel”. The main fatty acids in both falafel products were palmitic acid (1.3-2.9 g/100 g), oleic acid (11-13 g/100 g), LA (3.5-4.5 g/100 g) and ALA (0.87-1.1 g/100 g, Tab. 7.2) which is consistent with previously reported main fatty acids in falafel [28]. The total fat content varied between 17 g/100 g and 24 g/100 g. In the two vegan vegetable currys, the total amount of fat was different (curry one: 4.6 g/100 g *vs*. curry two: 13 g/100 g) as well as the fatty acid distribution. Lauric acid (0.70-3.1 g/100 g), oleic acid (2.4-4.9 g/100 g), LA (0.69-1.3 g/100 g) and myristic acid (0.22-1.3 g/100g) occurred in high amounts in both curry samples. The specific occurrence of mid-chain fatty acids (C10, C12, C14) indicates the use of coconut fat [29], as consumers would expect in a curry. The large differences in the concentrations between the mid-chain fatty acids, which are less pronounced in the C18 fatty acids, could indicate that more coconut milk was used in curry two. The higher total fat content also supports this. The two different “Sauce Bolognese” contained 4.0-4.6 g/100 g fat and the main fatty acids were palmitic acid (0.62-1.1 g/100 g), stearic acid (0.34-0.67 g/100 g) and oleic acid (1.6-2.4 g/100 g). The “Schnitzel” made from pork or chicken had a similar fatty acid distribution, with pork having as expected a higher fat content (13 g/100 g; chicken 7.5 g/100 g). The main fatty acids were palmitic acid (0.77-1.0 g/100 g), oleic acid (4.5-6.9 g/100 g), LA (1.3-2.1 g/100 g) and ALA (0.44-0.94 g/100 g, Tab. 7.2).

Falafel contained the highest oxylipin concentrations of all investigated food samples (Fig. 7.2, Tab. 11.12-11.18). The highest levels were found for 9(10)-epoxy stearic acid (270-400 nmol/g) as well as the epoxy-PUFA of LA and ALA (e.g. 9(10)-EpOME 280-360 nmol/g; 15(16)-EpODE around 180 nmol/g).

Additionally, the hydrolysis products 9,10-dihydroxy stearic acid (140-360 nmol/g) and 15,16-DiHODE (85-110 nmol/g) occurred in high amounts. This may be explained by the deep-frying of falafel in plant oil. During the frying process, the falafel made from mashed beans and chickpeas soak up the thermally stressed frying oil [30], explaining the high concentrations of *trans*-epoxy fatty acids and *E*,*E*-9- and *E*,*E*-13-HODE (Tab. 11.13) which are formed during frying [26]. Additionally, chickpeas contain high amounts of LA [31], which is also prone to oxidation especially during frying, leading to a complex pattern of oxylipins.

In the oxylipin profile of the vegan vegetable currys, the high concentration of 15-HODE (11 nmol/g and 21 nmol/g) and the high concentration of 15(16)-EpODE in curry one (27 nmol/g) were striking (Fig. 7.2). We previously reported high levels of 15(16)-EpODE and 15-HODE in rapeseed, soybean and flaxseed oils [13] Thus, the high concentrations might come from the plant oil(s) used for preparing the curry. Interestingly, we also found high concentrations of dihydroxy-PUFA, e.g. for 15,16-DiHODE in curry one: 17 nmol/g and in curry two: 36 nmol/g. indicating the use of refined oils (see above).

The "Schnitzel" showed higher oxylipin concentration than the "Sauce Bolognese" (Fig. 7.2). The main oxylipins derived from oleic acid, LA and ALA were all in the range of 40-80 nmol/g. Thus, the oxylipin/PUFA ratio was the highest for ALA-derived oxylipins followed by LA-derived and oleic acid-derived oxylipins. This order was also observed in other food samples such as sauce "Bolognese" and it reflects the susceptibility of the FA for autoxidation [32]. Sauce "Bolognese" contained rather low oxylipin concentrations which varied strongly between the two samples, with higher concentrations found in sample one. This is consistent with higher oleic acid and LA concentrations in sample one (Tab. 7.2). However, the oxylipin/FA ratio for oleic acid and ALA is higher in sample one indicating a higher FA oxidation rate.

Based on the analysis of the oleic acid rich food samples it can be concluded, that though oleic acid is the main fatty acid, ALA and LA are more oxidized (higher oxylipin/precursor-FA ratio). Frying of foods results in high concentrations of

oxylipins and the oxylipin pattern comprises specific heat induced oxylipins such as *E*,*E*-hydroxy-PUFA. Moreover, the oxylipin pattern allows do deduce the oil used for the preparation of complex meals as demonstrated for the analysis of the currys.

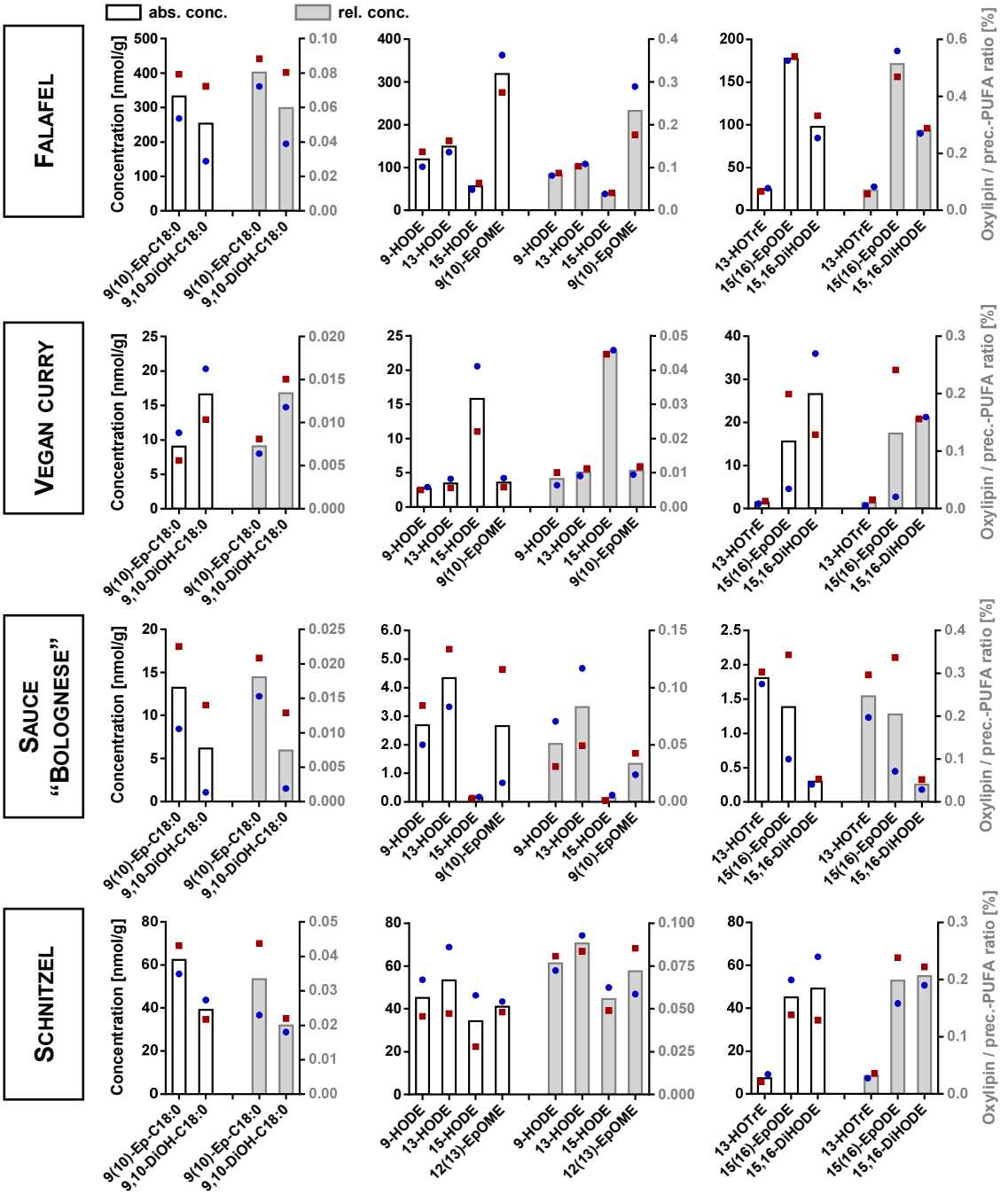

Fig. 7.2: Concentrations of oxylipins in food samples rich in oleic acid. Two different falafel, vegan currys, sauces type "Bolognese" and "Schnitzel" (one from pork (blue) and one from chicken (red)) were analyzed in duplicate. Shown are the determined oxylipin concentrations (black bars) as well as the ratio between oxylipin and precursor-FA concentration (grey bars) and the individual oxylipin concentration of the two samples (blue and red dots).

7.3.4 Oxylipin pattern in LA rich food samples

The main fatty acid in the analyzed meat replacement products (vegetarian sausage alternative (12 g/100 g fat) and vegetarian chicken filet (7 g/100 g fat)) was aside from oleic acid (both 2.6 g/100 g) LA (vegetarian chicken 0.91 g/100 g; vegetarian sausage 2.9 g/100 g; Tab. 7.2). Additionally, the vegetarian chicken contained high amounts of palmitic acid (0.38 g/100 g) and ALA (0.27 g/100 g), which is consistent with soybean and wheat proteins as well as rapeseed oil and sunflower oil being used for its production. The vegetarian sausage was made from pea protein as well as coconut oil and sunflower oil as fat source and thus, it contains high concentrations of lauric acid (2.9 g/100 g) and myristic acid (1.1 g/100 g).

In the third product, baked vegetarian fish fingers, high amounts of palmitic acid (0.87 g/100 g), oleic acid (3.0 g/100 g), LA (4.2 g/100 g) and ALA (1.0 g/100 g, Tab. 7.2) were determined. The vegetarian fish fingers were produced from salsify, partially oiled hempseed protein, sunflower oil and flaxseed oil according to manufacturer. The total fat content was declared to be 10 g/100 g. Due to the use of flaxseed oil, the vegetarian fish fingers contain a relevant level of n3-PUFA, namely ALA, but not EPA or DHA.

The vegetarian sausage contained relevant concentrations of 9- and 13-OH-LA and -ALA (e.g. 9-HODE 180 nmol/g; 13-HODE 270 nmol/g; 13-HOTrE: 34 nmol/g, Fig. 7.3). The oxylipin concentrations were similar to the concentrations in the fried falafel. Additionally, the oxylipin/FA ratio was high with up to 1.5% indicating a high degree of FA oxidation. On the contrary, lower oxylipin concentrations were found in vegetarian chicken (9-HODE 41 nmol/g; 13-HODE 53 nmol/g; 13-HOTrE: 6.2 nmol/g; Fig. 7.3, Tab. 11.13-11.14). Thus, one would assume that the oils in the sausage are more (thermally) stressed during production. However, the vegetarian chicken contained relevant amounts of *trans*-epoxy-FA, (e.g. *trans*-9(10)-epoxy stearic acid: sausage 12 nmol/g; chicken: 50 nmol/g), while the *cis*-epoxy-FA concentrations were similar (*cis*-9(10)-epoxy stearic acid 37 nmol/g vs. 38 nmol/g). While *cis*-epoxy-FA can result from enzymatical conversion [33], *trans*-epoxy-FA are formed autoxidatively particularly at high temperatures [26, 30],

suggesting that the oils used for the vegetarian chicken were strongly heated during production. Interestingly, the vegetarian chicken contained high amounts of 15,16-DiHODE (40 nmol/g), while the concentration was low in the sausage (2.8 nmol/g). This indicates the use of refined n3-PUFA containing oil (Tab. 7.1). Indeed, based on the list of ingredients, rapeseed oil (rich in ALA) was used for the vegetarian chicken, while the sausage was made of sunflower and coconut oil.

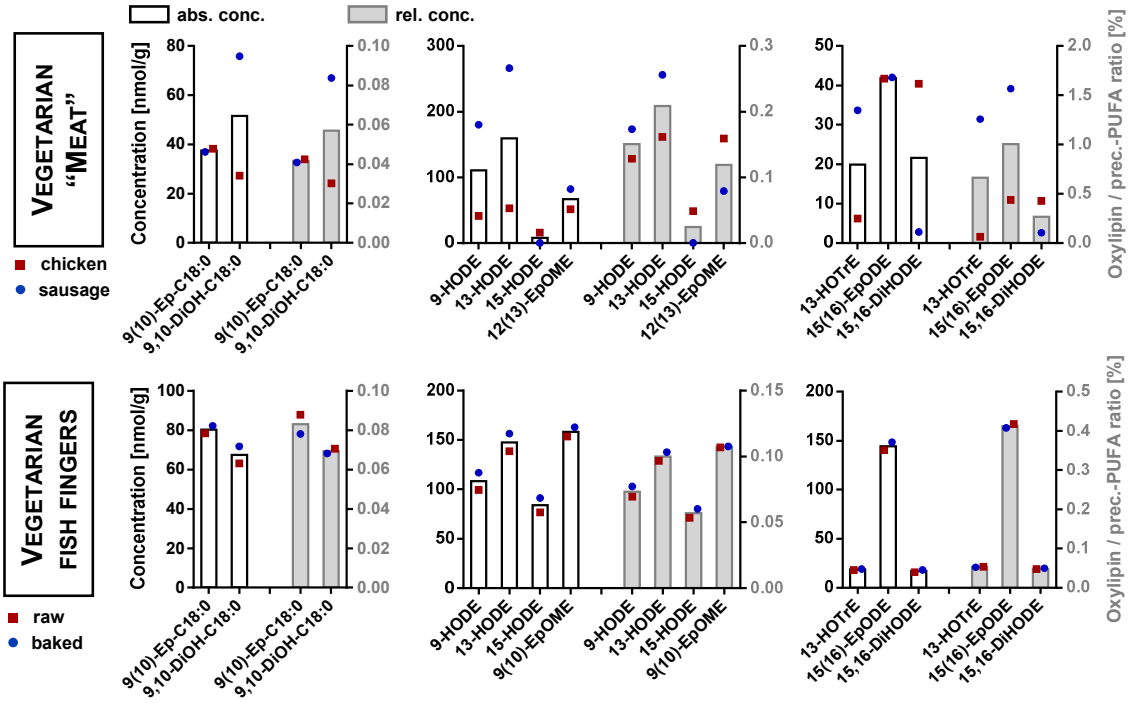

Fig. 7.3: Concentrations of oxylipins in vegetarian meat and fish alternatives rich in linoleic acid. Two different meat alternatives (type sausage (blue) and type chicken filet (red)) and vegetarian fish fingers (raw (red) and baked (blue)) were analyzed in duplicate. Shown are the determined oxylipin concentrations (black bars) as well as the ratio between oxylipin and precursor-FA concentration (grey bars).

The vegetarian fish finger alternatives contained high levels of LA derived oxylipins (Fig. 7.3, Tab. 11.13) with 120 nmol/g for 9-HODE and around 150 nmol/g for 13-HODE and 9(10)-EpOME. Additionally, they showed relevant levels of *E*,*E*-HODE (9-*E*,*E*-HODE 71 nmol/g and 13-*E*,*E*-HODE 130 nmol/g) and of *trans*-epoxy-FA (e.g. *trans*-9(10)-EpOME 63 nmol/g). We previously showed that *trans*-epoxy-FA and *E*,*E*-hydro(pero)xy-LA are formed during frying, i.e. oil heated to high temperature. Thus, similarly to the vegetarian meat replacements, the vegetarian fish fingers seem to be exposed to (strong) heat during production. Interestingly, the oxylipin concentration between the raw and baked vegetarian

fish fingers differed only slightly (e.g. 13-HODE: raw 140 nmol/g; baked 160 nmol/g, Fig. 7.3). Thus, baking of the fish fingers seems not to promote oxidation of the contained PUFA.

Overall, the LA containing vegetarian meat and fish finger alternatives can be made from different protein and fat sources resulting in a distinct fatty acid distribution. All products contain a relevant amount of LA and ALA oxidation products and particularly *E*,*E*-HODE and *trans*-epoxy-FA which are formed during strong heating of oils.

7.3.5 Concentrations of oxylipins in DHA and EPA containing fish products

The main fatty acids in baked fish fingers declared as “rich in omega-3” were palmitic acid (0.68 g/100 g), oleic acid (5.1 g/100 g), LA (1.5 g/100 g) and ALA (0.63 g/100 g, Tab. 7.2). The concentration of EPA was determined to be 0.079 g/100 g and of DHA 0.11 g/100 g (sum of EPA + DHA of 0.19 g/100 g). The manufacturer recommends a portion of five fish fingers which are 150 g. Based on this, an intake of around 280 mg EPA and DHA results from one meal which is slightly above the recommend intake of 250 mg EPA and DHA per day [34]. The baked traditional fish fingers also contained mainly palmitic acid (0.61 g/100 g), oleic acid (4.9 g/100 g), LA (1.5 g/100 g) and ALA (0.57 g/100 g, Tab. 7.2). EPA was determined to be 0.072 g/100 g and DHA 0.081 g/100 g (sum of EPA + DHA 0.15 g/100 g). Based on the portion of 5 fish fingers, the intake of EPA and DHA per meal would be 230 mg. Thus, fish fingers contain enough EPA and DHA to achieve a sufficient supply for the day. However, one meal of fish fingers contains only enough EPA and DHA to fulfill the recommendation for one day, while for oily cold-water fish like salmon, eating of 1-2 servings per weeks results in a sufficient supply [34].

The main fatty acids in tuna filet pickled in sunflower oil were palmitic acid (0.39-0.69 g/100 g), stearic acid (0.26-0.53 g/100 g), oleic acid (0.85-2.7 g/100 g) and LA (1.7-4.8 g/100 g). The concentration of EPA and DHA was found to be 0.017-0.025 g/100 g and 0.11-0.14 g/100 g (Tab. 7.2), respectively, resulting in a sixfold

higher concentration of DHA compared to EPA which was also reported previously for tuna [35]. Thus, the sum of EPA and DHA was 0.13-0.17 g/100 g.

The oxylipin patterns of the “n3-rich” and normal fish fingers were very comparable (Fig. 7.4). The main LA-derived oxylipins were around 40 nmol/g except for 13-HODE in normal fish fingers (baked: 67 nmol/g). For oxidized ALA, high concentrations were determined for 15(16)-EpODE (baked: 70-85 nmol/g) and 15,16-DiHODE (baked: 51-58 nmol/g). The high 15(16)-EpODE concentration may result from the rapeseed oil which is declared as ingredient, as we previously showed that it occurs in high concentration in rapeseed oil [13]. The highest concentration of EPA derived oxylipins were determined for 9-, 12- and 18-HEPE as well as 17(18)-EpETE and of DHA derived oxylipins for 4-, 11- and 20-HDHA as well as 19(20)-EpDPE (Fig. 7.4, Tab. 11.17-11.18). Interestingly, 9- and 18-HEPE as well as 11- and 20-HDHA, for which the highest concentrations among the EPA and DHA oxylipins were found, are formed autoxidatively [36-38]; however, their concentrations did not further increase during baking. A previous study even reported decreased concentrations of monohydroxylated ARA, EPA and DHA after baking of Atlantic salmon compared to the raw fish, while HODE concentrations increased [17]. This increase was lower when the salmon was coated with e.g. different sauces [17]. As the fish fingers are covered by a breadcrumb coating, this could explain, why there was no increase detectable in our study; however, we also did not see the previous described thermal degradation. The effect of baking on the oxylipin level might depend on the food, as a second study from the same group using farmed rainbow trout showed no decrease in monohydroxylated EPA and DHA after baking [15].

The tuna filets pickled in sunflower oil contained low amounts of oxylipins which varied strongly between the two samples (Fig. 7.4, Tab. 11.12-11.18). For LA derived oxylipins, in sample two, higher concentrations were determined (e.g. 13-HODE 14 nmol/g vs. sample two 4.0 nmol/g).

Fig. 7.4 (next page): Concentration of oxylipins in DHA and EPA containing fish products. Two different types of fish fingers (one declared as “omega-3 rich” and one without additional declaration; (raw (red) and baked (blue)) as well as two different tuna filet samples pickled in sunflower oil were analyzed in duplicate. Shown are the determined oxylipin concentrations (black bars) as well as the ratio between oxylipin and precursor-PUFA concentration (grey bars).

TUNA IN SUNFLOWER OIL
FISH FINGERS
"OMEGA-3" FISH FINGERS
Concentration [nmol/g]
Oxylipin / prec.-PUFA ratio [%]
abs. conc.
rel. conc.
raw
baked
9-HODE
13-HODE
15-HODE
9(10)-EpOME
12(13)-EpOME
9-HOTrE
13-HOTrE
15(16)-EpODE
15,16-DiHODE
5-HEPE
9-HEPE
12-HEPE
18-HEPE
17(18)-EpETE
4-HDHA
11-HDHA
20-HDHA
19(20)-EpDPE

Interestingly, sample one contained equal amounts of several LA and DHA derived oxylipins (e.g. 4-HDHA 4.0 nmol/g, 20-HDHA 4.1 nmol/g, Fig. 7.4) despite a much lower DHA level (0.14 g/100 g) compared to LA (1.7 g/100 g, Tab. 7.2). On the contrary, sample two having high LA oxylipin levels contained lower concentrations of 4-HDHA (1.6 nmol/g) and 20-HDHA (1.8 nmol/g) and also lower EPA derived oxylipin concentrations than sample one (e.g. 18-HEPE 0.42 nmol/g *vs*. 0.93 nmol/g). While the differences in the two samples for LA derived oxylipins could be explained by the different levels of LA (1.7 g/100 g *vs*. 4.8 g/100 g), the EPA (0.017 g/100 g *vs*. 0.025 g/100 g) and DHA (0.11 g/100 g *vs*. 0.14 g/100 g) concentrations were similar. Thus, it is likely that the tuna used for sample one was more oxidized maybe due to different processing techniques. Surprisingly, the concentration of most ALA derived oxylipins were similar in both samples (< 0.1 nmol/g), while 15,16-DiHODE was found in high concentrations (4.5-4.9 nmol/g). Similarly, concentrations were higher for other LA- and oleic acid-derived dihydroxy fatty acids compared to the corresponding epoxy fatty acids (Tab. 11.12-11.13). The high dihydroxy fatty acids probably results from the added (refined) sunflower oil (Tab. 7.1).

Fish fingers and tuna contain a relevant amount of EPA and DHA to achieve the recommended daily intake of EPA and DHA with one portion, however, they do not contain enough n3-PUFA to compensate for days without fish meals. Baking of coated fish is a gentle way of processing fish as it did not change n3-PUFA concentrations nor increases oxylipin concentrations. Tuna contains low amounts of oxidized fatty acids.

7.3.6 Total oxylipin concentrations in the food samples

The total oxylipin concentration per gram was estimated by summarizing the concentrations of all oxylipin which were covered by the method. The highest total oxylipin values were determined in falafel (0.83-1.2 mg/g) followed by vegetarian fish fingers (around 0.47 mg/g) and the vegetarian grilling sausage (around 0.34 mg/g; Tab. 7.3). The concentrations were dramatically lower for other food samples such as “Sauce Bolognese” with 0.0083-0.021 mg/g. Taking different portion sizes into account, the total oxylipin amount was calculated for one portion

either based on the information of the manufacturer or for falafel, curry and pizza based on the size of the meal.

Tab. 7.3: Total oxylipin amount in the food samples. Shown are the sums of all oxylipins derived from oleic acid, LA and ALA covered by the method. The determination of the size of one portion was made either based on the information of the manufacturer or for falafel, curry and pizza based on the size of the meal.

	Sum [µg/g food]	Portion size [g] [1]	Per portion [mg/portion]
Vegan vegetable curry	37 - 53	500	22 - 23
Falafel	1200 - 830	68	81 - 56
Sauce "Bolognese"	21 - 8.3	200	4.2 - 1.7
Schnitzel (chicken)	190	150	29
Schnitzel from pork	240	110	26
Vegetarian chicken filet	190	150	28
Vegetarian grilling sausage	340	80	27
Pork grilling sausage	34 - 34	150	4.5 - 5.6
Hamburger Patty	23 - 29	70	1.6 - 2.0
Tuna filet in sunflower oil	29 - 57	65	1.9 - 3.6
Pizza "Margherita"	35 - 41	145 [2]	5.1 - 6.0
Vegetarian fish fingers	470	95	45
Omega-3 fish fingers	240	125	30
Fish fingers	260	125	33

[1] according to manufacturer declaration (prepacked) or size of the meal (take-away)
[2] amount of cheese on one pizza

Based on this a relevant intake per portion results for falafel (56-81 mg/portion) and vegetarian fish fingers (around 45 mg/portion). For fish fingers, the vegetarian meat alternatives, the currys and the “Schnitzel”, an oxylipin intake of 22-30 mg/portion was calculated. The sauce “Bolognese”, the pizza “Margherita”, the hamburger patties, the grilling sausage and the tuna filet contained low oxylipins amounts with < 6 mg/portion.

Thus, the diet is a relevant source for oxylipins of which several are known to be physiologically active lipid mediators [4]. Further studies are needed to evaluate, to which percentage these oxylipins are actually absorbed from processed food in the gastrointestinal tract and whether the exogenously ingested oxylipins act in the same way like the endogenously formed oxylipins.

7.4 Conclusion

We investigated the concentration of oxidized fatty acids in different food samples which were rich in saturated fatty acids, oleic acid and PUFA. Refined oils contain relevant amounts of dihydroxy-FA presumably resulting from hydrolysis of epoxy-PUFA during the refining process, as concentrations were low in virgin oils. Thus, the epoxy-to-diol ratio seems to be a promising marker for refined oils independent from the oil source/species. While oxylipin concentrations were low in food samples containing high amounts of saturated fatty acids, foods rich in unsaturated fatty acids contained relevant amounts of oxidized fatty acids, especially highly processed and heated food such as falafel. Concentrations differ strongly between the different kind of foods and even within the same type of food which does not always correlate to the content of the precursor PUFA. By now, only limited data is available on the impact of processing on the formation of oxidized fatty acids. We could show that the final baking step of pre-fried vegetarian fish fingers and traditional fish fingers did not change n3-PUFA or oxylipin concentrations. However, considering that several oxylipins are physiologically potent lipid mediators and are absorbed through the diet, more information is needed on the occurrence, concentrations and formation of oxylipins in food.

7.5 References

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Chapter 8

Beyond autoxidation and lipoxygenases: Fatty acid oxidation products in plant oils *

*For decades, research on oxidation of linoleic acid (LA, C18:2 n6) and α-linolenic acid (ALA, C18:3 n3) in plant oils has focused on autoxidatively formed and lipoxygenase-derived 9-hydro(pero)xy- and 13-hydro(pero)xy-LA and -ALA. Here, using a non-targeted approach, we show that other hydroxy fatty acids are more abundant in plant oils. Liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry analyses unveiled highly abundant peaks in flaxseed and rapeseed oils. Using authentic reference standards, seven of the peaks were identified as 9-, 10-, 12-, 13- and 15-HODE as well as 9- and 13-HOTrE. Additionally, six peaks were characterized based on the retention time, the exact mass of the [M-H]⁻ ion and its fragment ions as 16-OH-C18:3, 18-OH-C18:3, three isomers of 12-OH-C18:2 and one of 15-OH-C18:2. 16-OH-C18:3 and 18-OH-C18:3 were tentatively identified as 16-OH-ALA and 18-OH-ALA, respectively, based on autoxidation and terminal hydroxylation of ALA using CYP4F2. Investigation of formation pathways suggests that fatty acid desaturase 3 is involved in the formation of the 12-OH-C18:2 isomers, 15-HODE and its isomer. The dominantly occurring 12-OH-C18:2 isomer was identified as 12*R,S*-OH-9Z,15Z-octadecadienoic acid (densipolic acid) based on a synthetic standard. The characterized oxylipins occurred in cold-pressed flaxseed and rapeseed oils at concentrations of up to 0.1 g/100 g and thus at about sixfold higher levels than the well-known 9-hydro(pero)xy- and 13-hydro(pero)xy-LA and -ALA. Concentrations in sunflower oil were lower but increased when oil was pressed from preheated seeds. Overall, this study provides fundamental new information about the occurrence of oxidized fatty acids in plant oils, having the potential to characterize their quality and authenticity.*

* modified from Koch, E.; Löwen, A.; Kampschulte, N.; Plitzko, K.; Wiebel, M.; Rund, K. M.; Willenberg, I.; Schebb, N. H. Beyond Autoxidation and Lipoxygenases: Fatty Acid Oxidation Products in Plant Oils. J Agric Food Chem 2023, 71 (35), 13092-13106.

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8.1 Introduction

Plant oils are the main source of the essential polyunsaturated fatty acids (PUFA) linoleic (LA) and α-linolenic acid (ALA) and thus play an important role in human nutrition [1, 2]. Flaxseed oil and rapeseed oil are important as one of the few oils containing relevant amounts of the n3-PUFA ALA [2]. Consumption of plant oils massively increased during the 20th century mainly due to the growing importance of soybean and rapeseed oil [1,3]. Besides these oils, also sunflower, palm and other oils are produced and consumed in foods in millions of tons per year worldwide [4].

Plant oils contain a large amount of C18-PUFA [2], which are precursors for a multitude of oxidation products, so-called oxylipins [5]. During and after oil pressing, the 1(*Z*),4(*Z*)-pentadiene system of the PUFA can be oxidized non-enzymatically by oxygen or by enzyme-catalyzed reactions of which lipoxygenases (LOX) are the best described (Fig. 1.1) [6, 7]. Non-enzymatic oxidation comprises autoxidation by radical triplet oxygen $^3O_2$ and photooxidation by singlet oxygen $^1O_2$ in a non-radical manner [7]. Autoxidation is a radical chain reaction starting in the case of LA with the abstraction of the bisallylic hydrogen atom at position 11, followed by delocalization of the radical and the subsequent formation of a peroxyl group exclusively at the outer positions of the double bond system as well as of a conjugated diene system. The abstraction of a hydrogen atom from e.g. another fatty acid finally leads to hydroperoxides at carbon atoms 9 and 13 in the case of LA as well as a dominant formation of 9- and 16-hydroperoxides in the case of ALA [8,9]. The configuration of the conjugated diene system depends mainly on the temperature and the availability of hydrogen donors [10, 11]. In contrast, photooxidation type 2 leads to the direct addition of activated $^1O_2$ to double bonds. This results not only in hydroperoxides at the outer positions but also in hydroperoxides in the middle of the double bond system at positions 10, 12 (and 15 for ALA), albeit at a lower percentage [7] due to cyclization because of double bonds in β,γ-position forming epidioxides and bicycloendoperoxides [8, 10]. 9- and 13-hydroperoxides are also formed by the catalytic activity of LOX, which occur in the

oilseeds [12–15]. LOX come into contact with the substrate during oil pressing through disruption of the cells. Thus, 9- and 13-hydro(pero)xy-LA and -ALA have been considered for decades to be the main PUFA oxidation products in oils.

That this is a limited view on the patterns of oxidized fatty acids in plant oils has become obvious at the latest in our recent study [16]. We found in virgin, cold-pressed flaxseed and rapeseed oils other oxylipins being more dominant than the expected and widely known autoxidation products: 15-HODE which was previously only described in oat seeds [17, 18] and 15(16)-EpODE. With a concentration of up to 0.1 g/100 g, they are relevant constituents of plant oils and they were found to be specific for each oil [16]. Thus, plant oils contain a diverse pattern of oxylipins. Its investigation is of high importance for a better characterization of oils in terms of occurrence of potentially biologically active oxylipins [19] and possibly new parameters for assessing oil quality and authenticity.

In the present study, we performed the first non-targeted, comprehensive analysis of oxylipins in flaxseed, rapeseed and sunflower oils aiming to characterize edible oils beyond the limited view on autoxidation products as it has been the state of the art for decades. Oil samples were hydrolyzed and analyzed by liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to investigate which oxidation products are present. Based on the retention time, the exact mass and the fragmentation pattern, information about their structure was gained. Oxylipins were further characterized using gas chromatography-mass spectrometry (GC-MS) following different derivatization techniques. Formation pathways were investigated by enzymatic and non-enzymatic incubation of fatty acids, analysis of flaxseeds and *Arabidopsis thaliana* (*A. thaliana*) seeds with different fatty acid desaturation activity as well as *Paysonia densipila* (*P. densipila*) and *Physaria gordonii* (*P. gordonii*) seeds. Finally, the new and known oxylipins were (semi-)quantitatively determined in cold-pressed flaxseed, rapeseed and sunflower oil as well as in sunflower oil from preheated seeds. The study provides unique data about the patterns of oxygenated PUFA, their concentrations and their possible formation pathways in seeds and oils. Thus, the present study does not only improve our understanding of the composition of plant oils which is of

fundamental importance for food chemistry. With the quantitative oxylipin pattern, we also provide a possible new parameter, which may be used in the future to evaluate the authenticity of plant oils.

8.2 Experimental

8.2.1 Plant oils

Oil was extracted from flaxseeds (*Linum usitatissimum*), sunflower seeds (*Helianthus annuus*) and rapeseeds *(Brassica napus*), which were provided by local oil mills using a Komet screw press type CA59G (IBG Monforts GmbH & 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. Heating of the sunflower seeds before pressing was performed using a drying oven (Heraeus, Thermo Scientific, USA). To achieve the desired core temperature of the seeds in an adequate time, the drying cabinets were preheated and operated at 75 °C (moderate heating) and 95 °C (high temperature). Batches of approximately 400 g seeds were placed in bowls in the preheated oven. This procedure resulted in a seed temperature (inner part of the bowls) of 60 °C within 60 min (moderate heating) and 80 °C within 100 min of heating (high temperature). The seeds were removed from the oven after 60 min and 100 min, respectively, and used for the pressing as described above.

8.2.2 Chemicals

Iso-propanol, methanol (MeOH) and acetic acid (HAc) were purchased from Fisher Scientific (Schwerte, Germany). Acetonitrile (ACN) was obtained from VWR (Darmstadt, Germany). Ultra-pure water was generated using a Barnstead Genpure Prosystem from Thermo Fisher Scientific (Langenselbold, Germany). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was bought from Carl Roth GmbH (Karlsruhe, Germany). All other chemicals were from Merck (Darmstadt, Germany). Densipolic acid was synthesized from the corresponding aldehyde and Wittig salt by the Wittig reaction and

subsequent deprotection of the functional groups (Fig. 11.21). Recombinant human CYP4F2 was purchased from Corning (Amsterdam, The Netherlands).

8.2.3 Sample preparation for the determination of the total oxylipin concentrations

Sample preparation for total oxylipin analysis was carried out as described [16]. In brief, 5 mg of oil was diluted with 2 ml of *iso*-propanol. 50 µl of this solution were used and 350 µl of *iso*-propanol, 10 µl of butylated hydroxytoluene (BHT, 0.2 mg/ml in MeOH) as antioxidant, 50 µl of water and 100 µl of 0.6 M potassium hydroxide in MeOH/H2O (75/25, *v*/*v*) were added. Samples were hydrolyzed for 30 min at 60 °C and neutralized. Oxylipins were extracted by SPE (C8/anion exchange material, bed weight 300 mg, volume 3 ml, Bond Elut Certify II, Agilent, Waldbronn, Germany). 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*). Samples were evaporated to dryness using a vacuum concentrator (1 mbar, ~60 min; Christ, Osterode am Harz, Germany) and subsequently reconstituted in 50 µl of MeOH.

8.2.4 LC-MS analysis

Fatty acid oxidation products were analyzed by liquid chromatography (Vanquish Horizon UHPLC) coupled to a high-resolution mass spectrometer (Orbitrap QExactive HF; Thermo Fisher Scientific, Dreieich, Germany). Chromatographic separation was carried out on a ZORBAX Eclipse Plus C18 analytical column (150 × 2.1 mm ID; 1.8 µm particle size; Agilent, Waldbronn, Germany) at 40 °C column oven temperature. A mixture of ACN/MeOH/HAc (80/15/0.1, *v*/*v*/*v*) was used as organic solvent B and solvent A was acidified ultra-pure water + 5% of solvent B. The flow rate was set to 0.3 ml/min and the binary gradient was as follows: 0-1 min isocratic 21% B, 1.0-1.5 min linear to 26% B, 1.5-10.0 min linear to 51% B, 10.0-19.0 min linear to 66% B, 19.0-25.1 min linear to 98% B, 25.1-27.6 min isocratic 98% B and 27.6-27.7 min back to 21% B followed by re-equilibration (27.7-31.1 min). The injection volume was 5 µl. Ionization was carried out using electrospray ionization in negative mode (ESI(-)). The source parameters

were set as follows: sheath gas flow rate 30; aux gas flow rate 5; sweep gas flow rate 4; spray voltage −3.5 kV; capillary temperature 280 °C; S-lens RF level 50; and aux gas heater temperature 400 °C. The full MS analysis was carried out with a resolution of 60,000, an automated gain control target of 106, a maximum injection time of 160 ms and a scan range of *m*/*z* 150−500. For data-dependent MS^2, the resolution was set to 15,000, the maximum injection time to 80 ms, the automated gain control target to 5×10^4 and the normalized collision energy to 30.

Targeted LC−MS analysis of oxylipins was performed using an Agilent 1290 Infinity II LC system (Agilent, Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer (QTRAP 5500, Sciex, Darmstadt, Germany) [20]. Detection was carried out in the scheduled selected reaction monitoring mode (SRM) following ESI(-). External calibration with internal standards was used for quantification as described [20, 21]. In order to collect fractions for GC-MS analysis (see below), the separation was carried out using a larger column diameter (ZORBAX Eclipse Plus C18; 150 × 3 mm ID; 3.5 µm particle size) and an adapted gradient. Oxylipins were extracted from the collected fractions using ethyl acetate and evaporated to dryness. Chiral analysis of fractions of monohydroxylated fatty acids was carried out using an amylose-based column (Chiralpak IA-U, 50 × 3.0 mm, particle size <2 µm; Daicel, Osaka, Japan) with a gradient of ACN and water with 0.1% HAc as described [22].

8.2.5 GC-MS analysis

Derivatization using BSTFA. For methylation, to 25 µl of an oxylipin standard (50 µM in MeOH) or of dried fractions dissolved in 25 µl of MeOH, 25 µl of 0.4 M trimethylsilyl diazomethane was added. Samples were vortexed and derivatization was carried out at room temperature for 10 min. Solvents and derivatization agents were removed under a gentle stream of nitrogen. For silylation, samples were reconstituted in 25 µl of pyridine and 25 µl of BSTFA + 1% TMCS were added. Samples were kept at room temperature for 20 min. Solvents and derivatization agents were removed under a gentle stream of nitrogen. Samples were reconstituted in 25 µl of *n*-hexane for GC−MS analysis.

Derivatization using DMDS. For activation of double bonds, the residue after methylation was dissolved in 100 µl of an iodine solution in methyl *tert*-butyl ether (100 mg/ml) and 200 µl of dimethyl disulfide (DMDS). Derivatization was carried out overnight at 0 °C. The reaction was stopped by the addition of 400 µl of 5% sodium thiosulfate solution. *n*-Hexane (200 µl) was added and samples were vortexed. The upper phase was collected, the solvent was removed under a gentle stream of nitrogen and the residue was reconstituted in 25 µl of *n*-hexane for GC-MS analysis.

GC-MS conditions. A Trace GC Ultra instrument was coupled to a DSQII single quadrupole MS instrument (Thermo Fisher Scientific, Dreieich, Germany) equipped with a Rxi-35Sil MS (30 m × 0.25 mm ID, 0.25 µm film thickness) analytical column. The carrier gas was helium with a flow rate of 1.5 ml/min. The injector temperature was set to 270 °C, the MS transfer line to 280 °C and the ion source to 250 °C. 1 µl of the samples were injected with a split ratio of 1:20. The temperature program for analysis of silylated oxylipin methyl esters was as follows: 0-1 min isotherm 180 °C, 20 °C/min to 240 °C, kept for 5 min and 20 °C/min to 280 °C. The temperature program for analysis of oxylipin methyl esters derivatized with DMDS was as follows: initial temperature 150 °C, 10 °C/min to 300 °C, kept for 5 min. Ionization was carried out using electron ionization (EI, 70 eV). A full-scan analysis was performed with a scan range of *m*/*z* 50-450 for BSTFA derivatization (scan rate 500 Da/s, cycle time 0.82 s) and *m*/*z* 50-500 for DMDS derivatization (scan rate 500 Da/s, cycle time 0.92 s).

8.2.6 Enzymatic and non-enzymatic formation of oxylipins

Incubation with CYP4F2. Incubation of fatty acids and CYP4F2 was carried out as described [23]. In brief, from 50 µl of 400 µM ALA solution, the solvent (MeOH) was removed. 175 µl of 0.1 M potassium hydrogen phosphate buffer (pH 7.4) and 5 µl of CYP4F2 (1 nmol CYP/ml) were added. Samples were pre-incubated for 10 min at 37 °C. The reaction was started by the addition of the NADPH generating system (glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $NADP^+$, magnesium chloride). After an incubation time of 30 min at 37 °C, the reaction was stopped by the addition of 600 µl of ice-cold ethyl acetate.

Metabolites were extracted twice with ethyl acetate and the solvent was removed. Samples were reconstituted in 50 µl of MeOH and analyzed by LC-MS. A negative control was prepared by incubation of ALA without the addition of CYP4F2.

Non-enzymatic formation. For autoxidation of ALA, 25 µl of a 50 µM ALA or LA solution in ethanol was mixed with 2 µl of *tert*-butyl hydroperoxide (7 mM). The solvent was removed using a gentle stream of nitrogen. Incubation was carried out at room temperature for 24 h. Samples were reconstituted in MeOH. For photooxidation, the fatty acid standards were exposed to sunlight in fused silica glass. Hydroperoxides were reduced using 10 µl of $SnCl_2$ (10 mg/ml). Samples were analyzed by LC−MS.

8.2.7 *Analysis of* Arabidopsis thaliana *seeds, golden and brown flax-seeds as well as* Paysonia densipila *and* Physaria gordonii *seeds*

The fatty acid desaturase 3 (FAD3) overexpressing, FAD3 deficient and the wild-type (Col-0) *A. thaliana* seeds were purchased from the Arabidopsis Stock Center (University of Nottingham, Nottingham, Great Britain). The brown flaxseeds were purchased from a local oil mill and the golden flaxseeds were bought in a local supermarket. *P. densipila* and *P. gordonii* seeds were obtained from the National Plant Germplasm System of the USDA-Agricultural Research Service (Parlier, CA, USA). Seeds were homogenized and extracted in *iso*-propanol. The lipid extracts were prepared for analysis by the addition of 10 µl of 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*). Samples were hydrolyzed and oxylipins were extracted by SPE and analyzed by LC−MS as described above.

8.3 Results and discussion

The fatty acid oxidation product patterns in different plant oils were comprehensively characterized unveiling that edible oils contain much more oxylipins than those derived by autoxidation or LOX activity. The new oxylipins were structurally

characterized and suggestions about their formation pathways were made. Finally, their concentrations were quantified in different plant oils.

8.3.1 PUFA oxidation products in virgin flaxseed, rapeseed and sunflower oil

LC-ESI(-)-MS analysis of hydrolyzed oils showed several peaks (4-7) with high intensity in the total ion chromatogram (TIC, *m*/*z* 150-500) between 18 min and 19.5 min (Fig. 8.1). The MS spectra unveiled that the peaks were mainly caused by ions at *m*/*z* 295.2271 (Fig. 11.22). Assuming fatty acid oxidation products, the molecules consist only of carbon, oxygen and hydrogen atoms. This results in a sum formula of $C_{18}H_{32}O_3$ with an exact mass of the $[M-H]^-$ ion of 295.2279 Da. With an error <0.5 ppm, it can be assumed that all four substances have this molecular formula (Tab. 8.1 and 8.2). From the sum formula, the number of double bond equivalents (DBE) can be calculated, which was found to be three. In the case of fatty acid oxidation products this means that one double bond belongs to the carboxy moiety and two double bonds are within the carbon backbone. Thus, the oxylipins giving rise to the peaks (4-7) between 18 min and 19.5 min have the basic structure of C18:2 + O. In this retention time window around 19 min, hydroxylated LA oxylipins (e.g. 13-HODE 19.49 min; Tab. 8.1 and 8.2) elute. This indicates that the new C18:2 + O derivatives bear also a hydroxy group.

Additionally, several smaller peaks (1-3) were detected between 16-18 min. They were caused by ions of *m*/*z* 293.2115−293.2129 (Fig. 11.22), which corresponds with an error <1 ppm to the sum formula of $C_{18}H_{30}O_3$ (exact mass $[M-H]^-$ 293.2122 Da; Tab. 8.1 and 8.2). The number of DBE was calculated to be four. Assuming that one belongs to the carboxy group, the precursor-PUFA of these oxylipins is C18:3. Comparison of the retention time window to hydroxylated ALA standards (e.g. 9-HOTrE 17.16 min; Tab. 8.1 and 8.2) indicated that the C18:3 + O derivatives also bear a hydroxy group.

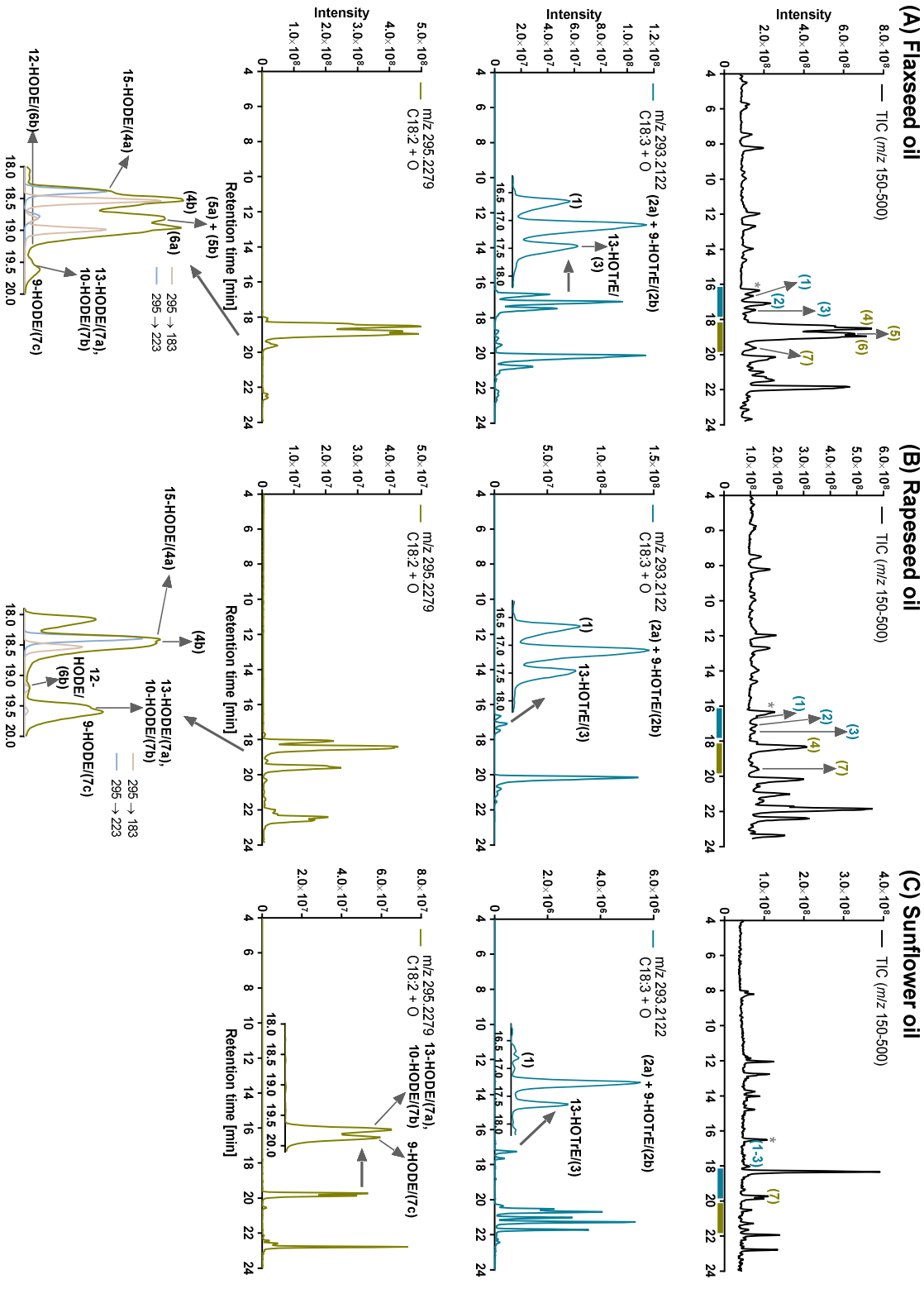

(A) Flaxseed oil
(B) Rapeseed oil
(C) Sunflower oil
TIC (m/z 150-500)
m/z 293.2122 C18:3 + O
(2a) + 9-HOTrE/(2b)
13-HOTrE/(3)
m/z 295.2279 C18:2 + O
15-HODE/(4a)
13-HODE/(7a), 10-HODE/(7b)
12-HODE/(6b)
9-HODE/(7c)
Intensity
Retention time [min]

Fig. 8.1 (previous page): Analysis of hydrolyzed flaxseed, rapeseed and sunflower oil by LC-ESI(-)-HRMS. Shown are the total ion chromatograms (*m*/*z* 150-500) as well as the extracted ion chromatograms (XIC) of C18:3 + O at *m*/*z* 293.2122 and of C18:2 + O at *m*/*z* 295.2279. The peaks are numbered and identified oxylipins are indicated. The mass spectra can be found in Fig. 11.22 and the corresponding product ion spectra in Fig. 8.2. The inserts of the XIC of C18:2 + O show SRM data of the transitions 295 → 183 (red) and 295 → 223 (blue) using a QqQ instrument. The peak indicated with * was also present in blank injections. The large peak in rapeseed oil at 20.1 min belongs to 15(16)-EpODE, a dominating oxylipin in this oil [16].

The signals (1-3) with the *m*/*z* of C18:3 + O (*m*/*z* 293.2122) were detected in all oils with retention times of (1) 16.61 min, (2) 17.07 min and (3) 17.46 min. The peak intensities were the highest in flaxseed oil, while in sunflower oil the peak at 16.61 min was almost absent. Within the three peaks (1-3) with *m*/*z* 293.2122 (Fig. 8.1), four different mass spectra resulting from collision-induced dissociation (CID) were found (Fig. 8.2A). All product ion spectra contained the precursor ion which is presumably the $[M-H]^-$ ion (*m*/*z* 293.2122−293.2124) and a fragment resulting probably from the loss of water (*m*/*z* 275.2016−275.2019). Additionally, peak (1) at 16.61 min showed a dominant fragment at *m*/*z* 263.2018, which seems to be caused by a single compound as the product ion spectra were consistent over the peak. For peak (2) at 17.07 min, two different product ion spectra over the peak were obtained with high intensities at *m*/*z* 235.1702 (2a) and at *m*/*z* 171.1024 (2b). Thus, two C18:3 + O derivatives co-elute, giving rise to only one broad peak. For peak (3) at 17.46 min, a fragment at *m*/*z* 195.1390 was detected. By comparing the retention times and fragment ions with authentic standards, one of the two compounds of peak (2) was assigned to 9-HOTrE (2b) and peak (3) to 13-HOTrE. Thus, two unknown hydroxylated C18:3 isomers with retention times of 16.61 min/(1) and 17.07 min/(2a) exist in the oils.

Hydroxy-PUFA show a characteristic α-cleavage in CID [24, 25] which helps to pinpoint the position of the hydroxy group. For peak (1) at 16.61 min, an α-cleavage fragment with *m*/*z* 263.2018 was detected. This indicated a hydroxy group at position 18. The *m*/*z* of the α-cleavage fragment of (2a) eluting together with 9-HOTrE was 235.1702 and thus, the hydroxy group is presumably at position 16. The determined and calculated exact masses of the fragments were consistent with an error <1 ppm (Tab. 8.2). In summary, (1) is probably 18-OH-C18:3, (2a) was assigned to 16-OH-C18:3 and 9-HOTrE/(2b) and 13-HOTrE/(3) were identified.

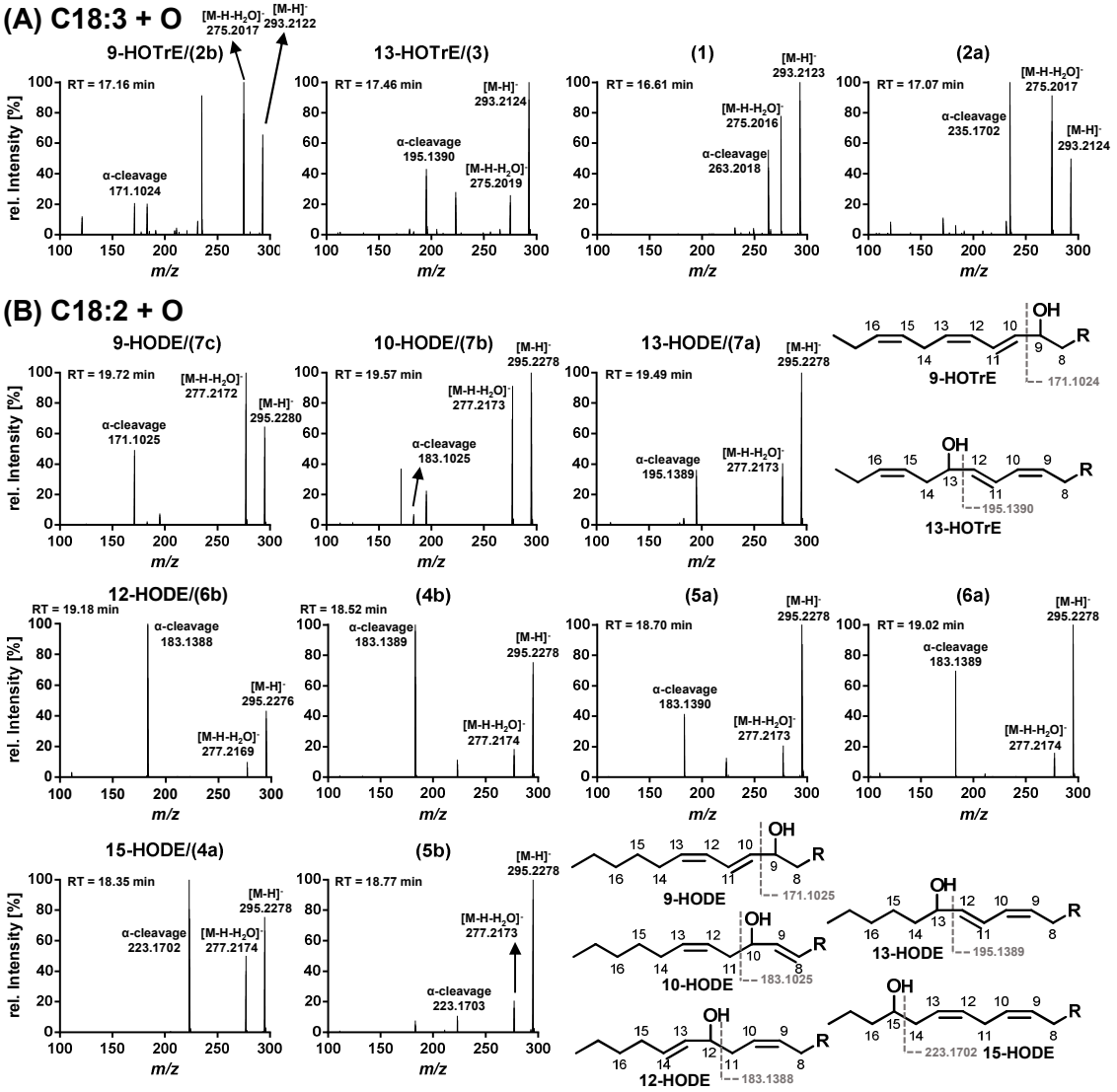


Fig. 8.2: LC-ESI(-)-CID high-resolution product ion spectra of the hydroxylated C18:3 + O and C18:2 + O fatty acids giving rise to the peaks 1-7 in Fig. 8.1. The exact mass of the $[M-H]^-$ ion, the loss of water and the exact mass of a fragment probably resulting from the α-cleavage next to the functional group are indicated. Identified oxylipins are labeled by the name and the unknown oxylipins are referred to their occurrence in the peaks (1-7) shown in Fig. 8.1. The structures of the standards indicate the suggested site of fragmentation. R = $(CH_2)_6$-COOH.

The extracted ion chromatogram (XIC) of *m*/*z* 295.2279 (C18:2 + O) of flaxseed oil contained at least four peaks at retention times of (4) 18.52 min, (5) 18.82 min, (6) 18.97 min and (7) 19.61 min (Fig. 8.1 A). In rapeseed oil, the peaks were distinct but smaller than in flaxseed oil and the large peaks (5) at 18.82 min as well as (6) at 18.97 min were not detected (Fig. 8.1 B). In sunflower oil, only small peaks were detected and the signals at 18.52 min (4), 18.82 min (5) and 18.97 min (6) were missing (Fig. 8.1 C).

Fragment spectra changed over the peaks (4-7), indicating co-eluting compounds (Fig. 8.1 and 8.2 B): two compounds were found for each peak (4), (5) and (6), which was supported by a SRM analysis. For peak (7), three different product ion spectra were detected, which could be assigned based on the MS/MS data, the retention times and authentic standards to 13-HODE (7a, 19.49 min), 10-HODE (7b, 19.57 min) and 9-HODE (7c, 19.72 min). 15-HODE/(4a) and 12-HODE/(6b, 12-OH-9*Z*,13*E*-octadecadienoic acid), for which a standard is commercially available, were one of two compounds of peak (4) at 18.35 min and of peak (6) at 19.18 min, respectively. Thus, at least four unknown hydroxylated C18:2 isomers remain: one compound of peak (4) at 18.52 min/(4b), the two coeluting compounds (5a) and (5b) of peak (5) at 18.82 min and one compound of peak (6) at 19.02 min/(6a).

In the product ion spectra of the peaks (4-6), the $[M-H]^-$ ion (*m*/*z* 295.2276-295.2280) and the loss of water (*m*/*z* 277.2169-277.2174) were detectable (Fig. 8.2 B). As expected, an additional dominant fragment was observed resulting presumably from an α-cleavage next to the hydroxy group. However, the same product ion spectra were found for (4b) at 18.52 min, (5a) at 18.70 min and (6a) at 19.02 min, which were identical to that of the 12-HODE standard (19.18 min, Fig. 8.2 B). In addition, the product ion spectrum of (5b) at 18.77 min contained the same ions as that of the 15-HODE (18.35 min/(4a)) standard. The determined exact mass of the α-cleavage fragments was consistent with the calculated mass of 12-OH-C18:2 and 15-OH-C18:2 (error < 1.1 ppm). Thus, it can be assumed that (4b), (5a) and (6a) are 12-OH-C18:2 isomers and (5b) is an isomer of 15-OH-C18:2. Based on the *m*/*z* of the fragments, the number of double bonds within the fragment and the rest of the molecule can be calculated. For the 12-OH-C18:2 isomers ((4b), (5a) and (6a)), one double bond is located between the hydroxy group and the methyl end and one between the hydroxy group and the carboxy end. In the 15-OH-C18:2 isomer (5b), both double bonds are located between the carboxy moiety and the hydroxy group (Tab. 8.2). Thus, the three 12-OH-C18:2 isomers bear isolated double bonds and they consequently show no UV absorption (Tab. 8.2). As (5b) did not show UV absorption (Tab. 8.2), it

can be concluded that the double bonds in (5b) are isolated as well and presumably present in a pentadiene system like in naturally occurring fatty acids.

Tab. 8.1: Characterization of oxylipin standards which were detected in the oils by LC-MS and GC-MS.

	9-HOTrE	13-HOTrE	9-HODE	10-HODE	12-HODE	13-HODE	15-HODE
LC-HRMS RT [min]	17.16	17.46	19.72	19.57	19.18	19.49	18.35
***m/z* [M-H]$^-$**	293.2122	293.2124	295.2280	295.2278	295.2276	295.2278	295.2278
Sum formula	$C_{18}H_{30}O_3$	$C_{18}H_{30}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$
calculated *m/z* [M-H]$^-$	293.2122	293.2122	295.2279	295.2279	295.2279	295.2279	295.2279
Error [M-H]$^-$ [ppm]	0.00	0.68	0.34	0.34	1.69	0.34	0.34
Double bond equivalent (DBE) [1]	4	4	3	3	3	3	3
Precursor fatty acids	ALA	ALA	LA	LA	LA	LA	LA
***m/z* fragment ion ESI(-)** [2]	171.1024	195.1390	171.1025	183.1025	183.1388	195.1389	223.1702
Sum formula fragment ion	$C_9H_{15}O_3^-$	$C_{12}H_{19}O_2^-$	$C_9H_{15}O_3^-$	$C_{10}H_{15}O_3^-$	$C_{11}H_{19}O_2^-$	$C_{12}H_{19}O_2^-$	$C_{14}H_{23}O_2^-$
calculated *m/z* fragment ion	171.1027	195.1391	171.1027	183.1027	183.1391	195.1391	223.1704
Error [ppm]	1.75	0.51	1.17	1.09	2.73	1.02	0.90
OH-Position [3]	9-OH	13-OH	9-OH	10-OH	12-OH	13-OH	15-OH
Double bond position [4]	10*E*,12*Z*,15*Z*	9*Z*,11*E*,15*Z*	10*E*,12*Z*	8*E*,12*Z*	9*Z*,13*E*	9*Z*,11*E*	9*Z*,12*Z*
GC-MS (derivatization using BSTFA)							
GC-MS RT [min]	6.60	6.62	6.38	6.03	6.06	6.63	6.51
***m/z* base peak**	73	73	73	271	185	73	145
***m/z* fragment ion of α-cleavage**	223	311	225	271	185	311	145
OH-Position	9-OH	13-OH	9-OH	10-OH	12-OH	13-OH	15-OH
GC-MS (derivatization using DMDS)							
GC-MS RT [min]	n.d. [5]	n.d. [5]	n.d. [5]	15.80	15.76	n.d. [5]	16.01
***m/z* base peak**	n.d. [5]	n.d. [5]	n.d. [5]	55	57	n.d. [5]	67
***m/z* fragment DB cleavage**	n.d. [5]	n.d. [5]	n.d. [5]	131	217	n.d. [5]	217
Double bond position (based on derivatization)	n.d. [5]	n.d. [5]	n.d. [5]	C12	C9	n.d. [5]	C9
UV-Abs. max [nm] [6]	226	227	232	none	none	226	none

[1] calculated by folmula (DBE = (2*C - H + 2)/2), one double bound belongs to the carboxy group
[2] main fragment besides loss of water
[3] based on based on the *m/z* of the main fragment which is assumed to result from a-cleavage
[4] according to manufacturer
[5] no peak detectable
[6] scan range 220-320 nm

Overall, we detected in hydrolyzed plant oils the (well-known) oxidation products 9-HOTrE/(2b), 13-HOTrE/(3), 9-HODE/(7c), 10-HODE/(7b), 12-HODE/(6b), 13-HODE/(7a) and the earlier described 15-HODE/(4a) [16]. Additional six new oxylipins could be detected: 18-OH-C18:3/(1); 16-OH-C18:3/(2a), 3 × 12-OH-C18:2/(4b), (5a), (6a) and 15-OH-C18:2/(5b). These compounds are highly abundant in rapeseed and flaxseed oil and their LC-MS signal intensities exceed by far those of the autoxidation and LOX products 9-HODE and 13-HODE as well as 9-HOTrE and 13-HOTrE. In the following, these new oxylipins are further structurally characterized in flaxseed oil containing the highest concentrations of all newly detected oxylipins.

8.3.2 Characterization of oxylipins by GC-MS using different derivatization strategies

GC-MS was used as an orthogonal chromatographic and complementary MS approach. Oxylipins in hydrolyzed flaxseed oil were fractionated by LC and derivatized to make them accessible to GC-MS analysis and to gain structural information.

Similar to the CID, the fragmentation induced by EI (70 eV) of the silylated methyl esters leads to prominent fragments resulting from the α-cleavage [26, 27]. For example, 12-HODE gives rise to ions at *m*/*z* 185, 15-HODE at *m*/*z* 145 and 13-HOTrE at *m*/*z* 311 (Fig. 8.3, 11.23 and Tab. 8.1). At which position the α-cleavage takes place and which part of the molecule is detected as a charged fragment ion depends at least on two structural features: (1) the α-cleavage is preferred between two sp^3 hybridized carbon atoms: for example, in 10-HODE (sp^2 hybridized at C9), the cleavage occurs between C10 and C11 and the carboxy end is the visible fragment (Fig. 8.3), while in 12-HODE (sp^2 hybridized at C13) the cleavage takes place between C12 and C11 and a charged fragment of the methyl end is a detectable fragment. (2) If both neighboring carbon atoms are sp^3 hybridized, the cleavage on the site of the carboxy end is preferred and the methyl end with the trimethylsilyl group results as charged fragment. For example, for 15-HODE (sp^3 hybridized at C14 and C16), the α-cleavage occurred in the direction of the carboxy-terminus between C15 and C14.

The direction of the α-cleavage can be used to further characterize the new oxylipins: for the two 12-OH-C18:2 isomers (4b) and (6a), a dominant signal at *m*/*z* 185 could be detected in the mass spectra, which was also found for 12-HODE (Fig. 8.3). This results most likely from an α-cleavage between C12 and C11, yielding a fragment of the methyl end bearing the trimethylsilyl group. Thus, (4b) and (6a) bear indeed a hydroxy group a position 12 and presumably a double bond at C9. For 12-OH-C18:2/(5a), *m*/*z* 299 was the base peak. This fragment is presumably also formed by an α-cleavage next to the hydroxy group at position 12, but in contrast to 12-HODE as well as (4b) and (6a), the cleavage occurred between C12 and C13, giving rise to a fragment of the carboxy end

bearing the silylated hydroxy group (Fig. 8.3). Thus, in 12-OH-C18:2/(5a), one double bond seems to be located between C10 and C11.

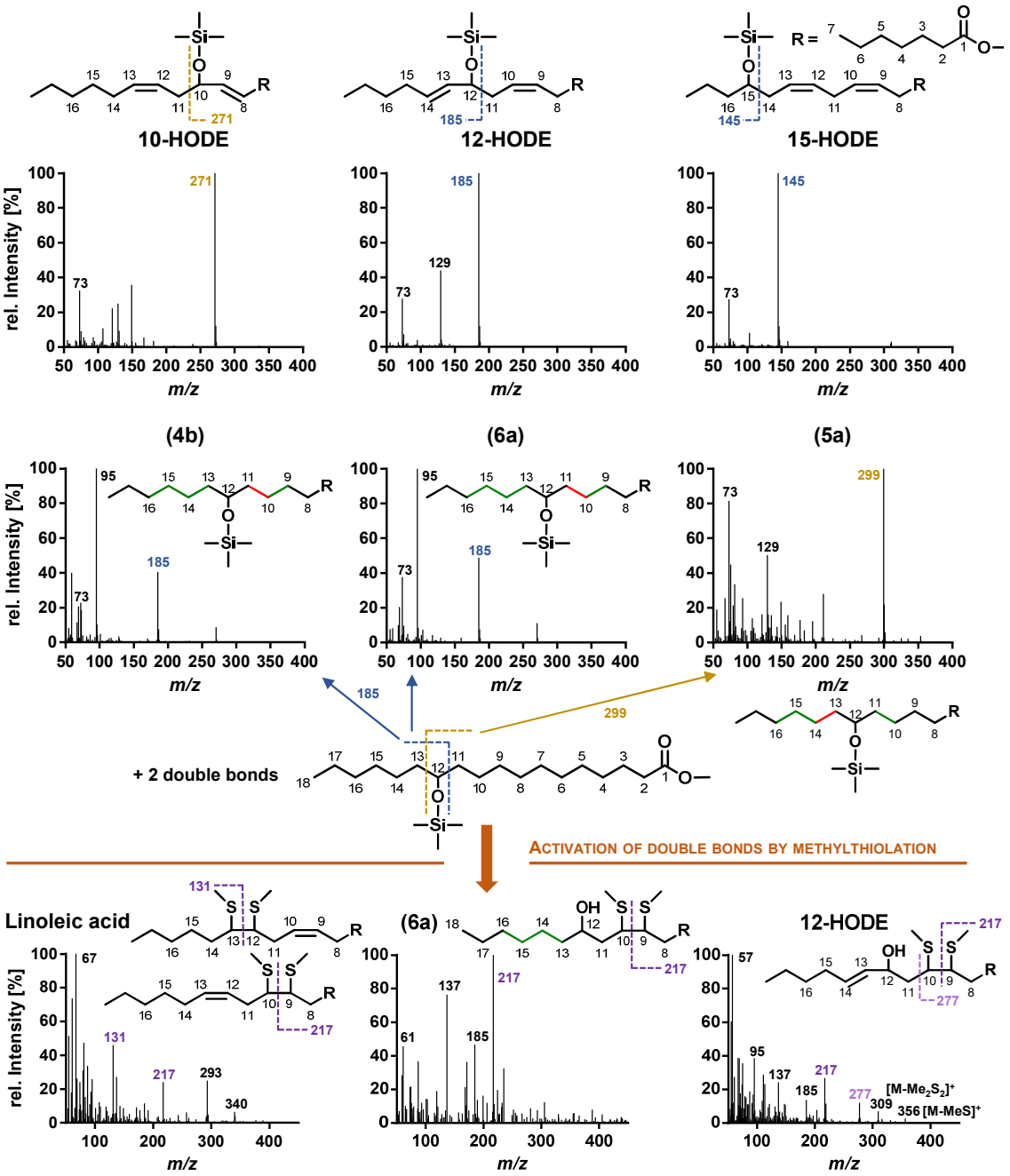


Fig. 8.3: GC-EI-mass spectra of hydroxylated linoleic acid standards and of unknown oxylipins. Shown are the mass spectra of different hydroxylated fatty acids after derivatization with trimethylsilyl diazomethane and BSTFA to the corresponding trimethylsilyl fatty acid methyl ester. The structures indicate the suggested site of the α-cleavage. The green bonds within the structures for the 12-OH-C18:2 isomers (4b), (5a) and (6a) represent possible double bond positions, while the red bonds are most likely not a double bond. Bottom: double bonds were activated using dimethyl disulfide to determine their position. R = $(CH_2)_6$-COOH.

For 15-OH-C18:2/(5b) showing a fragment at *m*/*z* 145 which is identical to the fragment of 15-HODE (Fig. 11.23), the position of the hydroxy group at C15 was supported. Additionally, the product ion spectra of the new C18:3 + O oxylipins supported the hydroxy position at C18 for (1) and C16 for (2a) (Fig. 11.23).

Methylthiolation of double bonds with DMDS is a valuable tool to specify their position [28, 29]. This activation of a double bond leads to a specific break of the former C-C double bond. This can be detected at *m*/*z* 217 for 9*Z*- and *m*/*z* 131 for 12*Z*-enoic acids such as LA (Fig. 8.3). Due to the steric reason, only one of the two double bonds (either 9*Z* or 12*Z*) is methylthiolated [28, 29]. In addition to the break of the carbon chain, the loss of one or two MeS-groups was found for LA (*m*/*z* 340 and *m*/*z* 293, Fig. 8.3) [29]. Consistently, for 10-HODE, a fragment at *m*/*z* 131 and for 12-HODE and 15-HODE, a fragment at *m*/*z* 217 was detectable (Fig. 8.3 and 11.24). In the case of hydroxy fatty acids, it is apparent that double bonds in proximity to the OH-group are not derivatized, maybe due to steric hindrance. In the fragment spectra of 15-HODE, ions at *m*/*z* 236 (Fig. 11.24) were visible, which might result from an α-cleavage after the loss of both MeS-groups. For 10-HODE and 12-HODE (Fig. 8.3 and 11.24), also the loss of one or two MeS-groups could be detected (*m*/*z* 356; *m*/*z* 309). The fragments with *m*/*z* 185 and *m*/*z* 137 in the fragment spectra of 15-HODE and 12-HODE were presumably due to further fragmentation of the *m*/*z* 217 fragment including the loss of the methoxy group of the methylated carboxy moiety [29] and the loss of the MeS-group. For two of the six new oxylipins, namely 12-OH-C18:2/(6a) and 15-OH-C18:2/(5b), signals after methylthiolation of the collected fractions of hydrolyzed flaxseed oil could be obtained (Fig. 8.3 and 11.24). Their MS spectra showed a signal with high intensity at *m*/*z* 217 revealing a double bond at position 9.

Different derivatization strategies and analysis by GC−MS allowed us to draw further conclusions about the structure of newly detected oxylipins. All compounds were methylated and silylated supporting their structure as hydroxylated fatty acids. For all six new compounds, the position of the hydroxy groups could be supported: (1) 18-OH-C18:3; (2a) 16-OH-C18:3; (4b), (5a) and (6a) 12-OH-C18:2 and (5b) 15-OH-C18:2. Additionally, (5a) seems to bear a double bond

between C10 and C11. (6a) was further characterized as 12-OH-9,x-C18:2 and (5b) as 15-OH-9,x-C18:2.

Tab. 8.2: Characterization of unknown oxylipins by LC-MS and GC-MS.

Compound	(1)	(2a)	(4b)	(5a)	(6a)	(5b)
LC-HRMS RT [min]	16.61	17.07	18.52	18.70	19.02	18.77
***m/z* [M-H]$^-$**	293.2123	293.2124	295.2278	295.2278	295.2278	295.2278
Suggested sum formula [(1)]	$C_{18}H_{30}O_3$	$C_{18}H_{30}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$	$C_{18}H_{32}O_3$
Calculated *m/z* [M-H]$^-$	293.2122	293.2122	295.2279	295.2279	295.2279	295.2279
Error [M-H]$^-$ [ppm]	0.34	0.68	0.34	0.34	0.34	0.34
Double bond (DB) equivalent [(2)]	4	4	3	3	3	3
***m/z* fragment ion ESI(-)** [(3)]	263.2018	235.1702	183.1389	183.1390	183.1389	223.1703
Suggested sum formula fragment ion [(1)]	$C_{17}H_{27}O_2^-$	$C_{15}H_{23}O_2^-$	$C_{11}H_{19}O_2^-$	$C_{11}H_{19}O_2^-$	$C_{11}H_{19}O_2^-$	$C_{14}H_{23}O_2^-$
Calculated *m/z* fragment ion	263.2017	235.1704	183.1391	183.1391	183.1391	223.1704
Error [ppm]	0.38	0.85	1.09	0.55	1.09	0.45
Suggested OH-Position [(4)]	18-OH	16-OH	12-OH	12-OH	12-OH	15-OH
Suggested DB position [(5)]	3 x C2-C16	3 x C2-C14	1 x C2-C10; 1 x C13-C17	1 x C2-C10; 1 x C13-C17	1 x C2-C10; 1 x C13-C17	2 x C2-C13
GC-MS (derivatization using BSTFA)						
GC-MS RT [min]	8.24	7.02	6.30	6.18	6.32	6.58
***m/z* base peak**	73	73	95	299	95	145
***m/z* fragment ion of α-cleavage**	103	351	185	299	185	145
Suggested OH-Position [(4)]	18-OH	16-OH	12-OH	12-OH	12-OH	15-OH
GC-MS (derivatization using DMDS)						
GC-MS RT [min]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	15.9	16.08
***m/z* base peak**	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	217	71
***m/z* fragment DB cleavage**	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	217	217
Suggested double bond position [(6)]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	n.d. [(7)]	C9	C9
UV-Abs. max. [nm] [(8)]	none	229	none	none	none	none
Formation by autoxidation [(9)]	✗	✓	✗	✗	✗	✗
Formation by photooxidation [(10)]	✗	✓	✗	✗	✗	✗
Tentative identification	18-HOTrE (9,12,15) [(11)]	16-HOTrE (9,12,14)	Densipolic acid	12-OH-C18:2	12-OH-C18:2 (9,x)	15-OH-C18:2 (9,x)

[(1)] assuming the component consists of C, H and O.

[(2)] calculated by folmula (DBE=(2*C-H+2)/2), one double bound belongs to the carboxy group

[(3)] main fragment besides loss of water

[(4)] based on based on the *m/z* of the main fragment which is assumed to result from a-cleavage

[(5)] based on exact mass of the fragment ion resulting from α-cleavage and the number of double bond equivalents

[(6)] based on observed fragment ion resulting from cleavage of the derivatized double bound

[(7)] no peak detectable

[(8)] scan range 220-320 nm

[(9)] treatment of ALA/LA with *tert*-butyl hydroperoxide

[(10)] treatment of ALA/LA with sunlight in quartz glas

[(11)] same oxylipin formed by conversion of ALA with CYP4F2, a selective C-terminal hydroxylating human enzyme.

8.3.3 Investigation of the formation of the new oxylipins and putative role of FAD3

Peak (2a) was characterized as 16-OH-C18:3 which is likely to be 16-HOTrE, an expected autoxidation product of ALA. In order to support this assumption, ALA was incubated with radical-generating *tert*-butyl hydroperoxide. LC-MS analysis of the autoxidized ALA revealed an intense peak at the same retention time with the same fragment spectrum as peak (2a) (Fig. 11.25). Thus, (2a) was tentatively identified as 16-HOTrE (16-OH-9*Z*,12*Z*,14*E*-octadecatrienoic acid).

Peak (1) was characterized to be 18-OH-C18:3 and thus might be a specific oxidation product of ALA resulting from ω-hydroxylation. In order to support this suggestion, ALA was incubated with recombinant human CYP4F2, which hydroxylates fatty acids exclusively at ω- and ω-1-position [23]. The LC-MS chromatogram of ALA incubated with CYP4F2 showed a high peak at the same retention time with the same fragment spectrum as peak (1) (Fig. 11.25). Thus, (1) was tentatively identified as 18-HOTrE (18-OH-9*Z*,12*Z*,15*Z*-octadecatrienoic acid).

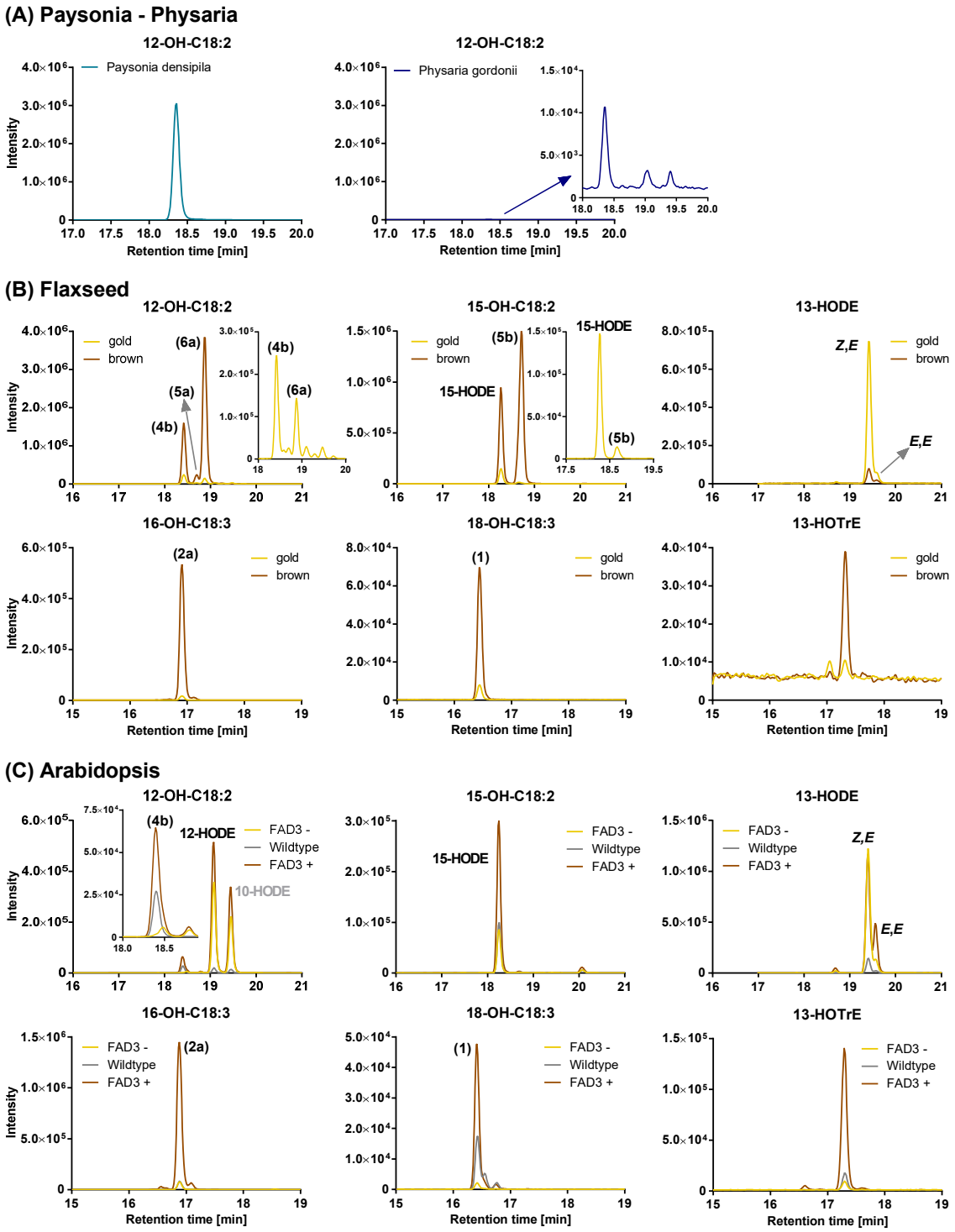
The three unknown 12-OH-C18:2 isomers (4b), (5a) and (6a) have other retention times than 12-HODE (12-OH-9*Z*,13*E*-octadecadienoic acid), a product of photooxidation type 2 of LA. Taking the reaction mechanisms of both radical as well as photooxidation into account, none of these non-enzymatic PUFA oxidation mechanisms can lead to other 12-OH-C18:2 products of LA [10]. Consistently, (4b), (5a) and (6a) were not formed when incubating LA with *tert*-butyl hydroperoxide or when LA was exposed to sunlight (Tab. 8.2). Thus, they result from distinct formation pathways.

Several studies describe the occurrence of non-autoxidatively formed 12-OH-9*Z*,15*Z*-C18:2 (densipolic acid) in some *Paysonia* species, which belong to the *Brassicaceae* family [30]. This oxylipin is putatively formed by the catalytic activity of the FAD3 converting LA to ALA [31]. LC-MS analysis of the oxylipin pattern from *P. densipila* seeds containing densipolic acid [30] showed an intense peak (4b) (Fig. 8.4 A). The peak was small in *P. gordonii* seeds, which are known to contain only low amounts of densipolic acid [30]. Using a synthetic standard, peak (4b) (12-OH-C18:2) could be identified as densipolic acid (12-OH-9*Z*,15*Z*-C18:2)

based on consistent retention times, exact masses of the $[M-H]^-$ ions and product ion spectra (Fig. 11.26). This oxylipin is suggested to be formed from ricinoleic acid (12-OH-9*Z*-C18:1) through the insertion of a double bond at ω-3 position by FAD3 [31, 32].

In order to test the hypothesis that FAD3 is involved in the formation of the newly detected oxylipins, different seeds with high and low FAD3 activity were analyzed. Golden flaxseeds carry point mutations in the LuFAD3A and LuFAD3B gene encoding for the microsomal desaturase [33] and thus have lower FAD3 activity and ALA content. Here, the signals of the 12-OH-C18:2 isomers densipolic acid, (5a) and (6a) were 7-fold, 9-fold and 27-fold lower compared to wild-type brown flaxseeds (Fig. 8.4 B). Similarly, for 15-HODE, the golden flaxseeds showed a 6-fold and for 15-OH-C18:2/(5b) a more than 100-fold lower signal intensity (Fig. 8.4 B). On the contrary, the LA-derived 13-HODE peak was higher for the golden flaxseeds than for the brown flaxseeds. This data show that in flaxseeds with an active FAD3, the 12-OH-C18:2 isomers densipolic acid, (5a), (6a), as well as 15-OH-C18:2/(5b) and 15-HODE are more abundant than in flaxseeds with low FAD3 activity. This suggests an involvement of FAD3 in the synthesis of these oxylipins. The hypothesis is supported by the higher signals for densipolic acid and 15-HODE in hydrolyzed lipid extracts from FAD3 overexpressing *A. thaliana* seeds (Fig. 8.4 C) compared to the wild-type and the absence of densipolic acid in FAD3 deficient *Arabidopsis* seeds.

Fig. 8.4 (next page): Semi-quantitative analysis of selected oxylipins in (A) *Paysonia densipila* and *Physaria gordonii* seeds, (B) brown and yellow flaxseeds and (C) *Arabidopsis* seeds with high and low FAD3 desaturase activity. Shown are SRM chromatograms of hydrolyzed seed lipid extracts analyzed by LC-MS. (A) *Paysonia densipila* and *Physaria gordonii* seeds were analyzed regarding the occurrence of densipolic acid (retention time 18.36 min); (B) Flaxseed with low FAD3 activity (golden flaxseeds) were analyzed regarding their oxylipin pattern and compared to wild-type brown flaxseeds. The total fatty acid concentrations were determined to be: gold: LA = 21 ± 2 g/100 g; ALA = 0.49 ± 0.08 g/100 g; brown: LA = 3.6 ± 0.3 g/100 g; ALA = 10 ± 1 g/100 g (mean ± SD, n = 3). (C) *Arabidopsis* seeds which are deficient in FAD3 (FAD -) or FAD3 overexpressing (FAD3 +) as well as the wild-type (Col-0) were analyzed. The total fatty acid concentrations were determined to be: FAD - : LA = 15 g/100 g; ALA = 0.28 g/100 g; wild-type: LA = 6.9 g/100 g; ALA = 4.3 g/100 g; FAD + : LA = 7.6 g/100 g; ALA = 7.6 g/100 g. The following transitions were used: 12-HODE 295 → 183; 15-HODE 295 → 223; 13-HODE: 295 → 195; 16-HOTrE/(2a): 293 → 235; 18-HOTrE/(1): 293 → 263; 13-HOTrE: 293 → 195.



The low signal intensities of 15-HODE in the golden flaxseeds as well as in the FAD3 deficient *Arabidopsis* seeds despite higher LA concentrations (flaxseeds golden 21 g/100 g, brown 3.6 g/100 g; *Arabidopsis* FAD3 deficient 15 g/100 g, overexpressing 7.6 g/100 g) are surprising. This indicates that (i) LA is not the precursor-PUFA for 15-HODE or (ii) FAD3 catalyzes the hydroxylation of LA.

Indeed, desaturases and hydroxylases share a high sequence homology: only four changes in the amino acid residues abutting the active site histidines are required to convert a desaturase without hydroxylase activity into a bifunctional hydroxylase-desaturase [34]. In addition, the mechanisms of hydroxylation and desaturation differ only in the last step where the intermediary formed fatty acid carbon radical either binds an oxygen from the oxo-diiron complex in the active site [35, 36] or disproportionates [37]. Our data suggests that flaxseeds possess a bifunctional hydroxylase-desaturase because (i) formation of 15-HODE is low in seeds with low FAD3 activity despite high LA levels and (ii) the hydroxy group is inserted in position 15, the initial position of abstraction of the hydrogen atom during the desaturation mechanism [36]. With the hydroxylation of LA by FAD3, we suggest here a new formation pathway of oxylipins occurring in edible oils.

The difference in the peak intensities in golden *vs*. brown flaxseeds was with up to 100-fold dramatically larger for peak 12-OH-C18:2/(6a) and 15-OH-C18:2/(5b) than for densipolic acid and 15-HODE. Both (6a) and (5b) bear a double bond between C9 and C10 (Fig. 8.3 and 11.24). One could assume that (6a) and (5b) result from C18:1 n9 and are either first hydroxylated at position 12 and subsequently desaturated (position unknown, (6a)) or first desaturated at position 12 (unknown configuration) and then hydroxylated at position 15 yielding (5b). Two observations support this hypothesis: (i) the suggested formation pathway for component 12-OH-C18:2/(6a) leads to a (*cis*-*trans*)-isomer at position C9 or C15 of densipolic acid and both showed identical product ion spectra in CID following ESI(-) as well as using EI (Fig. 8.2 and 8.3); (ii) 15-OH-C18:2/(5b) bears two double bonds between the hydroxy group at position 15 and the carboxy group of which one is located between C9 and C10. Thus, from a biochemical perspective, it is reasonable that the second double bond is at position C12-C13, which means that 15-OH-C18:2/(5b) is a (*cis*-*trans*)-isomer of 15-HODE.

Based on the formation route, we could tentatively identify several new oxylipins in plant oils: 18-OH-C18:3/(1) was tentatively identified as 18-HOTrE (18-OH-9*Z*,12*Z*,15*Z*-C18:3) and 16-OH-C18:3/(2a) as 16-HOTrE (16-OH-9*Z*,12*Z*,14*E*-C18:3). The highly abundant peak (4b) was identified as 12-OH-9*Z*,15*Z*-C18:2

(densipolic acid). It is assumed that it is formed from ricinoleic acid (12-OH-9*Z*-C18:1) by the catalytic activity of FAD3. For (6a) – the second relevant 12-OH-C18:2 isomer – no structure could be suggested; however, it seems to be a (*cis-trans*)-isomer of densipolic acid. Moreover, our data suggest that 15-HODE might be formed from LA by FAD3, which probably possesses a bifunctional hydroxylase-desaturase activity. Finally, for the other 15-OH-C18:2/(5b), no structure could be deduced; however, it is possibly an (*cis-trans*)-isomer of 15-HODE.

8.3.4 Chiral LC separation of the characterized oxylipins

Chiral analysis of 16-HOTrE/(2a) showed two peaks (Fig. 8.5). This supports its formation by stereorandom autoxidation. Two peaks were also detected for 15-HODE, densipolic acid, 12-OH-C18:2/(6a) and 15-OH-C18:2/(5b) in flaxseed oil. Interestingly, chiral analysis of densipolic acid in *P. densipila* seeds revealed only one peak. Densipolic acid is described to occur in *R*-configuration [38] based on its formation from ricinoleic acid occurring as *R*-enantiomer in castor oil [39]. Consistently, we found only one peak in the chiral analysis of ricinoleic acid in castor oil, flaxseeds and *P. densipila* seeds. However, for densipolic acid and its possible (*cis-trans*)-isomer 12-OH-C18:2/(6a), both the *R*- and the *S*-isomer were detected in flaxseed oil. Thus, the route of formation of densipolic acid in flaxseed oil seems to be different from that in *P. densipila*. Further research is needed to clarify whether the second isomer, i.e., *S*-densipolic acid, results from another formation pathway than *R*-densipolic acid or whether and how the FAD3 catalysis leads to both isomers.

In addition, for 15-HODE which we suggest to be formed by the hydroxylating activity of FAD3, two peaks and thus *R*- and *S*-15-HODE were observed (Fig. 8.5). Thus, it can be concluded that direct hydroxylation catalyzed by FAD3 is not stereoselective. It should be noted that 15-HODE has been found so far only in oat seeds, where its configuration was determined to be *R* based on GC analysis of the *N*-acyl-L-phenylalanine methyl ester derivative [17]. Here, we show not only its occurrence in flaxseed oil but demonstrate that both enantiomers are formed presumably by FAD3 activity. Further studies are needed regarding the catalytic mechanism of hydroxylation by FAD3 and the stereoselectivity of the enzyme.

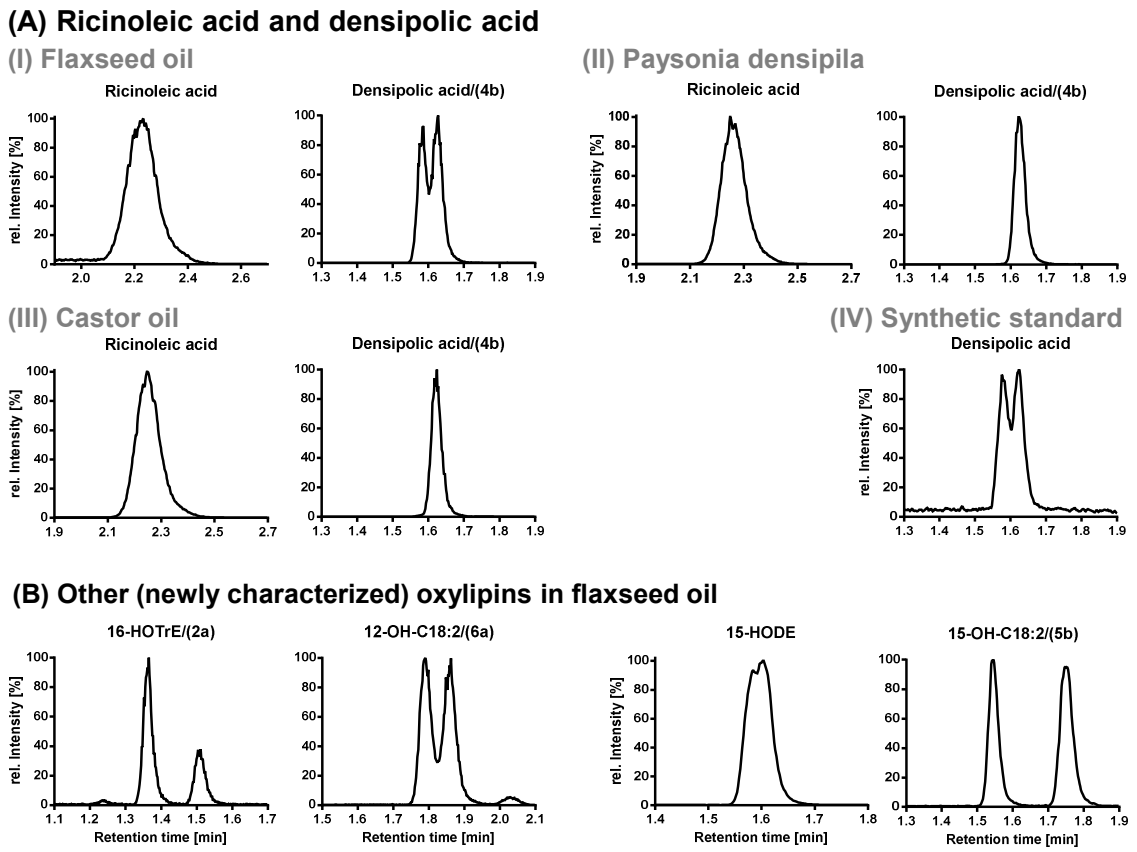


Fig. 8.5: Chiral LC analysis of oxylipins in different oils. Shown are the chromatograms of the chiral LC analysis of (A) ricinoleic acid and densipolic acid in flaxseed oil, Paysonia densipila seeds, castor oil and synthetized densipolic acid as well as (B) 16-HOTrE/(2a), 12-OH-C18:2/(6a), 15-HODE and 15-OH-C18:2/(5b), in flaxseed oil.

8.3.5 Oxylipin concentrations and its modulation by heating of seeds before pressing

The concentrations of the new and known oxylipins were determined in flaxseed, rapeseed and sunflower oil (Tab. 8.3). It should be noted that for the newly detected oxylipins, concentrations could only be determined semi-quantitatively using the calibration curve of 13-HOTrE for 16-HOTrE/(2a); 12-HODE for 12-OH-C18:2/(5a) and 12-OH-C18:2/(6a); and 15-HODE for 15-OH-C18:2/(5b) because no standards are yet available. Current work aims to synthesize these compounds for full structure elucidation as well as absolute quantification. In flaxseed oil, the highest concentrations were found for densipolic acid with 115 mg/100 g, for 12-OH-C18:2/(6a) with 74 mg/100 g and for 15-HODE with 76 mg/100 g. High levels were also found for 16-HOTrE with 98 mg/100 g. On the contrary, 9-HODE and 13-HODE as well as 9-HOTrE and 13-HOTrE were present at rather low

concentrations being the highest for 13-HOTrE (17 mg/100 g) [16]. Thus, in flaxseed oil, the newly described oxylipins dominate the oxylipin pattern.

Tab. 8.3: Concentrations of oxylipins in cold-pressed plant oils. Shown are the concentrations of different hydroxy-PUFA as well as 15(16)-EpODE in cold-pressed flaxseed, rapeseed and sunflower oil (mean ± SD, n = 3) determined by LC-MS/MS.

	[mg/100 g]	Flaxseed oil	Rapeseed oil	Sunflower oil
	9-HODE	12 ± 2	4.5 ± 0.2	14.38 ± 0.09
	10-HODE	0.274 ± 0.007	0.49 ± 0.02	0.0651 ± 0.0005
	12-HODE	interference	0.29 ± 0.02	0.036 ± 0.002
	Densipolic acid/(4b)	115 ± 5	5.9 ± 0.2	0.0037 ± 0.0003
C18:2	12-OH-C18:2/(5a) [(1)]	9.7 ± 0.3	not detected	not detected
	12-OH-9,x-C18:2/(6a) [(1)]	74 ± 3	not detected	not detected
	13-HODE	7.3 ± 0.6	9.3 ± 0.3	18.3 ± 0.1
	15-HODE	76 ± 4	14.8 ± 1.0	0.016 ± 0.001
	15-OH-9,x-C18:2/(5b) [(2)]	21.2 ± 0.6	not detected	not detected
	9-HOTrE	3.3 ± 0.1	1.43 ± 0.08	0.126 ± 0.007
	13-HOTrE	17 ± 1	2.7 ± 0.2	0.120 ± 0.006
C18:3	18-HOTrE/(1)	13.5 ± 0.2	2.3 ± 0.1	0.0089 ± 0.0002
	16-HOTrE/(2a) [(3)]	98 ± 3	16 ± 1	0.62 ± 0.04
	15(16)-EpODE	45 ± 2	70 ± 3	0.136 ± 0.007

[(1)] quantification based on calibration curve of 12-HODE
[(2)] quantification based on calibration curve of 15-HODE
[(3)] quantification based on calibration curve of 13-HOTrE

In rapeseed oil, the concentration of densipolic acid was in the same range as 9-HODE and 13-HODE (5.9 mg/100 g, 4.5 mg/100 g and 9.3 mg/100 g, respectively; Tab. 8.3). 15-HODE and 16-HOTrE showed slightly higher concentrations (15 mg/100 g and 16 mg/100 g). As previously reported by us, rapeseed oil contains specifically high concentration of the terminal epoxide of ALA (15(16)-EpODE, 70 mg/100 g) [16], which is also visible as the large peak in Fig. 8.1 at 20.1 min. Also in rapeseed oil, the newly described oxylipins are quantitatively important (Tab. 8.3). The substantial occurrence of these so far unknown PUFA oxidation products underlines the importance of a thorough characterization of our food and raises the question if these oxylipins might have a physiological function as it is described for e.g. (other) HODE [19, 40].

In sunflower oil, oxylipin concentrations were rather low and the highest concentrations were determined for 9-HODE and 13-HODE as already previously reported [16]. The autoxidation products of ALA, namely, 9-, 13- and 16-HOTrE occurred in low levels. Similarly, 15-HODE and densipolic acid were detected at low concentrations and (5a), (6a) and (5b) were absent. This is consistent with the hypothesis that these oxylipins are FAD3-derived because in the investigated high-oleic sunflower oil the amount of ALA was 0.04 g/100 g and thus, the activity of FAD3 is low.

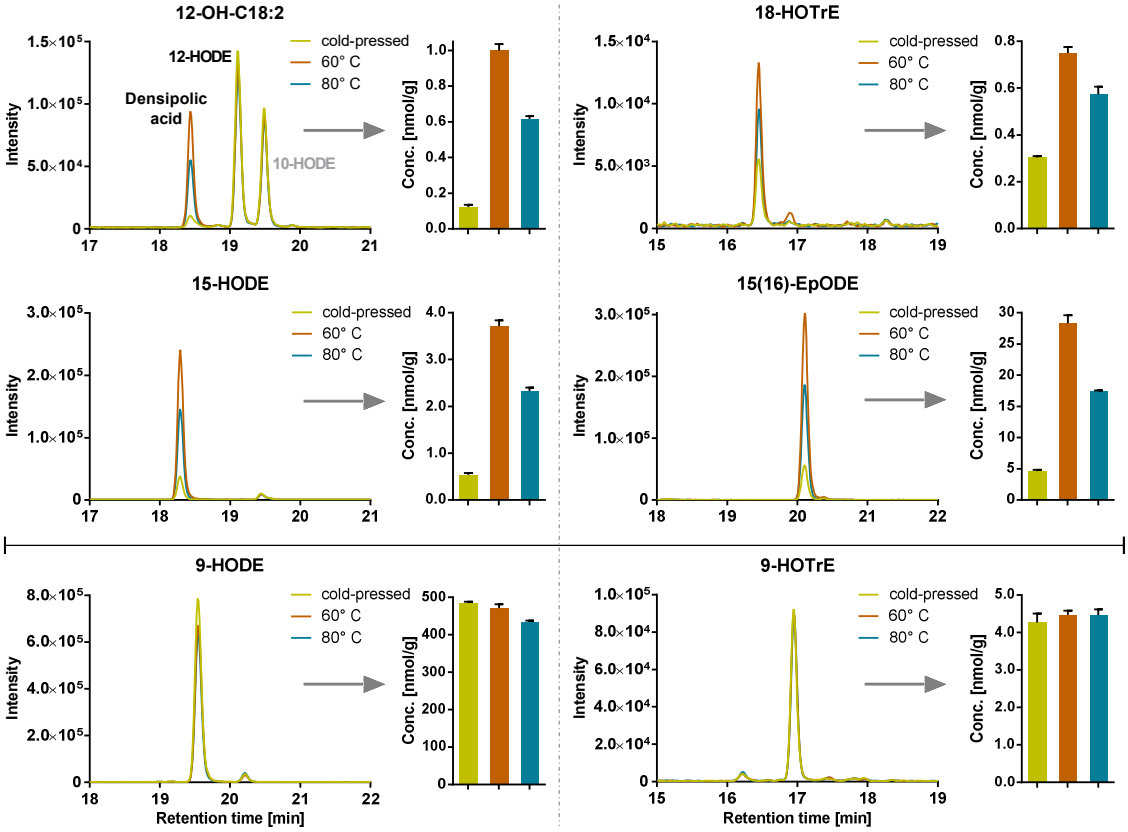


Fig. 8.6: Changes in oxylipin levels in sunflower oil obtained from non-heated and preheated seeds. Shown are LC-MS/MS chromatograms and concentrations of those oxylipins, whose concentrations changed after preheating of seeds as well as 9-HODE and 9-HOTrE for comparison. Concentrations were determined from hydrolyzed oils by LC-MS/MS (mean ± SD, n = 3).

Enzymatically catalyzed reactions are temperature-dependent: the speed of the reaction increases with higher temperature; however, reaching denaturing conditions, the reaction is stopped. Therefore, we hypothesized that moderate heating during/prior pressing would increase the concentration of enzymatically formed oxylipins, while higher temperatures would decrease the levels. In an initial

experiment, we tested this hypothesis for sunflower seeds: heating of the seeds to 60°C prior to oil pressing resulted in more than sevenfold higher concentrations for densipolic acid and 15-HODE (Fig. 8.6). When the seeds were heated to 80°C, the concentrations were higher compared to the cold-pressed oil, but lower compared to the concentrations in the oil obtained from seeds preheated to 60°C (e.g., densipolic acid: cold-pressed: 0.13 nmol/g, 60°C 1.0 nmol/g, 80°C: 0.62 nmol/g). During heating, it might first come to an increase in FAD3 activity, which is inhibited at temperatures of up to 80°C. This hypothesis is further supported by an increase of the ALA concentrations from 40 mg/100 g in the cold-pressed oil to 110 mg/100 g in the oil from seeds heated to 60 °C and 90 mg/100 g in the oil from seeds heated to 80 °C.

Interestingly, the concentrations of 18-HOTrE were increased as well by a factor of 2.5 (Fig. 8.6). It might be concluded that FAD3 also plays a role in the formation of 18-HOTrE, which is indeed conceivable, because it has three double bonds at C9, C12 and C15 and a hydroxy group at C18. Hence, 18-HOTrE is the C18:3-analogue of 15-HODE (C9, C12 and 15-OH). In addition to 15-HODE, densipolic acid and 18-HOTrE, it was surprising that 15(16)-EpODE – the terminal epoxide of ALA – occurred in sixfold higher levels in oil obtained from preheated seeds. Some formation pathways are known such as the involvement of CYPs [41], peroxygenases [42], or non-enzymatical oxygen transfer from a peroxy-radical [43, 44]. Additionally, reports exist about the formation of 12(13)-EpOME, the terminal epoxide of LA, in some *Asteraceae* species by a Δ12-desaturase-like enzyme [45]. It might be speculated that an ω3-desaturase-like enzyme exists which is responsible for the formation of 15(16)-EpODE. Similar to FAD3, this enzyme might be sensitive to heat and thus the concentration of 15(16)-EpODE is influenced by seed preheating. The results of the quantitative analysis of the comprehensive set of oxylipins in oils show that the so far unknown oxylipins are of high importance for the overall concentrations of oxylipins in rapeseed and flaxseed oils. Moreover, as demonstrated for sunflower oil, the formation of several oxylipins was found to be modulated by heat application as it is, for

example, used during (illegal) hot-pressing of virgin oils. This makes them a new potential marker in terms of authenticity of oils, warranting further investigations.

8.4 Conclusion

In the present study, we characterized the pattern of oxidized fatty acids in plant oils by LC-HRMS and GC-MS and suggest formation pathways. In flaxseed oil, several highly abundant peaks were detected that did not match the so far known oxidized fatty acids, i.e., oxylipins. Based on their retention times in RP-LC, the *m*/*z* of the $[M-H]^-$ ions as well as of the fragments resulting from an α-cleavage, the new peaks were characterized as 16-OH-C18:3, 18-OH-C18:3, 3 × 12-OH-C18:2 and 15-OH-C18:2. Further characterization using GC-MS supported the position of the hydroxy group and allowed to elucidate the position of the double bonds. Additionally, two of the 12-OH-C18:2 showed identical CID product ion and EI mass spectra suggesting that both are isomers, similarly found for 15-HODE and 15-OH-C18:2. 18-OH-9*Z*,12*Z*,15*Z*-C18:3 could be tentatively identified as 18-HOTrE and 16-OH-9*Z*,12*Z*,14*E*-C18:3 as 16-HOTrE by generation of standards using (non)enzymatical oxidation of ALA. Comparison of the dominating peak of 12-OH-C18:2 with the oxylipins of *P. densipila* seeds and a synthetic standard allows the identification of *R*- and *S*-densipolic acid (12-OH-9*Z*,15*Z*-C18:2) as a major oxylipin in flaxseed oil. Based on the analysis of different (genetically modified) plants we suggest that FAD3 is involved in the formation of densipolic acid, the other 12-OH-C18:2 isomers, as well as 15-HODE (15-OH-9*Z*,12*Z*-C18:2) and its isomer.

The newly characterized oxylipins occur with up to 0.1 g/100 g in considerably higher concentrations in some oils than the known major oxylipins 9- and 13-HODE as well as 9- and 13-HOTrE. This shows the importance of a comprehensive characterization of the oxylipin pattern in plant oils and other foods: on the one hand in order to understand the composition of our foods and on the other hand to find new parameters to evaluate processing (e.g., cold-pressed oils) and authenticity.

8.5 References

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Chapter 9

Concluding Remarks and Future Perspectives

Within this thesis, the occurrence and concentrations of oxylipins in plant oils, n3-PUFA supplements and fatty foods, the oxidative changes during storage and heating of oils and possible new markers for evaluation of oil quality and authenticity were investigated using comprehensive, state-of-the-art analytical methods. For reliable quantification of oxylipins and fatty acids, targeted LC-MS-based methods and sample preparation strategies were developed to analyze total (chapter two) and non-esterified oxylipins (chapter four) and fatty acids (chapter three) in plant oils. The presented sample preparation for the analysis of total oxylipins comprises alkaline hydrolysis as well as SPE and allows the simultaneous determination of the entire fatty acid distribution from the hydrolysate and thus from the same sample without additional sample preparation. Specific analysis of non-esterified fatty acids and oxylipins could be achieved by separating them from other lipid classes – mainly triacylglycerols – using normal-phase SPE. The developed methods can be used to quantify non-esterified oxylipins as well as total oxylipins. The binding form of the esterified oxylipins and the class of the lipid in which they are present are not considered. Thus, in addition to the differentiation of oxylipins in esterified and non-esterified, analysis of the distribution of the different oxylipins and their structural classes in different lipids, e.g. triacylglycerols, phospholipids, phytosterol ester and their position within one lipid class, e.g. triacylglycerols, could be a valuable tool. It could give a deeper insight in the progress of lipid peroxidation because it would allow to study specifically, which lipid classes are preferentially oxidized and which fatty acid distribution within a lipid molecule causes high or low oxidation.

The oxidative status of an oil is an essential parameter for its quality, as during lipid peroxidation, volatile, aroma-active aldehydes are formed [1]. LA and ALA which occur in high concentrations in oils, are prone to (aut)oxidation due to their double bonds. Hydroperoxides are formed in the first steps of autoxidation and were low concentrated in freshly pressed plant oils as shown in chapter five, although already considerable concentrations of the corresponding hydroxy-LA, i.e. 9- and 13-HODE as well as of the corresponding hydroxy-ALA, i.e. 9- and 13-HOTrE were quantified. However, plant oils are seldomly consumed directly after pressing. They are rather stored in the supermarket and at the consumers for several months or longer. Over time, the autoxidation progresses, causing firstly an increase in hydroperoxy-LA and -ALA concentrations [2]. Surprisingly, the ALA-rich flaxseed oil showed only slight hydroperoxide and secondary volatile aldehyde formation, despite the high susceptibility of ALA to autoxidation [3]. The sensory impairment that many consumers notice in flaxseed oil often already after a short storage period, and which was also the case in the study carried out during this thesis, can therefore not necessarily be regarded as "rancidity". Some authors suggest that an oxidation of methionine in so-called cyclolinopeptides in flaxseed oil are responsible for the bitter taste of stored flaxseed oil [4]. If they have also antioxidative properties, as some reports discuss [5], warrants further investigation.

Consumers should pay attention to the storage of virgin rapeseed oil which tends to massive autoxidation, particularly under high oxygen availability such as in half-full bottles. This was detectable in a strong increase in 9- and 13-hydroperoxy-LA and -ALA concentrations, and in the peroxide value (PV) which accounts for the entirety of hydroperoxides [6]. The PV is frequently used together with the anisidine value [7] – marker for α,β-unsaturated aldehydes – or the concentrations of the volatile aldehydes directly, to measure lipid peroxidation. Chapter five shows that oxylipins and PV correlate well, but there was only a marginal change in the concentration of volatile aldehydes despite a more than 500-fold increase in the PV. In sunflower oil, even a decrease in *E*-2-heptenal over time was quantified, although the concentrations of the precursor oxylipin 12-HODE increased.

Thus, concentrations of volatile aldehydes only poorly reflect ongoing oxidative changes in the used oil. In the future, the reliability and use of this parameter for assessing the oxidative status of edible oils should be discussed.

Autoxidation mechanisms at high temperatures such as during deep-frying differ from them taking place at room temperature (chapter six): The under moderate autoxidative conditions primarily formed *E*,*Z*-hydroperoxides are unstable and degrade to secondary oxidation products. On the contrary, the thermodynamically favored *E*,*E*-hydroperoxides and *E*,*E*-hydroxy-PUFA are formed at high temperatures. Strong heating of oils also causes the specific formation of *trans*-epoxy-PUFA and their hydrolysis products *erythro*-dihydroxy-PUFA, while the *cis*-epoxy-PUFA and the *threo*-dihydroxy-PUFA concentrations changed only slightly. Thus, high concentrations of *E*,*E*-hydroperoxides, *trans*-epoxy- and *erythro*-dihydroxy-PUFA could be markers for heated oils. However, as concentrations alone might not be reliable because they can depend largely on the oil, the ratio of *E*,*Z*-to-*E*,*E*-hydroxy-PUFA, *cis*-to-*trans*-epoxy-PUFA and *threo*-to-*erythro*-dihydroxy-PUFA can be a valuable tool. These ratios decrease over time and particularly the *E*,*Z*-to-*E*,*E*-hydroxy- and the *cis*-to-*trans*-epoxy-PUFA ratios are interesting because they fall below 1 after longer frying times. Moreover, the *cis*-to-*trans*-epoxy-PUFA ratio is promising because it can be determined directly from the LC-MS chromatogram by comparing the peak heights and no pure standard is necessary. Further studies are needed to validate in detail how ratios of i) *E*,*Z*-to-*E*,*E*-hydroxy-PUFA, ii) *cis*-to-*trans*-epoxy-PUFA and iii) *threo*-to-*erythro*-dihydroxy-PUFA can be applied as new markers for high-temperature treatment of plant oils. It would be interesting to investigate if these markers can also indicate the heating of an oil to around 100 °C as it is done during illegal soft-deodorization of virgin (olive) oils to remove unpleasant odors [8]. Thermal processing of virgin oils is not allowed [9]; however, this food fraud is not detectable so far.

The evaluation of authenticity of foods is one of the most challenging tasks in modern food chemistry. Valid parameters are needed to prove not only if the oil is refined or virgin, but also the pressing process (cold *vs*. hot pressing) and the

source of an oil, e.g. if an oil declared as olive oil was made from olives. In the thesis, several different parameters are discussed which might help to answer authenticity questions (chapter five and eight). The oxylipin patterns of oils are a result of both autoxidation and specific enzyme activity occurring in the oilseeds. The best-described enzymes are LOX, giving rise to 9- and 13-hydroperoxy-LA and -ALA as also autoxidation does [2, 10]. LOX activity can be considered to be a specific property of the individual oilseeds; however, this specific pattern is overlayed by non-specific autoxidation. Thus, 9- and 13-hydroperoxy-PUFA – or their reduced form hydroxy-PUFA – are not suitable as markers for the origin of oils. In chapter five, 15-HODE and 15(16)-EpODE were characterized to be the most abundant oxylipin in rapeseed and flaxseed oil with concentrations up to 0.1 g/100 g. 15-HODE has attracted particular attention because due to the homoallyl position of the hydroxy group, it can only be formed enzymatically. Therefore, its concentration is specific for the source of the oil. In addition, it is not removed by refining. Refining removes hydroxy-PUFA with the hydroxy group in allylic position, i.e. 9-, 10-, 12- and 13-HODE as well as 9- and 13-HOTrE, and epoxy-PUFA (chapter seven). The latter are hydrolyzed to their corresponding vicinal dihydroxy-PUFA, presumably during the degumming step by the added phosphoric acid. It might be conceivable that allylic hydroxy-PUFA are unstable at high temperatures and break down during the deodorization step to secondary oxidation products which are removed by the steam distillation. On the contrary, the homoallylic structure of 15-HODE may be more resistant to degradation. Therefore, 15-HODE might also be used in refined oils as a potential marker for the origin of the oil.

In the course of the thesis, it became obvious that several oxylipins exist in plant oils, which were less known or unknown: isomers of 12-hydroxy-C18:2 and 15-hydoxy-C18:2 as well as 18-HOTrE were identified, whose formation must be enzymatically catalyzed (chapter eight). Indeed, it turned out to be likely that FAD3 plays an important role in all their formation routes. The final proof of the involvement of FAD3 in the formation of the new oxylipin is still pending. Different approaches have been tried to develop a dynamic system to definitively

demonstrate the formation of the oxylipins: for example, *C. elegans*, possessing an active FAD3, was fed with ricinoleic acid, a potential precursor fatty acid. However, proof of formation was not possible because there was no negative control, as i) the ricinoleic acid standard already contains the target oxylipin in low amounts and ii) *E. coli* itself, the food source of the worm, contains high amounts of ricinoleic acid. Here, further research is needed to verify the role of FAD3 in the formation routes of the newly detected oxylipins.

The benefit of elucidating the formation pathways of these oxylipins becomes clear when considering their potential as a new marker for cold-pressed oils. These oils are obtained only by mechanical pressing of oilseeds [9] and are considered to be of "high quality", making it possible to sell them at higher prices. Thus, cold-pressed oils are a rewarding target of food fraud and so far, no parameter exists to prove cold-pressing. A pilot study in chapter eight showed that preheating of seeds to 60 °C resulted in higher concentrations of 15-HODE, 15(16)-EpODE, 12-OH-C18:2 and 18-HOTrE. It is likely that the exposure to heat activates the enzyme(s) being responsible for the oxylipin formation. Further increase of the temperature on the contrary caused moderately elevated levels of the oxylipins probably because of an inactivation of the enzyme(s) at high temperatures. Thus, enzymatic activity in the oilseeds and during oil pressing gives each oil its unique oxylipin fingerprint. More details about these oxylipin formation pathways in oilseeds and during oil pressing are needed to further improve the understanding of PUFA oxidation in oils and to pave the route to use the full potential of oxylipins for answering urgent questions of food fraud.

Plant oils, especially soybean and rapeseed oil, are the main fat sources in the Western diet which is thus characterized by a high consumption of n6-PUFA and a low n3-/n6-PUFA ratio [11-12]. As a sufficient supply of EPA and DHA is attributed to positive health effects [13], a multitude of n3-PUFA supplements based on fish, krill or micro-algae oil is available on the market (chapter four). The refined fish and micro-algae oil had rather low oxylipin concentrations and a relative oxidation rate (oxylipin/precursor PUFA) of < 0.1%, while concentrations were high in the non-refined krill oil. The positive effects of the consumption of n3-PUFA

supplements on the endogenous n3-PUFA status is demonstrated by numerous studies [14]. However, previous EPA and DHA supplementation studies did not test the oxidative status of the n3-PUFA capsules administered to the subjects. Thus, the extent to which the degree of oxidation and thus the uptake of physiologically relevant lipid mediators enhances or impairs the positive effect of the n3-PUFA supplements requires further investigation.

Oxidized fatty acids also occur in our food and meals: chapter seven showed that the diet is a relevant source of oxylipins. Different foods and meals containing vegetables, fish, meat and their vegetarian replacements were screened regarding their oxylipin concentrations and the degree of fatty acid oxidation. Foods with high amounts of saturated fatty acids contained low amounts of oxylipins. High concentrations and a high degree of oxidations were found in highly processed food, particularly if the processing included high-temperature applications such as deep-frying. Interestingly, baking turned out to be a gentle processing method. The estimated intake of oxylipin per portion was mainly 2-30 mg, but could reach up to 80 mg in fried food. However, more research is needed to evaluate, to which extent oxidized fatty acids are absorbed from complex, processed food as well as if the absorbed oxylipins circulating then in the plasma, have the same effect as the oxylipins which are formed endogenously.

Overall, using the developed sensitive and reproducible LC-MS methods for oxylipin and fatty acid analysis, this thesis throughout characterizes the patterns of oxidized fatty acids in oils. Based on this, it improves our understanding of the sources of oxylipins in oils, the oxidative processes and changes during oil pressing, oil heating and processing as well as storage of oils in the context of evaluating the quality and authenticity of oils.

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Summary

Plant oils consist largely of glycerol esters of monounsaturated, i.e. oleic acid, as well as of the polyunsaturated fatty acids (PUFA) linoleic acid (LA) and α-linolenic acid (ALA). They are prone to oxidation by non-enzymatical mechanisms – autoxidation and photooxidation – as well as the activity of different enzymes of which lipoxygenases (LOX) are the best described. From these reactions, a plethora of fatty acid oxidation products result whose analysis by liquid chromatography-mass spectrometry (LC-MS) is challenging: on the one hand, a high diversity of functional groups is formed and on the other hand, within e.g. hydroxy-PUFA, regioisomers and enantiomers exist. The entirety of hydroxy-, epoxy- as well as di- and multihydroxy-PUFA can be determined by releasing esterified oxylipins from lipids with potassium hydroxide and extraction of oxylipins from the matrix by solid phase extraction using silica-based reversed-phase/anion exchange cartridges (chapter two). Artificial formation of *cis*-epoxy-PUFA through prolonged drying of silica-based cartridges was identified as a critical step, highlighting the importance of well-characterized sample preparation strategies. The final sample preparation approach allows not only the reliable, efficient and reproducible quantification of total oxylipins, but also the determination of the precursor fatty acid concentrations without additional sample preparation (chapter three). The total fatty acids can be analyzed directly from the hydrolysate by LC-MS following dilution in ethanol. A C8 reversed-phase column (100 x 2.1 mm, 2.6 µm core-shell particle) allowed efficient chromatographic separation of 41 saturated and unsaturated fatty acids between C6:0 and C24:0 as well as 11 deuterated internal standards within 15 min run time. *Pseudo*-selected reaction monitoring mode, i.e. isolation of the *m*/*z* of the $[M-H]^-$ ion in Q1 and Q3, is a valuable tool for making low fragmenting analytes such as saturated and monounsaturated fatty acids accessible for tandem-MS analysis. The benefit of the developed LC-MS-based method for fatty acid analysis is that it does not

require derivatization. This makes it possible to quantify non-esterified fatty acids in the presence of other lipids. This is for example interesting to distinguish between virgin and refined oils.

The combined precursor-PUFA and oxylipin analysis from the same sample makes a fast quantification of their concentrations and additionally a determination of the degree of oxidation (oxylipin/precursor-PUFA ratio) possible. Using this approach, questions about the composition and oxidative status of oils can be answered. Both the amount of fatty acid – especially of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) – as well as how strong they are oxidized are important parameters for the quality of long-chain n3-PUFA containing supplements which are frequently consumed by people on a Western diet which is low in EPA and DHA (chapter four). Between different fish, krill and micro-algae oil-based supplements, the amount of EPA and DHA per capsule varied considerably, yet was in line with the declared content for all products. The n3-rich oils contained in addition to EPA and DHA different concentrations of saturated and monounsaturated fatty acids; however, on the package only limited information about other fatty acids was available for the consumer. Non-refined krill oil showed high oxylipin concentrations including the biologically active lipid mediators 15-HEPE, 18-HEPE and 17-HDHA and the highest degree of oxidation (EPA 0.7%; DHA 1.3%). Refined fish and micro-algae oils were characterized by a low oxidation and non-esterified fatty acid levels of ≤ 0.1% which might be used as novel quality markers for a good refining process of oils used in the n3-PUFA supplements. Micro-algae oils contained specifically high concentrations of terminal hydroxylated EPA (20-HEPE) and DHA (22-HDHA) which can serve as an authenticity marker for micro-algae oil. Thus, analysis of fatty acids and oxylipins provides new insights in the quality, origin and authenticity of n3-PUFA-rich oils.

The main PUFA in human nutrition are linoleic acid (LA) and α-linolenic acid (ALA), coming from the consumption of plant oils. Each plant oil has its characteristic oxylipin fingerprint coming on the one hand from the activity of fatty acid oxidizing enzymes in the seeds and on the other hand from oxidation during oil production and storage. Food chemistry research on fatty acid oxidation in plant

oils has focused on the oxidative changes of the final produced oils and thus on the autoxidatively formed 9-hydro(pero)xy- and 13-hydro(pero)xy-LA and -ALA, not being aware that other oxylipins dominate in flaxseed and rapeseed oil (chapter five): 15(16)-EpODE and the so far in oils unknown 15-HODE were found in concentrations up to 0.1 g/100 g. Despite the high abundance, the formation pathway(s) of 15-HODE is unknown. 15-HODE concentrations were found to be specific for the oil source and were not changed by the refining process (Fig. 10.1). Thus, it might be a new potential marker to prove the origin of virgin and refined oils. The fact that highly abundant, specific oxylipins were overlocked for decades, has risen the need for a comprehensive characterization of the patterns, concentrations and formation pathways of oxylipins in plant oils.

An untargeted screening for hydroxy-PUFA in flaxseed oil by LC-HRMS unveiled – in addition to the 9- and 13-hydroxy-AL and -ALA – several peaks with high intensities (chapter eight). They were characterized as 16-OH-C18:3, 18-OH-C18:3, 15-OH-C18:2 and three derivatives of 12-OH-C18:2. 16-OH-C18:3 was tentatively identified as 16-HOTrE and 18-OH-C18:3 as 18-HOTrE by autoxidation and terminal hydroxylation of ALA using CYP4F2, respectively. Additionally, one dominantly occurring 12-OH-C18:2 isomer was found to be densipolic acid (12-OH-9*Z*,15*Z*-octadecadienoic acid) which is presumably formed by desaturation of ricinoleic acid through fatty acid desaturase 3 (FAD3, Fig. 10.1). An involvement of FAD3 in the formation of the 12-OH-C18:2 isomers as well as 15-HODE and its isomer became evident because their peak intensities were considerably higher in seeds with high FAD3 activity, i.e. brown flaxseeds and FAD3 overexpressing *Arabidopsis thaliana* seeds, compared to seeds with low FAD3 activity (golden flaxseeds and FAD3 deficient *Arabidopsis* seeds). Golden flaxseeds carry point mutations in the two genes encoding for FAD3 and have thus high amounts LA and low amounts of ALA. That 15-HODE shows a sixfold smaller peak in golden compared to brown flaxseeds despite a 40-fold higher LA content indicates that FAD3 may be a bifunctional desaturase-hydroxylase, capable of hydroxylating LA directly in homoallyl position to 15-HODE (Fig. 10.1). This would be a new mechanism for formation of oxylipins in seeds. The

concentrations of i) the 15-OH-C18:2 and 12-OH-C18:2 isomers were with up to 0.1 g/100 g about sixfold higher than the known 9-/13-HODE and 9-/13-HOTrE in flaxseeds oil, ii) the 12-OH-C18:2 isomers were specific for the source of an oil and iii) densipolic acid, 15-HODE, 15(16)-EpODE and 18-HOTrE in sunflower oil were found to depend on the pressing conditions as concentrations were higher when oil was pressed from preheated seeds. Thus, the pattern of (enzymatically formed) oxylipins in plant oils might be a new attractive marker to prove the origin of an oil as well as to evaluate the authenticity of cold-pressed oils.

Changes in the profile of fatty acid oxidation products in plant oils after oil pressing occur exclusively non-enzymatically. Common treatments of oils are storage, heating and refining. The progress of lipid peroxidation can be assessed by the analysis of the peroxide value which is a parameter for the entirety of hydroperoxides and – more specifically – by the determination of the oxylipin patterns. A combined analysis of both parameters during storage of virgin flaxseed, rapeseed and sunflower oil revealed a surprisingly low lipid peroxidation in flaxseed oil despite the high amount of ALA, while the oxylipin concentrations and the peroxide value were massively increased in rapeseed oil (chapter five). However, volatile aldehydes which are commonly used to evaluate secondary lipid oxidation increased only slightly in rapeseed oil and did not reflect the ongoing oxidative changes. Thus, their use as oxidation markers is limited, while oxylipin concentrations and peroxide values correlated well and allowed deeper insights in oxidative changes of plant oils.

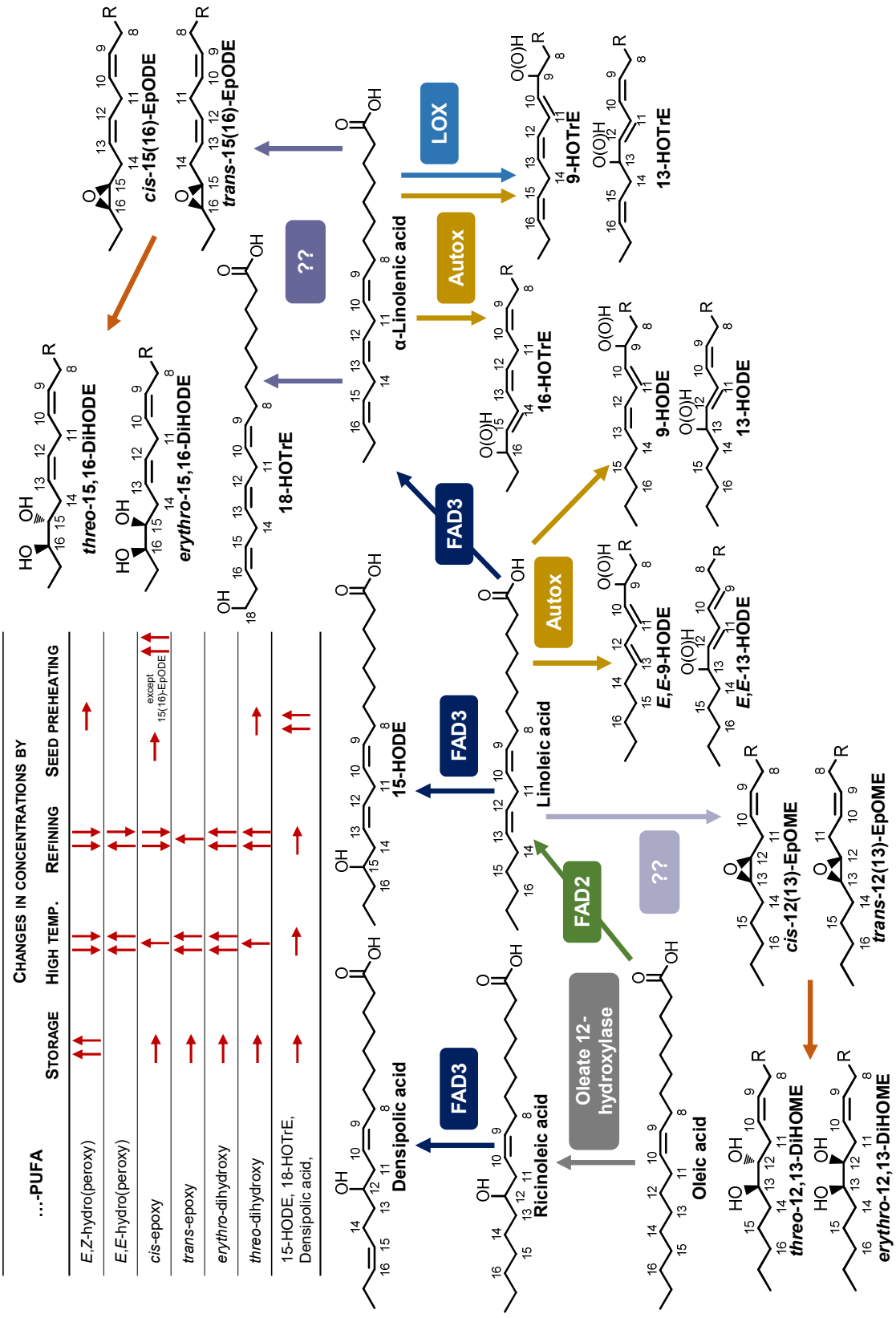

Fig. 10.1: Overview about the results within this thesis regarding the formation pathways of oxylipins in seeds and oils as well the influence of storage and processing of oils on the oxylipin concentrations.

The use of the *E*,*Z*-hydroperoxides to describe lipid peroxidation turned out to be not suitable when investigating fatty acid oxidation at high temperatures such as during deep-frying (chapter six). Analysis of oxylipins in high-oleic sunflower oil used for deep-frying of potato chips (175 °C) showed that under these conditions, the *E*,*Z*-hydroperoxides being formed at low temperatures, are degraded. On the contrary, the thermodynamically favored *E*,*E*-hydroperoxides and *E*,*E*-hydroxy-PUFA were formed. Different from oxidation at moderate temperature was also the specific increase in *trans*-epoxy-PUFA concentrations and in its hydrolysis product, *erythro*-dihydroxy-PUFA (Fig. 10.1). Thus, strong heating of oils caused changes in the ratios of *E*,*Z*-to-*E*,*E*-hydroxy-, *cis*-to-*trans*-epoxy- and *erythro*-to-*threo*-dihydroxy-PUFA. Especially the *cis*-to-*trans*-epoxy and the *E*,*Z*-to-*E*,*E*-hydroxy-PUFA ratio has the potential to be a new marker to evaluate the frying oil quality because it falls < 1 at longer frying times.

A third common treatment of oils is refining. This process considerably decreases concentrations of epoxy-PUFA and 9-, 10-, 12- and 13-HODE as well as 9- and 13-HOTrE (chapter seven), while 15-HODE concentrations are not altered. The loss of epoxy-PUFA probably is caused by hydrolysis of the epoxy ring to the corresponding vicinal dihydroxy-PUFA as their concentrations were massively higher in refined compared to virgin oils (Fig. 10.1). This results in a low epoxy-to-diol ratio in refined oils which might be a new potential marker for refining. Refined oils find wide application in processed foods which are commonly consumed in the Western diet and may thus be an important source of oxylipins. Food with high amounts of saturated fatty acids such as hamburger patties contained low oxylipin levels (2-6 mg/100 g), while foods rich in unsaturated fatty acids contained relevant amounts of oxidized fatty acids: in fried falafel and vegetarian meat as well as fish replacements, high concentrations (up to 120 mg/100 g, 80 mg per serving) and a high ratio of oxylipin to precursor-PUFA were found (chapter seven). Additionally, the oxylipin pattern of these thermally stressed products comprised specific heat-induced oxylipins such as *trans*-epoxy- and *E*,*E*-hydroxy-PUFA. Between different kinds of foods and even within the same type of food, the concentrations differed strongly making general

conclusions difficult. However, our daily meals do contain relevant amounts of oxylipins. More data about their occurrence and the impact of processing are needed as well as data about the absorption of oxylipins from processed food and the physiological role of these exogenously supplied oxylipins.

This thesis shows the continuing high importance of a thorough characterization of our food and provides fundamental information about the patterns of oxylipins in oils, their formation pathways in the seeds and their changes during storage, heating and processing. In addition, it suggests new attractive markers which might serve to evaluate the authenticity of cold-pressed oils and to detect food fraud: i) the specificity of the individual oxylipin fingerprint of oils for proving the oil source; ii) concentrations of densipolic acid, 15-HODE, 15(16)-EpODE and 18-HOTrE to evaluate the pressing conditions; iii) the ratios of *E*,*Z*-to-*E*,*E*-hydroxy-, *cis*-to-*trans*-epoxy- and *erythro*-to-*threo*-dihydroxy-PUFA to evaluate strong heating of oils and iv) the epoxy-to-diol ratio to distinguish between virgin and refined oils.

Appendix

11.1 Chapter 2

Detailed information on the characterization of sample preparation for the determination of total oxylipins:

1 Lipid and oxylipin extraction from plasma (prior to hydrolysis)

Extraction efficiencies using liquid-liquid extraction (LLE) and protein precipitation procedures were evaluated using aqueous bovine serum albumin (BSA) solution (50 mg/ml in phosphate buffered saline (PBS)) spiked with either alkali-stable deuterated oxylipins standards (internal standards (IS) for oxylipin quantification) or fatty acid lipids. Moreover, oxylipin profiles in 100 µl of human plasma with selected LLE and protein precipitation protocols were evaluated.

Three LLE procedures were tested: Extraction with i) ethyl acetate and *n*-hexane (EA/*n*Hex, 75/25, *v/v*), ii) chloroform and methanol ($CHCl_3$/MeOH, 2/1, *v/v*) [1] and iii) methyl *tert*-butyl ether and methanol (MTBE/MeOH, 3/1, *v/v*) [2] (see Tab. 11.1 for detailed procedures). Lipid extracts were evaporated in a vacuum concentrator (1 mbar, 30 °C; Christ, Osterode am Harz, Germany). For protein precipitation, plasma was extracted with a fourfold excess (100 µl sample + 400 µl organic solvent) of methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), MeOH/ACN, 3/2, *v/v*, or *iso*-propanol (iProp). Samples were frozen at -80 °C for at least 30 min and centrifuged (4 °C, 10 min, 20 000 x *g*).

Recovery of oxylipins was calculated via external calibration. Lipid samples were evaporated, derivatized to fatty acid methyl esters and analyzed via GC-FID [2]. For LLE, recovery of fatty acids was calculated against the directly derivatized lipid standard and for protein precipitation against a lipid standard which was diluted with *iso*-propanol and PBS before evaporation and derivatization.

2 Hydrolysis conditions

Experiments were carried out using lipid extracts obtained following LLE or protein precipitation with *iso*-propanol (see above). Dried lipid extracts obtained from LLE were dissolved in 400 µl of *iso*-propanol. For hydrolysis, 100 µl of water and 300 µl of 1.5 M

potassium hydroxide (KOH) in MeOH/water (75/25, *v/v*) were added and samples were hydrolyzed for 30 min at 60 °C. Supernatants from protein precipitation were hydrolyzed at 60 °C using KOH in methanol/water (75/25; *v/v*) and optimization steps comprised sample volume and base concentration during hydrolysis as well as hydrolysis time. After hydrolysis, samples were put on ice and neutralized (pH = 6) with diluted acetic acid (HAc) prior to further extraction.

3 Solid phase extraction (SPE) protocol for free oxylipins from hydrolyzed samples

After neutralization, hydrolyzed samples were diluted with 2 ml of 0.1 M aqueous disodium hydrogen phosphate buffer (Na_2HPO_4) adjusted to pH 6.0 with HAc. The pH in the samples was checked and adjusted to 6 before extraction of oxylipins by SPE on Bond Elut Certify II columns (200 mg, 3 ml, 47-60 µm particles, Agilent Technologies, Waldbronn, Germany) as described [3] or with a modified protocol using the same solvents and buffers on Oasis MAX cartridges (60 mg, 3 ml, 30 µm particles, Waters, Eschborn, Germany).

Epoxidation of PUFA using silica-based cartridges was investigated by extracting 100 µl of a methanolic standard solution containing IS (20 nM) and ARA (0.7 mM) with the final protocol. *Trans*- and *cis*-epoxy-PUFA concentrations were determined [4] and compared to a diluted standard which was analyzed directly.

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2. Ostermann, A. I.; Muller, M.; Willenberg, I.; Schebb, N. H., Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography - a comparison of different derivatization and extraction procedures. *Prostag Leukotr Ess* **2014**, *91* (6), 235-41.

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Tab. 11.1: Overview over the liquid-liquid extraction procedures

$CHCl_3$/MeOH [1] and $CHCl_3$/MeOH + HAc	**MTBE/MeOH** [2] and MTBE/(MeOH+HAc) [#]	**EA/*n*Hex** and EA/*n*Hex + HAc
Sample 100 µl 50 mg/ml BSA in PBS + 10 µl deuterated oxylipin standard (1 pmol) or + 10 µl lipid standard (10 nmol) + *Antiox*	Sample 100 µl 50 mg/ml BSA in PBS + 10 µl deuterated oxylipin standard (1 pmol) or + 10 µl lipid standard (10 nmol) + *Antiox*	Sample 100 µl 50 mg/ml BSA in PBS + 10 µl deuterated oxylipin standard (1 pmol) or + 10 µl lipid standard (10 nmol) + *Antiox*
Vortex sample	Vortex sample	Vortex sample
+ $CHCl_3$/MeOH (2/1, *v/v*) [+ HAc] to a final sample dilution of 20:1	+ 300 µl MeOH [+ HAc]	+ 750 µl EA/*n*Hex (75/25, *v/v*) [+ HAc]
Vortex sample	Vortex sample	Vortex sample for 5 min
Centrifugation (room temp., 10 min, 4000 x *g*)	+ 600 µl MTBE	Centrifugation (4 °C, 2 min, 20 000 x *g*)
+ 0.2 parts MeOH if phases are not separated and repeat centrifugation	Vortex Sample for 1.5 min	Collect organic phase
Transfer supernatant to new glass tube	Add 300 µl 0.15 M NH_4Ac	Repeat extraction with another 750 µl extraction solvent
+ $CHCl_3$ to $CHCl_3$:MeOH:water 8:4:3	Vortex sample	
+ 0.2fold vol. 0.73% NaCl of crude extract	Centrifugation (4 °C, 5 min, 3500 x*g*)	
Vortex sample	Collect upper organic phase	
Centrifugation (room temp., 5 min, 4000 x *g*)	Rewash lower aqueous phase with 300 µl MTBE and combine organic phases	
Remove upper aqueous phase		
Wash lower organic phase two times with low volumes of upper phase pure solvent ($CHCl_3$:MeOH:0.58% NaCl 3:48:47)		

References:

1. Folch, J.; Lees, M.; Stanley, G. H. S., A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *J Biol Chem* **1957**, *226* (1), 497-509.

2. Ostermann, A. I.; Muller, M.; Willenberg, I.; Schebb, N. H., Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography - a comparison of different derivatization and extraction procedures. *Prostag Leukotr Ess* **2014**, *91* (6), 235-41.

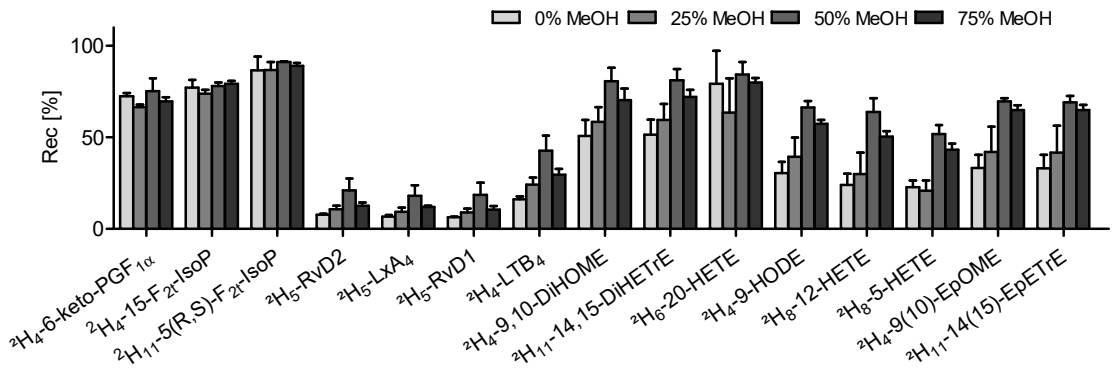
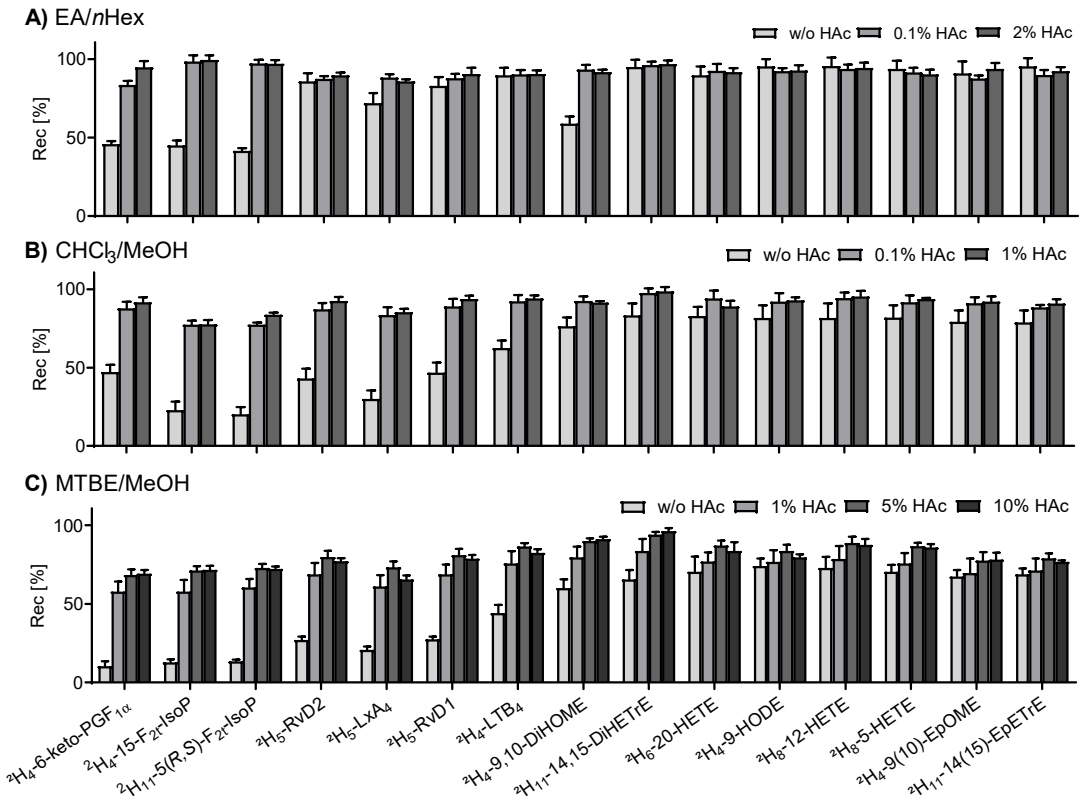


Fig. 11.1: Recovery rates of internal standards following direct hydrolysis of plasma. Shown are recoveries of deuterated oxylipin standards following direct hydrolysis of plasma without prior extraction of lipids (mean ± SD; n = 3). Plasma samples (100 µl) were hydrolyzed using 700 µl of 2 M aqueous sodium hydroxide solution with 0%, 29%, 57% and 86% methanol, resulting in 1.5 M sodium hydroxide during hydrolysis with an organic content of 0%, 25%, 50% and 75% (methanol). After neutralization, free oxylipins were extracted via SPE on Bond Elut Certify II material from the hydrolyzed sample and analyzed by LC-MS.

I) Deuterated oxylipin standards



II) Fatty acid lipids

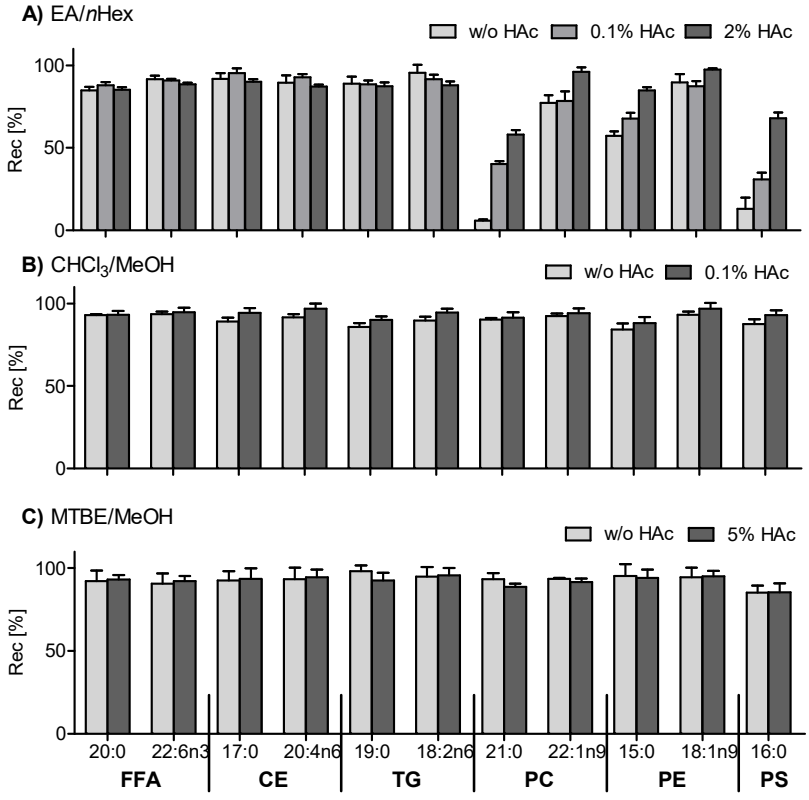


Fig. 11.2 (previous page): Optimization of LLE procedures for free oxylipins and lipids. Shown are recoveries of (I) deuterated oxylipin standards and (II) fatty acid lipids from BSA solution (100 µl, 50 mg/ml in PBS) following LLE with (A) EA/*n*Hex (75/25, *v/v*), (B) $CHCl_3$/MeOH (2/1, *v/v*) and (C) MTBE/MeOH (2/1, *v/v*, Tab. 11.1) with varying amount of HAc in the extraction solvent (mean ± SD; n = 4). For oxylipin analysis, samples were evaporated, reconstituted and analyzed by LC-MS. Lipid extracts were evaporated, derivatized to fatty acid methyl esters and analyzed by GC-FID. Recovery of fatty acids was calculated against a directly derivatized lipid standard. Concentration of HAc was selected based on the results for extraction of deuterated oxylipin standards.

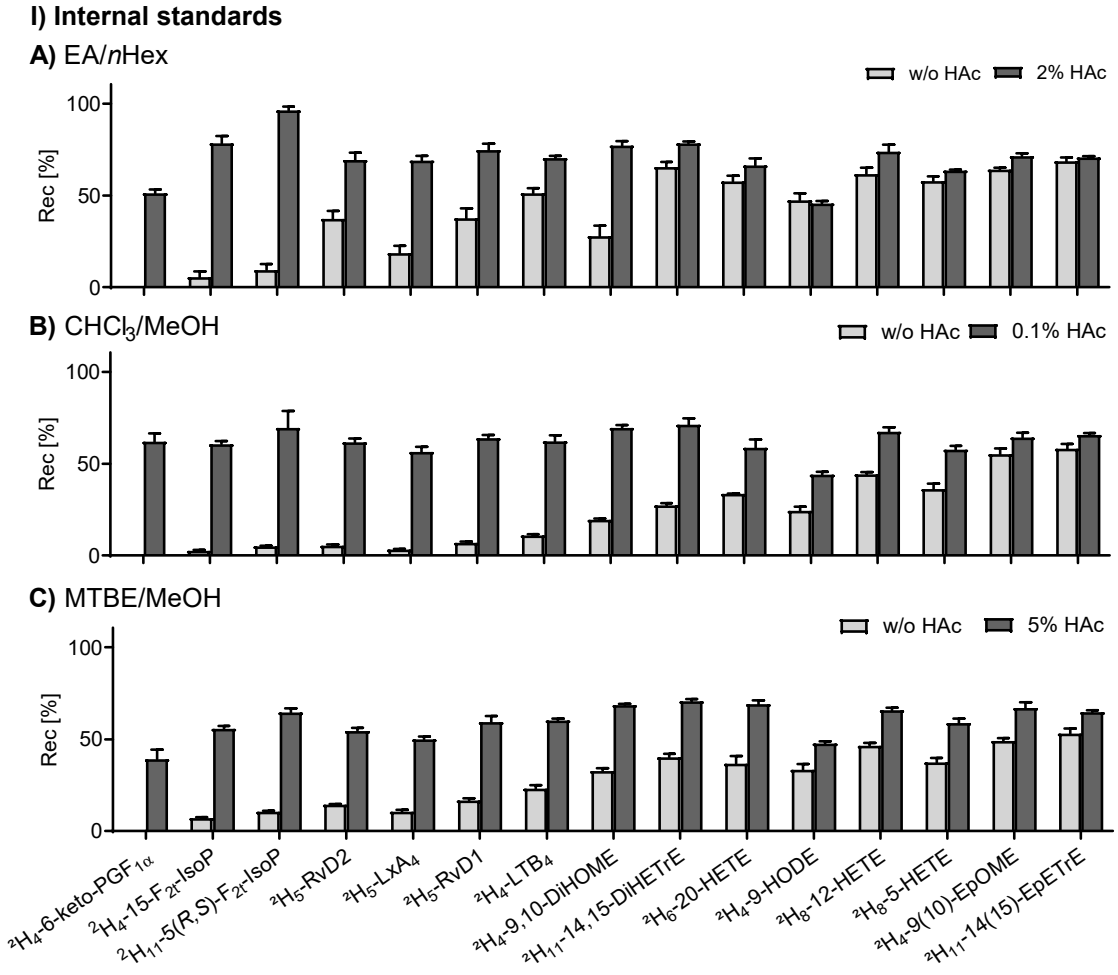
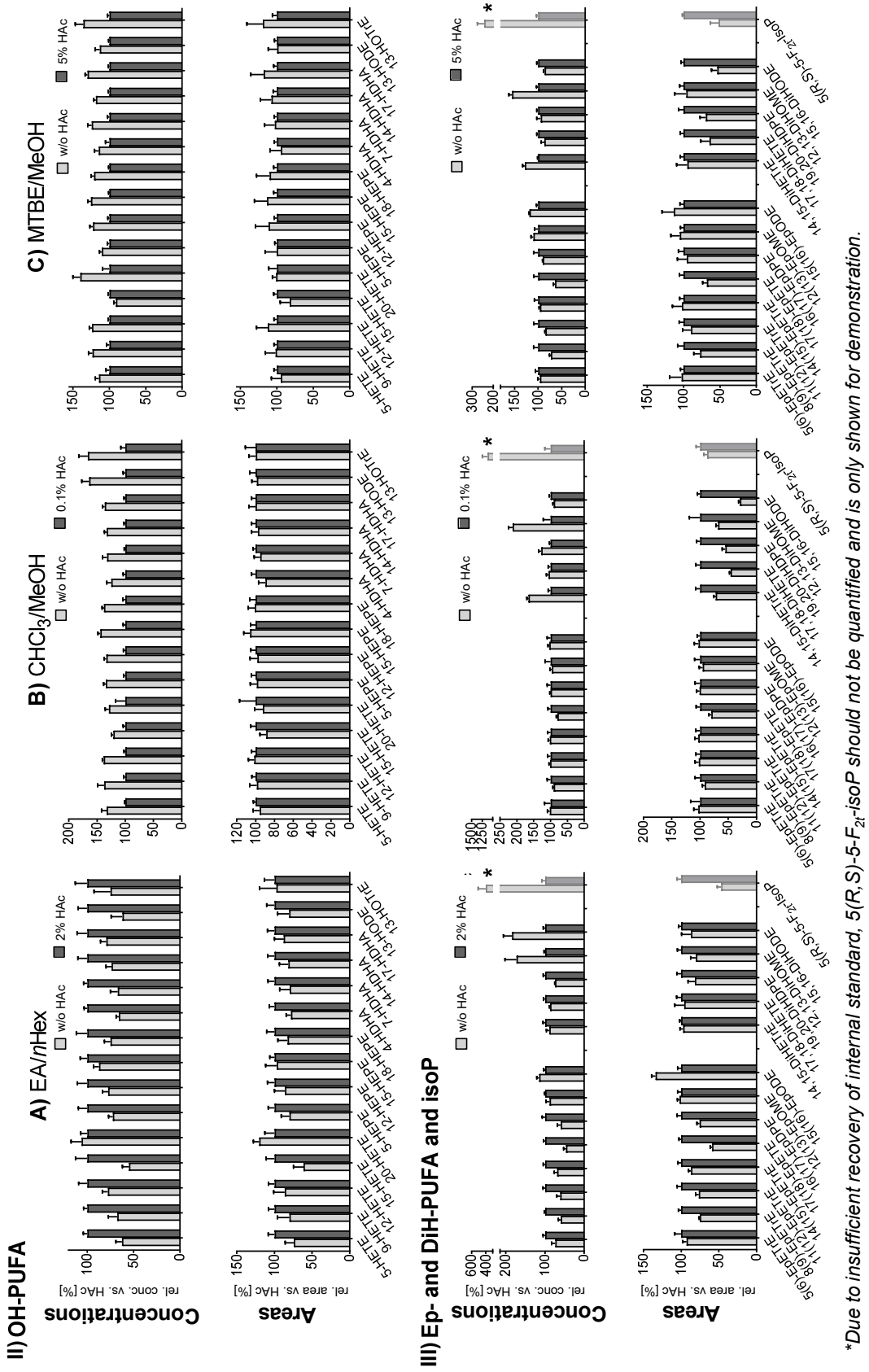


Fig. 11.3: LLE of plasma using acidified and non-acidified extraction solvents. Shown are (I) recoveries of deuterated oxylipin standards from plasma (100 µl) as well as relative concentrations and relative areas of representative (II) OH-PUFA and (III) Ep- and DiH-PUFA as well as isoP following LLE with (A) EA/*n*Hex (75/25, *v/v*), (B) $CHCl_3$/MeOH (2/1, *v/v*) and (C) MTBE/MeOH (2/1, *v/v*) (mean ± SD; n=3, following page). Lipid extracts after LLE were evaporated to dryness and dissolved in 400 µl of *iso*-propanol. Hydrolysis was carried out following addition of 100 µl of water and 300 µl of 1.5 M KOH in methanol/water (75/25, *v/v*) for 30 min at 60 °C. After neutralization, free oxylipins were extracted by SPE on Bond Elut Certify II material from the hydrolyzed sample and analyzed by LC-MS. Relative concentrations and areas in (II) and (III) were calculated against extraction with acidified solvents.



Due to insufficient recovery of internal standard, 5(R,S)-5-F_{2t}-isoP should not be quantified and is only shown for demonstration.

Fig. 11.3 continued.

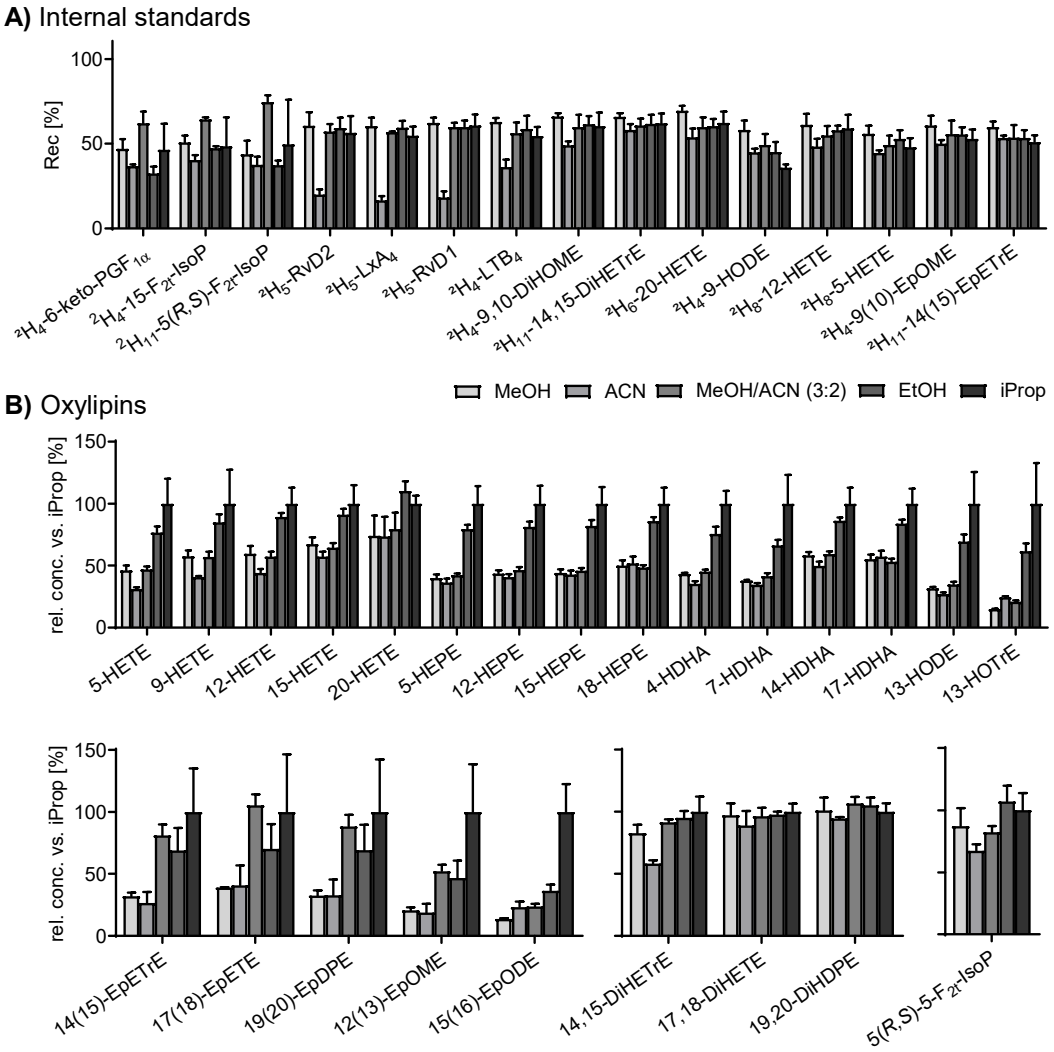


Fig. 11.4: Extraction of lipids from plasma using protein precipitation. Shown are (A) recoveries of deuterated oxylipin standards and (B) relative concentrations of a representative set of analytes in plasma (100 µl) following protein precipitation with 400 µl of methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), methanol/ acetonitrile (3/2, *v/v*, MeOH/ACN) or *iso*-propanol (iProp) (mean ± SD; n=4) and base hydrolysis (300 µl of 5.5 M NaOH (aq); 30 min, 60 °C). Free oxylipins were extracted by SPE (on Bond Elut Certify II material) from the hydrolyzed sample and analyzed by LC-MS. Relative plasma oxylipin concentrations were calculated against iProp.

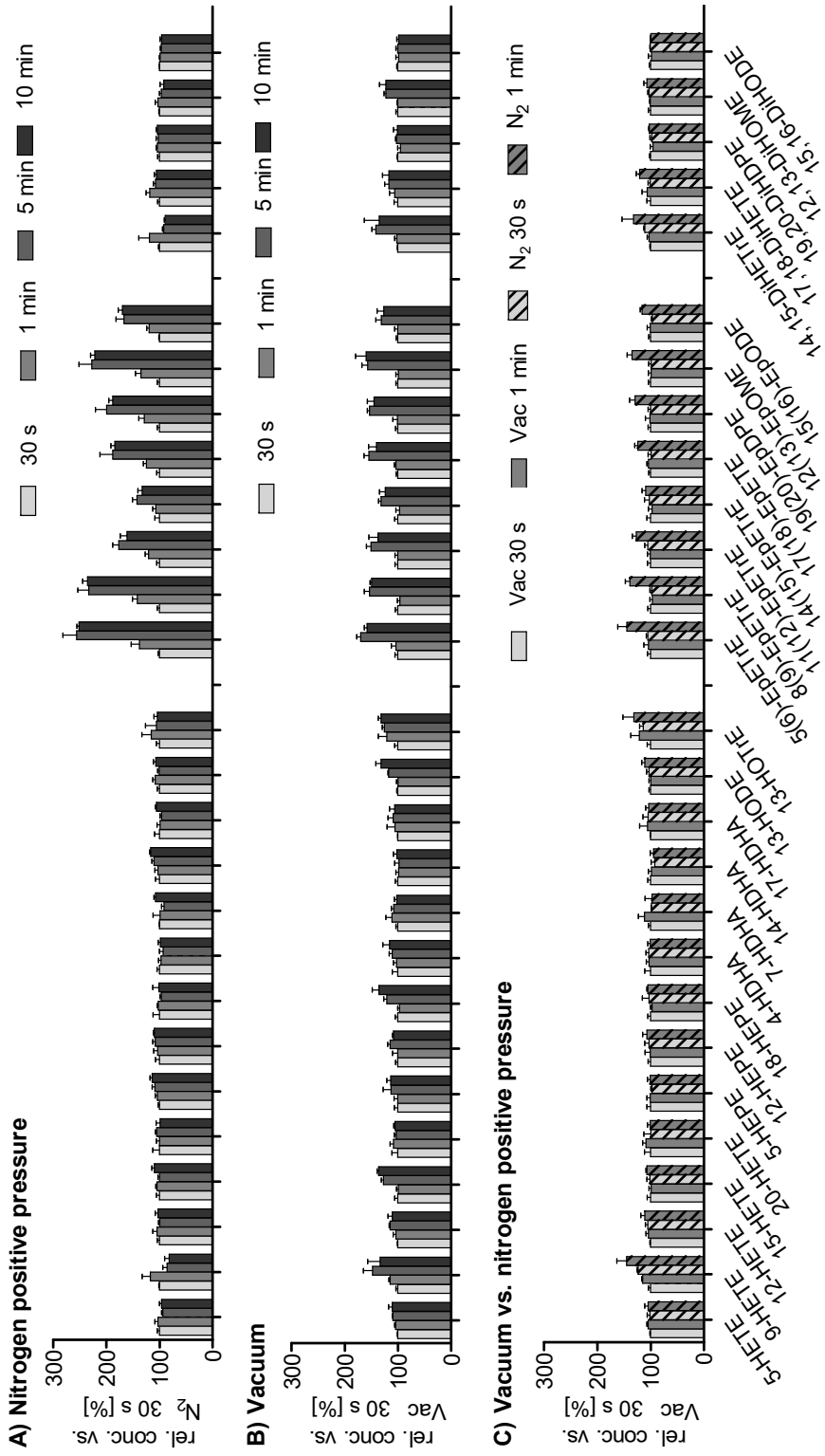


Fig. 11.5: Effect of sample drying during SPE before elution on the determined plasma oxylipin profile using Bond Elut Certify II cartridges. Shown are relative concentrations of a representative set of analytes following drying of samples with (A) nitrogen positive pressure and (B) vacuum (-200 mbar) for different time intervals (mean ± SD; n=3). Relative concentrations were calculated against the mean of the concentration following 30 s of drying. In (C) a comparison of drying with vacuum and nitrogen positive pressure is shown. Relative concentrations were calculated against drying with vacuum for 30 s. (D) shows the concentrations of *cis*- and *trans*-epoxy-PUFA in human plasma dried for either 30 s or 20 min prior to elution (following page).

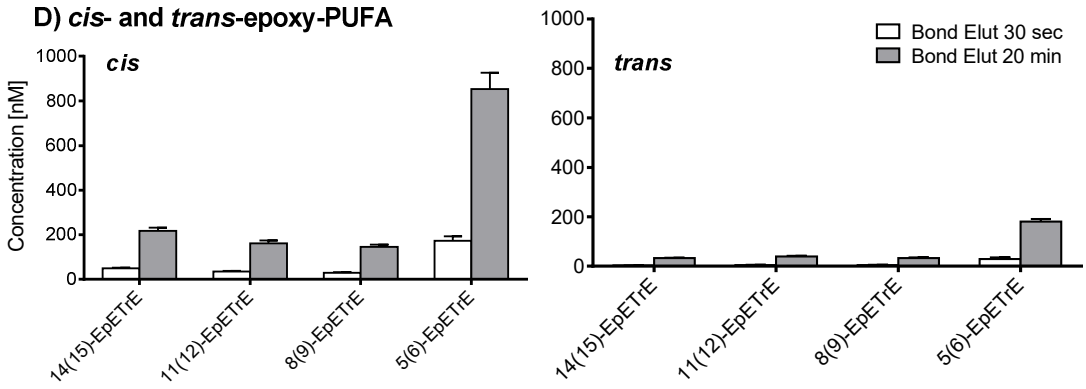


Fig. 11.5 continued.

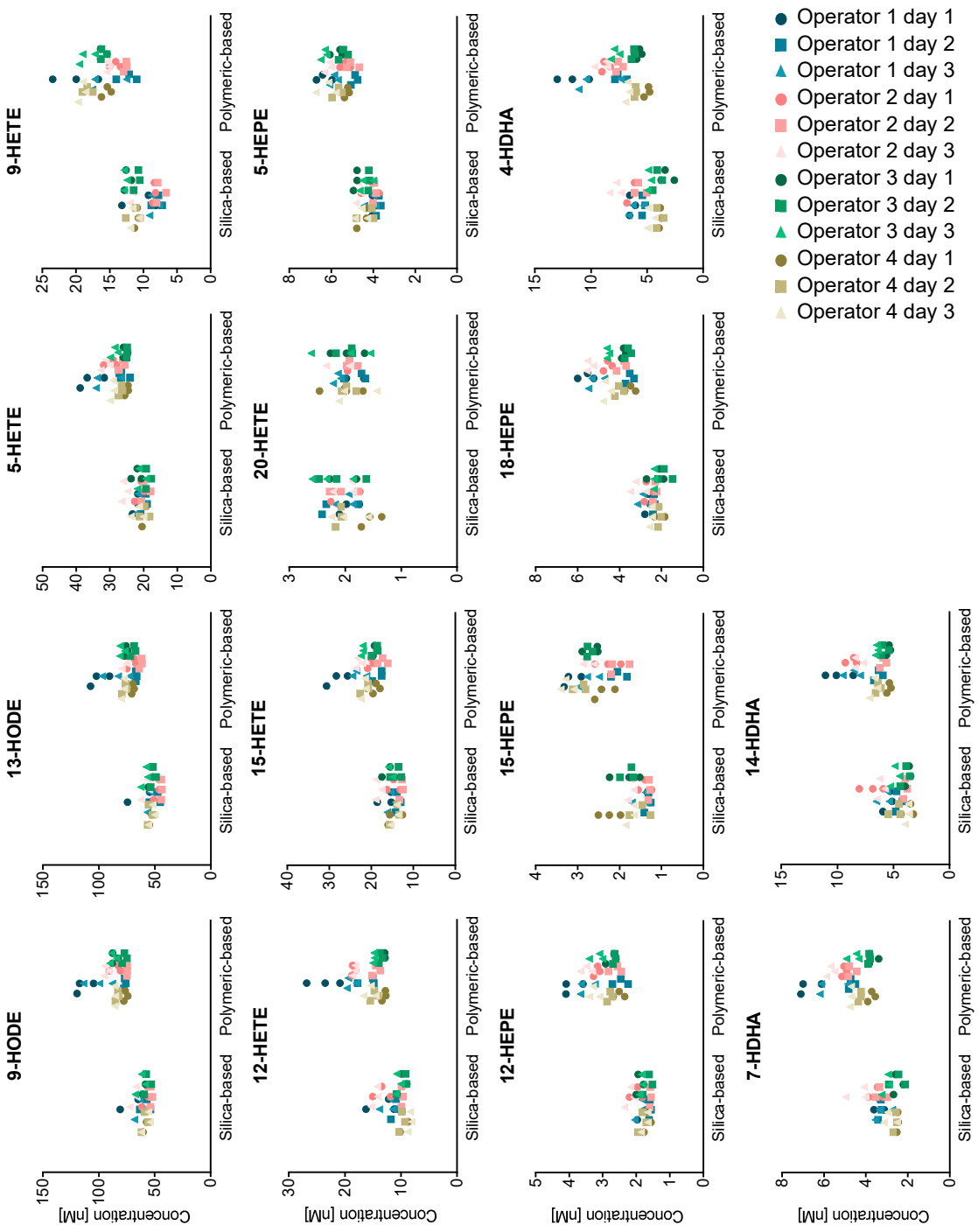


Fig. 11.6: Reproducibility using Bond Elut Certify II and Oasis MAX SPE for hydrolyzed plasma samples. Shown are individual concentrations of hydroxy-, epoxy- and dihydroxy-PUFA. Four different operators prepared a triplicate set of samples on three consecutive days with the final protocol on Bond Elut Certify II (left, silica-based) and Oasis MAX (right, polymeric-based) cartridges. Before elution, samples were dried with vacuum (-200 bar) for 30 s (Bond Elut) or 2 min (Oasis MAX). Shown are individual sample concentrations of a representative set of oxylipins.

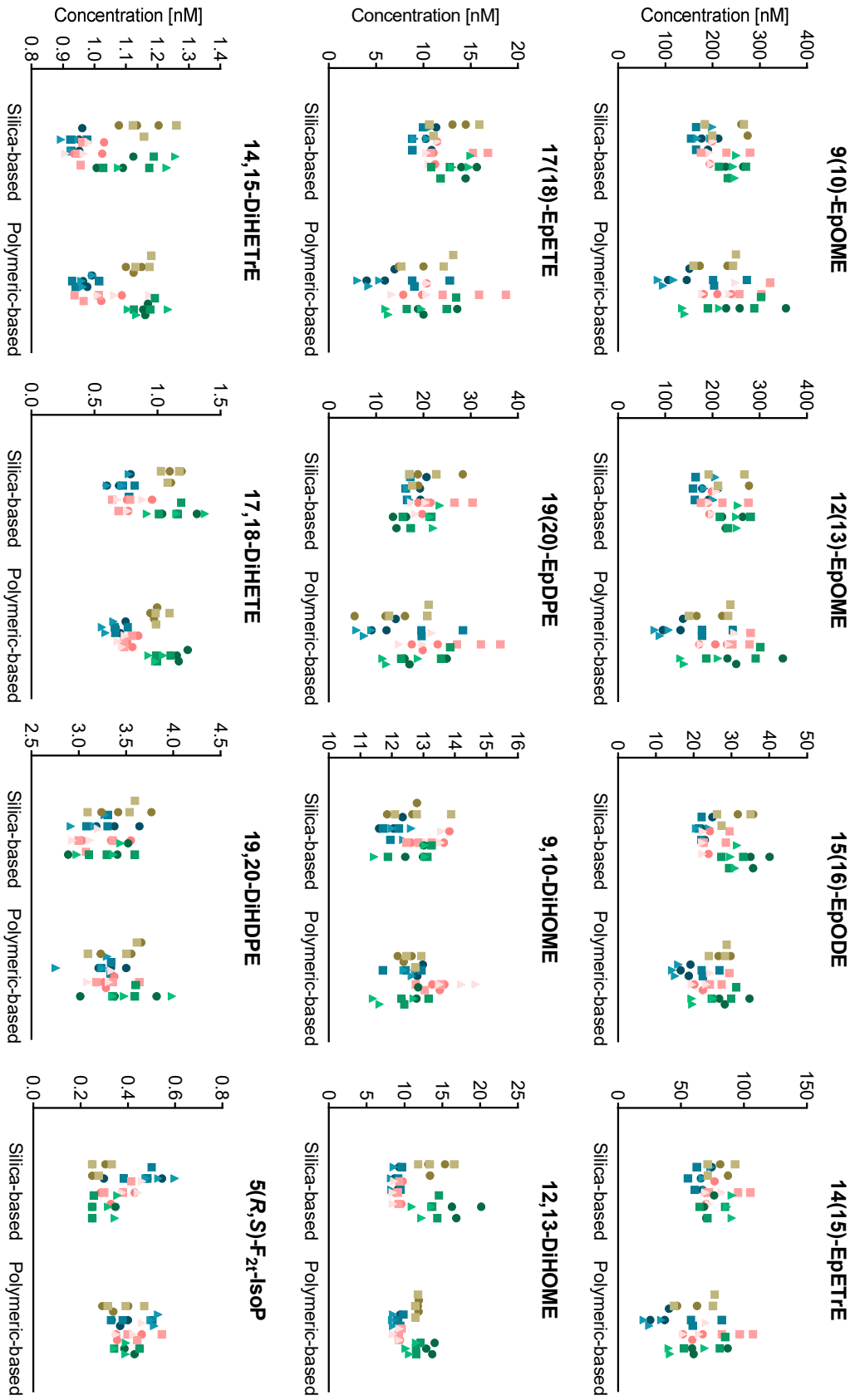


Fig. 11.6 continued.

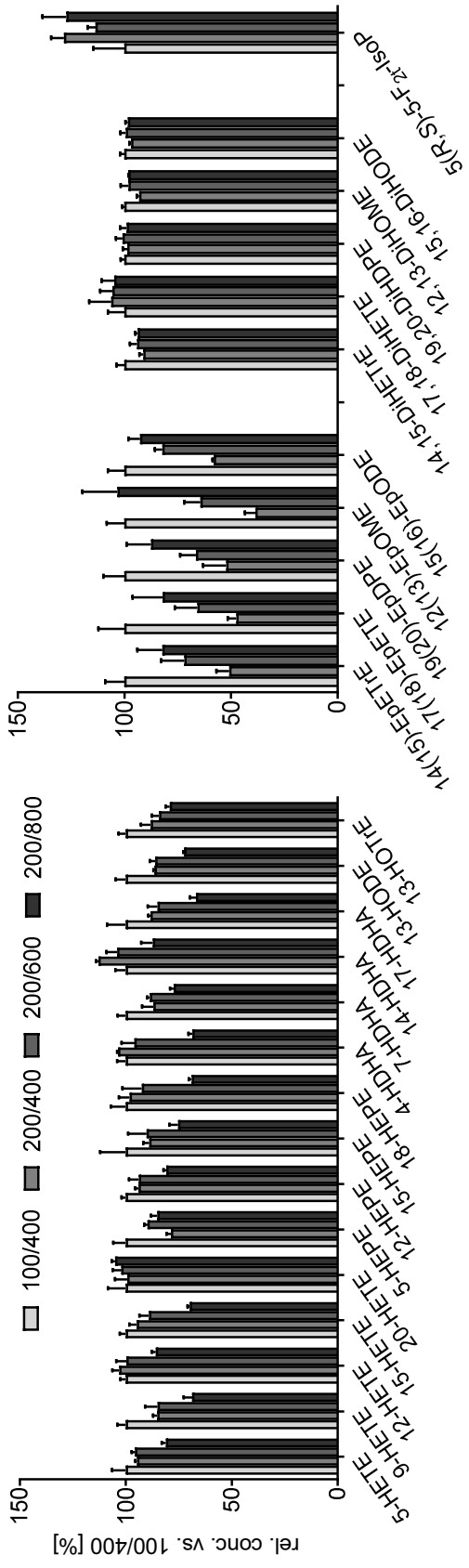


Fig. 11.7: Upscaling of plasma volume. For sample preparation, lipids were extracted from 100 µl or 200 µl plasma with *iso*-propanol (ratio sample+*iso*-propanol of 1+2 to 1+4). After hydrolysis, free oxylipins were extracted by SPE on Bond Elut Certify II material from the hydrolyzed sample and analyzed by LC-MS. Shown are relative concentrations of a representative set of analytes against sample preparation with 100 µl plasma and 400 µl of *iso*-propanol (mean ± SD; n=3).

Using 200 µl of plasma and 800 µl of *iso*-propanol (200/800), recovery rate of $^{2}H_4$-6-keto-$PGF_{1\alpha}$ was insufficient (< 40%). For the other setups, recovery rates of internal standards were acceptable and no differences were observed between the different experimental protocols.

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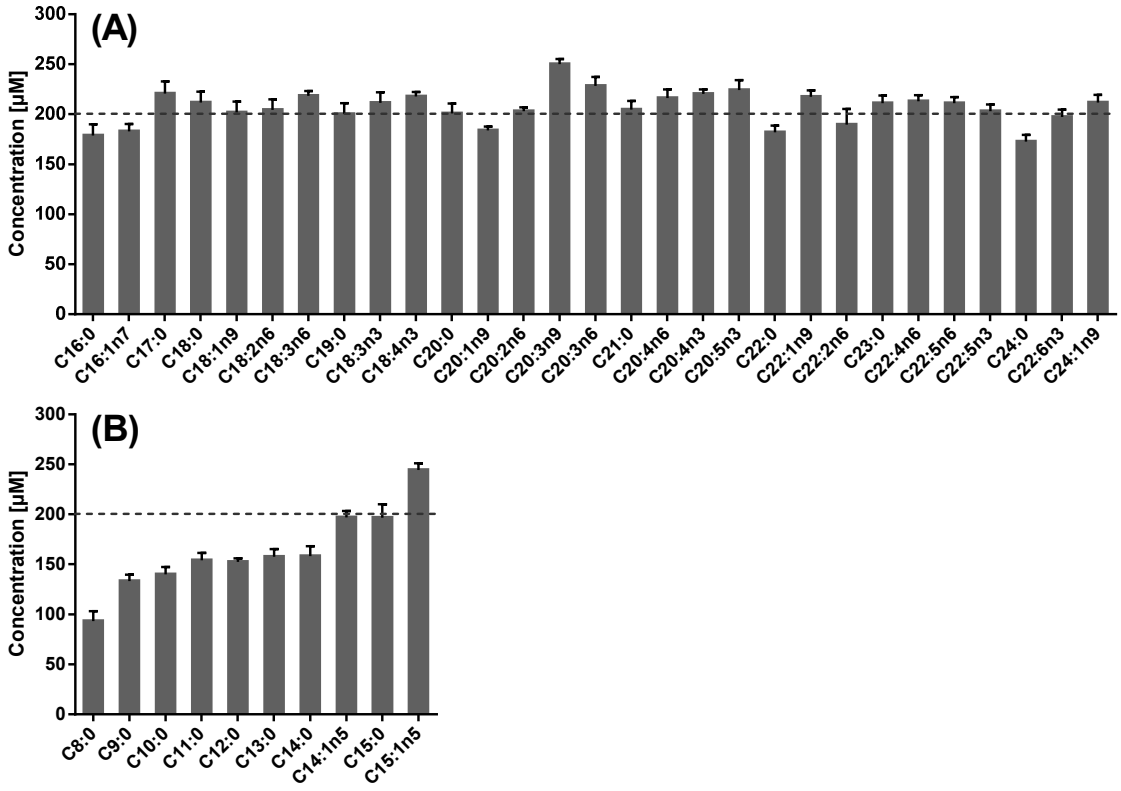

Fig. 11.8: Concentrations of fatty acids in calibration stock solutions determined by GC-FID (mean ± SD, n = 3). (A) 50 µl of the calibration stock solutions were evaporated to dryness, reconstituted in *n*-hexane and transesterified to methyl esters with methanolic HCl. C25:0 FAME was used as internal standard. (B) 50 µl of the calibration stock solutions were evaporated to dryness and reconstituted in methyl *tert*-butyl ether. 50 µl of trimethylsilyl sulfonium hydroxide (TMSH) was added and the samples were directly analyzed by GC-FID. C25:0 FAME was used as internal standard. Dashed lines indicate the nominal concentration of 200 µM. It should be noted that saturated fatty acids having ≤ 14 carbon atoms could not quantitively be transesterified to FAME neither by methanolic HCl nor by TMSH.

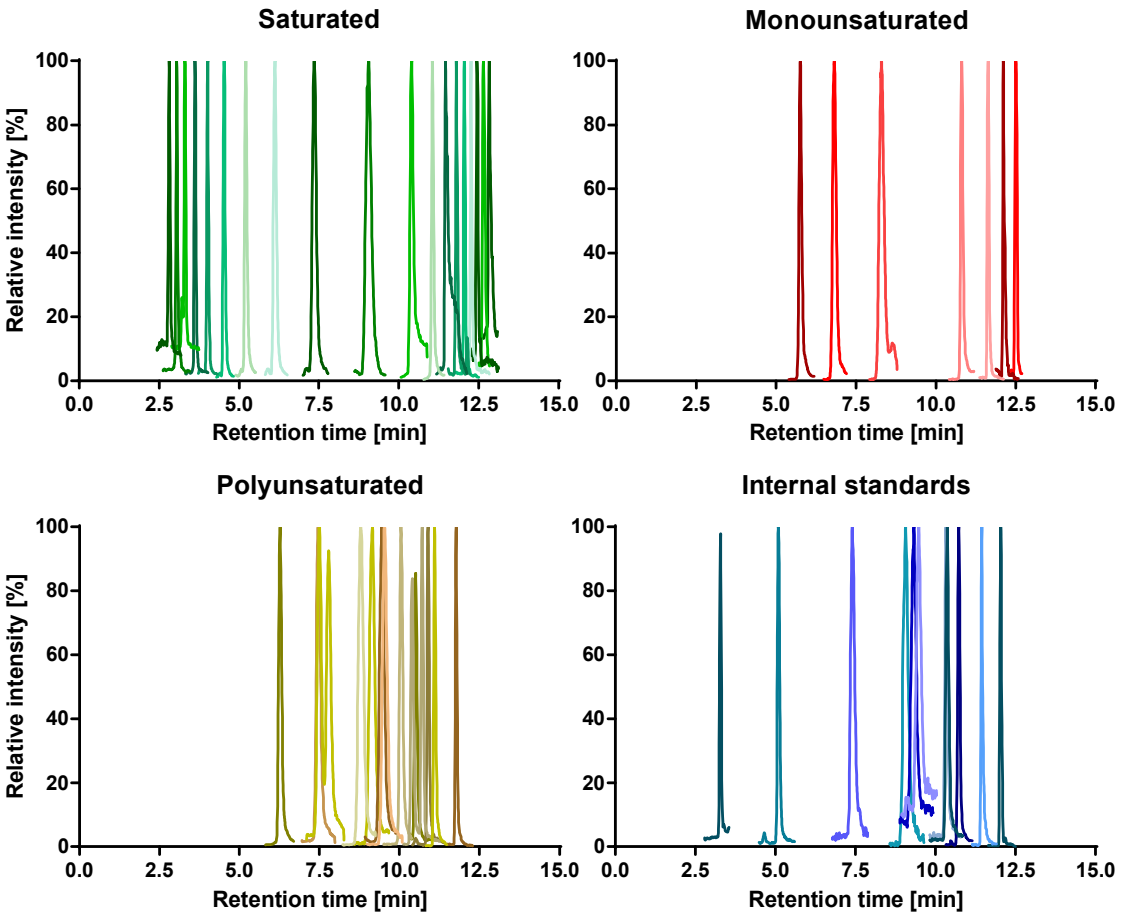

Fig. 11.9: Chromatographic separation of 41 fatty acids and 11 fatty acid internal standards. Shown are relative intensities of the *pseudo*-SRM transitions of the fatty acids after injection of 10 µl of fatty acid standard solutions (1 µM). Separation was carried out on RP-8 column (2.1 × 100 mm, particle size 2.6 µm (core-shell), pore size 10 nm) with a H_2O/ACN/MeOH/HAc gradient. Fatty acids are grouped according to their number of double bounds.

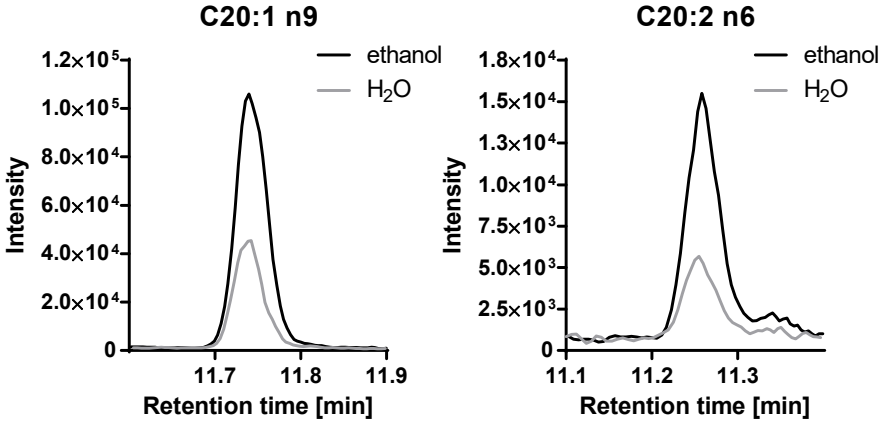

Fig. 11.10: Peak intensity of C20:1 n9 and C20:2 n6 in hydrolyzed rapeseed oil diluted in water or ethanol. Rapeseed oil was diluted in *iso*-propanol and hydrolyzed with 0.6 M KOH. The hydrolysate was diluted and injected (10 µl) in either water or in ethanol. Shown are exemplarily the *pseudo*-SRM signals of two long-chain fatty acids.

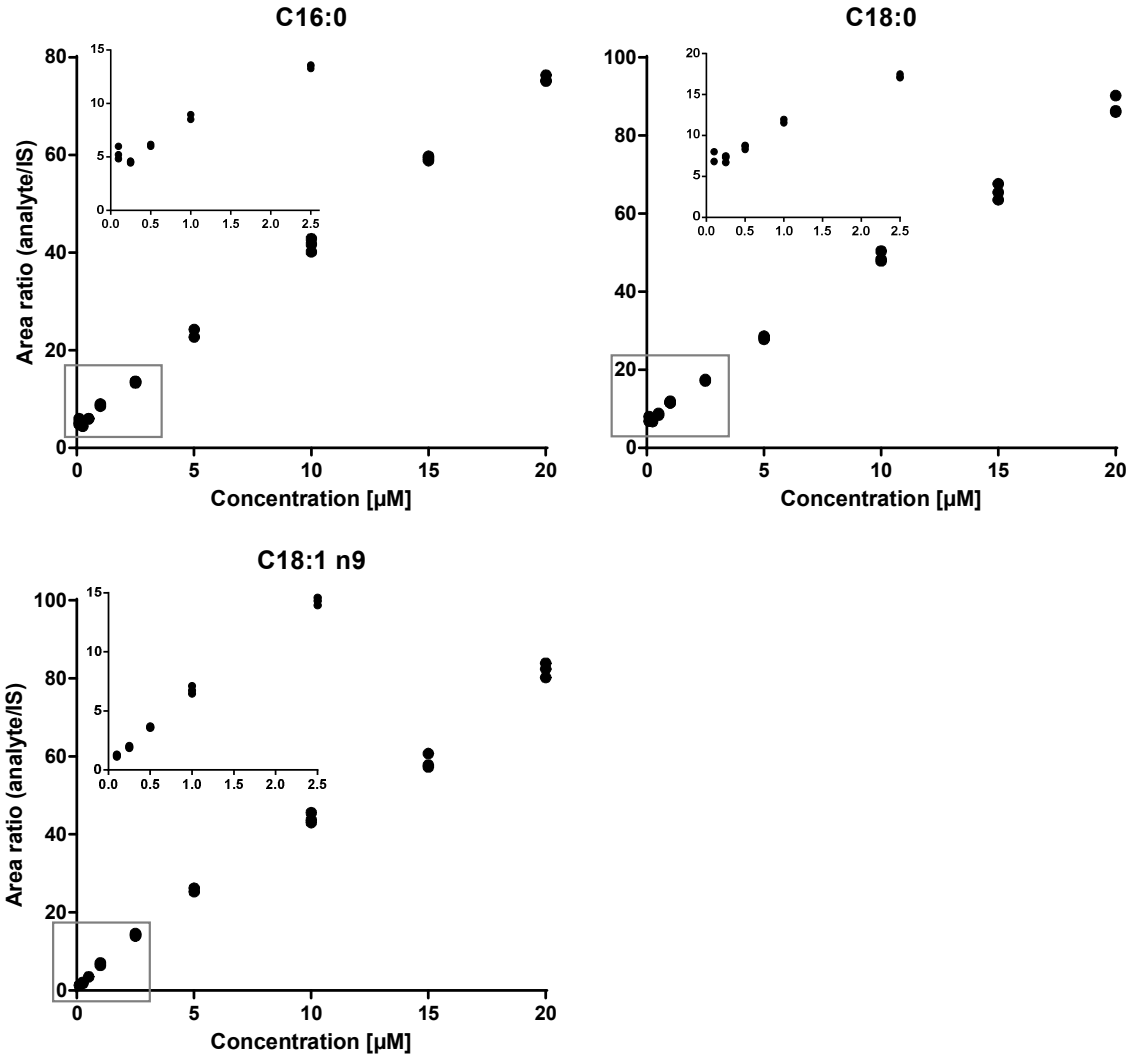

Fig. 11.11: Background levels of ubiquitously detectable fatty acids and their calibration curves. Area ratios (triple injections) are plotted against the concentration of the calibration standard. C16:0 and C18:0 also showed high peaks in blank injections and low concentrated calibration standards resulting in higher LLOQs. The LLOQ was set to the concentration yielding a peak height of at least twofold of the peak height in blank injections and an accuracy within the calibration curve of 80-120%. For C16:0 and C18:0 a linear regression could be used up to 20 µM and for C18:1 n9 quadratic regression up to 15 µM was applied.

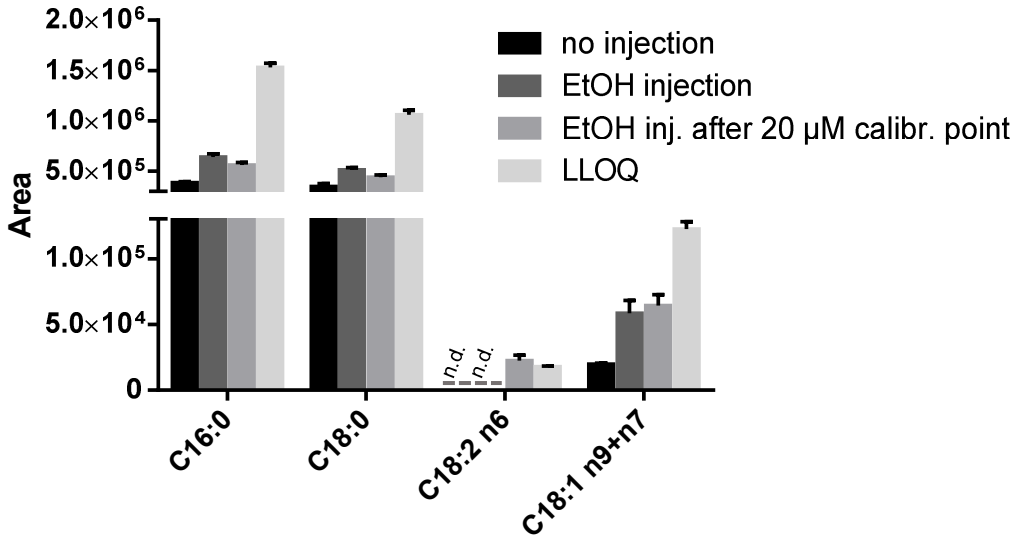

Fig. 11.12: Origin of background signals of ubiquitously detectable fatty acids. Shown are areas (mean ± SD, n = 3) of the fatty acids in LC-MS measurements without injection, injecting only pure ethanol as well as injection of pure ethanol after analysis of the highest calibration point (20 µM).

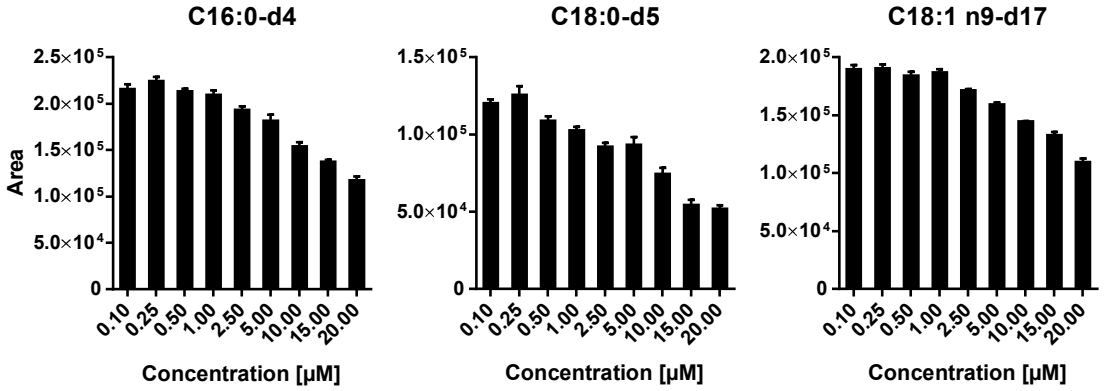

Fig. 11.13: Ion suppression of the internal standards C16:0-d4, C18:0-d5 and C18:1 n9-d17 (0.2 µM) with increasing fatty acid concentration in the calibrants. Areas (mean ± SD, n = 3) are plotted against the concentration in the calibration standard. The decreasing areas of the internal standards with increasing fatty acid concentrations indicates ion suppression.

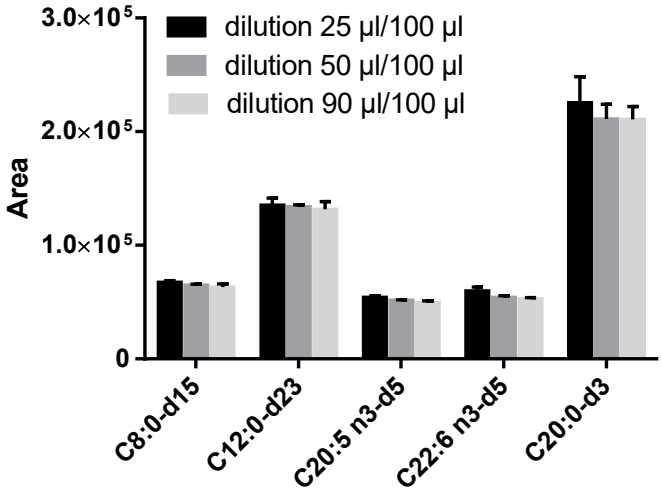

Fig. 11.14: Influence of dilution of human plasma samples on the areas of internal standards. Shown are areas (mean ± SD, n = 4) of internal standards in sequentially diluted hydrolyzed human plasma samples: 20 µl hydrolysate/500 µl ethanol. Subsequent dilutions were:
high: 25 µl/100 µl; medium: 50 µl/100 µl; low: 90 µl/100 µl.

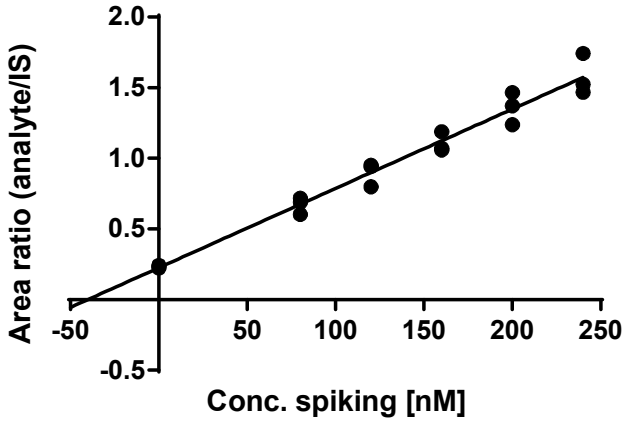

Fig. 11.15: Evaluation of accuracy of the determination of non-esterified fatty acids in human plasma using the standard addition procedure. Arachidonic acid was spiked at different levels in human plasma. 200 µl *iso*-propanol was added to 50 µl of plasma and 10 µl of the supernatant was diluted with ethanol (final volume: 100 µl). The x-intercept was determined using linear regression and had a best-fit value of -40.2 nM. The concentration in the vial of ARA in the non-spiked human plasma using the external concentration with internal standard was found to be 41.8 nM ± 1.9 nM (mean ± SD, n = 3).

Tab. 11.2: Intra- and inter-day precision of the total fatty acid concentrations in human plasma, sunflower oil and flaxseed oil. Total fatty acid concentrations were determined in triplicate on three separate days by means of LC-MS following hydrolysis. Intra- and inter-day variability was assessed by calculating the relative standard deviation on each single day and on all three days, respectively.
[1] The $[M-H-44]^-$ transition was used for quantification.

	Human plasma		**Sunflower oil**		**Flaxseed oil**	
	Intra-day RSD	Inter-day RSD	Intra-day RSD	Inter-day RSD	Intra-day RSD	Inter-day RSD
C14:1 n5	6.8	11				
C14:0	7.0	7.4				
C16:1 n7	4.1	8.1	1.4	20	7.6	8.6
C16:0	3.2	8.1	6.4	15	3.1	13
C18:4 n3	7.2	6.6				
C18:3 n6	5.5	4.8				
C18:3 n3	5.1	5.5	5.1	10	4.9	8.6
C18:2 n6	7.2	6.9	2.6	2.9	2.8	7.8
C18:1 n9+n7	4.6	7.9	2.5	8.7	7.9	14
C18:0	2.2	6.6	8.6	10	3.7	6.2
C20:5 n3	4.8	8.4				
C20:5 n3 [1]	4.6	8.3				
C20:4 n6	6.3	8.2				
C20:4 n6 [1]	3.6	9.5				
C20:4 n3	8.9	7.7				
C20:3 n9	6.1	15				
C20:3 n6+n3	10	7.5			11	12
C20:2 n6	4.7	10				
C20:1 n9	4.9	21	7.7	17	6.2	8.9
C20:0	14	18	6.9	10	7.7	13
C22:6 n3	4.2	6.4				
C22:6 n3 [1]	8.1	8.7				
C22:5 n3	4.6	7.7				
C22:5 n3 [1]	15	13				
C22:5 n6	5.7	19				
C22:5 n6 [1]	11	17				
C22:4 n6	3.9	16				
C22:1 n9	12	35			6.6	17
C22:0			4.9	18		

Tab. 11.3: Concentration of total oxylipins in human plasma. 100 µl of human plasma were diluted with *iso*-propanol. The supernatant after centrifugation was hydrolyzed with potassium hydroxide, neutralized and loaded onto pre-conditioned solid phase extraction (SPE) cartridges (C8/anion exchange). The eluate after SPE was evaporated, reconstituted and analyzed by LC-MS (mean ± SD, n = 4).

Precursor fatty acid	Analyte	Concentration [nM]
Oleic acid (C18:1 n9)	9(10)-Ep-stearic acid	90 ± 10
Linoleic acid (C18:2 n6)	9-HODE	1140 ± 30
	10-HODE	12.3 ± 0.5
	12-HODE	6.9 ± 0.5
	13-HODE	1930 ± 70
	15-HODE	16.4 ± 0.9
	9(10)-EpOME	95 ± 10
	12(13)-EpOME	78 ± 9
	9,10-DiHOME	18.7 ± 0.6
	12,13-DiHOME	6.4 ± 0.3
Linolenic acid (C18:3 n3)	9-HOTrE	26 ± 1
	13-HOTrE	60 ± 3
	9(10)-EpODE	4.3 ± 0.6
	12(13)-EpODE	2.6 ± 0.2
	15(16)-EpODE	30 ± 2
	9,10-DiHODE	0.75 ± 0.03
	12,13-DiHODE	0.49 ± 0.02
	15,16-DiHODE	23 ± 1
Mead acid (C20:3 n9)	5-HETrE	4.1 ± 0.2
Dihomo-γ-linoleic acid (C20:3 n6)	8-HETrE	21 ± 2
	12-HETrE	53 ± 2
	15-HETrE	36 ± 1
	14(15)-EpEDE	2.9 ± 0.4
Arachidonic acid (C20:4 n6)	5-HETE	94 ± 3
	8-HETE	123 ± 10
	9-HETE	240 ± 10
	11-HETE	240 ± 15
	12-HETE	200 ± 15
	15-HETE	163 ± 8
	16-HETE	1.5 ± 0.2
	17-HETE	0.35 ± 0.05
	18-HETE	0.75 ± 0.10
	20-HETE	1.46 ± 0.07
	12-HHTrE	2.4 ± 0.2
	tetranor-12-HETE	0.89 ± 0.04
	5(S),15(S)-DiHETE	9.48 ± 0.07
	8(S),15(S)-DiHETE	76 ± 2
	6-trans-LTB4	3.7 ± 0.1
	6-trans-12-epi-LTB4	7.1 ± 0.2
	8(9)-EpETrE	16 ± 2
	11(12)-EpETrE	18 ± 2
	14(15)-EpETrE	28 ± 3
	5,6-DiHETrE	13.8 ± 0.5
	8,9-DiHETrE	3.2 ± 0.2
	11,12-DiHETrE	1.07 ± 0.07
	14,15-DiHETrE	0.86 ± 0.05
	PGB2	1.8 ± 0.2
	5(*R,S*)-F_{2t}-IsoP	0.47 ± 0.05
	20-COOH-ARA	6.6 ± 0.3
Eicosa-pentaenoic acid (C20:5 n3)	5-HEPE	30 ± 2
	8-HEPE	46 ± 3
	9-HEPE	117 ± 6
	11-HEPE	52 ± 3
	12-HEPE	116 ± 7
	15-HEPE	102 ± 6
	18-HEPE	104 ± 5
	8(9)-EpETE	3.5 ± 0.4
	11(12)-EpETE	3.1 ± 0.4
	14(15)-EpETE	3.8 ± 0.4
	17(18)-EpETE	6.0 ± 0.7
	5,6-DiHETE	2.0 ± 0.2
	8,9-DiHETE	0.43 ± 0.01
	14,15-DiHETE	0.13 ± 0.02
	17,18-DiHETE	0.72 ± 0.03
Docosa-hexaenoic acid (C22:6 n3)	4-HDHA	45 ± 2
	7-HDHA	49 ± 2
	8-HDHA	74 ± 3
	10-HDHA	61 ± 5
	11-HDHA	108 ± 4
	13-HDHA	69 ± 5
	14-HDHA	82 ± 6
	16-HDHA	73 ± 4
	17-HDHA	79 ± 4
	20-HDHA	97 ± 6
	7(8)-EpDPE	6.3 ± 0.7
	10(11)-EpDPE	5.4 ± 0.6
	13(14)-EpDPE	5.3 ± 0.7
	16(17)-EpDPE	5.2 ± 0.7
	19(20)-EpDPE	9.7 ± 0.9
	7,8-DiHDPE	2.8 ± 0.1
	10,11-DiHDPE	0.56 ± 0.04
	13,14-DiHDPE	0.45 ± 0.02
	16,17-DiHDPE	1.26 ± 0.04
	19,20-DiHDPE	2.79 ± 0.07

Tab. 11.4: Concentrations of non-esterified fatty acids in human plasma. 100 µl of human plasma were diluted with *iso*-propanol. 10 µl or 40 µl of the supernatant were diluted in ethanol (10 µl/100 µl for high concentrated fatty acids and 40 µl/100 µl for low concentrated fatty acids; mean ± SD, n = 3).

Fatty acid	Concentration [µM]
C10:0	1.2 ± 0.2
C12:0	4.4 ± 0.5
C14:1 n5	1.7 ± 0.1
C14:0	15 ± 1
C15:0	1.4 ± 0.1
C16:1 n7	20 ± 1
C16:0	140 ± 10
C17:0	3.4 ± 0.2
C18:4 n3	0.12 ± 0.01
C18:3 n6	0.82 ± 0.09
C18:3 n3	12 ± 1
C18:2 n6	49 ± 5
C18:1 n9/7	190 ± 20
C18:0	46 ± 4
C20:5 n3	0.64 ± 0.07
C20:4 n6	2.7 ± 0.3
C20:4 n3	0.124 ± 0.005
C20:3 n9	0.06 ± 0.03
C20:3 n6/3	0.73 ± 0.07
C20:2 n6	0.70 ± 0.06
C20:1 n9	4.1 ± 0.3
C20:0	0.26 ± 0.05
C22:6 n3	2.9 ± 0.2
C22:5 n3	0.83 ± 0.07
C22:4 n6	0.34 ± 0.04
C22:1 n9	0.23 ± 0.07

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Tab. 11.5: Concentration of total fatty acids in the fish, micro-algae and krill oil supplements. Fatty acids were analyzed by LC-MS following hydrolysis (mean ± SD, n= 3).

[mg/g]	AO1	AO2	AO3	FO1	FO2	FO3	FO4	KO1	KO2	SPM	M+
C8:0	< LLOQ	< LLOQ	2.6 ± 0.3	1.53 ± 0.08	1.8 ± 0.3	< LLOQ	< LLOQ	< LLOQ	< LLOQ	3.9 ± 0.6	< LLOQ
C10:0	1.3 ± 0.5	< LLOQ	2.5 ± 0.2	1.3 ± 0.1	2.0 ± 0.3	1.4 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	< LLOQ	4.0 ± 0.4	< LLOQ
C12:0	1.5 ± 0.8	< LLOQ	0.58 ± 0.02	< LLOQ	1.2 ± 0.2	1.2 ± 0.2	< LLOQ	1.7 ± 0.6	1.38 ± 0.07	< LLOQ	< LLOQ
C13:0	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.49 ± 0.04	< LLOQ	< LLOQ	< LLOQ
C14:1 n5	< LLOQ	< LLOQ	< LLOQ	0.66 ± 0.04	n.d.	n.d.	< LLOQ	1.16 ± 0.07	1.18 ± 0.05	< LLOQ	n.d.
C14:0	11 ± 1	9.7 ± 0.6	11.2 ± 0.4	23.5 ± 0.5	55 ± 3	76 ± 9	15 ± 0.2	80 ± 20	74 ± 5	< LLOQ	18 ± 2
C15:1 n5	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.41 ± 0.02	< LLOQ	< LLOQ	< LLOQ
C15:0	1.3 ± 0.4	0.9 ± 0.1	1.06 ± 0.08	1.1 ± 0.2	2.6 ± 0.3	3.2 ± 0.2	< LLOQ	2.1 ± 0.5	2.21 ± 0.04	< LLOQ	2.8 ± 0.4
C16:1 n7	1.5 ± 0.3	0.94 ± 0.09	1.13 ± 0.05	45 ± 1	76 ± 6	89 ± 7	7.2 ± 0.5	58 ± 1	64 ± 3	2.7 ± 0.3	25 ± 2
C16:0	260 ± 20	210 ± 10	232 ± 6	126 ± 6	160 ± 20	190 ± 10	31 ± 5	190 ± 20	303 ± 30	40 ± 2	190 ± 20
C17:0	1.2 ± 0.4	0.8 ± 0.1	0.85 ± 0.06	1.1 ± 0.2	4.2 ± 0.3	4.7 ± 0.4	1.37 ± 0.06	< LLOQ	1.2 ± 0.1	0.29 ± 0.06	7.2 ± 0.4
C18:4 n3	1.5 ± 0.1	1.6 ± 0.1	1.56 ± 0.08	37 ± 1	33 ± 3	34 ± 1	27 ± 2	39 ± 3	42.5 ± 0.8	4.5 ± 0.4	12.3 ± 0.7
C18:3 n6	0.48 ± 0.04	0.35 ± 0.04	0.36 ± 0.03	1.05 ± 0.02	2.8 ± 0.3	2.92 ± 0.01	1.6 ± 0.1	2.22 ± 0.05	2.81 ± 0.08	0.29 ± 0.02	2.35 ± 0.06
C18:3 n3	< LLOQ	< LLOQ	< LLOQ	5.6 ± 0.2	7.4 ± 0.5	8.5 ± 0.4	4.3 ± 0.4	9.2 ± 0.1	16.5 ± 0.5	0.45 ± 0.08	10.0 ± 0.7
C18:2 n6	11.3 ± 0.8	7.9 ± 0.2	8.8 ± 0.5	12.4 ± 0.3	16 ± 1	17.9 ± 0.5	12.7 ± 0.2	14.8 ± 0.2	66 ± 3	2.0 ± 0.2	70 ± 5
C18:1 n9+n7	110 ± 20	90 ± 10	110 ± 20	140 ± 10	140 ± 10	210 ± 20	100 ± 20	150 ± 40	300 ± 40	15 ± 2	210 ± 10
C18:0	90 ± 10	27 ± 6	36 ± 3	41 ± 4	48 ± 8	48 ± 3	42 ± 3	n.d.	90 ± 20	33 ± 4	92 ± 3
C19:0	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.3 ± 0.2	1.1 ± 0.2	2.2 ± 0.1	< LLOQ	< LLOQ	< LLOQ	3.4 ± 0.4
C20:5 n3	190 ± 20	202 ± 7	190 ± 30	167 ± 6	192 ± 6	170 ± 20	400 ± 40	133 ± 8	132 ± 6	200 ± 30	59 ± 3
C20:4 n6	14 ± 1	14.0 ± 0.5	13.8 ± 0.6	3.7 ± 0.1	10.8 ± 0.8	9.4 ± 0.6	19 ± 1	2.52 ± 0.05	2.1 ± 0.2	11 ± 1	20 ± 1
C20:4 n3	8.8 ± 0.7	8.6 ± 0.6	8.8 ± 0.4	9.6 ± 0.4	9.9 ± 0.8	8.8 ± 0.5	19 ± 2	4.0 ± 0.2	3.5 ± 0.1	12 ± 1	3.5 ± 0.2
C20:3 n9	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.175 ± 0.003	0.11 ± 0.01	0.75 ± 0.04	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:3 n6+n3	0.63 ± 0.05	0.66 ± 0.05	0.65 ± 0.08	1.05 ± 0.04	2.4 ± 0.2	2.1 ± 0.1	4.48 ± 0.08	1.0 ± 0.1	0.65 ± 0.02	3.1 ± 0.1	1.90 ± 0.07
C20:2 n6	< LLOQ	< LLOQ	< LLOQ	2.61 ± 0.06	n.d.	n.d.	n.d.	0.63 ± 0.01	0.5 ± 0.1	n.d.	2.3 ± 0.2
C20:1 n9	1.4 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	109 ± 5	16 ± 1	15 ± 1	40 ± 4	7.4 ± 0.4	9.3 ± 0.9	27 ± 2	16 ± 2
C20:0	5.5 ± 0.6	5.0 ± 0.3	5.0 ± 0.3	0.93 ± 0.08	4.0 ± 0.2	< LLOQ	6.6 ± 0.3	< LLOQ	2.1 ± 0.3	6.4 ± 0.7	6.4 ± 0.6
C21:0	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.82 ± 0.07	< LLOQ	< LLOQ	< LLOQ	0.99 ± 0.08	1.4 ± 0.2
C22:6 n3	310 ± 20	293 ± 3	360 ± 60	103 ± 5	119 ± 6	110 ± 10	290 ± 30	65 ± 3	61.7 ± 0.9	300 ± 30	310 ± 30
C22:5 n3	49 ± 3	57.7 ± 0.4	41 ± 7	11.9 ± 0.3	21 ± 2	17 ± 1	51 ± 6	3.62 ± 0.07	3.25 ± 0.09	90 ± 10	16 ± 1
C22:5 n6	19 ± 2	16.0 ± 1.4	24 ± 2	1.23 ± 0.03	4.7 ± 0.2	3.5 ± 0.2	10.3 ± 0.4	0.26 ± 0.08	< LLOQ	14 ± 2	28 ± 2
C22:4 n6	4.9 ± 0.5	5.4 ± 0.3	4.4 ± 0.4	n.d.	1.34 ± 0.04	1.13 ± 0.07	3.1 ± 0.1	< LLOQ	< LLOQ	n.d.	2.52 ± 0.09
C22:2 n6	< LLOQ	< LLOQ	< LLOQ	0.94 ± 0.05	1.1 ± 0.1	0.9 ± 0.1	2.4 ± 0.1	< LLOQ	< LLOQ	7.4 ± 1.0	< LLOQ
C22:1 n9	< LLOQ	< LLOQ	< LLOQ	115 ± 8	11.9 ± 0.2	12 ± 2	39 ± 4	8.80 ± 0.01	10.52 ± 0.06	43 ± 3	8.7 ± 0.8
C22:0	1.88 ± 0.09	1.4 ± 0.2	1.5 ± 0.2	< LLOQ	0.8 ± 0.1	0.9 ± 0.1	2.1 ± 0.2	< LLOQ	< LLOQ	2.9 ± 0.4	3.7 ± 0.6
C24:1 n9	< LLOQ	< LLOQ	< LLOQ	2.6 ± 0.5	2.6 ± 0.3	2.9 ± 0.4	3.1 ± 0.7	< LLOQ	< LLOQ	9 ± 2	< LLOQ

Tab. 11.6: Concentration of total (esterified and non-esterified) oxylipins in the fish, micro-algae and krill oil supplements. Oxylipins were analyzed by LC-MS following hydrolysis and solid phase extraction (mean ± SD, n= 3).

Precursor	Oxylipin conc. [nmol/g]	KO1	KO2	AO1	AO2	AO3
C18:1 n9	9(10)-Ep-stearic acid	24 ± 3	101 ± 5	40 ± 2	25 ± 2	46 ± 2
	9,10-DiH-stearic acid	9 ± 2	83 ± 2	8.1 ± 0.9	6.1 ± 0.5	7.8 ± 0.6
C18:2 n6	9-HODE	77 ± 3	191 ± 5	6.4 ± 0.4	6.6 ± 0.2	6.9 ± 0.5
	10-HODE	2.0 ± 0.1	5.5 ± 0.1	0.44 ± 0.01	0.30 ± 0.02	0.42 ± 0.07
	12-HODE	1.22 ± 0.08	3.6 ± 0.1	0.24 ± 0.03	0.19 ± 0.01	0.25 ± 0.03
	13-HODE	112 ± 4	400 ± 10	10.6 ± 0.4	9.8 ± 0.3	10.7 ± 0.8
	15-HODE	2.60 ± 0.09	13.5 ± 0.7	0.72 ± 0.03	0.24 ± 0.04	0.41 ± 0.03
	9(10)-EpOME	3.3 ± 0.5	168 ± 6	8.9 ± 0.1	6.8 ± 0.2	8.1 ± 0.3
	12(13)-EpOME	2.6 ± 0.5	97 ± 2	4.6 ± 0.3	2.0 ± 0.2	2.8 ± 0.1
	9,10-DiHOME	1.5 ± 0.4	262 ± 1	21.1 ± 0.8	7.60 ± 0.07	9.2 ± 0.3
	12,13-DiHOME	0.5 ± 0.1	89.5 ± 0.8	2.3 ± 0.7	1.09 ± 0.04	1.26 ± 0.07
	9,10,11-TriHOME	0.47 ± 0.02	3.4 ± 0.2	< LLOQ	< LLOQ	< LLOQ
C18:3 n3	9-HOTrE	62 ± 1	78 ± 2	0.21 ± 0.02	0.30 ± 0.05	0.28 ± 0.03
	13-HOTrE	76 ± 2	116 ± 3	< LLOQ	< LLOQ	< LLOQ
	9(10)-EpODE	2.9 ± 0.4	50.4 ± 0.9	< LLOQ	< LLOQ	< LLOQ
	12(13)-EpODE	1.5 ± 0.2	8.1 ± 0.3	< LLOQ	< LLOQ	< LLOQ
	15(16)-EpODE	5.2 ± 0.8	199.5 ± 1.0	< LLOQ	< LLOQ	< LLOQ
	9,10-DiHODE	0.225 ± 0.003	39.3 ± 0.2	0.75 ± 0.03	0.36 ± 0.01	0.44 ± 0.02
	12,13-DiHODE	< LLOQ	5.2 ± 0.2	< LLOQ	< LLOQ	< LLOQ
	15,16-DiHODE	0.34 ± 0.03	198 ± 2	1.14 ± 0.06	1.39 ± 0.04	1.45 ± 0.07
	9,10,11-TriHODE	0.17 ± 0.02	0.41 ± 0.03	< LLOQ	< LLOQ	< LLOQ
	9,12,13-TriHODE	n.d.	13.4 ± 0.4	< LLOQ	< LLOQ	< LLOQ
C18:3 n6	13-gamma-HOTrE	15.8 ± 0.6	15.9 ± 0.3	< LLOQ	< LLOQ	< LLOQ
C20:3 n9	5-HETrE	0.16 ± 0.01	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:3 n6	8-HETrE	2.0 ± 0.8	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	12-HETrE	2.7 ± 0.1	2.9 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	15-HETrE	4.2 ± 0.2	4.1 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	14(15)-EpEDE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:4 n6	5-HETE	10.9 ± 0.5	8.56 ± 0.06	1.6 ± 0.3	1.30 ± 0.06	1.01 ± 0.02
	8-HETE	9.4 ± 0.5	8.5 ± 0.8	1.2 ± 0.2	0.79 ± 0.02	0.60 ± 0.05
	9-HETE	11 ± 1	10.0 ± 0.4	1.2 ± 0.3	< LLOQ	< LLOQ
	11-HETE	10.3 ± 0.6	9.8 ± 0.9	1.5 ± 0.4	0.92 ± 0.01	0.69 ± 0.05
	12-HETE	9.1 ± 0.7	8.9 ± 0.6	1.0 ± 0.2	0.65 ± 0.04	0.47 ± 0.06
	15-HETE	12.5 ± 0.4	13.0 ± 0.9	1.4 ± 0.3	0.84 ± 0.06	0.64 ± 0.05
	17-HETE	2.8 ± 0.1	3.0 ± 0.4	11.3 ± 0.3	10.8 ± 0.1	13.2 ± 0.4
	18-HETE	7.2 ± 0.3	7.8 ± 0.6	1.88 ± 0.08	1.37 ± 0.06	1.462 ± 0.09
	20-HETE	< LLOQ	< LLOQ	4.52 ± 0.42	4.4 ± 0.3	4.2 ± 0.6
	12-HHTrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	tetranor-12-HETE	0.35 ± 0.02	0.29 ± 0.03	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETrE	< LLOQ	< LLOQ	n.d.	1.4 ± 0.2	n.d.
	11(12)-EpETrE	0.39 ± 0.04	0.37 ± 0.03	1.7 ± 0.2	1.42 ± 0.09	1.7 ± 0.1
	14(15)-EpETrE	0.60 ± 0.06	< LLOQ	3.1 ± 0.3	2.6 ± 0.3	3.5 ± 0.1
	5,6-DiHETrE	0.303 ± 0.003	< LLOQ	0.41 ± 0.04	0.26 ± 0.01	0.30 ± 0.01
	8,9-DiHETrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	11,12-DiHETrE	< LLOQ	< LLOQ	0.068 ± 0.001	0.08 ± 0.01	0.09 ± 0.01
	14,15-DiHETrE	0.04 ± 0.01	< LLOQ	0.056 ± 0.003	0.05 ± 0.01	0.077 ± 0.001
	PGB_2	0.70 ± 0.06	0.8 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	$PGF_{2\alpha}$	1.99 ± 0.07	1.6 ± 0.3	< LLOQ	< LLOQ	< LLOQ
	20-COOH-ARA	< LLOQ	< LLOQ	2.6 ± 0.2	3.21 ± 0.06	2.6 ± 0.2

Tab. 11.6 continued.

Precursor	Oxylipin conc. [nmol/g]	KO1	KO2	AO1	AO2	AO3
	5-HEPE	339 ± 4	300 ± 20	10.6 ± 1.9	9.1 ± 0.3	6.7 ± 0.4
	8-HEPE	228 ± 4	230 ± 10	9.4 ± 1.8	6.3 ± 0.2	4.0 ± 0.2
	9-HEPE	440 ± 10	450 ± 30	18.4 ± 3.0	13.6 ± 0.6	9.6 ± 0.2
	11-HEPE	261 ± 7	260 ± 10	10.1 ± 1.8	6.31 ± 0.06	3.77 ± 0.01
	12-HEPE	500 ± 10	490 ± 30	15.4 ± 2.8	10.0 ± 0.2	6.2 ± 0.2
	15-HEPE	430 ± 3	440 ± 30	13.0 ± 2.2	8.6 ± 0.3	4.9 ± 0.3
	18-HEPE	678 ± 9	740 ± 40	22.8 ± 3.7	15.6 ± 0.6	10.2 ± 0.4
	19-HEPE	28.8 ± 0.9	27 ± 2	3.6 ± 0.3	4.1 ± 0.2	4.5 ± 0.1
	20-HEPE	10.3 ± 0.9	12 ± 1	270 ± 30	300 ± 10	281 ± 8
C20:5 n3	5(*S*),18(*R*)-diHEPE (RvE2)	12.5 ± 0.9	10.1 ± 0.4	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETE	22 ± 2	17.5 ± 0.8	29 ± 3	25 ± 2	25 ± 2
	11(12)-EpETE	28 ± 5	24 ± 4	36 ± 3	34 ± 3	35 ± 1
	14(15)-EpETE	28 ± 6	24 ± 6	36 ± 4	33 ± 4	38 ± 2
	17(18)-EpETE	53 ± 9	42 ± 2	76 ± 3	72 ± 8	77.0 ± 0.9
	5,6-DiHETE	15.6 ± 1.0	16.1 ± 0.5	6.7 ± 0.6	5.5 ± 0.1	5.3 ± 0.2
	8,9-DiHETE	2.26 ± 0.05	2.5 ± 0.3	0.94 ± 0.08	0.93 ± 0.08	1.16 ± 0.05
	11,12-DiHETE	1.86 ± 0.04	2.0 ± 0.1	2.2 ± 0.2	2.31 ± 0.02	2.3 ± 0.2
	14,15-DiHETE	1.38 ± 0.07	1.4 ± 0.1	0.7 ± 0.1	0.69 ± 0.05	0.90 ± 0.05
	17,18-DiHETE	2.63 ± 0.08	2.9 ± 0.2	2.8 ± 0.3	2.86 ± 0.04	3.7 ± 0.2
	PGB_3	2.9 ± 0.1	3.5 ± 0.4	< LLOQ	< LLOQ	< LLOQ
	4-HDHA	317 ± 8	342 ± 3	33 ± 5	31.7 ± 0.9	31.7 ± 0.3
	7-HDHA	156 ± 9	145 ± 6	26 ± 6	20.4 ± 0.8	20 ± 1
	8-HDHA	168 ± 6	157 ± 4	23 ± 5	16.3 ± 1.0	15.4 ± 1.0
	10-HDHA	180 ± 10	180 ± 10	26 ± 6	14.9 ± 0.2	12.9 ± 0.6
	11-HDHA	305 ± 3	298 ± 3	34 ± 5	23 ± 2	17 ± 1
	13-HDHA	210 ± 10	210 ± 20	26 ± 7	15.58 ± 0.06	13.0 ± 0.2
	14-HDHA	190 ± 10	180 ± 10	18 ± 5	11.3 ± 0.5	9.3 ± 0.1
	16-HDHA	161 ± 3	160 ± 10	19 ± 3	12.4 ± 0.4	11.4 ± 0.3
	17-HDHA	235 ± 4	210 ± 20	16 ± 2	10.2 ± 0.4	8.4 ± 0.6
	20-HDHA	332 ± 4	350 ± 30	35 ± 5	22.0 ± 0.6	19.7 ± 0.7
	21-HDHA	12.3 ± 0.1	12 ± 1	8.0 ± 0.6	8.6 ± 0.4	11.3 ± 0.6
C22:6 n3	22-HDHA	7.3 ± 0.2	7.1 ± 0.6	170 ± 10	166 ± 4	202 ± 5
	7(8)-EpDPE	8.1 ± 1.2	7.3 ± 0.7	29 ± 2	24 ± 1	31.0 ± 0.9
	10(11)-EpDPE	6 ± 1	5.2 ± 0.8	30 ± 2	25 ± 2	32.5 ± 0.7
	13(14)-EpDPE	9.2 ± 2.1	8 ± 1	41 ± 4	33 ± 3	47 ± 2
	16(17)-EpDPE	9.0 ± 1.6	7.3 ± 0.9	40 ± 3	32 ± 3	47.1 ± 0.5
	19(20)-EpDPE	71.4 ± 6.9	71.3 ± 0.7	72 ± 2	58 ± 5	81 ± 4
	4,5-DiHDPE	30.7 ± 1.7	34 ± 1	26.0 ± 0.7	18 ± 1	16.4 ± 0.8
	7,8-DiHDPE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	10,11-DiHDPE	0.78 ± 0.02	0.84 ± 0.09	1.82 ± 0.04	1.53 ± 0.01	2.69 ± 0.07
	13,14-DiHDPE	0.81 ± 0.02	0.9 ± 0.2	3.13 ± 0.07	2.77 ± 0.05	4.25 ± 0.04
	16,17-DiHDPE	0.76 ± 0.03	0.9 ± 0.2	1.52 ± 0.04	1.36 ± 0.06	2.13 ± 0.03
	19,20-DiHDPE	1.62 ± 0.07	1.8 ± 0.2	5.45 ± 0.04	5.1 ± 0.2	7.8 ± 0.2

Tab. 11.6 continued.

Precursor	Oxylipin conc. [nmol/g]	FO1	FO2	FO3	FO4	SPM	M+
C18:1 n9	9(10)-Ep-stearic acid	24 ± 2	37 ± 5	42 ± 6	36 ± 9	12 ± 3	100 ± 10
	9,10-DiH-stearic acid	7.2 ± 0.7	19.2 ± 0.4	15.0 ± 0.9	23 ± 2	26 ± 1	135 ± 9
C18:2 n6	9-HODE	4.4 ± 0.3	6.0 ± 0.2	7.9 ± 0.6	17.5 ± 0.9	2.3 ± 0.3	220 ± 20
	10-HODE	0.41 ± 0.05	0.58 ± 0.09	0.67 ± 0.02	0.68 ± 0.03	0.31 ± 0.07	6.3 ± 0.3
	12-HODE	0.34 ± 0.03	0.40 ± 0.05	0.46 ± 0.03	0.43 ± 0.01	0.17 ± 0.03	4.17 ± 0.20
	13-HODE	6.8 ± 0.5	9.1 ± 0.4	12 ± 1	26.2 ± 0.6	5.0 ± 0.7	470 ± 30
	15-HODE	0.73 ± 0.05	2.49 ± 0.05	3.5 ± 0.3	2.8 ± 0.1	4.4 ± 0.2	13.3 ± 0.5
	9(10)-EpOME	8.2 ± 0.2	3.0 ± 0.3	3.6 ± 0.3	15.2 ± 0.8	0.8 ± 0.1	170 ± 20
	12(13)-EpOME	2.3 ± 0.1	2.6 ± 0.1	3.27 ± 0.08	4.3 ± 0.3	0.56 ± 0.06	100 ± 10
	9,10-DiHOME	6.1 ± 0.5	4.5 ± 0.2	3.3 ± 0.2	11.1 ± 0.5	3.5 ± 0.2	330 ± 20
	12,13-DiHOME	1.3 ± 0.3	1.48 ± 0.01	1.5 ± 0.1	2.2 ± 0.2	1.83 ± 0.09	111 ± 9
	9,10,11-TriHOME	< LLOQ	0.26 ± 0.06	< LLOQ	< LLOQ	< LLOQ	4.1 ± 0.2
C18:3 n3	9-HOTrE	0.8 ± 0.1	2.5 ± 0.1	3.0 ± 0.3	2.8 ± 0.3	< LLOQ	29 ± 2
	13-HOTrE	< LLOQ	2.91 ± 0.08	4.1 ± 0.3	4.2 ± 0.2	< LLOQ	38 ± 3
	9(10)-EpODE	1.3 ± 0.1	2.2 ± 0.4	3.24 ± 0.07	2.4 ± 0.2	< LLOQ	42 ± 5
	12(13)-EpODE	0.76 ± 0.09	1.2 ± 0.2	1.4 ± 0.1	1.3 ± 0.2	< LLOQ	7 ± 1
	15(16)-EpODE	2.7 ± 0.6	3.6 ± 0.4	4.3 ± 0.2	3.9 ± 0.4	< LLOQ	160 ± 10
	9,10-DiHODE	0.13 ± 0.02	0.74 ± 0.05	0.48 ± 0.03	0.95 ± 0.02	0.59 ± 0.06	49 ± 3
	12,13-DiHODE	< LLOQ	< LLOQ	< LLOQ	0.30 ± 0.01	0.20 ± 0.03	5.9 ± 0.7
	15,16-DiHODE	0.63 ± 0.02	3.68 ± 0.08	4.0 ± 0.3	2.21 ± 0.08	5.2 ± 0.2	250 ± 20
	9,10,11-TriHODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.3 ± 0.1
	9,12,13-TriHODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	17.2 ± 1.1
C18:3 n6	13-gamma-HOTrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:3 n9	5-HETrE	< LLOQ	0.13 ± 0.02	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:3 n6	8-HETrE	< LLOQ	±	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	12-HETrE	0.66 ± 0.08	2.4 ± 0.1	2.6 ± 0.1	6.1 ± 0.3	12.4 ± 0.5	7.9 ± 0.6
	15-HETrE	0.35 ± 0.06	4.5 ± 0.3	4.5 ± 0.3	9.6 ± 0.3	30.7 ± 0.6	3.7 ± 0.2
	14(15)-EpEDE	< LLOQ	0.32 ± 0.04	0.31 ± 0.03	0.47 ± 0.03	< LLOQ	< LLOQ
C20:4 n6	5-HETE	0.37 ± 0.05	2.7 ± 0.2	2.4 ± 0.2	6.6 ± 0.1	n.d.	5.3 ± 0.4
	8-HETE	< LLOQ	2.2 ± 0.2	2.6 ± 0.3	7.3 ± 0.3	1.5 ± 0.3	6.3 ± 0.4
	9-HETE	< LLOQ	2.7 ± 0.4	3.1 ± 0.3	9.2 ± 0.6	< LLOQ	6.6 ± 0.6
	11-HETE	0.56 ± 0.05	3.3 ± 0.2	3.9 ± 0.3	10.7 ± 0.4	1.2 ± 0.2	8.1 ± 0.9
	12-HETE	0.33 ± 0.06	2.18 ± 0.02	2.3 ± 0.2	6.7 ± 0.2	1.2 ± 0.2	5.8 ± 0.7
	15-HETE	0.40 ± 0.01	2.66 ± 0.07	2.9 ± 0.2	10.0 ± 0.1	1.29 ± 0.09	6.8 ± 0.8
	17-HETE	n.d.	17.4 ± 0.5	17 ± 1	29.8 ± 1.0	62 ± 5	n.d.
	18-HETE	10.3 ± 0.1	19.5 ± 0.6	19 ± 2	37 ± 1	116 ± 9	12 ± 1
	20-HETE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	12-HHTrE	< LLOQ	< LLOQ	< LLOQ	0.76 ± 0.06	< LLOQ	< LLOQ
	tetranor-12-HETE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETrE	< LLOQ	1.6 ± 0.2	1.6 ± 0.3	3.8 ± 0.2	0.99 ± 0.01	2.1 ± 0.2
	11(12)-EpETrE	0.53 ± 0.09	1.76 ± 0.03	1.8 ± 0.1	4.4 ± 0.3	1.3 ± 0.2	3.3 ± 0.7
	14(15)-EpETrE	0.8 ± 0.2	3.0 ± 0.3	2.9 ± 0.3	5.9 ± 0.8	1.8 ± 0.1	4 ± 1
	5,6-DiHETrE	< LLOQ	1.30 ± 0.10	1.09 ± 0.08	2.13 ± 0.04	4.7 ± 0.4	0.58 ± 0.05
	8,9-DiHETrE	< LLOQ	0.49 ± 0.03	0.33 ± 0.04	0.52 ± 0.01	2.0 ± 0.2	0.61 ± 0.07
	11,12-DiHETrE	< LLOQ	0.293 ± 0.004	0.20 ± 0.02	0.40 ± 0.01	1.02 ± 0.08	0.49 ± 0.06
	14,15-DiHETrE	0.06 ± 0.01	0.68 ± 0.01	0.43 ± 0.03	0.76 ± 0.05	2.5 ± 0.2	1.0 ± 0.1
	PGB_2	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	$PGF_{2\alpha}$	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	20-COOH-ARA	23 ± 1	5.20 ± 0.03	5.6 ± 0.6	7.2 ± 0.5	40 ± 3	3.4 ± 0.6

Tab. 11.6 continued.

Precursor	Oxylipin conc. [nmol/g]	FO1	FO2	FO3	FO4	SPM	M+
	5-HEPE	9.3 ± 0.5	25.6 ± 0.7	26 ± 3	84 ± 2	14 ± 2	11 ± 1
	8-HEPE	9.6 ± 0.9	24.7 ± 0.7	27 ± 3	78.9 ± 0.2	12 ± 1	12 ± 1
	9-HEPE	22 ± 1	52.5 ± 0.8	56 ± 6	140 ± 2	20 ± 2	21 ± 2
	11-HEPE	12 ± 1	31 ± 1	31 ± 3	89 ± 1	12 ± 1	14 ± 1
	12-HEPE	16 ± 1	44 ± 1	45 ± 5	129 ± 2	20 ± 2	21 ± 3
	15-HEPE	15 ± 1	39 ± 2	40 ± 4	121.9 ± 0.4	17 ± 2	19 ± 2
	18-HEPE	23 ± 2	65 ± 1	66 ± 7	214 ± 4	32 ± 3	25 ± 2
	19-HEPE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	20-HEPE	< LLOQ	7.06 ± 0.51	7.7 ± 0.6	7.6 ± 0.3	21 ± 3	9 ± 1
C20:5 n3	5(*S*),18(*R*)-diHEPE (RvE2)	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETE	27 ± 4	35 ± 2	38 ± 4	92 ± 7	21 ± 1	9 ± 2
	11(12)-EpETE	34 ± 4	43 ± 3	42 ± 4	100 ± 10	28 ± 0	12 ± 4
	14(15)-EpETE	35 ± 5	41 ± 3	41 ± 4	90 ± 20	32 ± 1	13 ± 4
	17(18)-EpETE	70 ± 7	78 ± 6	77 ± 10	180 ± 20	58 ± 4	22 ± 5
	5,6-DiHETE	4.18 ± 0.09	28 ± 2	23 ± 3	49 ± 3	87 ± 7	< LLOQ
	8,9-DiHETE	2.3 ± 0.2	9.9 ± 0.5	7.0 ± 0.9	12.4 ± 0.8	35 ± 2	2.7 ± 0.3
	11,12-DiHETE	2.0 ± 0.1	8.5 ± 0.4	5.8 ± 0.6	11.0 ± 0.8	25 ± 2	2.6 ± 0.3
	14,15-DiHETE	1.7 ± 0.2	6.4 ± 0.4	4.3 ± 0.3	8.5 ± 0.5	18 ± 2	2.0 ± 0.1
	17,18-DiHETE	3.6 ± 0.3	18 ± 1	12 ± 1	19 ± 1	49 ± 4	4.5 ± 0.5
	PGB_3	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	4-HDHA	10.12 ± 0.06	35 ± 2	36 ± 4	122 ± 6	59 ± 2	120 ± 9
	7-HDHA	8.9 ± 0.7	23.8 ± 0.8	27 ± 3	92 ± 5	29.6 ± 1.0	91 ± 6
	8-HDHA	9 ± 1	23 ± 1	26 ± 3	70 ± 3	20 ± 2	72 ± 7
	10-HDHA	11 ± 2	28.0 ± 0.9	29 ± 3	95 ± 3	31 ± 4	100 ± 10
	11-HDHA	13 ± 1	36 ± 2	39 ± 3	122 ± 7	40 ± 2	120 ± 8
	13-HDHA	12 ± 1	31.2 ± 1.0	32 ± 3	108 ± 4	33 ± 6	108 ± 12
	14-HDHA	8.2 ± 0.9	22 ± 1	23 ± 1	80 ± 2	25 ± 4	83 ± 8
	16-HDHA	9.8 ± 1.0	24.1 ± 0.4	27 ± 4	79 ± 2	23 ± 3	81 ± 7
	17-HDHA	6.5 ± 0.7	28 ± 1	28 ± 3	72.9 ± 0.7	21 ± 3	75 ± 7
	20-HDHA	13.0 ± 0.8	37.6 ± 1.0	40 ± 5	150 ± 5	46 ± 4	120.0 ± 10
	21-HDHA	< LLOQ	3.0 ± 0.2	3.1 ± 0.4	n.d.	1.3 ± 0.3	2.40 ± 0.06
C22:6 n3	22-HDHA	< LLOQ	1.5 ± 0.2	1.6 ± 0.4	1.4 ± 0.2	2.7 ± 0.4	14 ± 1
	7(8)-EpDPE	10.8 ± 0.8	14.5 ± 0.6	15.2 ± 0.9	42 ± 2	24.8 ± 0.4	30 ± 7
	10(11)-EpDPE	11.0 ± 1.1	15.0 ± 0.7	16 ± 1	44 ± 1	27 ± 1	34 ± 8
	13(14)-EpDPE	13.9 ± 1.0	17 ± 1	18 ± 2	47 ± 6	30.6 ± 0.2	40 ± 10
	16(17)-EpDPE	13.7 ± 1.5	17 ± 2	17 ± 1	45 ± 5	30 ± 1	40 ± 10
	19(20)-EpDPE	23.7 ± 2.1	32 ± 2	32 ± 3	88 ± 8	56 ± 2	80 ± 20
	4,5-DiHDPE	9.7 ± 0.4	50 ± 2	46 ± 3	94 ± 6	180 ± 10	27 ± 2
	7,8-DiHDPE	< LLOQ	2.93 ± 0.07	2.2 ± 0.1	3.1 ± 0.1	12 ± 1	6.1 ± 0.4
	10,11-DiHDPE	1.19 ± 0.03	4.2 ± 0.1	3.2 ± 0.2	5.1 ± 0.2	16 ± 2	8.7 ± 0.6
	13,14-DiHDPE	1.07 ± 0.02	4.4 ± 0.1	3.3 ± 0.3	5.2 ± 0.2	17 ± 1	10.0 ± 0.6
	16,17-DiHDPE	1.17 ± 0.04	3.7 ± 0.1	2.6 ± 0.1	4.3 ± 0.1	13.0 ± 1.0	8.9 ± 0.7
	19,20-DiHDPE	2.59 ± 0.03	13.1 ± 0.4	9.6 ± 0.8	10.6 ± 0.2	47 ± 4	20 ± 1

Tab. 11.7: Concentrations of non-esterified fatty acids in fish, micro-algae and krill oil supplements. Non-esterified fatty acids were extracted from oils by solid phase extraction and analyzed by LC-MS (mean ± SD, n= 3).

[µg/g]	KO1	KO2	AO1	AO2	AO3	FO1	FO2	FO3	FO4	SPM	M+
C10:0	2.8 ± 0.7	4.0 ± 0.8	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C12:0	12 ± 1	18 ± 2	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C13:0	4.8 ± 0.6	5.2 ± 0.9	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C14:0	800 ± 90	1100 ± 110	< LLOQ	< LLOQ	< LLOQ	< LLOQ	7 ± 1	5.9 ± 0.2	1.7 ± 0.9	9.7 ± 0.8	18 ± 2
C15:1 n5	6.0 ± 0.9	8 ± 1	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C15:0	21 ± 3	32 ± 4	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.3 ± 0.1	0.22 ± 0.04	< LLOQ	< LLOQ	1.9 ± 0.7
C16:1 n7	720 ± 90	900 ± 200	< LLOQ	< LLOQ	< LLOQ	0.50 ± 0.04	19 ± 2	24.2 ± 0.8	3.5 ± 0.6	19 ± 1	47 ± 5
C16:0	6800 ± 900	11000 ± 2500	30 ± 10	36.4 ± 0.9	13 ± 2	< LLOQ	90 ± 10	84.3 ± 5.0	29 ± 4	60 ± 10	570 ± 60
C17:0	80 ± 10	120 ± 20	3 ± 3	< LLOQ	< LLOQ	< LLOQ	7 ± 2	7.1 ± 1.7	2.1 ± 0.5	4 ± 2	16 ± 2
C18:4 n3	870 ± 90	1400 ± 500	< LLOQ	< LLOQ	< LLOQ	0.47 ± 0.05	18.3 ± 0.7	18.3 ± 0.2	10.1 ± 0.3	21 ± 2	20 ± 1
C18:3 n6	60 ± 10	110 ± 30	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.15 ± 0.08	1.37 ± 0.06	< LLOQ	1.5 ± 0.1	3.56 ± 0.06
C18:3 n3	370 ± 30	700 ± 200	< LLOQ	< LLOQ	< LLOQ	< LLOQ	3.7 ± 0.4	4.2 ± 0.2	1.8 ± 0.1	2.6 ± 0.3	106 ± 2
C18:2 n6	520 ± 60	1300 ± 400	21 ± 2	20.8 ± 1.4	17 ± 2	2.0 ± 0.2	9.5 ± 0.6	11.5 ± 0.5	8.9 ± 0.1	11 ± 2	760 ± 20
C18:1 n9+n7	4400 ± 400	7000 ± 1700	25 ± 7	20.7 ± 1.0	20 ± 2	1.1 ± 0.4	94 ± 9	95 ± 2	45 ± 2	71 ± 6	700 ± 100
C18:0	270 ± 40	600 ± 100	< LLOQ	< LLOQ	< LLOQ	< LLOQ	50 ± 10	40 ± 1	32 ± 7	40 ± 20	160 ± 40
C19:0	21 ± 3	29 ± 8	< LLOQ	< LLOQ	< LLOQ	< LLOQ	2.2 ± 0.5	2.5 ± 0.7	3.2 ± 0.3	2.9 ± 0.7	7.2 ± 0.9
C20:5 n3	6600 ± 600	11000 ± 3500	37 ± 1	87.0 ± 2.2	49 ± 4	8.6 ± 0.7	175 ± 9	165 ± 5	290 ± 20	390 ± 30	131 ± 8
C20:4 n6	160 ± 20	270 ± 80	< LLOQ	< LLOQ	< LLOQ	< LLOQ	12.3 ± 0.5	13.2 ± 0.7	16.6 ± 0.7	24 ± 2	< LLOQ
C20:4 n3	130 ± 30	240 ± 80	0.95 ± 0.09	2.4 ± 0.2	1.7 ± 0.1	< LLOQ	7.1 ± 0.4	8.2 ± 0.2	13.5 ± 0.3	16 ± 1	±
C20:3 n9	4.92 ± 0.98	7 ± 2	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.8 ± 0.2	0.9 ± 0.1	< LLOQ	< LLOQ	< LLOQ
C20:3 n6+n3	33 ± 6	60 ± 20	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.86 ± 0.1	2.28 ± 0.09	4.1 ± 0.1	5.1 ± 0.4	3.9 ± 0.2
C20:2 n6	46 ± 5	40 ± 10	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.67 ± 0.2	1.90 ± 0.1	5.0 ± 0.2	4.7 ± 0.4	6.6 ± 0.7
C20:1 n9	500 ± 100	750 ± 150	1.0 ± 0.8	0.63 ± 0.07	< LLOQ	4.2 ± 0.3	42 ± 6	43 ± 6	86 ± 3	110 ± 10	48.9 ± 0.6
C20:0	10.3 ± 0.6	31 ± 8	0.5 ± 0.4	1.5 ± 0.4	0.9 ± 0.3	< LLOQ	3.5 ± 0.4	4.4 ± 0.8	9.9 ± 1.0	9.17 ± 0.63	15 ± 2
C22:6 n3	6000 ± 600	9000 ± 2000	176 ± 5	320 ± 10	190 ± 20	13.2 ± 0.6	150 ± 10	159 ± 7	530 ± 40	260 ± 30	740 ± 10
C22:5 n3	110 ± 20	250 ± 100	17.0 ± 0.4	41 ± 3	17.2 ± 0.6	0.92 ± 0.01	18 ± 2	23 ± 1	67 ± 4	80 ± 7	35 ± 2
C22:5 n6	65 ± 7	84 ± 18	8.43 ± 0.09	16.8 ± 0.8	9.6 ± 0.1	< LLOQ	3.8 ± 0.5	4.8 ± 0.1	22.9 ± 0.3	10 ± 1	58 ± 3
C22:4 n6	3.3 ± 0.5	6 ± 3	1.30 ± 0.08	3.7 ± 0.4	1.35 ± 0.08	< LLOQ	1.5 ± 0.3	1.69 ± 0.09	6.2 ± 0.2	6.0 ± 0.5	6.2 ± 0.5
C22:2 n6	10 ± 2	15 ± 6	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	4.6 ± 0.3	3.8 ± 0.3	< LLOQ
C22:1 n9	370 ± 20	440 ± 90	< LLOQ	< LLOQ	< LLOQ	7.4 ± 0.3	6.5 ± 0.2	9 ± 1	45 ± 4	64 ± 8	21 ± 2
C22:0	< LLOQ	13 ± 4	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	17 ± 4

Table 11.8: Concentrations of non-esterified oxylipins in fish, micro-algae and krill oil supplements. Non-esterified oxylipins were extracted from oils by solid phase extraction and analyzed by LC-MS (mean ± SD, n = 3).

Precursor	Oxylipin conc. [nmol/g]	KO1	KO2	AO1	AO2	AO3
C18:1 n9	9(10)-Ep-stearic acid	2.8 ± 0.3	13.2 ± 0.4	0.06 ± 0.01	0.07 ± 0.01	0.073 ± 0.003
	9,10-DiOH-stearic acid	0.71 ± 0.04	3.5 ± 0.3	0.029 ± 0.001	0.021 ± 0.004	0.024 ± 0.003
C18:2 n6	9-HODE	4.00 ± 0.07	22.3 ± 0.7	0.138 ± 0.010	0.19 ± 0.01	0.096 ± 0.008
	10-HODE	0.137 ± 0.005	0.44 ± 0.02	0.0099 ± 0.0001	0.0084 ± 0.0007	0.009 ± 0.002
	12-HODE	0.080 ± 0.005	0.27 ± 0.03	0.0056 ± 0.0003	0.0054 ± 0.0005	0.007 ± 0.002
	13-HODE	6.2 ± 0.2	47.9 ± 2.4	0.199 ± 0.004	0.28 ± 0.01	0.137 ± 0.010
	15-HODE	0.040 ± 0.003	0.20 ± 0.01	< LLOQ	0.0020 ± 0.0006	0.0031 ± 0.0005
	9-oxo-ODE	0.53 ± 0.02	0.6 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	13-oxo-ODE	0.78 ± 0.07	1.2 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	9(10)-EpOME	0.36 ± 0.03	2.6 ± 0.3	0.033 ± 0.004	0.033 ± 0.002	0.0272 ± 0.0001
	12(13)-EpOME	< LLOQ	4.4 ± 0.2	0.022 ± 0.002	0.019 ± 0.003	0.015 ± 0.002
	9,10-DiHOME	0.125 ± 0.009	44 ± 2	0.03 ± 0.01	0.039 ± 0.005	0.034 ± 0.003
	12,13-DiHOME	0.082 ± 0.009	14.9 ± 0.8	0.010 ± 0.003	0.016 ± 0.002	0.018 ± 0.001
	9,10,11-TriHOME	< LLOQ	0.73 ± 0.05	< LLOQ	< LLOQ	0.008 ± 0.002
C18:3 n3	9-HOTrE	4.2 ± 0.1	8.8 ± 0.6	0.007 ± 0.001	0.009 ± 0.002	0.0096 ± 0.0001
	13-HOTrE	6.89 ± 0.10	14.6 ± 1.1	0.030 ± 0.001	0.033 ± 0.006	0.048 ± 0.006
	9(10)-EpODE	0.09 ± 0.02	0.49 ± 0.02	< LLOQ	< LLOQ	< LLOQ
	12(13)-EpODE	0.067 ± 0.007	0.42 ± 0.08	< LLOQ	< LLOQ	< LLOQ
	15(16)-EpODE	0.17 ± 0.02	2.04 ± 0.02	< LLOQ	< LLOQ	< LLOQ
	9,10-DiHODE	0.023 ± 0.001	4.5 ± 0.2	0.0013 ± 0.0007	0.0019 ± 0.0004	0.0018 ± 0.0002
	12,13-DiHODE	< LLOQ	1.07 ± 0.06	< LLOQ	< LLOQ	< LLOQ
	15,16-DiHODE	0.026 ± 0.008	24 ± 1	0.018 ± 0.007	0.024 ± 0.004	0.060 ± 0.006
	9,10,11-TriHODE	< LLOQ	0.061 ± 0.008	< LLOQ	< LLOQ	< LLOQ
	9,12,13-TriHODE	n.d.	0.69 ± 0.09	< LLOQ	< LLOQ	< LLOQ
C18:3 n6	13-gamma-HOTrE	0.81 ± 0.07	1.0 ± 0.2	< LLOQ	< LLOQ	< LLOQ
C20:3 n9	5-HETrE	0.032 ± 0.001	0.046 ± 0.002	< LLOQ	< LLOQ	< LLOQ
C20:3 n6	8-HETrE	0.20 ± 0.04	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	12-HETrE	0.18 ± 0.03	0.27 ± 0.06	< LLOQ	< LLOQ	< LLOQ
	15-HETrE	0.199 ± 0.003	0.23 ± 0.04	< LLOQ	< LLOQ	< LLOQ
	14(15)-EpEDE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:4 n6	5-HETE	4.8 ± 0.1	6.9 ± 0.6	0.0040 ± 0.0009	0.0051 ± 0.0007	0.005 ± 0.002
	8-HETE	3.12 ± 0.01	3.9 ± 0.3	< LLOQ	< LLOQ	< LLOQ
	9-HETE	1.44 ± 0.08	2.1 ± 0.2	< LLOQ	< LLOQ	< LLOQ
	11-HETE	1.39 ± 0.03	1.92 ± 0.08	< LLOQ	< LLOQ	< LLOQ
	12-HETE	1.45 ± 0.04	2.0 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	15-HETE	2.51 ± 0.08	3.6 ± 0.2	< LLOQ	< LLOQ	< LLOQ
	16-HETE	0.029 ± 0.004	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	17-HETE	0.37 ± 0.01	0.56 ± 0.05	0.0057 ± 0.0007	0.008 ± 0.001	0.0086 ± 0.0001
	18-HETE	0.50 ± 0.02	0.77 ± 0.06	< LLOQ	< LLOQ	0.0061 ± 0.0004
	12-HHTrE	0.227 ± 0.009	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	11(12)-EpETrE	0.044 ± 0.008	0.09 ± 0.03	< LLOQ	0.0023 ± 0.0004	< LLOQ
	14(15)-EpETrE	< LLOQ	< LLOQ	< LLOQ	0.004 ± 0.001	< LLOQ
	5,6-DiHETrE	0.096 ± 0.008	0.153 ± 0.008	< LLOQ	< LLOQ	0.0079 ± 0.0006
	8,9-DiHETrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	11,12-DiHETrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	14,15-DiHETrE	0.0080 ± 0.0004	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	PGB_2	0.49 ± 0.02	0.61 ± 0.01	< LLOQ	< LLOQ	< LLOQ
	$PGF_{2\alpha}$	1.84 ± 0.06	2.5 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	5(*R*,*S*)-5-F_{2t}-IsoP	0.058 ± 0.007	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	20-COOH-ARA	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ

Table 11.8 continued.

Precursor	Oxylipin conc. [nmol/g]	KO1	KO2	AO1	AO2	AO3
	5-HEPE	144.5 ± 2.6	220 ± 20	0.031 ± 0.002	0.050 ± 0.007	0.029 ± 0.005
	8-HEPE	34.3 ± 1.1	47 ± 3	< LLOQ	0.007 ± 0.001	< LLOQ
	9-HEPE	52.9 ± 0.9	78 ± 5	< LLOQ	< LLOQ	< LLOQ
	11-HEPE	32.8 ± 0.7	47 ± 3	0.0050 ± 0.0003	0.010 ± 0.001	0.0032 ± 0.0005
	12-HEPE	59 ± 1	87 ± 6	0.017 ± 0.002	0.022 ± 0.003	< LLOQ
	15-HEPE	58 ± 2	82 ± 5	0.0127 ± 0.0007	0.023 ± 0.003	< LLOQ
	18-HEPE	126 ± 4	190 ± 10	0.0109 ± 0.0007	0.027 ± 0.001	0.0080 ± 0.0008
	19-HEPE	4.0 ± 0.2	6.2 ± 0.5	< LLOQ	< LLOQ	< LLOQ
	20-HEPE	0.38 ± 0.06	0.48 ± 0.05	< LLOQ	< LLOQ	< LLOQ
C20:5 n3	5(*S*),18(*R*)-diHEPE (RvE2)	5.7 ± 0.2	7.6 ± 0.5	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETE	1.8 ± 0.3	3.6 ± 0.6	< LLOQ	< LLOQ	< LLOQ
	11(12)-EpETE	1.4 ± 0.1	3.0 ± 0.2	0.032 ± 0.003	0.052 ± 0.009	0.015 ± 0.002
	14(15)-EpETE	1.6 ± 0.1	3.7 ± 0.3	0.052 ± 0.002	0.09 ± 0.02	0.019 ± 0.001
	17(18)-EpETE	2.3 ± 0.3	4.7 ± 0.3	0.039 ± 0.003	0.11 ± 0.03	0.015 ± 0.005
	5,6-DiHETE	5.36 ± 0.05	8.3 ± 0.5	< LLOQ	0.035 ± 0.008	0.125 ± 0.005
	8,9-DiHETE	0.39 ± 0.01	0.56 ± 0.04	< LLOQ	< LLOQ	< LLOQ
	11,12-DiHETE	0.318 ± 0.006	0.48 ± 0.03	< LLOQ	< LLOQ	< LLOQ
	14,15-DiHETE	0.236 ± 0.007	0.34 ± 0.03	< LLOQ	< LLOQ	< LLOQ
	17,18-DiHETE	0.426 ± 0.005	0.63 ± 0.01	< LLOQ	< LLOQ	< LLOQ
	PGB_3	0.47 ± 0.02	0.61 ± 0.03	< LLOQ	< LLOQ	< LLOQ
	4-HDHA	23 ± 1	26 ± 2	0.030 ± 0.002	0.040 ± 0.002	0.024 ± 0.001
	7-HDHA	17.4 ± 0.5	24 ± 1	0.019 ± 0.001	0.038 ± 0.002	0.016 ± 0.002
	8-HDHA	25.7 ± 0.9	39 ± 3	0.013 ± 0.002	0.0275 ± 0.0008	0.01303 ± 0.00004
	10-HDHA	29.0 ± 0.7	41 ± 2	0.0381 ± 0.0009	0.056 ± 0.003	0.024 ± 0.003
	11-HDHA	45 ± 1	63 ± 5	0.052 ± 0.004	0.067 ± 0.003	0.0249 ± 0.0002
	13-HDHA	37.10 ± 0.07	51 ± 2	0.0490 ± 0.0009	0.063 ± 0.005	0.021 ± 0.002
	14-HDHA	35.9 ± 0.4	49 ± 3	0.047 ± 0.003	0.057 ± 0.004	0.022 ± 0.004
	16-HDHA	29.9 ± 0.4	39 ± 2	0.047 ± 0.004	0.056 ± 0.009	0.022 ± 0.004
	17-HDHA	34.6 ± 0.5	52 ± 3	< LLOQ	< LLOQ	< LLOQ
	20-HDHA	n.d.	< LLOQ	0.063 ± 0.004	0.092 ± 0.008	n.d.
	21-HDHA	2.2 ± 0.1	3.0 ± 0.1	< LLOQ	< LLOQ	< LLOQ
C22:6 n3	22-HDHA	0.16 ± 0.04	n.d.	0.047 ± 0.001	0.026 ± 0.002	0.032 ± 0.001
	7(8)-EpDPE	7.0 ± 0.6	8.5 ± 0.7	0.036 ± 0.002	0.050 ± 0.008	0.022 ± 0.002
	10(11)-EpDPE	0.71 ± 0.04	1.4 ± 0.2	0.096 ± 0.009	0.13 ± 0.02	0.030 ± 0.002
	13(14)-EpDPE	0.9 ± 0.1	1.8 ± 0.2	0.111 ± 0.008	0.16 ± 0.03	0.039 ± 0.001
	16(17)-EpDPE	0.9 ± 0.1	1.7 ± 0.1	0.15 ± 0.01	0.23 ± 0.05	0.052 ± 0.003
	19(20)-EpDPE	4.5 ± 0.5	5.8 ± 0.4	0.16 ± 0.01	0.25 ± 0.08	0.057 ± 0.008
	4,5-DiHDPE	0.72 ± 0.03	1.7 ± 0.1	< LLOQ	< LLOQ	< LLOQ
	7,8-DiHDPE	0.23 ± 0.03	0.35 ± 0.05	< LLOQ	< LLOQ	< LLOQ
	10,11-DiHDPE	0.142 ± 0.005	0.22 ± 0.02	< LLOQ	< LLOQ	0.0022 ± 0.0002
	13,14-DiHDPE	0.172 ± 0.002	0.24 ± 0.02	< LLOQ	< LLOQ	0.0035 ± 0.0003
	16,17-DiHDPE	0.132 ± 0.006	0.204 ± 0.007	< LLOQ	< LLOQ	0.0035 ± 0.0006
	19,20-DiHDPE	0.28 ± 0.01	0.44 ± 0.03	< LLOQ	< LLOQ	< LLOQ

Table 11.8 continued.

Precursor	Oxylipin conc. [nmol/g]	FO1	FO2	FO3	FO4	SPM	M+
C18:1 n9	9(10)-Ep-stearic acid	0.073 ± 0.003	0.10 ± 0.01	0.11 ± 0.02	0.12 ± 0.02	0.071 ± 0.005	1.2 ± 0.2
	9,10-DiOH-stearic acid	0.036 ± 0.002	0.17 ± 0.01	0.21 ± 0.02	0.059 ± 0.008	0.092 ± 0.006	1.7 ± 0.1
C18:2 n6	9-HODE	0.230 ± 0.002	0.032 ± 0.003	0.031 ± 0.006	0.51 ± 0.06	0.18 ± 0.07	37 ± 5
	10-HODE	0.0068 ± 0.0004	0.0029 ± 0.0003	0.0033 ± 0.0004	0.0123 ± 0.0008	0.024 ± 0.009	1.2 ± 0.2
	12-HODE	0.0045 ± 0.0005	0.0028 ± 0.0002	0.004 ± 0.001	0.0083 ± 0.0007	0.016 ± 0.007	0.61 ± 0.04
	13-HODE	0.329 ± 0.010	0.057 ± 0.009	0.06 ± 0.01	0.74 ± 0.09	0.3 ± 0.1	53 ± 6
	15-HODE	< LLOQ	0.0041 ± 0.0005	0.0058 ± 0.0004	0.0030 ± 0.0006	0.0118 ± 0.0008	0.27 ± 0.02
	9-oxo-ODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	13-oxo-ODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	9(10)-EpOME	0.0278 ± 0.0007	0.0076 ± 0.0009	0.009 ± 0.002	0.049 ± 0.006	0.015 ± 0.004	1.0 ± 0.2
	12(13)-EpOME	0.021 ± 0.003	0.009 ± 0.001	0.014 ± 0.004	0.035 ± 0.004	0.0119 ± 0.0007	1.8 ± 0.2
	9,10-DiHOME	0.047 ± 0.001	0.0249 ± 0.0006	0.0135 ± 0.0009	0.063 ± 0.006	0.020 ± 0.002	37 ± 1
	12,13-DiHOME	0.0130 ± 0.0009	0.00794 ± 0.00006	0.0043 ± 0.0005	0.019 ± 0.002	0.0090 ± 0.0006	12.9 ± 0.6
	9,10,11-TriHOME	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.9 ± 0.2
C18:3 n3	9-HOTrE	< LLOQ	< LLOQ	< LLOQ	0.0066 ± 0.0008	0.015 ± 0.007	3.7 ± 0.2
	13-HOTrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.05 ± 0.03	5.0 ± 0.4
	9(10)-EpODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.30 ± 0.03
	12(13)-EpODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.30 ± 0.04
	15(16)-EpODE	< LLOQ	< LLOQ	0.014 ± 0.004	< LLOQ	< LLOQ	1.17 ± 0.09
	9,10-DiHODE	0.0007 ± 0.0001	0.0017 ± 0.0003	0.00049 ± 0.00007	0.0029 ± 0.0001	0.0019 ± 0.0005	3.8 ± 0.2
	12,13-DiHODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.83 ± 0.05
	15,16-DiHODE	0.0061 ± 0.0001	0.0091 ± 0.0001	0.006 ± 0.001	0.009 ± 0.001	0.0156 ± 0.0004	21.3 ± 1.0
	9,10,11-TriHODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.18 ± 0.03
	9,12,13-TriHODE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	1.9 ± 0.2
C18:3 n6	13-gamma-HOTrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:3 n9	5-HETrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.046 ± 0.008
C20:3 n6	8-HETrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	12-HETrE	< LLOQ	0.016 ± 0.001	0.019 ± 0.002	0.011 ± 0.001	0.040 ± 0.004	0.09 ± 0.02
	15-HETrE	< LLOQ	0.007 ± 0.002	0.009 ± 0.002	0.0164 ± 0.0003	0.150 ± 0.006	0.062 ± 0.009
	14(15)-EpEDE	< LLOQ	0.0027 ± 0.0004	0.0040 ± 0.0003	0.006 ± 0.001	< LLOQ	< LLOQ
C20:4 n6	5-HETE	< LLOQ	0.034 ± 0.003	0.034 ± 0.002	0.20 ± 0.02	0.074 ± 0.002	2.3 ± 0.4
	8-HETE	< LLOQ	< LLOQ	< LLOQ	0.0107 ± 0.0001	< LLOQ	< LLOQ
	9-HETE	< LLOQ	< LLOQ	< LLOQ	±	< LLOQ	0.04 ± 0.01
	11-HETE	< LLOQ	0.006 ± 0.001	0.010 ± 0.001	0.023 ± 0.004	0.0058 ± 0.0006	0.048 ± 0.002
	12-HETE	< LLOQ	0.008 ± 0.001	0.011 ± 0.001	0.0221 ± 0.0008	0.0063 ± 0.0004	0.029 ± 0.005
	15-HETE	< LLOQ	0.0086 ± 0.0007	0.015 ± 0.002	0.049 ± 0.006	0.012 ± 0.002	0.04 ± 0.01
	16-HETE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	17-HETE	0.0054 ± 0.0004	0.077 ± 0.005	0.116 ± 0.009	0.061 ± 0.009	0.16 ± 0.01	0.24 ± 0.02
	18-HETE	0.0038 ± 0.0004	0.072 ± 0.002	0.10 ± 0.01	0.064 ± 0.008	0.24 ± 0.02	0.18 ± 0.03
	12-HHTrE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	11(12)-EpETrE	< LLOQ	0.019 ± 0.002	0.028 ± 0.002	0.033 ± 0.004	0.010 ± 0.003	0.012 ± 0.001
	14(15)-EpETrE	< LLOQ	0.039 ± 0.005	0.060 ± 0.003	0.06 ± 0.01	0.024 ± 0.003	< LLOQ
	5,6-DiHETrE	< LLOQ	0.056 ± 0.003	0.028 ± 0.003	0.032 ± 0.003	0.340 ± 0.033	0.19 ± 0.02
	8,9-DiHETrE	< LLOQ	< LLOQ	< LLOQ	0.006 ± 0.001	< LLOQ	0.0098 ± 0.0008
	11,12-DiHETrE	< LLOQ	< LLOQ	< LLOQ	0.0024 ± 0.0001	0.0032 ± 0.0005	0.010 ± 0.002
	14,15-DiHETrE	< LLOQ	0.001 ± 0.000	< LLOQ	0.0033 ± 0.0004	0.0118 ± 0.0005	0.022 ± 0.002
	PGB_2	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	$PGF_{2\alpha}$	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	5(*R*,*S*)-5-F_{2t}-IsoP	< LLOQ	< LLOQ	< LLOQ	0.0049 ± 0.0008	< LLOQ	0.017 ± 0.003
	20-COOH-ARA	< LLOQ	< LLOQ	< LLOQ	0.021 ± 0.003	< LLOQ	< LLOQ

Table 11.8 continued.

Precursor	Oxylipin conc. [nmol/g]	FO1	FO2	FO3	FO4	SPM	M+
	5-HEPE	0.0046 ± 0.0009	0.322 ± 0.002	0.41 ± 0.02	2.8 ± 0.4	0.54 ± 0.08	5.0 ± 0.7
	8-HEPE	< LLOQ	0.0255 ± 0.0009	0.037 ± 0.003	0.15 ± 0.02	0.032 ± 0.001	0.09 ± 0.01
	9-HEPE	< LLOQ	0.050 ± 0.002	0.073 ± 0.004	0.29 ± 0.04	0.052 ± 0.008	0.21 ± 0.02
	11-HEPE	< LLOQ	0.043 ± 0.001	0.057 ± 0.001	0.22 ± 0.03	0.068 ± 0.009	0.120 ± 0.009
	12-HEPE	< LLOQ	0.110 ± 0.001	0.158 ± 0.005	0.42 ± 0.05	0.14 ± 0.02	0.20 ± 0.02
	15-HEPE	< LLOQ	0.122 ± 0.004	0.172 ± 0.003	0.39 ± 0.05	0.18 ± 0.03	0.19 ± 0.02
	18-HEPE	< LLOQ	0.155 ± 0.001	0.207 ± 0.008	0.9 ± 0.1	0.22 ± 0.02	0.28 ± 0.02
	19-HEPE	< LLOQ	< LLOQ	< LLOQ	n.d.	< LLOQ	< LLOQ
	20-HEPE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
C20:5 n3	5(*S*),18(*R*)-diHEPE (RvE2)	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	8(9)-EpETE	< LLOQ	0.055 ± 0.005	0.079 ± 0.003	0.28 ± 0.02	< LLOQ	< LLOQ
	11(12)-EpETE	< LLOQ	0.31 ± 0.01	0.415 ± 0.008	0.71 ± 0.10	0.22 ± 0.01	< LLOQ
	14(15)-EpETE	< LLOQ	0.445 ± 0.009	0.66 ± 0.04	0.9 ± 0.1	0.39 ± 0.03	0.038 ± 0.010
	17(18)-EpETE	< LLOQ	0.47 ± 0.03	0.72 ± 0.05	1.1 ± 0.2	0.43 ± 0.04	< LLOQ
	5,6-DiHETE	< LLOQ	1.09 ± 0.06	0.55 ± 0.05	0.86 ± 0.08	6.1 ± 0.2	1.5 ± 0.2
	8,9-DiHETE	< LLOQ	0.0106 ± 0.0005	0.00830 ± 0.00007	0.103 ± 0.008	0.043 ± 0.002	0.06 ± 0.01
	11,12-DiHETE	< LLOQ	0.0141 ± 0.0004	0.010 ± 0.001	0.065 ± 0.006	0.088 ± 0.002	0.08 ± 0.02
	14,15-DiHETE	< LLOQ	0.0124 ± 0.0004	0.0067 ± 0.0005	0.041 ± 0.004	0.100 ± 0.002	0.08 ± 0.01
	17,18-DiHETE	< LLOQ	0.0178 ± 0.0007	0.014 ± 0.002	0.059 ± 0.008	0.108 ± 0.002	0.13 ± 0.02
	PGB_3	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	4-HDHA	0.016 ± 0.005	0.0416 ± 0.0009	0.06 ± 0.03	0.36 ± 0.03	0.09 ± 0.01	4.2 ± 0.5
	7-HDHA	< LLOQ	0.025 ± 0.004	0.05 ± 0.02	0.26 ± 0.03	0.026 ± 0.002	0.90 ± 0.03
	8-HDHA	< LLOQ	0.022 ± 0.003	0.038 ± 0.006	0.20 ± 0.02	0.021 ± 0.002	0.8 ± 0.1
	10-HDHA	0.006 ± 0.002	0.057 ± 0.002	0.096 ± 0.002	0.34 ± 0.04	0.07 ± 0.01	0.87 ± 0.08
	11-HDHA	< LLOQ	0.056 ± 0.004	0.10 ± 0.01	0.40 ± 0.04	0.065 ± 0.004	0.9 ± 0.1
	13-HDHA	< LLOQ	0.070 ± 0.003	0.118 ± 0.001	0.42 ± 0.05	0.070 ± 0.005	0.82 ± 0.07
	14-HDHA	< LLOQ	0.0556 ± 0.0001	0.100 ± 0.001	0.33 ± 0.04	0.061 ± 0.003	0.74 ± 0.04
	16-HDHA	0.0044 ± 0.0003	0.065 ± 0.002	0.108 ± 0.004	0.37 ± 0.05	0.055 ± 0.007	0.62 ± 0.07
	17-HDHA	< LLOQ	< LLOQ	0.052 ± 0.005	0.34 ± 0.04	n.d.	0.60 ± 0.08
	20-HDHA	< LLOQ	0.093 ± 0.005	0.164 ± 0.007	n.d.	0.094 ± 0.010	1.08 ± 0.07
	21-HDHA	< LLOQ	< LLOQ	< LLOQ	n.d.	< LLOQ	< LLOQ
C22:6 n3	22-HDHA	< LLOQ	0.032 ± 0.003	< LLOQ	n.d.	0.027 ± 0.005	< LLOQ
	7(8)-EpDPE	< LLOQ	0.067 ± 0.003	0.097 ± 0.005	0.35 ± 0.04	0.031 ± 0.005	0.13 ± 0.03
	10(11)-EpDPE	0.0031 ± 0.0008	0.21 ± 0.01	0.35 ± 0.02	0.91 ± 0.08	0.13 ± 0.02	0.09 ± 0.03
	13(14)-EpDPE	< LLOQ	0.212 ± 0.004	0.32 ± 0.02	0.8 ± 0.1	0.10 ± 0.01	0.14 ± 0.03
	16(17)-EpDPE	< LLOQ	0.243 ± 0.007	0.37 ± 0.02	1.0 ± 0.1	0.13 ± 0.01	0.14 ± 0.03
	19(20)-EpDPE	< LLOQ	0.246 ± 0.002	0.37 ± 0.01	1.0 ± 0.1	0.15 ± 0.03	0.8 ± 0.2
	4,5-DiHDPE	< LLOQ	< LLOQ	< LLOQ	< LLOQ	0.151 ± 0.008	1.5 ± 0.1
	7,8-DiHDPE	< LLOQ	< LLOQ	< LLOQ	0.08 ± 0.01	< LLOQ	0.076 ± 0.005
	10,11-DiHDPE	< LLOQ	0.0074 ± 0.0007	0.0053 ± 0.0002	0.049 ± 0.006	0.019 ± 0.002	0.107 ± 0.009
	13,14-DiHDPE	< LLOQ	0.0089 ± 0.0002	0.0056 ± 0.0004	0.043 ± 0.004	0.028 ± 0.001	0.14 ± 0.01
	16,17-DiHDPE	< LLOQ	0.0075 ± 0.0006	0.0054 ± 0.0006	0.030 ± 0.004	0.021 ± 0.001	0.126 ± 0.007
	19,20-DiHDPE	< LLOQ	0.0149 ± 0.0010	0.0124 ± 0.0007	0.047 ± 0.005	0.050 ± 0.002	0.223 ± 0.008

11.4 Chapter 5

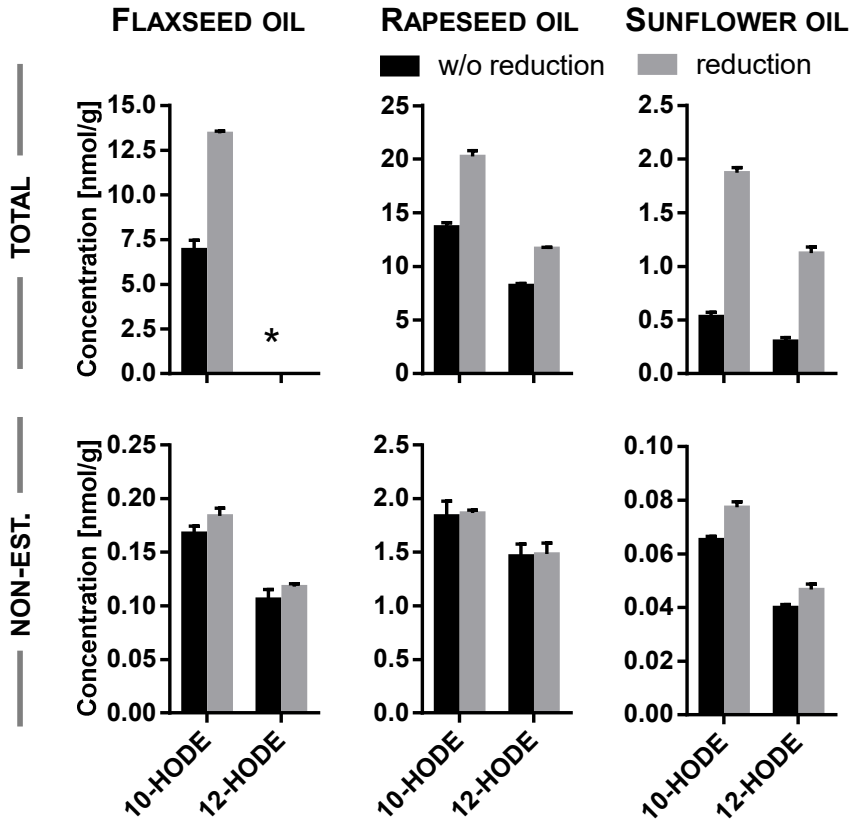

Fig. 11.16: Concentrations of total as well as non-esterified 10- and 12-hydroxy-linoleic acid with and without reduction of hydroperoxides in freshly pressed oils. Total (non-esterified and esterified oxylipins) as well as non-esterified oxylipins were analyzed in freshly cold-pressed oils by LC-MS (mean ± SD, n = 3) with and without reduction (w/o reduction) of hydroperoxides by $SnCl_2$.

* Concentration could not be determined due to matrix interference.

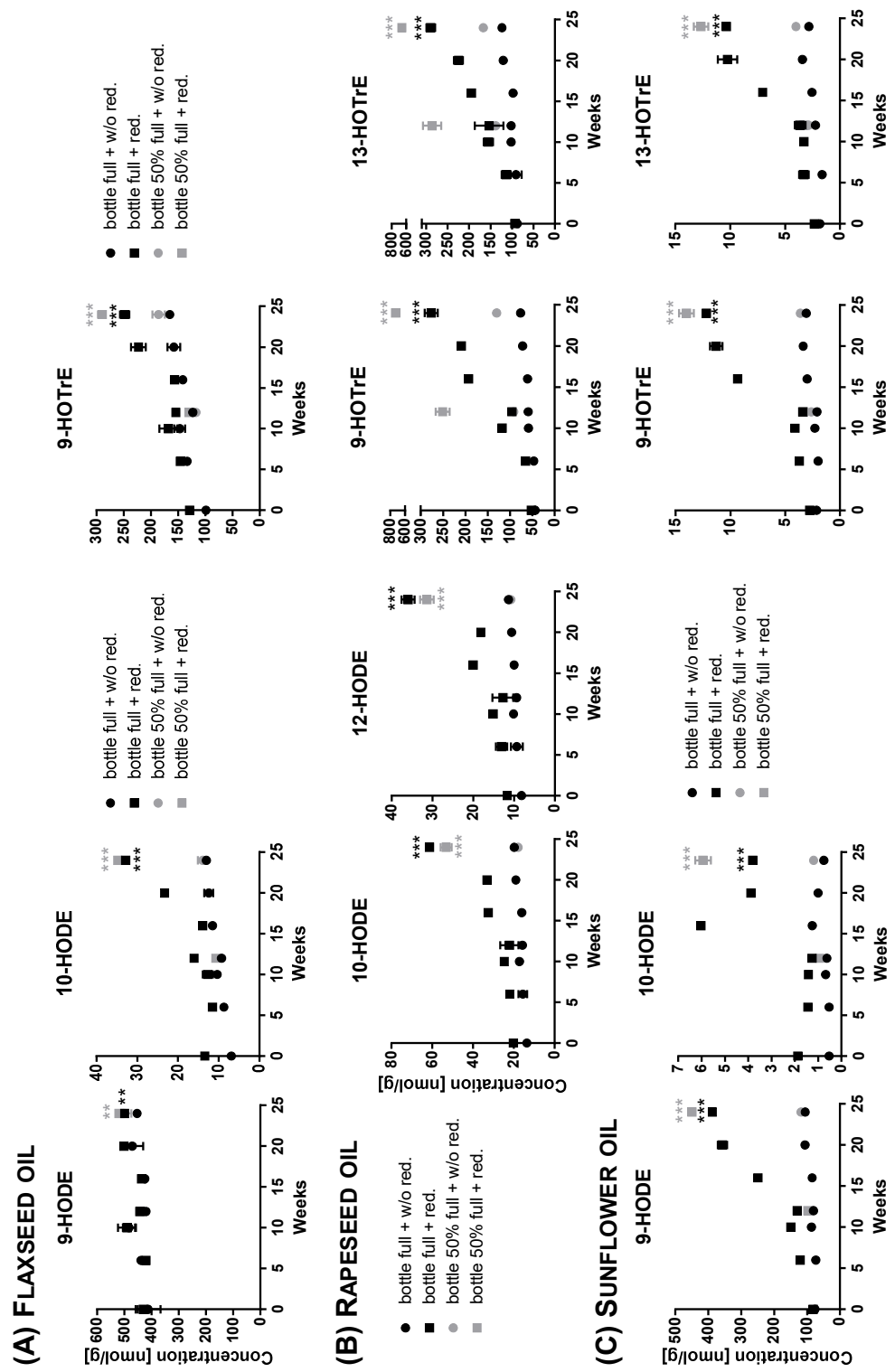

Fig. 11.17: Concentrations of total oxylipins in (A) flaxseed oil, (B) rapeseed oil as well as (C) sunflower oil at different time points of storage. Freshly pressed oils were stored for 24 weeks and analyzed regularly regarding the total oxylipin concentrations (with/without reduction of hydroperoxides by $SnCl_2$; mean ± SD, n = 3). At each time point, the concentrations 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 also carried out. A new bottle was opened at every time point. Statistic evaluation of the concentration at week 24 (reduction; full and half-filled) *versus* concentration at week 0 (reduction) was performed using multiple t-test with Holm-Sidak correction for multiple comparison (α = 0.05; p value < 0.05 (*), p < 0.01 (**) or p < 0.001 (***)).

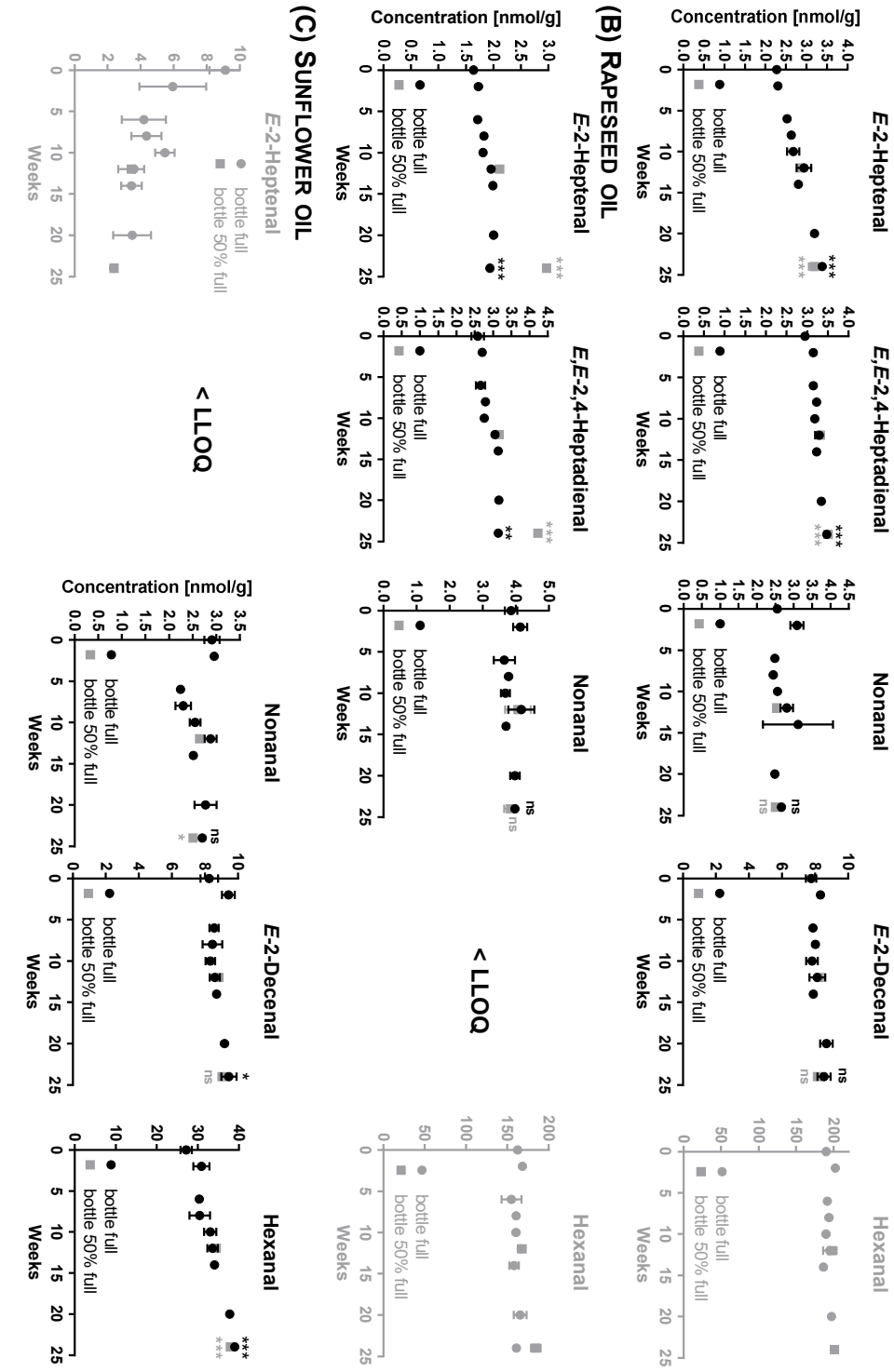

Fig. 11.18: Concentrations of volatile aldehydes in (A) flaxseed oil, (B) rapeseed oil as well as (C) sunflower oil (bottom) at different time points of storage. Freshly pressed oils were stored for 24 weeks and analyzed regularly regarding the volatile aldehyde concentrations (mean ± SD, n = 3). At each time point, the concentrations 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 also carried out. A new bottle was opened at every time point. For multipanel illustration, the data already shown in chapter 5 are shown here again in gray. Statistic evaluation of the concentration at week 24 (full and half-filled) *versus* concentration at week 0 was performed using multiple t-test with Holm-Sidak correction for multiple comparison ($\alpha = 0.05$; p value < 0.05 (*), $p < 0.01$ (**) or $p < 0.001$ (***), ns = not significant).

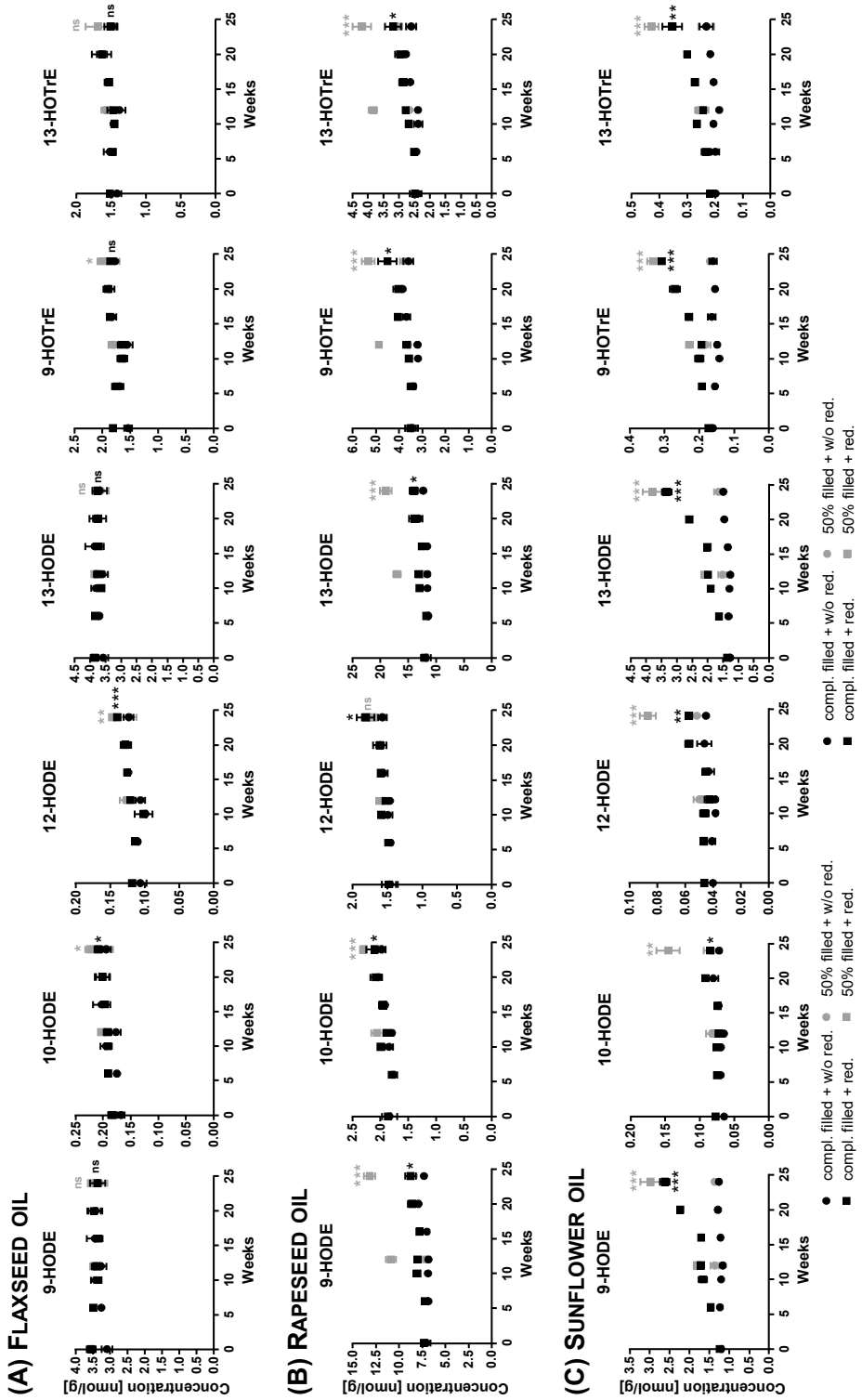

Fig. 11.19: Concentrations of non-esterified oxylipins in (A) flaxseed oil, (B) rapeseed oil as well as (C) sunflower oil at different time points of storage. Freshly pressed oils were stored for 6 months and analyzed regularly (with/without reduction of hydroperoxides by $SnCl_2$; mean ± SD, n = 3). At each time point, the concentrations were determined for the oils stored in full bottles. After 12 weeks and 24 weeks, an analysis of the oils stored in half-full bottles was also carried out. A new bottle was opened at every time point. Statistic evaluation of the concentration at week 24 (reduction; full and half-filled) *versus* concentration at week 0 (reduction) was performed using multiple t-test with Holm-Sidak correction for multiple comparison (α = 0.05; p value < 0.05 (*), p < 0.01 (**) or p < 0.001 (***), ns = not significant).

Tab. 11.9: Concentrations of total oxylipins in freshly pressed flaxseed, rapeseed and sunflower oils. Total oxylipins were analyzed by LC-MS (mean ± SD, n = 3) with and without reduction of hydroperoxides by $SnCl_2$. n.d.: not determinable due to matrix interference; 9,10,11-TriHOME, 9,10,13-TriHOME: matrix interference in all samples; 9,10,11-TriHODE: < LLOQ in all samples.

[nmol/g]			Flaxseed oil w/o reduction	Flaxseed oil reduced	Rapeseed oil w/o reduction	Rapeseed oil reduced	Sunflower oil w/o reduction	Sunflower oil reduced
Oleic acid (18:1 n9)	epoxy-PUFA	9(10)-Ep-stearic acid	30 ± 2		127 ± 9		243 ± 6	
	vic dihydroxy-PUFA	9,10-DiH-stearic acid	38 ± 3		215 ± 9		30.5 ± 1.0	
Linoleic acid (C18:2 n6)	hydroxy-PUFA	9-HODE	400 ± 50	430 ± 20	143 ± 4	169 ± 4	76 ± 5	81 ± 1
		10-HODE	6.9 ± 0.5	13.4 ± 0.1	13.7 ± 0.4	20.3 ± 0.5	0.53 ± 0.04	1.88 ± 0.05
		12-HODE	n.d.	n.d.	8.2 ± 0.2	11.70 ± 0.08	0.30 ± 0.03	1.120 ± 0.06
		13-HODE	260 ± 30	307 ± 8	297 ± 5	338 ± 3	44.3 ± 0.7	56.7 ± 0.5
		15-HODE	2700 ± 200	2700 ± 100	580 ± 10	590 ± 20	2.4 ± 0.1	2.21 ± 0.04
	epoxy-PUFA	9(10)-EpOME	23 ± 2		32 ± 3		25.4 ± 0.3	
		12(13)-EpOME	25 ± 2		87 ± 3		4.9 ± 0.1	
	vic dihydroxy-PUFA	9,10-DiHOME	1.6 ± 0.2		4.5 ± 0.2		12.8 ± 0.3	
		12,13-DiHOME	0.63 ± 0.06		2.9 ± 0.1		0.29 ± 0.02	
	multi hydroxy-PUFA	9,12,13-TriHOME	7.5 ± 0.5		40.9 ± 0.9		2.73 ± 0.05	
α-Linolenic acid (C18:3 n3)	hydroxy-PUFA	9-HOTrE	99 ± 7	129 ± 4	43.9 ± 0.6	52.35 ± 0.27	2.13 ± 0.05	2.7 ± 0.1
		13-HOTrE	630 ± 40	650 ± 10	88 ± 2	93.13 ± 1.29	1.84 ± 0.07	2.3 ± 0.2
		9(10)-EpODE	56 ± 4		18 ± 1		0.86 ± 0.04	
	epoxy-PUFA	12(13)-EpODE	30 ± 3		13.1 ± 0.7		0.23 ± 0.02	
		15(16)-EpODE	1520 ± 80		2370 ± 100		8.8 ± 0.2	
	vic dihydroxy-PUFA	9,10-DiHODE	1.2 ± 0.1		0.67 ± 0.02		0.80 ± 0.02	
		12,13-DiHODE	1.59 ± 0.08		0.71 ± 0.07		0.06 ± 0.01	
		15,16-DiHODE	30 ± 6		71 ± 2		0.92 ± 0.04	
	multi hydroxy-PUFA	9,10,13-TriHODE	n.d.		4.8 ± 0.2		< 0.16	
		9,12,13-TriHODE	n.d.		12.3 ± 0.3		< 0.016	

Tab. 11.10: Concentrations of total hydroxy-PUFA in a second freshly cold-pressed flaxseed oil using other flaxseeds as well as commercially available flaxseed oils obtained from an oil mill and a local supermarket. Total oxylipins were analyzed by LC-MS (mean ± SD, n = 3) without reduction of hydroperoxides. 12-HODE could not be evaluated due to matrix interference.

[nmol/g]	Freshly pressed virgin flaxseed oil	Virgin flaxseed oil from oil mill	Virgin flaxseed oil from supermarket
9-HODE	600 ± 30	680 ± 40	590 ± 50
10-HODE	13.7 ± 0.7	18.6 ± 0.9	4.9 ± 0.2
13-HODE	290 ± 20	350 ± 20	270 ± 20
15-HODE	3100 ± 100	4700 ± 200	4200 ± 600
9-HOTrE	239 ± 10	246 ± 8	180 ± 20
13-HOTrE	410 ± 10	790 ± 20	700 ± 70

Tab. 11.11: Concentrations of non-esterified fatty acids in the pressed oils. Analysis of non-esterified fatty acid concentrations was carried out by LC-MS after solid phase extraction using aminopropyl cartridges to remove the excess of triacylglycerols (mean ± SD, n = 3). Oil samples were stored in completely filled bottles for 24 weeks and analyzed at time point 0, 12 and 24 weeks.

[µg/g]	Flaxseed oil			Rapeseed oil			Sunflower oil		
	week 0	week 12	week 24	week 0	week 12	week 24	week 0	week 12	week 24
C16:1 n7	0.84 ± 0.04	1.6 ± 0.2	1.3 ± 0.2	9.9 ± 0.6	16.0 ± 0.2	14.4 ± 0.5	2.7 ± 0.1	4.3 ± 0.8	3.4 ± 0.3
C16:0	75 ± 4	99 ± 1	100 ± 10	116 ± 7	144 ± 8	137 ± 9	87 ± 4	100 ± 10	91 ± 4
C18:3 n3	250 ± 20	227 ± 2	230 ± 2	200 ± 20	180 ± 10	191 ± 9	6 ± 1	6.0 ± 0.3	4.8 ± 0.1
C18:2 n6	92 ± 6	92.5 ± 1.0	99 ± 3	400 ± 30	380 ± 20	430 ± 20	65 ± 8	57 ± 4	71 ± 10
C18:1 n9 + n7	200 ± 10	233 ± 6	219 ± 4	1100 ± 160	1480 ± 60	1270 ± 70	1300 ± 200	1300 ± 50	1300 ± 70
C18:0	48 ± 2	65 ± 2	60 ± 10	34 ± 1	51.1 ± 0.4	37 ± 8	69 ± 5	70 ± 10	56 ± 5
C20:2 n6	0.39 ± 0.02	0.27 ± 0.02	0.20 ± 0.02	2.10 ± 0.06	1.72 ± 0.05	1.55 ± 0.03		< LLOQ	
C20:1 n9	2.21 ± 0.06	4.11 ± 0.06	9.6 ± 0.2	15 ± 3	32 ± 2	73 ± 3	5.1 ± 0.4	8.1 ± 0.7	18 ± 1
C20:0	22 ± 2	21.9 ± 0.4	23.1 ± 0.5	13 ± 2	13.1 ± 0.7	12.4 ± 0.7	13 ± 1	10.6 ± 0.7	12.1 ± 0.5
C22:1 n9	1.97 ± 0.03	1.08 ± 0.02	1.2 ± 0.3	19 ± 2	11 ± 1	8.5 ± 0.3	1.50 ± 0.04	0.60 ± 0.09	0.7 ± 0.1

11.5 Chapter 7

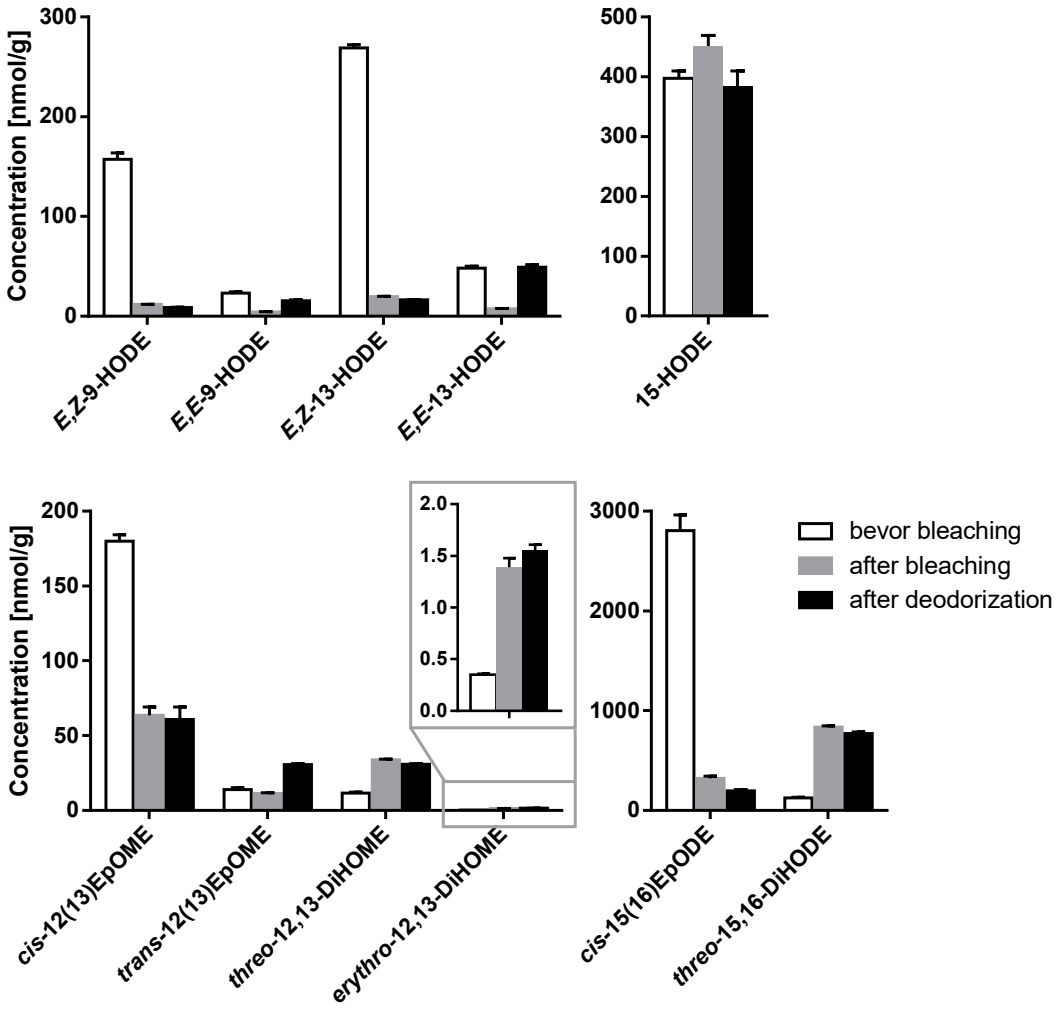

Fig. 11.20: Influence of bleaching and refining of rapeseed oil on oxylipin concentrations. Shown are the concentrations (mean ± SD, n = 3) of selected oxylipins in rapeseed oil bevor the bleaching step of the refining process, after bleaching and in fully refined rapeseed oil. Oxylipins were analyzed by LC-MS.

Tab. 11.12: Concentrations of oleic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples. The *trans*-epoxy-FA were quantified using the *cis*-epoxy-FA calibration curve.

[nmol/g]	Oleic Acid (18:1 n9)			
	epoxy-FA		vic dihydroxy-FA	
	9(10)-epoxy-stearic acid	*t*-9(10)-epoxy-stearic acid	*erythro*-9,10-DiOH-stearic acid	*threo*-9,10-DiOH-stearic acid
Grilled sausage 1-1	26.3	5.88	0.871	5.05
Grilled sausage 1-2	20.9	5.80	0.933	4.88
Grilled sausage 2-1	17.5	5.36	0.825	3.97
Grilled sausage 2-2	20.0	6.62	1.00	4.14
Pizza "Margherita" 1-1	35.0	6.40	2.25	9.66
Pizza "Margherita" 1-2	32.8	6.34	2.29	9.52
Pizza "Margherita" 2-1	39.9	5.38	3.23	10.3
Pizza "Margherita" 2-2	28.9	5.31	2.64	8.77
Hamburger Patty 1-1	36.0	12.0	0.82	3.02
Hamburger Patty 1-2	38.6	9.72	0.76	2.84
Hamburger Patty 2-1	31.6	23.0	2.01	5.21
Hamburger Patty 2-2	43.6	27.5	2.56	6.40
Falafel 1-1	379	556	169	358
Falafel 1-2	391	551	166	366
Falafel 2-1	246	293	83.6	137
Falafel 2-2	255	327	92.7	151
Curry 1-1	6.68	6.10	13.0	12.5
Curry 1-2	7.35	6.82	14.8	13.4
Curry 2-1	10.2	6.53	42.1	18.5
Curry 2-2	11.8	8.09	47.6	22.1
Sauce "Bolognese" 1-1	20.5	4.96	1.42	13.3
Sauce "Bolognese" 1-2	15.5	3.83	0.99	9.09
Sauce "Bolognese" 2-1	7.68	1.86	0.26	1.16
Sauce "Bolognese" 2-2	9.20	1.92	0.26	0.97
Schnitzel (chicken) 1-1	70.5	69.2	20.2	32.2
Schnitzel (chicken) 1-2	67.4	66.7	21.8	37.3
Schnitzel (pork) 2-1	49.1	50.7	27.1	41.5
Schnitzel (pork) 2-2	62.5	53.5	29.8	45.9
Veg. Chicken filet 1-1	40.0	47.0	13.4	27.2
Veg. Chicken filet 1-2	36.5	53.0	14.7	27.4
Veg. sausage 2-1	36.8	11.7	38.5	76.4
Veg. sausage 2-2	37.0	12.1	37.3	75.2
Veg. Fish finger raw 1-1	85.5	40.8	10.5	68.6
Veg. Fish finger raw 1-2	71.5	34.0	8.55	57.7
Veg. Fish finger baked 1-1	90.1	44.7	12.7	77.4
Veg. Fish finger baked 1-2	74.5	36.1	10.8	66.1
n3 fish finger raw 1-1	57.4	60.9	29.2	44.0
n3 fish finger raw 1-2	53.0	55.9	29.4	42.5
n3 fish finger baked 1-1	64.6	70.5	33.8	50.3
n3 fish finger baked 1-2	54.1	59.0	31.1	45.6
Fish fingers raw 1-1	53.3	63.1	24.0	37.2
Fish fingers raw 1-2	55.1	63.6	25.6	39.6
Fish fingers baked 1-1	59.6	69.9	30.9	47.9
Fish fingers baked 1-2	59.0	70.3	29.5	45.6
Tuna filet 1-1	2.60	1.80	1.92	22.2
Tuna filet 1-2	2.24	1.70	1.71	19.9
Tuna filet 2-1	5.72	2.33	3.71	55.5
Tuna filet 2-2	3.58	1.93	3.02	46.3

Tab. 11.13 (next page): Concentrations of linoleic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples. The *trans*-epoxy PUFA were quantified using the *cis*-epoxy-PUFA calibration curve. n.d.: not determinable due to matrix interference.

[nmol/g]	Linoleic Acid (LA; 18:2 n6)														
	hydroxy-PUFA							epoxy-FA				vic dihydroxy-FA		multi-hydroxy-FA	
	9-HODE	*E*,*E*-9-HODE	10-HODE	12-HODE	13-HODE	*E*,*E*-13-HODE	15-HODE	9(10)-EpOME	*t*-9(10)-EpOME	12(13)-EpOME	*t*-12(13)-EpOME	9,10-DiHOME	12,13-DiHOME	9,10,11-TriHOME	9,12,13-TriHOME
Grilled sausage 1-1	7.43	2.06	0.514	0.339	18.0	3.08	0.258	8.30	1.29	9.92	1.09	2.01	1.05	0.249	1.41
Grilled sausage 1-2	7.56	2.08	0.521	0.315	18.7	3.28	0.180	7.21	1.29	9.15	1.06	1.99	1.00	0.273	1.64
Grilled sausage 2-1	9.05	1.53	0.375	0.256	22.5	2.53	0.192	6.97	1.16	9.65	0.960	1.85	0.970	0.173	0.59
Grilled sausage 2-2	8.82	1.60	0.409	0.281	21.7	2.48	0.185	7.70	1.24	10.4	1.07	1.78	0.981	0.183	0.66
Pizza "Margherita" 1-1	6.22	2.48	0.388	0.329	8.66	2.52	0.748	4.81	0.554	4.66	0.536	3.41	1.83	0.119	8.10
Pizza "Margherita" 1-2	6.62	2.34	0.374	0.287	8.29	2.39	0.675	4.27	0.582	4.74	0.580	3.47	1.92	0.125	13.5
Pizza "Margherita" 2-1	8.59	1.94	0.394	0.327	8.13	1.98	1.94	5.01	0.659	5.20	0.647	3.23	2.31	0.214	12.1
Pizza "Margherita" 2-2	12.9	2.16	0.363	0.290	9.28	2.18	1.59	5.70	0.518	7.66	0.520	3.53	3.02	0.258	21.0
Hamburger Patty 1-1	2.35	0.962	0.130	0.131	4.42	1.34	0.563	2.14	0.539	2.10	0.422	0.155	0.151	0.055	0.594
Hamburger Patty 1-2	2.73	1.00	0.152	0.137	4.95	1.37	0.609	2.60	0.496	2.49	0.433	0.103	0.112	0.059	0.715
Hamburger Patty 2-1	1.98	1.14	0.141	0.129	3.45	1.55	0.420	2.17	1.45	2.00	1.26	0.201	0.142	0.051	0.892
Hamburger Patty 2-2	2.02	1.26	0.142	0.135	3.53	1.76	0.432	2.74	1.82	2.42	1.38	0.232	0.155	0.046	0.755
Falafel 1-1	135	105	54.3	34.9	160	169	63.8	260	261	183	187	54.4	26.6	3.39	n.d.
Falafel 1-2	139	106	53.1	35.2	165	166	64.3	291	281	186	177	52.4	26.5	4.35	
Falafel 2-1	98.1	77.5	36.7	24.7	129	134	46.2	369	124	168	73.0	25.3	12.4	2.12	
Falafel 2-2	105	85.8	41.2	26.6	144	146	50.7	357	138	198	80.4	27.4	13.7	2.22	
Curry 1-1	2.55	1.43	0.580	0.364	2.87	2.81	10.9	2.81	1.76	2.11	1.71	0.635	0.600	0.286	0.750
Curry 1-2	2.41	1.46	0.655	0.379	2.67	2.82	11.2	3.02	1.97	2.30	1.84	0.667	0.642	0.307	0.828
Curry 2-1	2.44	0.784	1.22	0.796	3.58	1.45	19.7	3.96	1.72	2.32	1.67	0.98	1.06	0.089	0.630
Curry 2-2	3.37	0.880	1.73	1.05	4.65	1.78	21.4	4.51	2.09	2.76	1.98	1.14	1.27	0.077	0.558
Sauce "Bolognese" 1-1	3.66	2.87	0.398	0.257	5.88	4.03	0.156	5.57	0.768	2.70	0.689	1.69	0.304	0.423	1.73
Sauce "Bolognese" 1-2	3.08	2.62	0.266	0.159	4.82	3.62	0.107	3.72	0.647	2.09	0.600	1.26	0.265	0.480	1.74
Sauce "Bolognese" 2-1	2.00	0.959	0.156	0.103	3.45	1.23	0.174	0.609	0.150	0.63	0.152	0.228	0.077	0.324	0.977
Sauce "Bolognese" 2-2	2.00	0.946	0.183	0.128	3.21	1.18	0.165	0.732	0.166	0.64	0.149	0.152	0.075	0.374	1.06
Schnitzel (chicken) 1-1	37.0	11.5	5.68	3.57	38.4	20.9	21.3	35.2	19.8	39.4	16.2	5.08	3.93	0.561	54.5
Schnitzel (chicken) 1-2	36.0	12.3	5.37	3.72	37.3	20.9	23.4	34.5	21.1	37.7	14.7	5.15	4.05	0.608	58.2
Schnitzel (pork) 2-1	47.1	13.8	7.00	4.21	63.8	26.3	43.7	31.0	16.1	39.6	12.0	4.48	4.98	0.786	73.5
Schnitzel (pork) 2-2	60.1	14.3	6.87	4.77	73.8	26.5	48.9	38.6	17.5	47.3	11.8	4.98	5.82	0.899	97.0
Veg. Chicken filet 1-1	41.6	21.0	2.73	1.84	46.5	35.5	15.6	31.4	13.5	47.6	11.9	7.19	6.10	1.12	76.5
Veg. Chicken filet 1-2	41.4	22.5	3.29	2.10	59.3	38.3	16.2	33.3	15.9	56.3	12.7	6.23	6.49	1.09	50.1
Veg. sausage 2-1	177	67.4	3.70	2.14	265	101	0.495	51.4	10.2	80.2	9.49	27.5	4.07	1.24	20.0
Veg. sausage 2-2	183	69.3	4.04	2.41	267	103	0.401	56.8	11.8	84.6	10.5	29.6	4.57	1.29	23.7
Veg. Fish finger raw 1-1	111	66.0	25.4	15.0	158	127	85.3	161	68.2	103	30.9	65.1	15.2	1.57	84.5
Veg. Fish finger raw 1-2	88.5	52.0	18.8	10.8	120	98.1	68.2	146	53.9	88.3	25.5	50.7	11.9	1.21	66.4
Veg. Fish finger baked 1-1	122	73.3	22.2	13.4	164	135	103	166	68.5	107	35.5	64.8	15.9	1.81	94.2
Veg. Fish finger baked 1-2	112	70.0	19.6	11.0	149	119	79.9	160	57.8	97.1	26.4	58.0	13.4	1.40	79.2
n3 fish finger raw 1-1	39.4	15.0	7.47	5.03	34.9	24.4	36.5	31.1	25.1	36.0	17.2	6.32	7.01	2.10	59.1
n3 fish finger raw 1-2	34.9	14.9	6.53	4.11	31.1	23.3	34.1	25.6	20.9	26.3	15.8	5.59	6.30	1.50	44.8
n3 fish finger baked 1-1	47.1	18.7	7.89	4.97	41.7	29.9	40.5	34.3	28.6	38.1	19.6	6.54	7.62	1.95	59.8
n3 fish finger baked 1-2	40.5	16.5	6.95	4.28	35.7	26.0	34.7	27.4	23.1	31.2	16.3	6.08	6.85	1.66	49.3
Fish fingers raw 1-1	38.7	15.8	7.12	4.09	59.0	27.2	27.7	31.1	26.0	38.6	19.1	4.83	4.59	1.45	59.1
Fish fingers raw 1-2	36.2	15.8	7.36	4.52	54.1	27.0	31.4	30.1	25.6	35.2	20.7	5.27	5.16	1.41	55.7
Fish fingers baked 1-1	47.3	20.9	9.37	5.01	65.4	34.9	33.7	34.1	29.1	39.9	21.4	5.82	5.77	1.61	67.6
Fish fingers baked 1-2	45.0	20.0	8.48	4.90	67.7	33.9	33.1	33.9	28.0	41.1	20.4	6.06	6.09	1.76	68.2
Tuna filet 1-1	2.05	2.69	0.228	0.140	4.20	3.85	0.225	4.48	1.13	3.19	1.07	16.3	2.18	0.0359	0.306
Tuna filet 1-2	1.80	2.44	0.210	0.128	3.88	3.43	0.198	3.47	0.957	2.56	0.945	14.8	1.87	0.0375	0.315
Tuna filet 2-1	7.42	12.4	0.509	0.273	15.6	23.7	0.311	9.75	2.43	5.75	2.32	36.5	3.49	0.0785	0.934
Tuna filet 2-2	6.08	10.2	0.414	0.234	12.9	18.8	0.257	7.04	1.97	3.98	1.80	32.3	2.97	0.0601	0.538

Tab. 11.14: Concentrations of α-linolenic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples. The *trans*-epoxy PUFA were quantified using the *cis*-epoxy-PUFA calibration curve. n.d.: not determinable due to matrix interference.

	α-Linolenic Acid (ALA; 18:3 n3)													
	hydroxy-PUFA		epoxy-PUFA					vic dihydroxy-PUFA			multi-hydroxy-PUFA	
[nmol/g]	9-HOTrE	13-HOTrE	9(10)-EpODE	*t*-9(10)-EpODE	12(13)-EpODE	*t*-12(13)-EpODE	15(16)-EpODE	*t*-15(16)-EpODE	9,10-DiHODE	12,13-DiHODE	15,16-DiHODE	9,10,11-TriHODE	9,12,13-TriHODE
Grilled sausage 1-1	0.469	0.760	0.660	0.0777	0.543	0.0490	13.5		0.0964	0.0993	0.920	0.0438	
Grilled sausage 1-2	0.421	0.754	0.577	0.0752	0.529	0.0435	14.7		0.0934	0.0927	0.789	0.0537	
Grilled sausage 2-1	0.647	1.12	0.594	0.0834	0.598	0.0504	17.9	n.d.	0.0919	0.100	0.701	0.0429	n.d.
Grilled sausage 2-2	0.623	1.15	0.643	0.0989	0.585	0.0588	18.6		0.0908	0.101	0.731	0.0444	
Pizza "Margherita" 1-1	1.30	2.52	0.961	0.124	0.590	0.0563	5.41	0.201	0.434	0.297	1.38	0.0225	0.649
Pizza "Margherita" 1-2	1.28	2.26	0.877	0.102	0.523	0.0601	5.40	0.176	0.435	0.289	1.41	0.0253	1.08
Pizza "Margherita" 2-1	2.09	4.45	1.86	0.235	1.05	0.115	6.14	0.301	0.475	0.337	4.68	0.0418	0.941
Pizza "Margherita" 2-2	2.27	3.90	1.24	0.190	0.843	0.0794	6.29	0.234	0.436	0.373	3.96	0.0464	1.72
Hamburger Patty 1-1	0.351	1.00	0.467	0.0729	0.233	0.0428	1.37	0.145	0.0182		0.161		
Hamburger Patty 1-2	0.384	1.13	0.534	0.0697	0.272	0.0407	1.54	0.138	0.0163	< LLOQ	0.156	n.d.	n.d.
Hamburger Patty 2-1	0.291	0.783	0.368	0.146	0.177	0.0952	1.13	0.220	0.0147		0.174		
Hamburger Patty 2-2	0.300	0.876	0.503	0.152	0.251	0.0875	1.37	0.238	0.0147		0.157		
Falafel 1-1	4.06	21.3	80.4	106	43.6	59.8	175	132	8.46	4.10	109	0.760	2.33
Falafel 1-2	4.62	22.8	85.4	114	43.9	63.5	185	145	8.50	4.10	113	1.14	2.19
Falafel 2-1	5.32	25.0	75.3	62.1	30.7	35.4	165	75.2	3.58	1.61	80.2	n.d.	1.39
Falafel 2-2	5.47	26.9	84.8	70.7	33.9	38.5	185	85.5	3.93	1.76	88.9		1.46
Curry 1-1	0.399	1.68	0.973	0.765	0.541	0.400	25.6	0.861	0.111	0.059	16.6	0.0631	0.0577
Curry 1-2	0.430	1.71	1.02	0.847	0.598	0.435	27.6	0.923	0.125	0.065	17.7	0.0695	0.0665
Curry 2-1	0.868	1.05	1.43	0.971	0.848	0.443	4.32	1.16	0.287	0.0989	33.4	0.0385	0.0984
Curry 2-2	1.11	1.26	1.50	1.18	1.02	0.560	4.87	1.30	0.320	0.118	38.5	0.0260	0.0678
Sauce "Bolognese" 1-1	0.911	2.21	0.250	0.113	0.148	0.0620	2.36	0.150	0.0529	0.0308	0.406	0.113	0.345
Sauce "Bolognese" 1-2	0.580	1.58	0.145	0.0610	0.0923	0.0414	1.93	0.109	0.0424	0.0252	0.260	0.121	0.363
Sauce "Bolognese" 2-1	0.665	1.75	0.139	0.0361	0.0825	0.0272	0.658	0.0717	0.0360	0.0200	0.290	0.0840	0.330
Sauce "Bolognese" 2-2	0.660	1.69	0.157	0.0414	0.0884	0.0263	0.593	0.0670	0.0257	0.0173	0.222	0.0985	0.356
Schnitzel (chicken) 1-1	3.82	5.38	10.9	10.9	7.18	5.52	37.2	12.0	0.836	0.444	34.4		3.76
Schnitzel (chicken) 1-2	4.31	5.86	10.8	10.9	7.16	5.62	36.7	12.4	0.852	0.453	34.5	n.d.	3.78
Schnitzel (pork) 2-1	8.16	8.58	8.77	7.72	5.97	3.94	47.8	9.29	0.670	0.363	60.0		6.00
Schnitzel (pork) 2-2	10.4	9.92	10.5	8.61	6.77	4.44	58.6	9.80	0.741	0.452	67.9		7.70
Veg. Chicken filet 1-1	3.75	5.73	6.83	6.61	5.60	3.60	38.1	8.22	0.604	0.448	40.0		6.44
Veg. Chicken filet 1-2	3.48	6.71	6.75	7.22	6.43	4.09	45.2	8.66	0.619	0.447	40.7	n.d.	3.93
Veg. sausage 2-1	18.6	32.2	7.47	0.783	14.5	0.610	41.1	1.04	0.489	0.552	2.71		
Veg. sausage 2-2	20.5	35.1	8.24	0.841	16.2	0.663	42.9	1.15	0.554	0.637	2.94		n.d.
Veg. Fish finger raw 1-1	13.9	20.4	7.08	0.881	7.18	0.441	147		0.774		17.7	0.210	7.28
Veg. Fish finger raw 1-2	10.9	15.5	6.21	0.782	5.98	0.409	134	n.d.	0.661	n.d.	14.1	0.178	5.95
Veg. Fish finger baked 1-1	15.5	22.0	7.88	1.41	8.20	0.693	160		0.875		19.9	0.239	8.50
Veg. Fish finger baked 1-2	12.6	16.3	7.11	1.25	6.99	0.655	137		0.736		16.3	0.198	6.92
n3 fish finger raw 1-1	5.11	9.45	11.2	13.4	8.48	6.44	84.0	17.3	0.790	0.649	54.1	0.285	4.96
n3 fish finger raw 1-2	4.16	8.43	10.3	12.4	7.78	5.76	72.5	15.5	0.772	0.638	50.8	0.240	3.96
n3 fish finger baked 1-1	5.30	11.1	12.3	16.1	9.84	7.48	92.5	19.3	0.870	0.735	60.6	0.324	5.43
n3 fish finger baked 1-2	4.70	9.74	10.4	13.1	8.11	6.02	77.5	15.5	0.780	0.685	55.1	0.270	4.61
Fish fingers raw 1-1	4.22	9.70	11.3	14.5	8.74	7.09	66.0	19.4	0.743	0.555	43.0	0.291	5.62
Fish fingers raw 1-2	4.51	10.7	11.4	14.8	8.54	7.14	60.8	18.7	0.790	0.583	45.5	0.227	4.37
Fish fingers baked 1-1	4.81	12.3	12.4	17.1	10.0	7.96	70.7	21.5	0.877	0.674	52.7	0.233	5.38
Fish fingers baked 1-2	4.89	12.7	12.1	16.2	9.92	7.96	69.4	21.6	0.909	0.672	49.3	0.321	6.44
Tuna filet 1-1	0.0757	0.0370	0.0778	0.0294	0.0293	0.0100	0.202	0.0338	1.19		4.81		
Tuna filet 1-2	0.0623	0.0334	0.0631	0.0252	0.0238	0.0132	0.148	0.0316	1.05	n.d.	4.22	< LLOQ	n.d.
Tuna filet 2-1	0.0670	0.0356	0.130	0.0290	0.0229	0.0103	0.487	0.0365	1.38		5.28		
Tuna filet 2-2	0.0543	0.0296	0.099	0.0249	0.0187	0.0095	0.379	0.0292	1.15		4.52		

Tab. 11.15: Concentrations of γ-linolenic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples.

[nmol/g]	dihomo-γ-Linolenic Acid (DGLA; 20:3 n6)				
	hydroxy-PUFA				epoxy-PUFA
	5-HETrE	8-HETrE	12-HETrE	15-HETrE	14(15)-EpEDE
Grilled sausage 1-1	0.0433	0.0139	0.0576	0.0844	0.0633
Grilled sausage 1-2	0.0398	0.0198	0.0583	0.0898	0.0431
Grilled sausage 2-1	0.0294	< LLOQ	0.0444	0.0690	0.0351
Grilled sausage 2-2	0.0272		0.0434	0.0758	0.0408
Pizza "Margherita" 1-1	0.104	0.0514	0.0704	0.0362	0.106
Pizza "Margherita" 1-2	0.0928	0.0625	0.0695	0.0463	0.106
Pizza "Margherita" 2-1	0.0705	< LLOQ	0.0501	0.0356	0.105
Pizza "Margherita" 2-2	0.0537		0.0522	0.0368	0.0701
Hamburger Patty 1-1	0.0482	0.103	0.0858	0.168	0.107
Hamburger Patty 1-2	0.0572	0.100	0.107	0.210	0.160
Hamburger Patty 2-1	0.0400	0.108	0.0852	0.157	0.101
Hamburger Patty 2-2	0.0401	0.055	0.0704	0.132	0.122
Sauce "Bolognese" 1-1	0.00658	< LLOQ	0.0128	0.0152	0.0100
Sauce "Bolognese" 1-2	0.00671		0.00948	0.0138	0.0104
Sauce "Bolognese" 2-1	0.0122	0.022	0.0268	0.0314	0.0150
Sauce "Bolognese" 2-2	0.0134	0.026	0.0278	0.0340	0.0206
Schnitzel (chicken) 1-1	0.0486	0.0347	0.0211	0.0619	0.0225
Schnitzel (chicken) 1-2	0.0388	0.0222	0.0155	0.0457	0.0142
Schnitzel (pork) 2-1	0.0467	0.0587	0.0330	0.0913	0.0436
Schnitzel (pork) 2-2	0.0406	0.0386	0.0351	0.0735	0.0436
Veg. Chicken filet 1-1	< LLOQ		< LLOQ		< LLOQ
Veg. Chicken filet 1-2					
Veg. sausage 2-1		0.0361	0.0350	0.0352	0.0174
Veg. sausage 2-2		0.0320	0.0281	0.0343	0.0179
n3 fish finger raw 1-1	< LLOQ		0.00503	< LLOQ	0.00238
n3 fish finger raw 1-2			0.00693		0.00176
n3 fish finger baked 1-1			0.00991		0.00243
n3 fish finger baked 1-2			0.00567		0.00212
Fish fingers raw 1-1	< LLOQ			0.00480	0.00134
Fish fingers raw 1-2				0.00321	0.00174
Fish fingers baked 1-1				0.00354	0.00132
Fish fingers baked 1-2				0.00511	0.000903
Tuna filet 1-1	< LLOQ	0.00515	0.0119	0.0192	< LLOQ
Tuna filet 1-2		0.00746	0.0158	0.0154	
Tuna filet 2-1		< LLOQ	0.00921	0.0107	
Tuna filet 2-2			0.00533	0.00562	

Tab. 11.16 (next page): Concentrations of arachidonic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples.

[nmol/g]	Arachidonic Acid (ARA; 20:4 n6)																	
	hydroxy-PUFA										epoxy-PUFA			vic dihydroxy-PUFA				prostanoids	isoprostanes
	5-HETE	8-HETE	9-HETE	11-HETE	12-HETE	15-HETE	16-HETE	17-HETE	18-HETE	12-HHTrE	8(9)-EpETrE	11(12)-EpETrE	14(15)-EpETrE	5,6-DiHETrE	8,9-DiHETrE	11,12-DiHETrE	14,15-DiHETrE	PGB2	5(*R,S*)-F_{2t}-IsoP
Grilled sausage 1-1	0.446	0.147	0.188	0.227	0.238	0.462	0.0242	< LLOQ		0.0567	0.258	0.328	0.512	0.0942	0.00427	0.00463	0.00670	0.0174	0.0176
Grilled sausage 1-2	0.438	0.130	0.185	0.214	0.229	0.472	0.0227			0.0467	0.205	0.252	0.376	0.0859	0.00486	0.00463	0.00579	0.0141	0.0127
Grilled sausage 2-1	0.358	0.085	0.116	0.141	0.172	0.279	0.0095			0.0135	0.133	0.189	0.289	0.0414	0.00281	0.00240	0.00260	0.00557	0.00684
Grilled sausage 2-2	0.359	0.117	0.132	0.175	0.215	0.328	0.0109			0.0129	0.160	0.195	0.303	0.0561	0.00383	0.00262	0.00297	0.00491	0.00430
Pizza "Margherita" 1-1	0.159	0.0181	0.0258	0.0397	0.0422	0.0584	< LLOQ			0.0664	0.0727	0.168	0.181	0.0554	< LLOQ	0.00988	0.00919	< LLOQ	< LLOQ
Pizza "Margherita" 1-2	0.142	0.0151	0.0209	0.0401	0.0484	0.0613				0.0582	0.0693	0.159	0.186	0.0530		0.00908	0.00910		
Pizza "Margherita" 2-1	0.112	< LLOQ		0.0350	0.0347	0.0403				0.0420	0.0714	0.137	0.147	0.0348		0.00642	0.00653	0.0158	
Pizza "Margherita" 2-2	0.099			0.0259	0.0286	0.0338				0.0419	0.0361	0.104	0.101	0.0315		0.00631	0.00529	0.0199	
Hamburger Patty 1-1	0.508	0.140	0.207	0.220	0.211	0.443	0.0190	< LLOQ		0.0639	0.197	0.314	0.320	0.130	0.00469	0.00370	0.00528	0.0153	0.0195
Hamburger Patty 1-2	0.509	0.166	0.241	0.287	0.248	0.537	0.0259			0.0706	0.256	0.421	0.518	0.175	0.00245	0.00371	0.00510	0.0197	0.0203
Hamburger Patty 2-1	0.404	0.122	0.184	0.177	0.171	0.354	0.0158			0.0542	0.173	0.317	0.333	0.141	0.00342	0.00245	0.00435	0.0147	0.0137
Hamburger Patty 2-2	0.345	0.111	0.191	0.170	0.171	0.309	0.0148			0.0486	0.229	0.354	0.379	0.128	0.00377	0.00309	0.00523	0.0138	0.0102
Sauce "Bolognese" 1-1	0.0628	0.0199	0.0254	0.0257	0.0285	0.0416	< LLOQ			0.0082	0.0213	0.0297	0.0319	0.0112	0.00140	0.00139	0.00116	0.0047	< LLOQ
Sauce "Bolognese" 1-2	0.0644	0.0193	0.0254	0.0268	0.0326	0.0525				0.0107	0.0287	0.0355	0.0402	0.0168	0.00171	0.00164	0.00157	0.0060	
Sauce "Bolognese" 2-1	0.103	0.0381	0.0558	0.0479	0.0513	0.0676				0.0055	0.0237	0.0340	0.0362	0.0195	0.00219	0.00138	0.00157	0.0267	0.00346
Sauce "Bolognese" 2-2	0.111	0.0367	0.0646	0.0521	0.0555	0.0816				0.0050	0.0347	0.0481	0.0555	0.0258	0.00252	0.00191	0.00186	0.0225	0.00317
Schnitzel (chicken) 1-1	0.421	0.0829	0.175	0.159	0.139	0.583	0.0191	< LLOQ		0.0936	0.162	0.211	0.209	0.0923	< LLOQ		0.00313	0.0359	0.0232
Schnitzel (chicken) 1-2	0.348	0.0632	0.118	0.123	0.112	0.454	0.0152			0.0685	0.136	0.190	0.184	0.0630			0.00295	0.0241	0.0212
Schnitzel (pork) 2-1	0.474	0.165	0.264	0.230	0.214	0.614	0.0187			0.0709	0.226	0.291	0.301	0.147	0.00796	0.00563	0.0161	0.0137	0.0118
Schnitzel (pork) 2-2	0.407	0.167	0.213	0.220	0.214	0.554	0.0146			0.0565	0.225	0.253	0.336	0.144	0.00648	0.00488	0.0120	0.0096	0.0114
Veg. Chicken filet 1-1	0.0146	< LLOQ		0.0085	0.0061	0.0265	< LLOQ	< LLOQ		< LLOQ	< LLOQ	0.00410	< LLOQ	< LLOQ				< LLOQ	< LLOQ
Veg. Chicken filet 1-2	0.0138			0.0125	0.0105	0.0368						0.00347							
Veg. sausage 2-1	0.380	0.171	0.258	0.258	0.166	0.469	0.0229	< LLOQ		0.0173	0.107	0.146	0.184	0.0845	0.00428	0.00459	0.00690	0.0373	0.0114
Veg. sausage 2-2	0.373	0.190	0.238	0.269	0.165	0.480	0.0254			0.0150	0.113	0.146	0.194	0.0734	0.00304	0.00421	0.00730	0.0379	0.0123
n3 fish finger raw 1-1	0.0296	0.0161	0.0335	0.0402	0.0265	0.0445	< LLOQ	0.0454	0.0631	0.0342	0.0222	0.0292	0.0359	0.0088	< LLOQ			0.0204	< LLOQ
n3 fish finger raw 1-2	0.0327	0.0169	0.0258	0.0506	0.0316	0.0530		0.0379	0.0604	0.0557	0.0160	0.0311	0.0384	0.0108				0.0248	
n3 fish finger baked 1-1	0.0270	0.0159	0.0187	0.0345	0.0272	0.0406		0.0493	0.0803	0.0184	0.0250	0.0319	0.0420	0.00862				0.0118	
n3 fish finger baked 1-2	0.0245	0.0150	0.0158	0.0291	0.0217	0.0447		0.0433	0.0730	0.0168	0.0187	0.0303	0.0348	0.00653				0.00949	
Fish fingers raw 1-1	0.0349	0.0168	0.0347	0.0424	0.0307	0.0510	< LLOQ			0.0305	0.0208	0.0178	0.0256	0.00861	< LLOQ			0.0138	< LLOQ
Fish fingers raw 1-2	0.0263	0.0176	0.0258	0.0434	0.0240	0.0359				0.0452	0.0160	0.0261	0.0273	0.0140				0.0115	
Fish fingers baked 1-1	0.0256	0.0124	0.0233	0.0288	0.0205	0.0332				0.0101	< LLOQ	0.0230	0.0279	0.00463				0.00572	
Fish fingers baked 1-2	0.0235	0.0129	0.0219	0.0276	0.0241	0.0406				0.0101		0.0190	0.0221	0.00447				0.00766	
Tuna filet 1-1	0.280	0.117	0.152	0.158	0.138	0.351	0.0150	0.0125	0.00574	0.0522	0.0771	0.0774	0.0846	0.1068	0.00739	0.00515	0.00726	0.0102	0.0229
Tuna filet 1-2	0.286	0.105	0.154	0.144	0.119	0.312	0.0141	0.0131	0.00728	0.0506	0.0557	0.0639	0.0758	0.0928	0.00776	0.00451	0.00653	0.00992	0.0241
Tuna filet 2-1	0.0904	0.0455	0.0689	0.0575	0.0466	0.126	0.00683	0.00737	< LLOQ	0.0226	0.0297	0.0301	0.0347	0.0380	0.00245	0.00180	0.00227	< LLOQ	0.0121
Tuna filet 2-2	0.0815	0.0366	0.0449	0.0514	0.0408	0.105	0.00593	0.00624		0.0169	0.0294	0.0272	0.0315	0.0423	0.00207	0.00203	0.00256		0.00993

Tab. 11.17: Concentrations of eicosapentaenoic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples.

[nmol/g]	Eicosapentaenoic Acid (EPA; 20:5 n3)														
	hydroxy-PUFA										epoxy-PUFA			vic dihydroxy-PUFA			prostanoids
	5-HEPE	8-HEPE	9-HEPE	11-HEPE	12-HEPE	15-HEPE	18-HEPE	19-HEPE	20-HEPE	8(9)-EpETE	11(12)-EpETE	14(15)-EpETE	17(18)-EpETE	5,6-DiHETE	8,9-DiHETE	11,12-DiHETE	14,15-DiHETE	17,18-DiHETE	PGB3
n3 fish finger raw 1-1	0.237	0.127	0.314	0.158	0.347	0.337	0.531			0.378	0.310	0.389	1.57	0.144	0.00961	0.00743	0.00869	0.0583	0.0185
n3 fish finger raw 1-2	0.237	0.114	0.293	0.141	0.306	0.290	0.519	< LLOQ	< LLOQ	0.372	0.309	0.353	1.23	0.142	0.00880	0.00677	0.00789	0.0347	0.0131
n3 fish finger baked 1-1	0.239	0.116	0.310	0.148	0.343	0.308	0.568			0.471	0.364	0.404	1.39	0.124	0.00721	0.00627	0.00745	0.0392	0.00837
n3 fish finger baked 1-2	0.214	0.120	0.294	0.130	0.318	0.292	0.507			0.366	0.358	0.389	1.29	0.114	0.00826	0.00578	0.00667	0.0384	0.00696
Fish fingers raw 1-1	0.472	0.218	0.632	0.272	0.617	0.486	0.988			0.398	0.360	0.346	0.993	0.222	0.0113	0.00844	0.00859	0.0266	0.0219
Fish fingers raw 1-2	0.334	0.161	0.486	0.202	0.479	0.347	0.657			0.411	0.357	0.342	0.962	0.240	0.0102	0.00742	0.00714	0.0409	0.0155
Fish fingers baked 1-1	0.328	0.143	0.552	0.186	0.451	0.322	0.684	< LLOQ	< LLOQ	0.397	0.360	0.379	0.867	0.118	0.00682	0.00393	0.00533	0.0289	0.0105
Fish fingers baked 1-2	0.325	0.137	0.466	0.174	0.446	0.308	0.635			0.374	0.313	0.323	0.701	0.114	0.00641	0.00499	0.00530	0.0316	0.0114
Tuna filet 1-1	0.325	0.111	0.240	0.0992	0.190	0.133	0.966		0.00742	0.107	0.0945	0.0860	0.168	0.177	0.0126	0.00909	0.00692	0.0144	
Tuna filet 1-2	0.308	0.106	0.214	0.0914	0.170	0.125	0.898	< LLOQ	0.00658	0.0715	0.0818	0.0692	0.128	0.159	0.0125	0.00772	0.00730	0.0146	< LLOQ
Tuna filet 2-1	0.141	0.0642	0.136	0.0553	0.115	0.0801	0.481		0.00507	< LLOQ	0.0487	0.0387	0.0789	0.0784	0.00646	0.00457	0.00381	0.00879	
Tuna filet 2-2	0.147	0.0531	0.110	0.0445	0.0897	0.0624	0.368		0.00571	< LLOQ	0.0460	0.0377	0.0682	0.0851	0.00624	0.00484	0.00393	0.00952	

Tab. 11.18: Concentrations of docosahexaenoic acid derived oxylipins in the food samples. Lipids were extracted from food samples by using acidified methyl *tert*-butyl ether. Total oxylipin concentrations were determined by LC-MS after hydrolyzation of esterified oxylipins. Concentrations were determined in duplicate from two representative food samples.

	Docosahexaenoic Acid (DHA; 22:6 n3)																			
	hydroxy-PUFA																			
[nmol/g]	4-HDHA	7-HDHA	8-HDHA	10-HDHA	11-HDHA	13-HDHA	14-HDHA	16-HDHA	17-HDHA	20-HDHA	21-HDHA	22-HDHA								
n3 fish finger raw 1-1	0.812	0.253	0.307	0.329	0.567	0.350	0.325	0.295	0.259	0.685	0.0160									
n3 fish finger raw 1-2	0.821	0.234	0.289	0.341	0.534	0.388	0.372	0.319	0.298	0.744	0.0241									
n3 fish finger baked 1-1	0.778	0.215	0.281	0.295	0.485	0.348	0.336	0.266	0.286	0.682	0.0212	< LLOQ								
n3 fish finger baked 1-2	0.686	0.198	0.249	0.267	0.423	0.315	0.305	0.274	0.270	0.646	0.0220									
Fish fingers raw 1-1	1.17	0.362	0.469	0.465	0.911	0.564	0.491	0.485	0.477	1.023	0.0292									
Fish fingers raw 1-2	0.845	0.255	0.301	0.377	0.628	0.432	0.402	0.354	0.359	0.733	0.0252	< LLOQ								
Fish fingers baked 1-1	0.814	0.210	0.273	0.283	0.958	0.336	0.337	0.290	0.286	0.634	0.0246									
Fish fingers baked 1-2	0.779	0.214	0.259	0.291	0.640	0.343	0.334	0.278	0.296	0.607	0.0166									
Tuna filet 1-1	3.98	0.782	0.742	0.955	1.13	0.953	0.833	0.767	0.761	4.19		0.0619								
Tuna filet 1-2	4.07	0.781	0.703	0.854	1.08	0.886	0.751	0.684	0.686	3.95	< LLOQ	0.0540								
Tuna filet 2-1	1.74	0.444	0.437	0.536	0.647	0.529	0.487	0.437	0.490	2.05		0.0487								
Tuna filet 2-2	1.48	0.353	0.331	0.458	0.541	0.464	0.439	0.383	0.405	1.65		0.0418								
	epoxy-PUFA					vic dihydroxy-PUFA														
	7(8)-EpDPE	10(11)-EpDPE	13(14)-EpDPE	16(17)-EpDPE	19(20)-EpDPE	4,5-DiHDPE	7,8-DiHDPE	10,11-DiHDPE	13,14-DiHDPE	16,17-DiHDPE	19,20-DiHDPE									
n3 fish finger raw 1-1	0.279	0.319	0.305	0.367	1.50	0.150		0.00761	0.0102	0.0106	0.174									
n3 fish finger raw 1-2	0.313	0.347	0.320	0.369	1.30	0.162	< LLOQ	0.00823	0.00754	0.0096	0.0963									
n3 fish finger baked 1-1	0.393	0.428	0.342	0.424	1.42	0.133		0.00640	0.00787	0.0106	0.0977									
n3 fish finger baked 1-2	0.362	0.410	0.384	0.433	1.24	0.145		0.00612	0.00712	0.0139	0.103									
Fish fingers raw 1-1	0.354	0.422	0.429	0.448	1.29	0.311		0.00840	0.00562	0.00928	0.0749									
Fish fingers raw 1-2	0.403	0.466	0.420	0.449	1.24	0.677	< LLOQ	0.00836	0.00810	0.0139	0.124									
Fish fingers baked 1-1	0.298	0.390	0.349	0.372	0.937	0.0967		0.00453	0.00389	0.00580	0.0590									
Fish fingers baked 1-2	0.281	0.318	0.298	0.295	0.812	0.0936		0.00484	0.00394	0.00562	0.0668									
Tuna filet 1-1	0.605	0.561	0.384	0.362	0.821	1.03	0.0873	0.0497	0.0439	0.0432	0.0845									
Tuna filet 1-2	0.491	0.443	0.304	0.293	0.715	0.950	0.0850	0.0472	0.0406	0.0407	0.0833									
Tuna filet 2-1	0.380	0.350	0.247	0.221	0.536	0.594	0.0494	0.0250	0.0241	0.0230	0.0474									
Tuna filet 2-2	0.339	0.328	0.225	0.204	0.466	0.669	0.0381	0.0271	0.0246	0.0244	0.0516									

11.6 Chapter 8

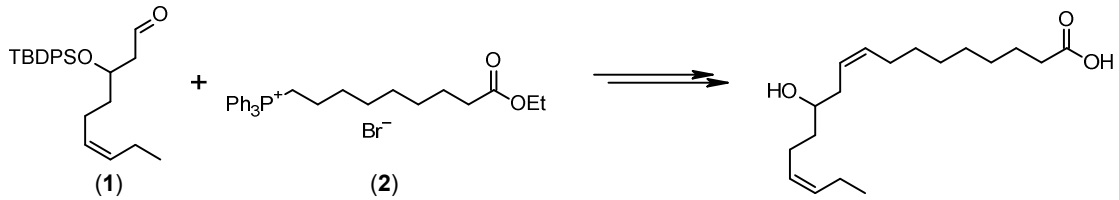

Fig. 11.21: Synthesis of densipolic acid from the corresponding aldehyde (**1**) and Wittig salt (**2**). For the synthesis of (**1**), *cis*-4-heptenal was reduced with a cyclic acetal containing Grignard reagent. The resulting hydroxy group was protected and the acetal group was deprotected. For (**2**), 9-bromononanol was oxidized to the acid, esterified and converted with triphenylphosphine.

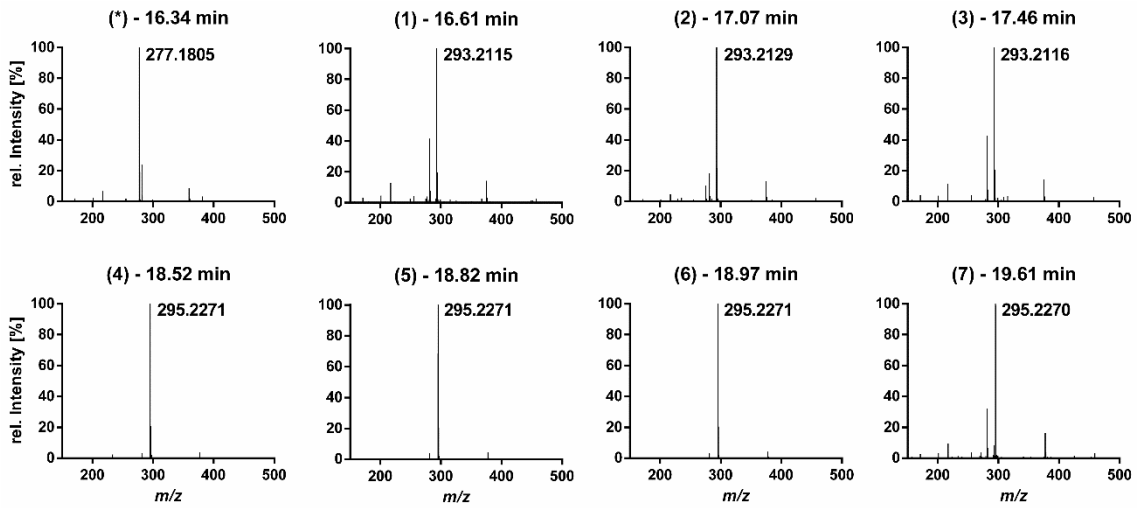

Fig. 11.22: LC-ESI(-)-MS spectra of the main peaks eluting between 16 min and 20 min. Shown are the full-scan spectra (*m*/*z* 150-500) of the dominating peaks (1-7, Fig. 8.1) in hydrolyzed oils. Indicated is the exact mass of the base peak.

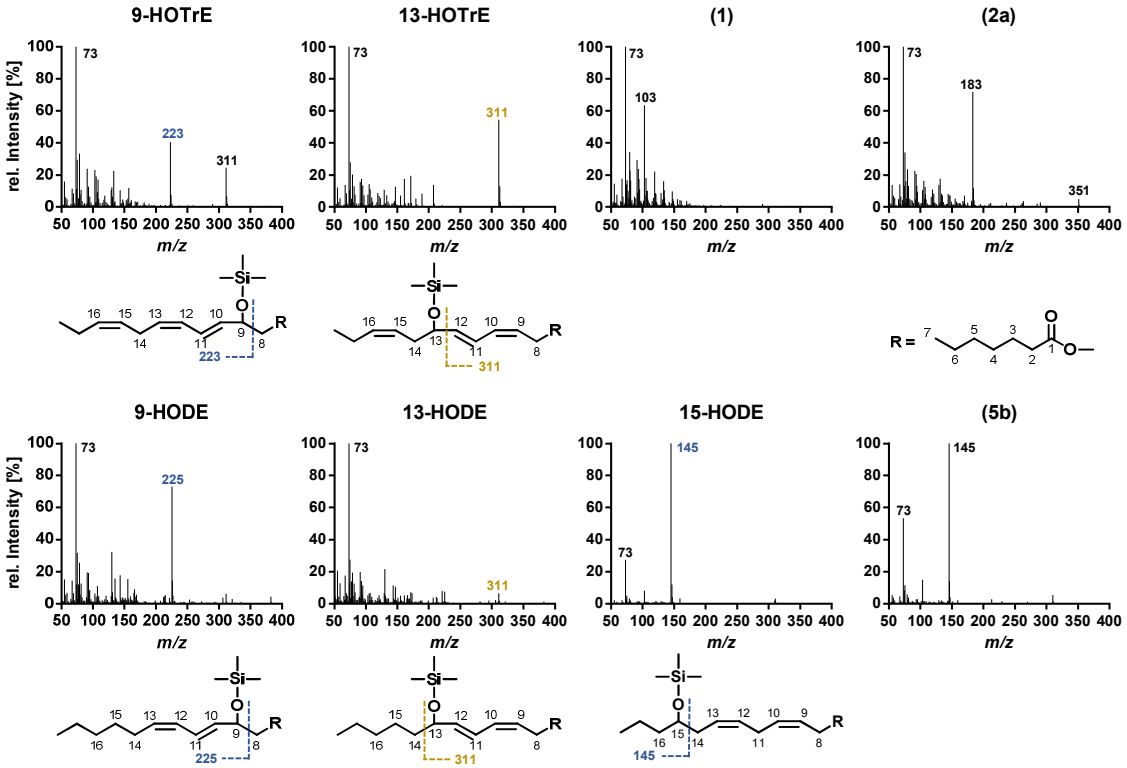

Fig. 11.23: Mass spectra of hydroxy linoleic acid and hydroxy α-linolenic acid standards as well as of (1), (2a) and (5b) obtained by electron ionization. Shown are the mass spectra of oxylipins after derivatization with trimethylsilyl diazomethane and BSTFA to the corresponding trimethylsilyl fatty acid methyl ester. Electron ionization was carried out using 70 eV. The structures show the suggested site of the α-cleavage.

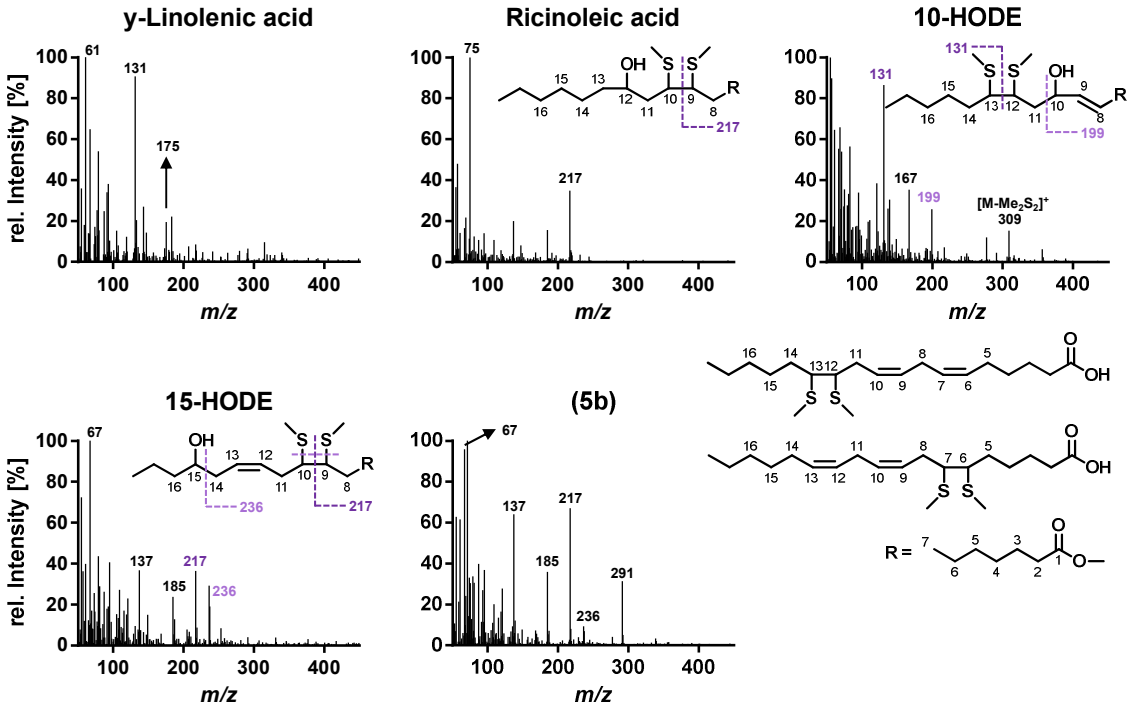

Fig. 11.24: Mass spectra of (hydroxy) fatty acids with activated double bonds following derivatization with dimethyl disulfide to the corresponding methylthiolated adducts. Electron ionization was carried out using 70 eV. In the structures, the suggested sites of fragmentation are indicated.

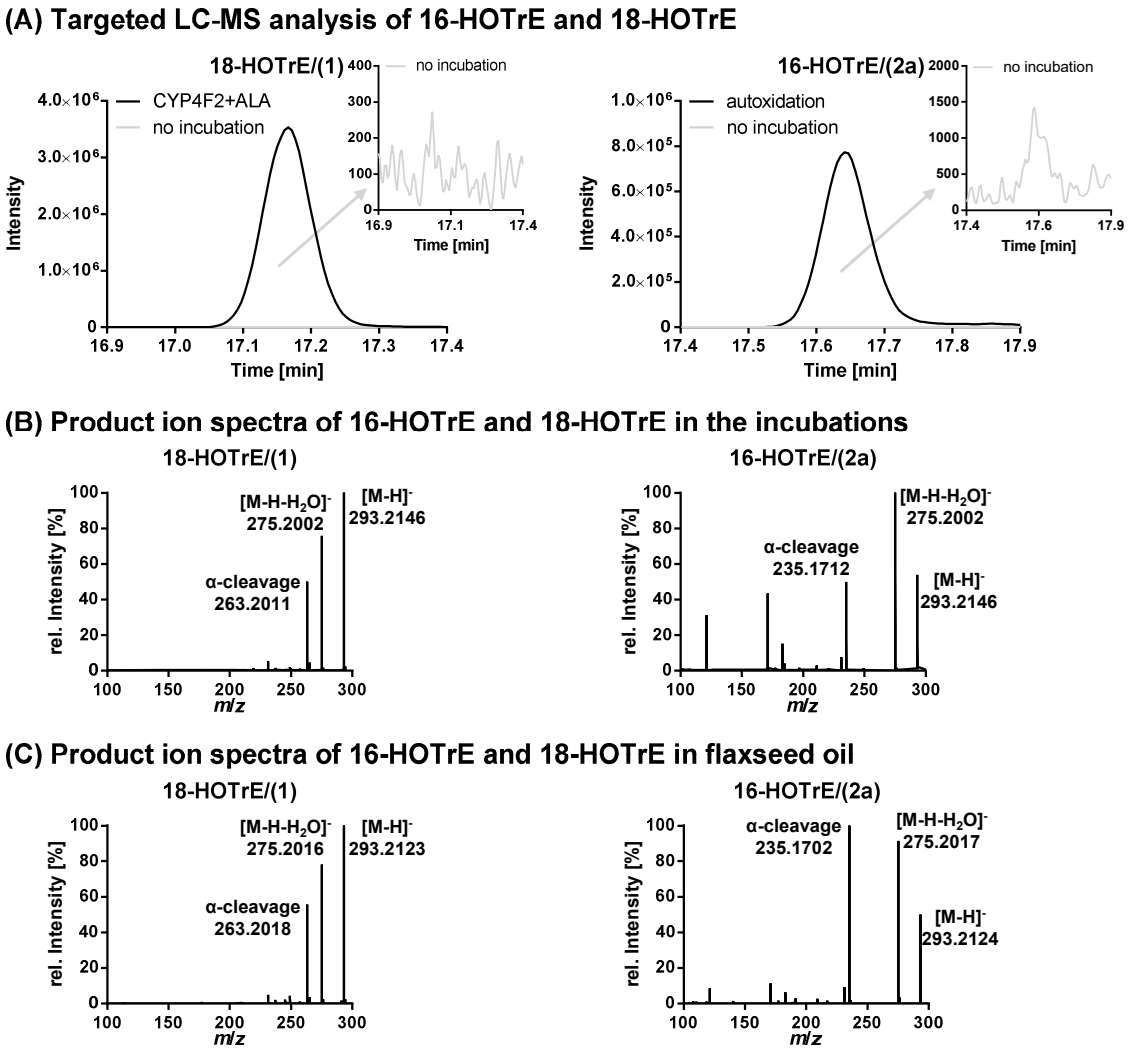

Fig. 11.25: Tentative identification of 18-HOTrE and 16-HOTrE. Shown is the formation of 18-HOTrE by incubation of α-linolenic acid with cytochrome P450 monooxygenase 4F2 specifically catalyzing the terminal hydroxylation of fatty acids and 16-HOTrE by autoxidation of α-linolenic acid induced by addition of *tert*-butyl hydroperoxide.

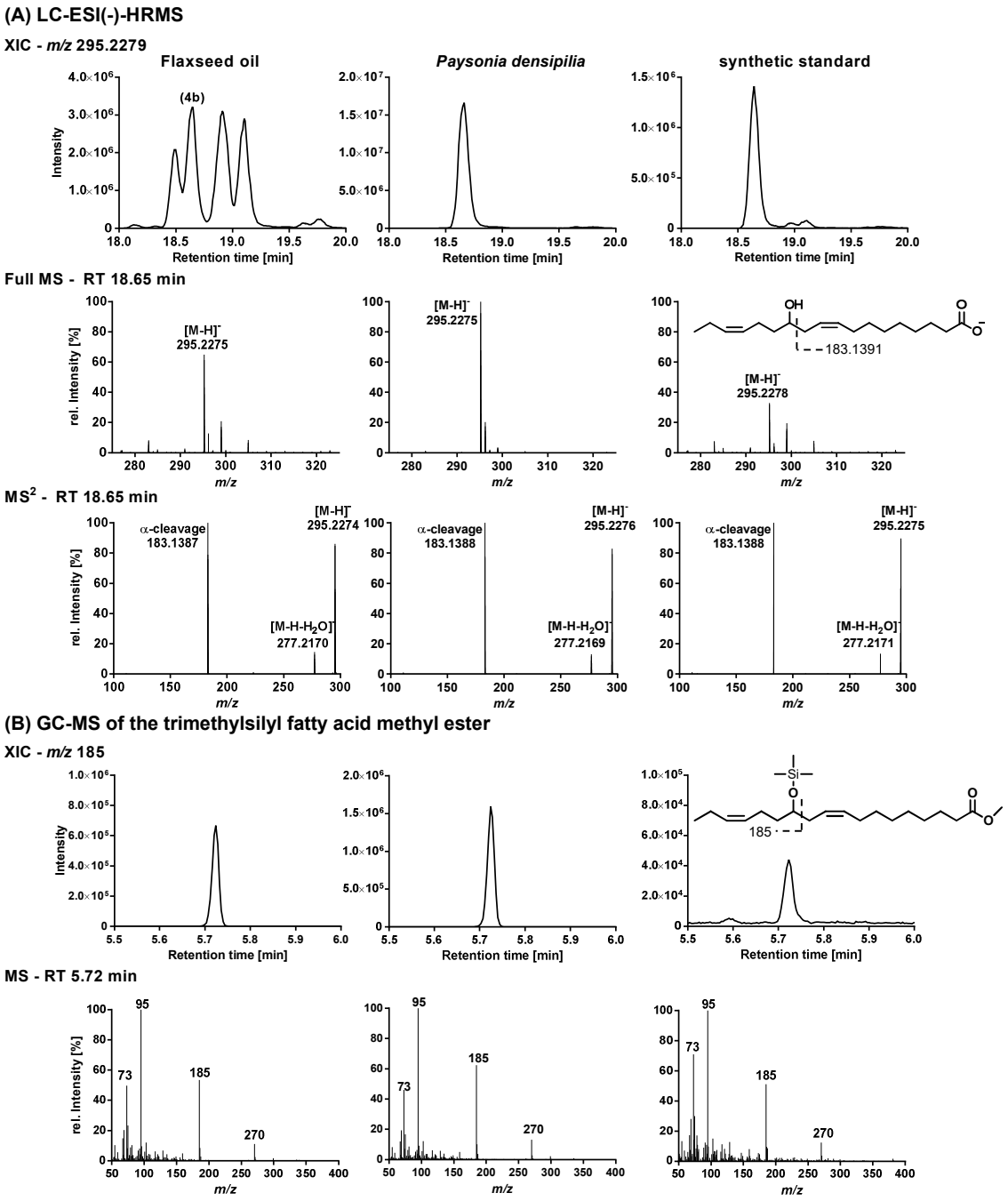

Fig. 11.26: Identification of densipolic acid. Shown is in (A) the extracted ion chromatogram (XIC) at *m/z* 295.2279 (C18:2 + O) of the LC-HRMS analysis (scan range *m*/*z* 150-500) of hydrolyzed flaxseed oil, hydrolyzed *Paysonia densipila* seed lipid extract and a synthetic densipolic acid standard. Additionally, the MS spectra and product ion spectra of the peak at 18.65 min are shown. In (B) a XIC at *m/z* 185 of the GC-MS analysis after derivatization with trimethylsilyl diazomethane and BSTFA to the corresponding trimethylsilyl fatty acid methyl ester and their corresponding mass spectra at 5.72 min are shown.

Abbreviations

ACN	acetonitrile
ALA	α-linolenic acid (C18:3 n3)
ARA	arachidonic acid (C20:4 n6)
BHT	butylated hydroxytoluene
BSA	bovine serum albumine
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CE	cholesterol ester
CE	collision energy
CID	collision-induced dissociation
COX	cyclooxygenase
CYP	cytochrome P450 monooxygenase
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid (C22:6 n3)
DiHDPE	dihydroxy docosapentaenoic acid
DiHETE	dihydroxy eicosatetraenoic acid
DiHETrE	dihydroxy eicosatrienoic acid
DiHODE	dihydroxy octadecadienoic acid
DiHOME	dihydroxy octadecenoic acid
DMDS	dimethyl disulfide
DP	declustering potential
n3-DPA	docosapenatenoic acid (C22:5 n3)
n6-DPA	docosapenatenoic acid (C22:5 n6)
EA	ethyl acetate
EDTA	ethylenediaminetetraacetic acid
EE	ethyl ester

EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	eicosapentaenoic acid (C20:5 n3)
EpDPE	epoxy docosapentaenoic acid
EpEDE	epoxy eicosadienoic acid
EpETE	epoxy eicosatetraenoic acid
EpETrE	epoxy eicosatrienoic acid
EpODE	epoxy octadecadienoic acid
EpOME	epoxy octadecenoic acid
ESI	electrospray ionization
EtOH	ethanol
FA	fatty acid
FAD3	fatty acid desaturase 3
FFA	free fatty acid
FAME	fatty acid methyl ester
FID	flame ionization detector
FWHM	full width at half maximum
GC	gas chromatography
GLA	γ-linolenic acid
HAc	acetic acid
HDHA	hydroxy docosahexaenoic acid
HEPE	hydroxy eicosapentaenoic acid
HETE	hydroxy eicosatetraenoic acid
HETrE	hydroxy eicosatrienoic acid
HODE	hydroxy octadecadienoic acid
HOTrE	hydroxy octadecatrienoic acid
HpODE	hydroperoxy octadecadienoic acid
HpOTrE	hydroperoxy octadecatrienoic acid
HRMS	high resolution mass spectrometry
*i*Prop	*iso*-propanol
IS	internal standard

IsoP	isoprostane
k	retention factor
KOH	potassium hydroxide
LA	linoleic acid
LC	liquid chromatography
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
LOD	limit of detection
LOX	lipoxygenase
LT	leukotriene
Lx	lipoxin
m/z	mass to charge ratio
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
NEFA	non-esterified fatty acids
*n*Hex	*n*-hexane
oxo-ETE	oxo eicosatetraenoic acid
oxo-ODE	oxo octadecadienoic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin
PL	phospholipid
PP	protein precipitation
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
PV	peroxide value
QC	quality control
R	resolution

RP	reversed-phase
RSD	relative standard deviation
RT	retention time
Rv	resolvin
S/N	signal to noise ratio
SD	standard deviation
sEH	soluble epoxide hydrolase
SEM	standard error of the mean
SPE	solid phase extraction
SPM	specialized pro-resolving mediator
SRM	selected reaction monitoring
t-AUCB	*trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
t-BOOH	*tert*-butyl hydroperoxide
TG	triacylglycerole
TMS	trimethylsilyl
TMSH	trimethylsilyl sulfonium hydroxide
TriHODE	trihydroxy octadecadienoic acid
TriHOME	trihydroxy octadecenoic acid
Tx	thromboxane

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Curriculum Vitae

Der Lebenslauf ist in der elektronischen Version aus Datenschutzgründen nicht enthalten.

List of Publications

Publications in peer-reviewed Journals

Within the Scope of this Thesis

Elisabeth Koch, Ariane Löwen and Nils Helge Schebb. *Do meals contain a relevant amount of oxylipins? LC-MS-based analysis of oxidized fatty acids in food*. Food Chem **2024**, *438*, 137941.

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