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Human lipoxygenase isoforms form complex patterns of double and triple oxygenated compounds from eicosapentaenoic acid

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ABSTRACT

Lipoxygenases (ALOX) are lipid peroxidizing enzymes that catalyze the biosynthesis of pro- and anti-inflammatory lipid mediators and have been implicated in (patho-)physiological processes. In humans, six functional ALOX isoforms exist and their arachidonic acid oxygenation products have been characterized. Products include leukotrienes and lipoxins which are involved in the regulation of inflammation and resolution. Oxygenation of n3-polyunsaturated fatty acids gives rise to specialized pro-resolving mediators, *e.g.* resolvins. However, the catalytic activity of different ALOX isoforms can lead to a multitude of potentially bioactive products. Here, we characterized the patterns of oxygenation products formed by human recombinant ALOX5, ALOX15, ALOX15B and ALOX12 from eicosapentaenoic acid (EPA) and its 18-hydroxy derivative 18-HEPE with particular emphasis on double and triple oxygenation products. ALOX15 and ALOX5 formed a complex mixture of various double oxygenation products from EPA, which include 5,15-diHEPE and various 8,15-diHEPE isomers. Their biosynthetic mechanisms were explored using heavy oxygen isotopes ($H_2{}^{18}O$, $^{18}O_2$ gas) and three catalytic activities contributed to product formation: i) fatty acid oxygenase activity, ii) leukotriene synthase activity, iii) lipohydroperoxidase activity. For ALOX15B and ALOX12 more specific product patterns were identified, which was also the case when these enzymes reacted in concert with ALOX5. Several double oxygenated compounds were formed from 18-HEPE by ALOX5, ALOX15B and ALOX12 including previously identified resolvins (RvE2, RvE3), while formation of triple oxygenation products, *e.g.* 5,17,18-triHEPE, required ALOX5. Taken together our data show that EPA can be converted by human ALOX isoforms to a large number of secondary oxygenation products, which might exhibit bioactivity.

1. Introduction

Lipoxygenases (ALOX isoforms) are non-heme iron containing enzymes that catalyze the oxygenation of polyunsaturated fatty acids (PUFA) by a sequence of steps involving hydrogen abstraction, rearrangement of double bonds, insertion of molecular oxygen and peroxy radical reduction leading to the formation of hydroperoxy PUFA. These primary oxygenation products can either be reduced to the respective hydroxy derivatives or can be converted to more complex oxygenation products *via* alternative secondary reactions [1]. Structural

Abbreviations: ACN, acetonitrile; ALOX/LOX, lipoxygenase(s); ARA, arachidonic acid; CE, collision energy; CID, collision induced dissociation; CXP, collision cell exit potential; diHEPE, dihydroxy eicosapentaenoic acid; diHETE, dihydroxy eicosatetraenoic acid; DP, declustering potential; EP, entrance potential; EPA, eicosapentaenoic acid; EpETE, epoxy eicosatetraenoic acid; HOAc, acetic acid; H(*p*)EPE, hydro(pero)xy eicosapentaenoic acid; H(*p*)ETE, hydro(pero)xy eicosatetraenoic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LT, leukotriene; LX, lipoxin(s); MeOH, methanol; MRM, multiple reaction monitoring; n3/n6/n9, omega-3/6/9; PUFA, polyunsaturated fatty acid(s); Rv, resolvin(s); SIM, selected ion monitoring; SPM, specialized pro-resolving lipid mediator; TIC, total ion chromatogram; triHEPE, trihydroxy eicosapentaenoic acid; triHETE, trihydroxy eicosatetraenoic acid

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prerequisite for a fatty acid to be oxygenized by an ALOX isoform is the presence of at least one bisallylic methylene. Therefore, PUFAs such as arachidonic acid (ARA) carrying several bisallylic methylenes can be oxidized by different ALOX isoforms to several hydroperoxy products. There are six functional *ALOX* genes in the human genome (*ALOX5*, *ALOX12*, *ALOX12B*, *ALOX15*, *ALOX15B*, *ALOXE3*) encoding for six functionally distinct ALOX isoforms. The nomenclature of ALOX isoforms is still a matter of discussion. However, according to the gene-based nomenclature the enzymes can be named as ALOX5, ALOX12, ALOX12B, ALOX15, ALOX15B and ALOXE3 [1].

Although the major ARA lipoxygenation products formed by the different human ALOX isoforms are well characterized, the overall product pattern is more complex. Some ALOX isoforms exhibit dual reaction specificity, *e.g.* human ALOX15 catalyzes the synthesis of both 15- and 12-HETE in a ratio of about 10:1 [2]. Furthermore, some ALOX isoforms exhibit multiple catalytic activities, by which hydroperoxy PUFA formed during the oxygenase reaction can be converted to secondary decomposition products [3]. For instance, human ALOX5 exhibits in addition to its fatty acid dioxygenase activity a 5,6-leukotriene A_4 (LTA_4) synthase activity [4]. For rabbit ALOX15 additional lipohydroperoxidase [5] and 14,15-LTA_4 synthase [6] activities were observed. Alternative alignments of substrate fatty acids at the active site of a given ALOX isoform may also contribute to the formation of more complex product patterns [7]. Since the primary ALOX products of most PUFA such as 15-H(*p*)ETE and 5-H(*p*)ETE still carry bisallylic methylenes they also constitute ALOX substrates. Their enzyme catalyzed dioxygenation can also contribute to the formation of a complex product mixture. The formation of double lipoxygenation products was first described for soybean LOX-1, which oxygenated its primary ARA oxygenation product 15(*S*)-HpETE to a mixture of 8(*S*),15(*S*)- and 5(*S*),15(*S*)-diHpETE at lower rates [8,9]. Recombinant human ALOX15 also accepts hydro(pero)xy PUFA as substrate and incubation of this enzyme with the ALOX5 product 5-HpETE led to the formation of 5,15- and 5,12-diH(*p*)ETE isomers [10]. Moreover, rabbit and pig ALOX15 orthologs exhibit LTA_4 synthase activities leading to the formation of epoxy PUFA that spontaneously hydrolyze to form dihydroxy eicosanoids [3]. When pure native rabbit ALOX15 was incubated *in vitro* with 15-HETE derivatives large amounts of conjugated trienes such as 8,15-, 5,15- and 14,15-diHETE were formed [11]. In addition, a mixture of conjugated tetraenes was detected [12] and 5,14,15-triHETE was identified as major representative. Experiments with $^{17}O_2$ gas indicated that the oxygen atoms at C5 and C14 originated from atmospheric oxygen [11]. Such trihydroxy PUFA were also described by Serhan et al. [13,14] as a result of interactions between the ALOX5 and ALOX15 pathways leading to the formation of lipoxin A_4 (5,6,15-triHETE; LXA_4) and lipoxin B_4 (5,14,15-triHETE; LXB_4). However, as opposed to the triple oxygenation pathway the epoxide hydrolysis pathway has been suggested as major biosynthetic route in these experiments. Another biosynthetic route for lipoxins involves a coordinated action of ALOX5 and ALOX12, which converts the ALOX5 product 5,6-LTA_4 to LXA_4 or LXB_4 [15].

In order to appreciate the capacity of human ALOX isoforms for the formation of bioactive multiple hydroxylated PUFA, it is crucial to understand their capability to form di- and trihydroxylated PUFA. The formation of such products from ARA was previously explored in different cellular and subcellular *in vitro* assays [16]. Apart from the early recognized chemotactic properties of LTB_4 [17], biological activities have also been described for double lipoxygenation products from ARA. For example, 8(*S*),15(*S*)-diHETE, which is formed by ALOX15, attenuated LTB_4-induced hyperalgesia [18] and 5(*S*),12(*S*)-diHETE, which is formed by the consecutive action of ALOX5 and ALOX12, showed inhibitory effects on platelet aggregation [19]. Moreover, double and triple oxygenated products of n3-PUFA *e.g.* eicosapentaenoic acid (EPA), such as the E-series resolvins, exhibit anti-inflammatory, pro-resolving activities in nanogram doses [20,21]. For ARA 12- and 15-lipoxygenating ALOX isoforms (ALOX15, ALOX15B, ALOX12) as well as for human ALOX5, EPA constitutes an effective substrate [22,23]. Enzymatic oxygenation of this PUFA may lead to the formation of structurally similar multiple hydroxylated products, which might exhibit biological activities. When EPA was incubated *in vitro* with human PMNs the formation of multiple hydroxylated products, such as 5,6-diHEPE and various LTB_5 isomers (5,12-diHEPE) was observed [24]. Furthermore, as described for ARA, the interaction of different ALOX isoforms leads to the formation of bioactive di- or trihydroxylated fatty acids from EPA, such as the lipoxenes (EPA derived lipoxins) [25].

In order to further investigate the biosynthesis of potentially bioactive EPA derived double and triple oxygenated compounds, we analyzed the product patterns formed from EPA by the catalytic activities of four recombinant human ALOX isoforms (ALOX15, ALOX15B, ALOX12, ALOX5) under identical experimental conditions. Moreover, we compared the product patterns produced during single enzyme incubations with those formed during consecutive and simultaneous incubations of two different ALOX isoforms. Finally, synthetic 18(*R*,*S*)-HEPE was applied as substrate for the ALOX isoforms in order to investigate the formation of specialized pro-resolving mediators (SPMs) by ALOX catalyzed oxygenation of 18-HEPE.

2. Material and methods

2.1. Chemicals

Fatty acids (*cis*-5,8,11,14,17-eicosapentaenoic acid, EPA), oxylipin standard substances and deuterated internal standards (IS) were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany) except $^2H_5$-*cis*-5,8,11,14,17-eicosapentaenoic acid, which was purchased from Sigma Aldrich (Taufkirchen, Germany). Standards for RvE2, 18(*S*)- and 18(*R*)-RvE3 were a kind gift of the lab of Makoto Arita (RIKEN Center for Integrative Medical Sciences, Japan). 18(*R*,*S*)-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid (18(*R*,*S*)-HEPE) was synthesized as described elsewhere. $H_2{}^{18}O$ (97% isotopic purity) and $^{18}O_2$ gas (99% isotopic purity) were purchased from Sigma Aldrich (Taufkirchen, Germany). LC-MS grade acetic acid (HOAc), methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific (Schwerte, Germany). Disodium hydrogen phosphate dihydrate and *n*-hexane were purchased from Carl Roth (Karlsruhe, Germany). All other chemicals were obtained from Sigma Aldrich (Schnelldorf, Germany) or VWR (Darmstadt, Germany).

2.2. Enzyme expression

Human ALOX isoforms were expressed as N-terminal His-tag fusion proteins in *E. coli* using the protocols described previously [26]. In brief, the coding regions of human ALOX cDNAs were sub-cloned into the bacterial expression plasmid pET28b. Competent bacteria [Rosetta 2 DE3 pLysS] were transformed with 100–400 ng of the pET28b-ALOX plasmids and cells were grown on kanamycin containing agar plates. Two well-separated bacterial clones were selected for each enzyme and five 1 mL bacterial pre-cultures (LB medium with 50 μg/mL kanamycin and 35 μg/mL chloramphenicol) were grown at 37 °C for 6 h and 180 rpm agitation. When the pre-culture reached an optical density (OD) at 600 nm of 0.1–0.15 at 1:50 dilution an appropriate amount of the pre-culture was added to a 50 mL main culture to reach an OD600 of 0.15. The main culture was grown overnight at 30 °C and the culture was continuously shaken at 250 rpm in Ultra Yield flasks (Thomson Instrument Company, Oceanside, USA) covered with an air top seal (Fisher Scientific, Schwerte, Germany). The culture medium was prepared from glucose-free minimal essential medium by the addition of trace elements and was supplemented with 40 g/L dextrin, 0.24 g/L tryptone/peptone and 0.48 g/L yeast extract. Before starting the bacterial culture, antibiotics and glucose were added. Finally, 100 μL 1:20 diluted antifoam 204 (Sigma, Deisenhofen, Germany) and 50 μL glycoamylase from Aspergillus niger (Amylase AG 300 L, Novozymes,

Bagsværd, Denmark) were also supplied. Expression of the recombinant enzymes was induced by the addition of 1 mM (final concentration) isopropyl-β-thiogalactopyranoside (IPTG). The cultures were incubated for 24 h at 22 °C and 230–250 rpm agitation. Bacteria were harvested by centrifugation and the resulting pellet was reconstituted in 5 mL PBS. Cells were lysed by sonication, cell debris was spun down and the lysis supernatant was used for direct activity assays without further purification.

2.3. Enzyme incubation

Enzyme preparations were incubated with ARA (100 μM, 3 min) and main products (monohydroxy PUFA) were quantified with LC-UV. Enzyme preparations were adjusted to equal ARA oxygenase activity and used for EPA or 18(*R*,*S*)-HEPE incubations.

To explore patterns of oxygenation products aliquots of enzyme preparations were incubated alone or in combination with ALOX5 in 250 μL PBS containing a final concentration of 10 μM EPA or 18(*R*,*S*)-HEPE. For incubations involving ALOX5, buffer contained additionally ATP (0.1 mM), $CaCl_2$ (0.4 mM), EDTA (0.1 mM) and dipalmitoylphosphatidylcholine (1.4 μg/mL). For the single enzyme incubations, the reaction mixture was incubated for 15 min at room temperature (RT). For the combined enzyme incubations, after 15 min an aliquot of the ALOX5 preparation was added to the reaction mixture and the samples were incubated for additional 15 min. Simultaneous incubations and incubations in reversed order were carried out for the combinations ALOX12/ALOX5 and ALOX15B/ALOX5. No-enzyme control incubations were performed using 250 μL PBS and 10 μM substrate. The hydroperoxides were reduced with 25 μL methanolic $SnCl_2$ (10 mg/mL), or by the addition of 1 mg of solid $NaBH_4$ and afterwards acidified with 15 μL HOAc. 250 μL of ice-cold MeOH were added, protein precipitates were spun down and the protein free supernatants were stored at −80 °C until analysis.

In selected experiments ALOX-catalyzed fatty acid oxygenation was carried out under $^{18}O_2$ atmosphere. For this purpose, we first anaerobized an appropriate volume (5 mL) of PBS containing the substrate fatty acid by flushing the solution extensively (30 min) with argon gas. Then, we slowly bubbled $^{18}O_2$ gas through this solution so that the sample was saturated with $^{18}O_2$ gas. 250 μL of this solution were transferred to a 500 μL reaction tube, which was previously filled with argon gas. Next, $^{18}O_2$ gas was bubbled through this solution to form an $^{18}O_2$ atmosphere above the sample. Finally, the reaction was initiated by the addition of small volumes of partly anaerobized enzyme solutions (stored for 1 h under argon atmosphere).

To carry out incubations in $H_2{}^{18}O$ buffer 50 μL of 20-fold concentrated PBS were added to 1 mL of $H_2{}^{18}O$. Small amounts of a methanolic stock solution of the substrate were added to 250 μL $H_2{}^{18}O$ buffer and the reaction was initiated by the addition of small volumes of enzyme preparation. Fatty acid hydroperoxides were reduced by the addition of 1 mg $NaBH_4$ and after acidification (addition of 25 μL HOAc) proteins were precipitated by the addition of 500 μL ACN. Precipitated proteins were spun down and the protein free supernatants were stored at −80 °C until analysis.

2.4. MS/MS experiments

For LC-MS/MS analysis, an aliquot of the sample was diluted with MeOH, 10 μL antioxidant mixture were added (containing 0.2 mg/mL BHT, 10 μM indomethacin and 10 μM *t*-AUCB in MeOH) as well as 10 μL of deuterated oxylipin internal standards (IS; 100 nM in MeOH). The sample mixture was vortexed and centrifuged (10 min, 4 °C, 20,000 ×*g*), supernatant was diluted with 0.1 M disodium hydrogen phosphate buffer (pH 6), adjusted to pH 6 and a MeOH content of approximately 15% and applied to the preconditioned SPE cartridge (Bond Elut Certify II, Agilent, Waldbronn, Germany). Cartridges were prepared and samples applied as described and free oxylipins were eluted with ethyl acetate/*n*-hexane (75:25) containing 1% HOAc [27,28]. Oxylipins were quantified in scheduled multiple reaction monitoring (MRM) mode on an AB Sciex QTRAP instrument (5500/6500; SCIEX, Darmstadt, Germany) using external calibration and 20 deuterated IS [27,28]. Additionally, EPA was quantified using the same LC-MS/MS method as for oxylipin analysis (*m/z* 301.3 ➔ 257.1, DP -80 V, CE -16 V, CXP -6 V) with $^2H_5$-EPA as IS (*m/z* 306.1 ➔ 262.2, DP -80 V, CE -16 V, CXP -6 V). Analyst 1.6.2 (SCIEX) was used for operating the instrument and qualitative analyses and Multiquant 2.1.1 (SCIEX) was used for data quantification.

For semi-quantitative measurement of the sum of EPA derived ALOX products, sample extracts were measured in selected ion monitoring (SIM) mode: *m/z* 317.2 and DP -75 V for mono-, *m/z* 333.2 and DP -90 V for double, and *m/z* 349.2 and DP -80 V for triple oxygenated products in different dilutions of the sample extract. For monohydroxy fatty acids, only the abundance of major products (5-, 12-, 15-HEPE) that were identified based on retention time of authentic standards were included. For double and triple oxygenated compounds, peaks eluting between 9.0–17.0 min (di-OH) or 5.0–13.0 min (tri-OH), exceeding a height of 5×10^5 cps and signal of no-enzyme incubation were included. Obtained peak areas of mono- as well as putative double and triple oxygenated products were summed with consideration of the dilution factor and expressed as relative share of total products detected for each sample.

For qualitative evaluation of formed products with no authentic standards commercially available to date, collision induced dissociation (CID) product ion spectra were recorded for double (*m/z* 333.2) and triple oxygenated (*m/z* 349.2) products with the following electronic parameters: CE ramp from −18 V to −26 V, DP -80 V, EP -10 V, CXP -11 V, a scan rate of 1000 Da/s and a scan range of 100–340/350 Da. In the EPA and 18(*R*,*S*)-HEPE incubations those peaks were labeled as products, which largely exceeded the no enzyme and no substrate control incubations. Molecular structures (position of OH-groups) were concluded from fragmentation patterns based on typical fragmentation mechanisms described for eicosanoids [29]. For unexpected formation of ions with even numbered *m/z*, sites of fragmentation were suggested despite the deviation of 1 Da between expected and observed fragment ion mass. To support several of the suggested structures of the major double oxygenated ALOX5 and ALOX15 products as well as their dominant formation route product ion spectra were obtained for $^{18}O_2$- and $H_2{}^{18}O$-incubation mixtures: *m/z* 333.2 for $^{16}O/^{16}O$, *m/z* 335.2 for $^{16}O/^{18}O$ and *m/z* 337.2 for $^{18}O/^{18}O$ (CE ramp −18 V to −26 V, DP -80 V, EP -10 V, CXP -11 V, scan rate 1000 Da/s, scan range 55–340 Da).

2.5. Chiral LC-MS/MS analysis of 18-HEPE

Separation of 18-HEPE enantiomers in ALOX incubation mixtures was carried out as described by Blum et al. [30]. In brief, an Agilent ZORBAX SB-C8 column was coupled to a Lux-Amylose-1 column. 10 μL sample extract (as described above) were injected and analytes were separated with a linear gradient of ACN/MeOH/H_2O/glacial HOAc starting at 27:3:70:0.05 and ending at 63:7:30:0.05 after 30 min with a flow rate of 0.4 mL/min utilizing an Agilent 1290 infinity UHPLC system. Detection was carried out using an Agilent 6490 Triple Quad MS (Agilent, Waldbronn, Germany) operated in negative ESI-mode using multiple reaction monitoring (MRM).

2.6. Data analysis

Incubations of enzymes were carried out as independent replicates as indicated in the figure legend. For quantitative evaluation of enzyme incubations, mean ± SD (n = 3) were calculated using Microsoft Office Excel 2016 software (Redmond, WA, USA). Figures and graphs were generated with GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA).

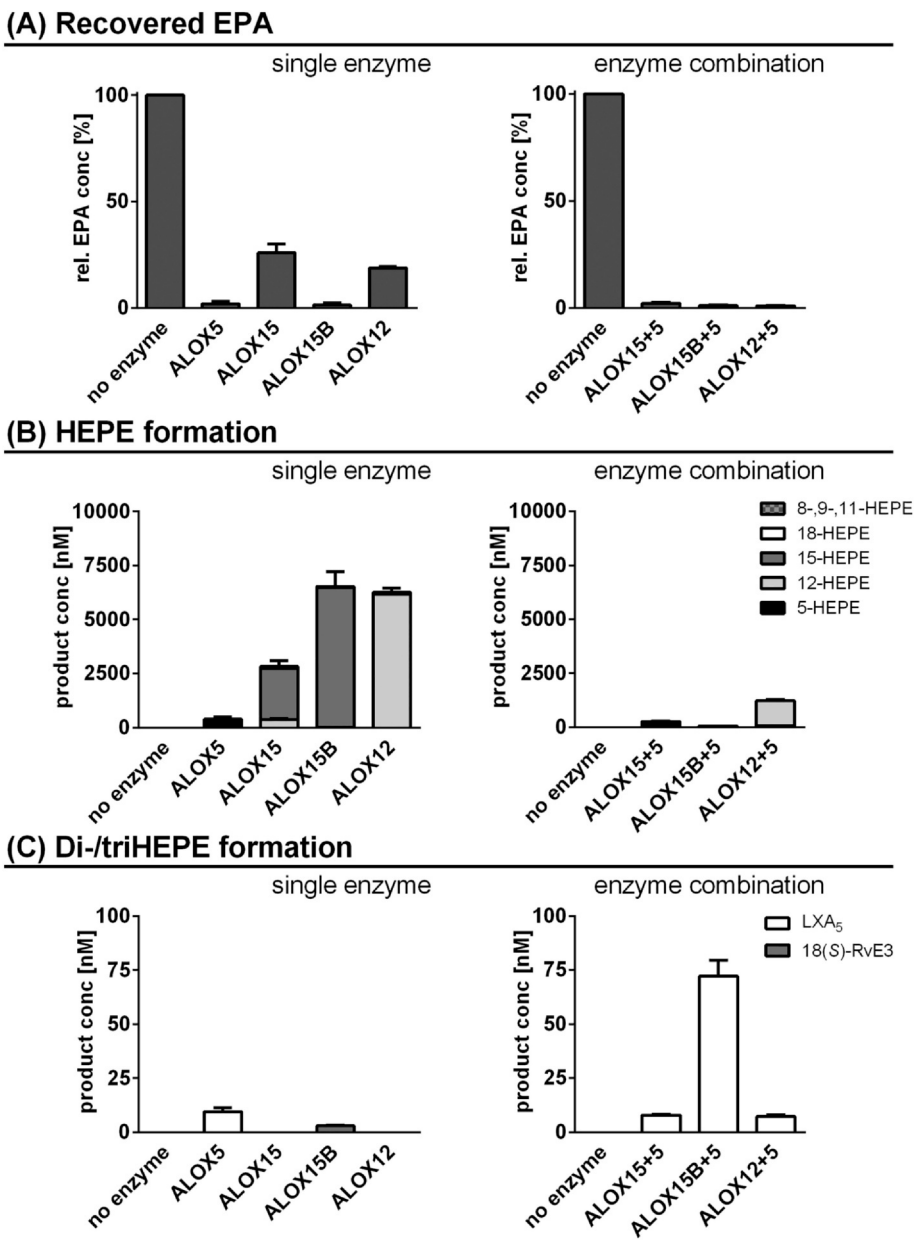

Fig. 1. Formation and quantification of well-established single, double and triple oxygenation products from EPA by recombinant human ALOX isoforms. Single and combined enzyme incubations were carried out as described in Materials and methods (10 μM EPA, 15 min, RT; n = 3) and remaining substrate (EPA) as well as specific mono-, di- and tri-HEPEs that could be identified based on authentic standards were quantified by LC-MS/MS. (A) Recovered EPA relative to no enzyme incubation in %; (B) assay concentration of EPA derived hydroxy fatty acids (HEPEs); (C) assay concentration of EPA derived specialized pro-resolving mediators comprising di- and trihydroxy fatty acids (di-/triHEPEs).

3. Results

3.1. Product specificity of human ALOX isoforms with EPA alone and in combination with ALOX5

In previous studies we have identified the major oxygenation products formed from EPA by different human ALOX isoforms but we did not exactly quantify product formation [23]. To fill this gap, we first quantified the ARA oxygenase activity of our enzyme preparations (ALOX15, ALOX15B, ALOX12, ALOX5) in separate activity assays and adjusted the enzyme concentrations to similar ARA oxygenase activities. These normalized enzyme preparations were then employed for the EPA oxygenase assays and residual EPA as well as formed mono-hydro(pero)xy fatty acids were quantified. Recovered EPA varied between 1.4 and 26% (Fig. 1A, left) and these data demonstrate that EPA is accepted as substrate by all tested enzymes. When we incubated ALOX15, ALOX15B and ALOX12 consecutively with ALOX5 only 1–2% of EPA were recovered (Fig. 1A, right). These data indicate that the remaining EPA, which was not oxygenated during the first incubation period, was completely oxygenated by ALOX5 during the second incubation period. Next, we quantified the formation of major products 5-, 12- and 15-HpEPE, which were analyzed as their respective hydroxy derivatives (HEPEs) (Fig. 1B). Here, we confirmed our previous findings [23] that the ARA 15-lipoxygenating ALOX15B forms almost exclusively 15-HEPE (n6/n9 ratio > 100) and thus, the enzyme exhibits a singular reaction specificity with this particular substrate. In contrast, ALOX15 converted EPA to a mixture of 15- and 12-HEPE (n6/n9 ratio 6.2) and this data is consistent with our previous report [23]. Dominant 12-HEPE formation was shown for ALOX12 (n6/n9 ratio < 0.01) and as expected 5-HEPE was the major ALOX5 oxygenation product (Fig. 1B, left). Notably, formation of HEPEs differed considerably between the ALOX isoforms and compared to ALOX12 and ALOX15B significantly lower levels of mono-oxygenation products were quantified for ALOX15 and ALOX5 (Fig. 1B, left). It should be stressed at this point that minor amounts of other HEPEs, *i.e.* 8-, 9-, 11- and 18-HEPE, were also detected. However, the relative shares of these products varied between 0.08%–2% of total mono-oxygenation products.

Interestingly, HEPE formation during consecutive incubations of human ALOX15, ALOX15B and ALOX12 with human ALOX5 was clearly lower compared to single enzyme incubation (Fig. 1B, right),

while the recovered EPA indicated complete substrate consumption. Moreover, the ratios of 5-lipoxygenation product *vs.* 12/15-lipoxygenation product(s) were different for each enzyme combination. For the ALOX12 + ALOX5 combination 12-HEPE levels were higher than 5-HEPE levels (5-HEPE:12-HEPE 1:18). For the ALOX15 + ALOX5 combination 5-HEPE was the major mono-oxygenation product, whereas 12-HEPE and 15-HEPE were detected in lower amounts (5-HEPE > 12-HEPE > 15-HEPE = 32:11:1). For the ALOX15B + ALOX5 combination only small amounts of mono-HEPEs were detected with 15-HEPE being the major reaction product, while 5-HEPE was not detected (Fig. 1B, right).

Apparent EPA consumption and formation of monohydroxy PUFA indicate that ALOX isoforms are capable of converting EPA to further products other than HEPEs, which was particularly evident for ALOX5 single enzyme and combined incubations (Fig. 1A + B).

3.2. Formation of secondary and tertiary oxygenation products

In order to further explore the product pattern of human ALOX isoforms, we next investigated the formation of secondary and tertiary oxygenation products in the single and combined enzyme incubations. For instance, 15-HpEPE formed during the incubation of ALOX15 and ALOX15B can further be converted by ALOX5 to 5,15-diHpEPE. The same product could also be formed when ALOX15 or ALOX15B employ their own EPA oxygenation product (15-HpEPE) as substrate for secondary oxygenation. When we quantified specific di- and triHEPE compounds, we found the formation of small amounts of lipoxin A_5 [5(*S*),6(*R*),15(*S*)-triHEPE] in the ALOX5 sample (Fig. 1C, left), which was also formed when ALOX5 was combined with ALOX15, ALOX15B or ALOX12 (Fig. 1C, right). Only small amounts of 18(*S*)-RvE3 [17(*R*),18(*S*)-diHEPE] were detected in the ALOX15B incubation sample (Fig. 1C, left), while detected levels of RvE2 [5(*S*),18(*R*)-diHEPE] were not considered because MRM transitions were inconsistent (Supplement, Fig. S1).

Due to the low formation of these specific double and triple oxygenated compounds, we next screened for EPA derivatives containing two or three oxygen atoms (*m/z* 333.2 or *m/z* 349.2, respectively). An estimation for the relative shares of mono-, double and triple oxygenated EPA derivatives in the single and combined enzyme incubation samples based on peak areas (SIM, without fragmentation) is summarized in Table 1. We found that for ALOX12 and ALOX15B the relative shares of the mono-oxygenated EPA derivatives were dominant. Only minor amounts (less than 5%) of presumed double and triple oxygenated EPA derivatives (*m/z* 333.2, *m/z* 349.2) were formed. For ALOX15 the monohydroxy derivatives contributed 73% to the product mixture. With this enzyme about 25% double oxygenated EPA derivatives were formed. Surprisingly, for ALOX5 an inverse relation was determined. Here, the mono-oxygenated EPA derivative (5-HEPE) only contributed 29% to the product mixture, whereas the relative share of double oxygenated products was 70%. In the consecutive incubations the relative shares of mono-oxygenation products were lower when compared with the single enzyme incubations. Here, double oxygenation products prevailed for most enzyme combinations. In fact, for ALOX15B + ALOX5 combination almost 90% of the reaction products were double oxygenated derivatives and with this enzyme combination we observed up to 8% triple oxygenation products (Table 1). A similar share of triple oxygenation products was also found for ALOX15 + ALOX5 combination.

Table 1
Relative distribution of primary and secondary oxygenation products for ALOX5, ALOX15, ALOX15B and ALOX12 incubations as well as combined incubations of these enzymes with ALOX5. Estimates for the share of mono-, double and triple oxygenated compounds were determined based on relative abundance (peak areas) at *m/z* 317.2, 333.2 and 349.2 in selected ion monitoring mode. Monohydroxy fatty acids at *m/z* 317.2 (5-HEPE, 12-HEPE, 15-HEPE) were identified by comparing their retention times with those of authentic standards. For double oxygenated compounds (*m/z* 333.2, retention time between 9.0 and 17 min) and triple oxygenated compounds (*m/z* 349.2, retention time between 5.0 and 13 min) areas of peaks exceeding a height of 5×10^5 cps and signal of no-enzyme incubation were summed ($n = 3$).

	m/z 317.2 [%]	*m/z* 333.2 [%]	*m/z* 349.2 [%]
ALOX5	29 ± 4	70 ± 4	1.1 ± 0.1
ALOX15	73 ± 3	25 ± 3	2.1 ± 0.05
ALOX15B	97 ± 0.5	3.0 ± 0.5	0.30 ± 0.04
ALOX12	98 ± 0.4	1.6 ± 0.2	0.79 ± 0.14
ALOX15 + 5	23 ± 4	73 ± 3	4.8 ± 0.7
ALOX15B + 5	3.0 ± 0.6	89 ± 1	8.1 ± 0.4
ALOX12 + 5	44 ± 2	53 ± 2	2.9 ± 0.03

3.3. Structural elucidation of secondary ALOX products of EPA

Suggested structures of the oxygenated EPA derivatives were tentatively identified based on their fragmentation patterns *via* LC-MS/MS of the most abundant double (Fig. 2) and selected triple oxygenated products (Supplement, Fig. S8). These products were absent in the no-enzyme controls, in which PBS was added to the incubation mixture instead of the enzyme preparation (Fig. 2). No-substrate controls, in which MeOH was added instead of EPA, were also run (Supplement, Fig. S13). The fragmentation patterns indicate the position of the different OH-groups as shown for representative fragmentation patterns in Fig. 3 (MS/MS data of all indicated compounds are summarized in the Supplement, Fig. S2). It should be noted that neither the configuration of the stereo-centers, *i.e.* of the hydroxy group bearing C-atoms (*S* or *R*) nor the configuration (*E* or *Z*) of the double bonds or their position can be deduced from fragmentation patterns.

First, we analyzed the double oxygenation EPA derivatives formed by ALOX5 (Fig. 2). Here, we found that two 5,12-diHEPE isomers (compounds 8, 9) were dominant and 5,6-diHEPE isomers (compounds 16, 19) were also present (Table 2). The most plausible explanation for the formation of these compounds is hydrolysis of a 5,6-LTA_5 intermediate. ALOX5 exhibits a leukotriene synthase activity [31] and thus is capable of converting ARA to 5,6-epoxy LTA_4. Our data indicate that a similar reaction does also proceed with EPA. Incubations of EPA with $^{18}O_2$ gas and in $H_2{}^{18}O$ buffer support the formation of 5,6- and 5,12-diHEPE *via* hydrolysis of an epoxide intermediate (Supplement, Fig. S3). Surprisingly, we also detected the formation of 5,15-diHEPE (compound 11) in the single enzyme incubation sample (Fig. 2, Table 2). Although we did not look in detail into the biosynthetic mechanism of this product, it might be possible that 5-HpEPE, which is formed as major mono-oxygenation product of EPA, is used as substrate by the enzyme for subsequent C15 oxygenation. This mechanistic scenario is supported by the formation of this product during incubations with $^{18}O_2$ gas and in $H_2{}^{18}O$ buffer (Supplement, Fig. S3). For effective oxygenation of C15 the substrate should be aligned inversely at the active site of the enzyme when compared with EPA.

When we analyzed the double oxygenated products formed by human ALOX15 we observed a rather complex product mixture and compounds 3, 4, 6, 14, 17 and 20 were dominant (Fig. 2). The suggested chemical structures of these compounds, which were deduced from their fragmentation patterns (Fig. 3; Supplement, Fig. S2), are summarized in Table 2. It can be seen that 8,15-dihydroxy isomers (compound 3, 4, 6) were the major double oxygenated products. These compounds can either be formed *via* oxygenation of 15-HpEPE by the enzyme or by spontaneous hydrolysis of a 14,15-LTA_5 intermediate. The formation of a 14,15-LTA_4 intermediate from ARA has previously been shown for purified rabbit ALOX15 [6]. Incubation of EPA and ALOX15 in $H_2{}^{18}O$ buffer indicate that while compounds 3 and 4 are formed by hydrolysis of an epoxide intermediate, compound 6 is more likely an oxygenation product of 15-HpEPE (Supplement, Figs. S4–5). In addition, we observed the formation of 14,15-dihydroxy EPA (compound 14) and this product can also be formed *via* hydrolysis of a

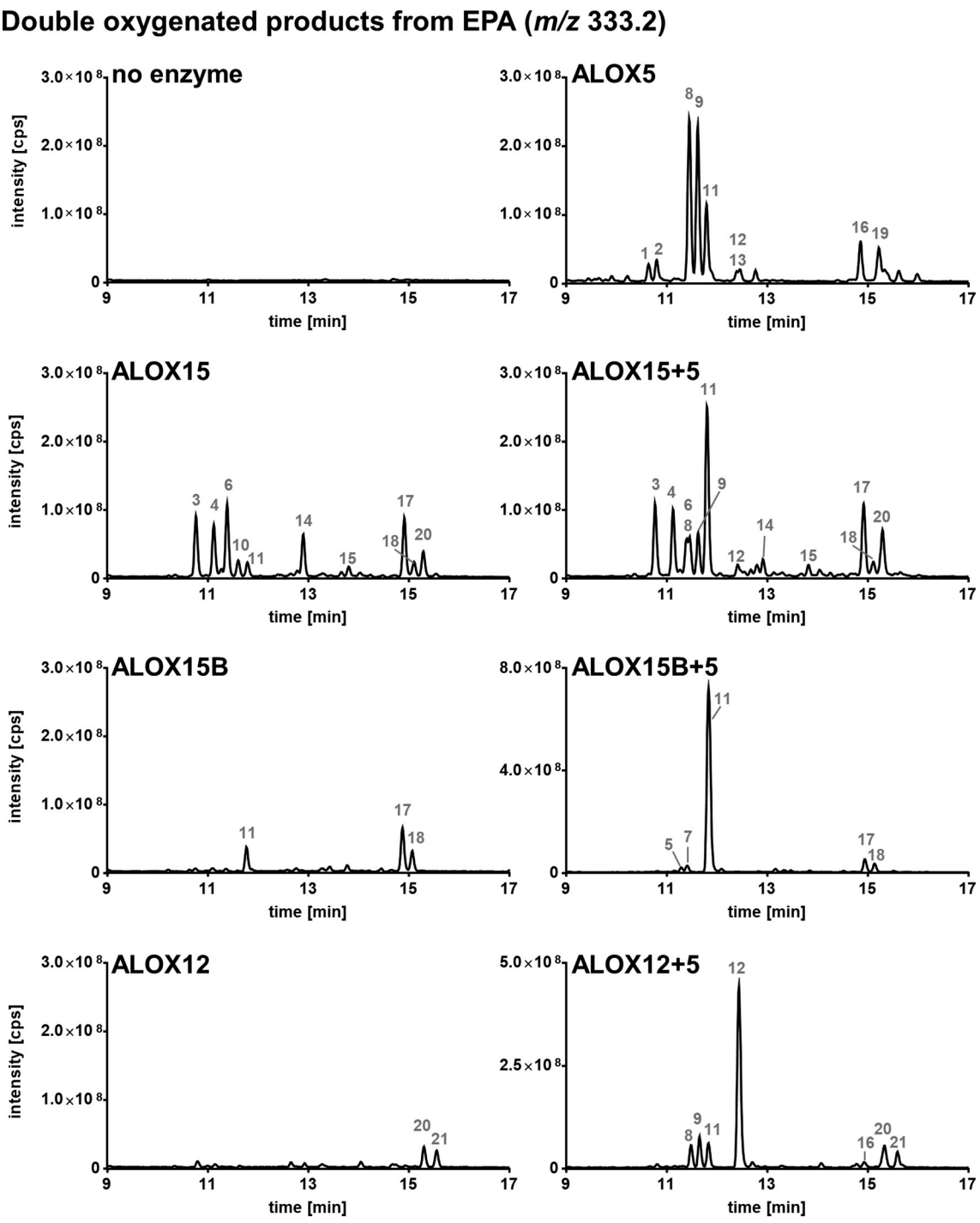

Fig. 2. Formation of double oxygenation products from EPA by recombinant human ALOX isoforms. Incubations were carried out as described in Materials and methods (10 μM EPA, 15 min, RT) and since only low levels of specific di- and triHEPEs were quantified (Fig. 1), single and combined enzyme incubations were screened for formation of further double oxygenated compounds from EPA. Shown are total ion chromatograms (TIC) of product ion scans of *m/z* 333.2 (100–350 Da, CE ramp −18 to −26 V). Major double oxygenation products are labeled as compounds 1–21 and listed in Table 2.

14,15-epoxide intermediate or *via* sequential double oxygenation of EPA, which seems to be the prevailing formation route under our experimental conditions (Supplement, Fig. S4). Finally, we observed the formation of two epoxy-hydroxy isomers [13-OH-14(15)-epoxy EPA (compound 17) and 10-OH-11(12)-epoxy EPA (compound 20)]. Epoxy-hydroxy fatty acids are typical hydroperoxidase products, which are formed when ALOX isoforms decompose their primary oxygenation products. This reaction is initiated by homolytic cleavage of the hydroperoxy group and secondary rearrangement of the alkoxy radical. This formation route is supported by the incubation of ALOX15 with $^{18}O_2$ gas indicating the insertion of two ^{18}O atoms (Supplement, Fig. S4). A similar pattern of secondary products has previously been described when ARA was incubated with pure rabbit ALOX15 [6].

Next, we analyzed the product pattern formed by consecutive incubation of ALOX15 and ALOX5 and observed a similarly complex product pattern (Fig. 2). We also detected 8,15-dihydroxy (compounds 3, 4, 6) and epoxy-hydroxy (compounds 17, 20) EPA derivatives. The major difference to the single ALOX15 incubation was that in the

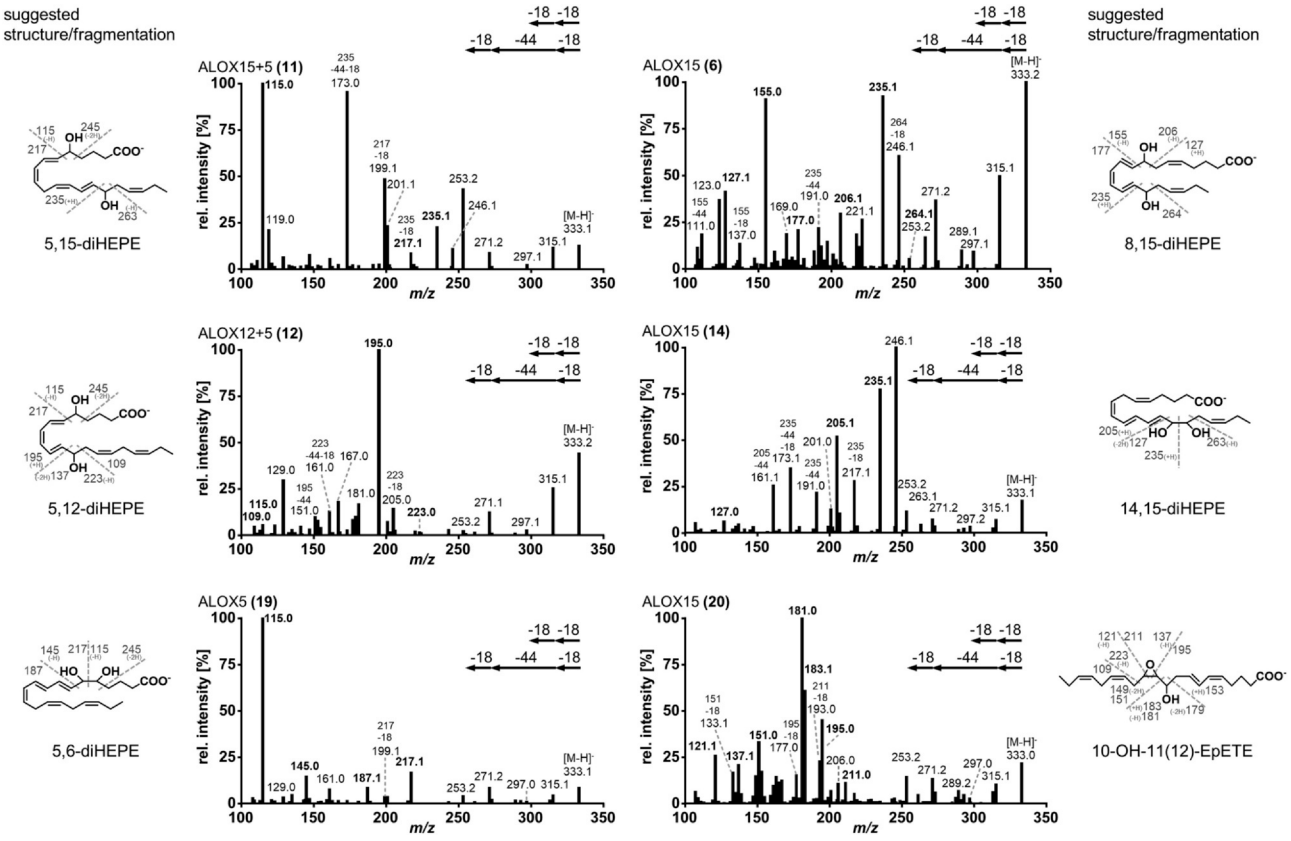

Fig. 3. Fragmentation patterns and suggested structures of representative double oxygenation products formed from EPA by different recombinant human ALOX isoforms. Human ALOX isoforms were incubated with EPA (10 μM, 15 min, RT) and the major double oxygenation products shown in Fig. 2 (no reference compound) were analyzed by mass spectrometry. Shown are collision induced dissociation product ion spectra of *m/z* 333.2 (100–350 Da, CE ramp −18 to −26 V) alongside suggested structure and fragmentation sites for representative compounds formed during incubation of ALOX15 + ALOX5 (compound 11), ALOX12 + ALOX5 (compound 12), ALOX5 (compound 19) as well as ALOX15 (compound 6, 14, 20). Note that only one of several possible double bond configurations is shown.

Table 2
Tentative structures of EPA derived double oxygenated products formed by different recombinant human ALOX isoforms. Chemical structures for EPA derived products (Fig. 2) were suggested based on their fragmentation patterns (Fig. 3, supplement Fig. S2). Compounds with similar fragmentation patterns and retention times were regarded as the same product. Marks (x) indicate enzyme incubations in which the respective compound was detected.

Compound	suggested positional isomer	ALOX						
		5	15	15+5	15B	15B+5	12	12+5
1	–	×						
2		×						
3	8,15-diHEPE		×	×				
4			×	×				
6			×	×				
10			×					
5	5,15-diHEPE					×		
7						×		
11		×	×	×	×	×		×
8	5,12-diHEPE	×		×				×
9		×		×				×
12		×		×				×
13		×						
14	14,15-diHEPE		×	×				
16	5,6-diHEPE	×						×
19		×						
15	13-OH-14(15)-EpETE		×	×				
17			×	×	×	×		
18			×	×	×	×		
20	10-OH-11(12)-EpETE		×	×			×	×
21							×	×

combined incubation large amounts of 5,15-diHEPE (compound 11) were formed. This product was only present in small amounts in the ALOX15 single incubation and originated from either ALOX5-catalyzed oxygenation of 15-H(p)EPE or ALOX15-catalyzed oxygenation of 5-H(p)EPE. In both cases, this product represents a double oxygenation product of EPA, which is supported by incubations in $^{18}O_2$ atmosphere and $H_2{}^{18}O$ buffer (Supplement, Fig. S6).

The pattern of the double oxygenated EPA derivatives formed by human ALOX15B was much less complex regardless of whether this enzyme reacted alone or in combination with ALOX5 (Fig. 2). The major products identified in the single enzyme incubation were two isomers of 13-OH-14(15)-epoxy EPA (compound 17, 18). These data indicate that ALOX15B does also exhibit a lipohydroperoxidase activity and thus, the enzyme is capable of converting its major EPA oxygenation product (15-HpEPE) to secondary decomposition products. To the best of our knowledge this has not been reported before for this enzyme. In addition, we observed the formation of 5,15-diHEPE (compound 11) and these data indicate that this enzyme is also capable of catalyzing C5 oxygenation. This result was somewhat surprising since ALOX15B exhibits a rather narrow substrate specificity [32]. The most plausible scenario for the formation of this compound is that after the enzyme has completely consumed EPA (Fig. 1A) it may bind 15-HpEPE at the active site in an inverse head-to-tail like fashion and then introduces dioxygen at C5. Similar products/mechanisms have previously been suggested for ARA conversion by soybean LOX-1 [9] and rabbit ALOX15 [6]. When ALOX15B acted in concert with ALOX5 (Fig. 2) 5,15-diHEPE was identified as dominant oxygenation product and the biosynthetic pathway (sequential double oxygenation of EPA by ALOX15B and ALOX5) of this product is straightforward.

Analyzing the pattern of double oxygenated compounds formed by ALOX12 we observed small amounts of 10-OH-11(12)-epoxy EPA (compounds 20, 21; Fig. 2). These compounds constitute secondary decomposition products of 12-HpEPE and their existence indicates the lipohydroperoxidase or lipoperoxide isomerase activity of ALOX12. 10-OH-11(12)-epoxy EPA derivatives have previously been named hepoxilin B4 [33]. Acting in concert with ALOX5, 5,12-diHEPE was the dominant reaction product (compound 12; Fig. 2) and its biosynthesis involves most probably sequential oxygenation of EPA by ALOX12 and ALOX5.

A similar pattern of products was observed in combined incubations when ALOX12 and ALOX5 were incubated simultaneously (simultaneous addition of both enzymes) and in inverse-order (starting the consecutive incubation with ALOX5 prior ALOX12 addition). However, the relative abundances of the products varied. When we initiated product formation by the addition of ALOX12 and subsequently added ALOX5 compound 12 (5,12-diHEPE, Fig. 2) was dominant. In contrast, in simultaneous ALOX12 + ALOX5 incubations higher amounts of ALOX5 derived products (compounds 8, 9, 11) were present. These products prevailed when the oxygenation reaction was initiated by the addition of ALOX5 prior to ALOX12. Similarly, ALOX5 products (compounds 8, 9) were formed in higher abundances in ALOX15B/ALOX5 assays, when the reaction was initiated by the addition of ALOX5 (Supplement, Fig. S7).

Formation of triple oxygenated compounds from EPA was one order of magnitude lower than formation of double oxygenated products (Supplement, Fig. S8). Particularly, consecutive incubation of ALOX15B and ALOX5 led to diverse triple oxygenated products including LXA_5 and various 5,6,15-triHEPE isomers as well as 5,14,15-triHEPE (LXB_5) isomers (Supplement, Fig. S9). Consistent with product specificity of tested ALOX isoforms, 18-HEPE derived E-series resolvins were mostly < LLOQ.

3.4. 18-HEPE formation and ALOX-catalyzed 18-HEPE oxygenation

First, we explored whether 18-HpEPE, an important pathway marker and precursor for E-series resolvins, can be formed from EPA by different ALOX isoforms. 18-HEPE was detected in the incubation mixtures of ALOX15, ALOX15B and ALOX12 exceeding the no-enzyme control 4–22-fold, while in the combined enzyme incubations 18-HEPE levels were lower (Fig. 4A). To test the stereochemistry of 18-HEPE formation we analyzed the products by chiral-phase HPLC. For 18-HEPE formed by ALOX15 we observed a preponderance of the *S*-enantiomer (18(*R*)-HEPE:18(*S*)-HEPE = 1:2.5), which was less pronounced for the incubation samples of ALOX15B and ALOX12 (Fig. 4B).

Next, we tested whether 18(*R*,*S*)-HEPE constitutes a suitable substrate for the different ALOX isoforms and as read-out parameters we employed i) the consumption of 18-HEPE (Fig. 5A) and ii) the formation of double and triple oxygenated reaction products (Fig. 5B + C). Quantifying 18-HEPE we found that ALOX5 induced major disappearance of 18-HEPE (Fig. 5A, left). In fact, more than 70% of the initial substrate was consumed during the incubation period. In contrast, for ALOX15, ALOX15B and ALOX12 only minor substrate conversion was observed. When we quantified the substrate disappearance of 18-HEPE in the combined enzyme incubations we found significant substrate conversion in all samples (Fig. 5A, right). This finding was not surprising since ALOX5, which effectively oxygenates 18-HEPE, was present in all samples. It was, however, surprising that the pre-incubation with ALOX15 reduced the capacity of ALOX5 for 18-HEPE oxygenation. The molecular basis for this regulatory activity has not been explored in detail. However, possible mechanisms are discussed later on in this paper (see discussion).

In principle, 18-HEPE can be converted to three different types of resolvins of the E-series: RvE1 [5(*S*),12(*R*),18(*R*)-trihydroxy EPA], RvE2 [5(*S*),18(*R*)-dihydroxy EPA], and RvE3 [17(*R*),18(*R*/*S*)-dihydroxy EPA]. When we searched our incubation samples for the formation of these products we detected significant amounts of RvE2 in the ALOX5 incubation (Fig. 5B, left). In the other samples RvE2 synthesis was minimal. However, significant amounts of RvE3 were present in the ALOX15B incubation (Fig. 5B, left). In the combined incubation samples significant amounts of RvE2 were detected in the ALOX15B + ALOX5 incubation and in the ALOX12 + ALOX5 incubation (Fig. 5B, right). Interestingly, in the ALOX15 + ALOX5 incubation we did not observe substantial RvE2 and RvE3 formation (Fig. 5B, right). Thus, here again the 18-HEPE oxygenase activity of ALOX5 was inhibited by pre-incubation with ALOX15. When we tested the formation of RvE1 in the incubation samples, we detected highest RvE1 formation in the ALOX5 sample and in the combined ALOX15B + ALOX5 and ALOX12 + ALOX5 samples (Fig. 5C, Supplement, Fig. S10). Finally, similar to EPA incubations, we screened 18(*R*,*S*)-HEPE incubations for other double and triple oxygenated compounds (*m/z* 333.2 and *m/z* 349.2, respectively; Fig. 6). We suggested different structures based on the fragmentation patterns of the formed products as shown for representative fragmentation patterns in Fig. 7 (MS/MS data of all indicated compounds are summarized in the Supplement, Figs. S11–12). ALOX15 was the only enzyme for which no double or triple oxygenated compounds formed from 18-HEPE were detected, which significantly exceeded the no-enzyme incubation (Fig. 6). ALOX5 catalyzed the formation of another 5,18- as well as 8,18-diHEPE-isomer (compound a, b; Table 3). 18-HEPE was also accepted as substrate by ALOX12 and different 12,18-diHEPE isomers (compound c, d) were major products. Combined incubations with ALOX5 did not result in additional products (Fig. 6A). In contrast to EPA incubations, ALOX5 catalyzed the formation of relatively high levels of several triple oxygenated products and combination of ALOX5 with ALOX15B or ALOX12 showed a similar product pattern (Fig. 6B). Suggested structures comprise 5,12,18-triHEPE (compound e, f; RvE1 isomers), 5,17,18-triHEPE (compound g, i) and 5,6,18-triHEPE (compound h, j) (Table 3, Fig. 7; Supplement, Fig. S12).

4. Discussion

4.1. ALOX5 and ALOX15 form a complex mixture of oxygenation products from EPA

Multiple hydroxylated PUFA, such as leukotrienes, lipoxins and resolvins, exhibit both pro- and anti-inflammatory bioactivities. These compounds can be formed from EPA, such as chemoattractant LTB_5 [34], lipoxins LXA_5 and LXB_5 [35] or 18-HEPE derived pro-resolving E-series resolvins [21,36]. Since 18-HEPE is not a main product of ALOX catalyzed EPA oxygenation (Fig. 4), ALOX isoforms were not capable of catalyzing substantial formation of E-series resolvins on their own or in combination with ALOX5 (Fig. 1C). However, structurally similar dihydroxylated compounds formed by a single or two consecutively acting ALOX isoforms might also be able to mediate physiological processes. We found that the spectrum of such products – especially for ALOX5 and ALOX15 alone or in combination – is rather complex with major products being 5,6-, 5,12-, 5,15-, 8,15-, 14,15-diHEPE isomers as well as epoxy-hydroxy-derivatives. Compared to ALOX12 and ALOX15B, ALOX5 and ALOX15 give rise to a broader product pattern: Both enzymes are capable of catalyzing hydrogen abstraction from different bisallylic methylenes of ARA (C7 and C10 for ALOX5, C13 and C10 for ALOX15) and this property is the mechanistic basis for the leukotriene synthase activities of these two enzymes [6,31]. In other words, ALOX5 and ALOX15 are more flexible when binding their substrates at the active site and this flexibility likely contributes to the complex patterns of oxygenation products we observed in this study.

We found that ALOX5 forms at least two 5,12-diHEPE isomers (compound 8, 9; Fig. 2), which likely originate from hydrolysis of a 5,6-epoxy-leukotriene intermediate. This mechanistic scenario is supported by the fragmentation patterns of compounds 8 and 9 formed in the presence of heavy oxygen isotopes (as shown for compound 8;

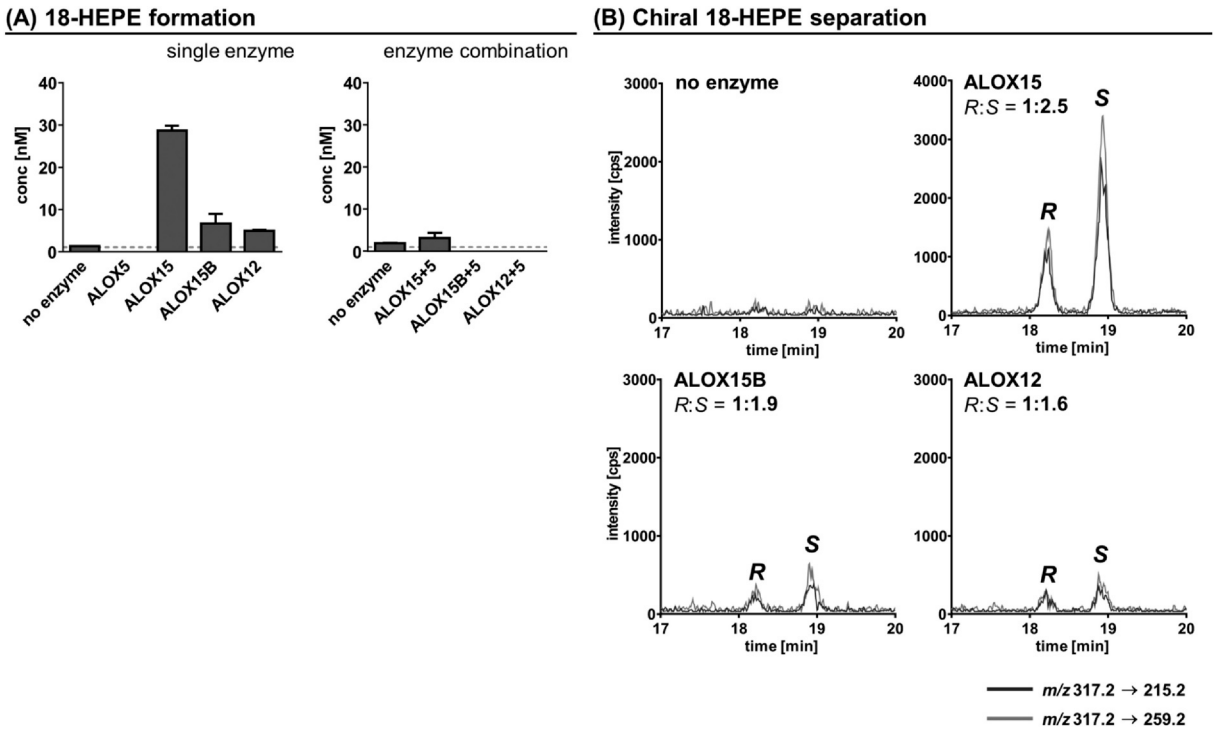

Fig. 4. Formation of 18-HEPE during incubation of different recombinant human ALOX isoforms with EPA. EPA was incubated with human ALOX isoforms (ALOX5, ALOX15, ALOX15B and ALOX12) alone and in combination with ALOX5 (10 μM EPA, 15 min, RT; $n = 3$). (A) Assay concentration of 18-HEPE; (B) Chiral analysis of 18-HEPE formed during single enzyme incubations. Chromatographic separation was carried out with an Agilent ZORBAX SB-C8 column coupled to a Lux-Amylose-1 column (ACN/MeOH/H_2O/glacial HOAc linear gradient) and the chromatograms were recorded at two different MS/MS transitions (*m/z* 317.2 ➔ 215.2, 259.2).

Supplement, Fig. S3). Under $^{18}O_2$ atmosphere no products carrying two ^{18}O-atoms ($[M\text{-}H]^-$ at *m/z* 337.2) were observed (Supplement, Fig. S3A). In fact, products carrying one ^{18}OH- and one ^{16}OH-group ($[M\text{-}H]^-$ at *m/z* 335.2) were formed and fragmentation spectra indicate that the ^{18}OH-group was located at C5. This oxygen atom should thus be incorporated during C5 oxygenation by ALOX5 (fragment *m/z* 335.2 ➔ 197.0). Consistent with this data, we found that when the reaction was carried out in $H_2{}^{18}O$ buffer the fragmentation pattern (fragment *m/z* 335.2 ➔ 195.0) indicated that the ^{18}O-atom is introduced at C12 (Supplement, Fig. S3B). This oxygen atom must thus originate from $H_2{}^{18}O$. A similar mechanism involving hydrolysis of a 5,6-epoxide intermediate leads to the formation of 5,6-diHEPE isomers (compounds 16, 19).

In contrast to the 5,12-diHEPE isomers (compounds 8, 9), compound 11 (5,15-diHEPE) is likely to be formed *via* the double lipoxygenation pathway and three lines of experimental evidence support this conclusion: i) This product is also formed in the combined ALOX15 + ALOX5 incubation showing a consistent retention time and fragmentation pattern. ii) When EPA oxygenation was carried out under $^{18}O_2$ atmosphere two ^{18}OH-groups were present ($[M\text{-}H]^-$ at *m/z* 337.2). Furthermore, the fragmentation pattern indicates an ^{18}OH-group at C5 (*m/z* 337.2 ➔ 117.0) and at C15 (*m/z* 337.2 ➔ 99.1; Supplement, Fig. S3A). iii) When the reaction was carried out in $H_2{}^{18}O$ buffer compound 11 carries two ^{16}OH-groups [*m/z* 333.2 ➔ 115.0 (^{16}OH-group at C5) and *m/z* 333.2 ➔ 97.1 (^{16}OH-group at C15)] and these data indicate the lack of incorporation of ^{18}O from $H_2{}^{18}O$ (Supplement, Fig. S3B). In contrast to the dominating dihydroxy EPA derivatives, only few fragments were observed for the two smaller but distinct compounds 1 and 2. Fragment spectra indicate the presence of an OH-group at C5 (*m/z* 333.2 ➔ 115.0) and C18 (*m/z* 333.2 ➔ 275.1). However, incubation with $^{18}O_2$ gas indicates incorporation of only one ^{18}O atom at C5 and incubation in $H_2{}^{18}O$ buffer supports formation involving hydrolysis of an epoxide intermediate. Since no mechanism for such a radical rearrangement is known, these compounds might constitute structures differing from the commonly observed diHEPE derivatives. As unexpectedly the majority of the detected EPA derived compounds by ALOX5 are secondary oxygenation products, it might be possible that further oxygenation or decomposition products are formed, which were not covered by the analyzed double and triple oxygenated compounds. More detailed experiments are required to address this question.

For ALOX15 we observed the most complex pattern of reaction products, which may result from its catalytic multiplicity: i) ALOX15 exhibits a pronounced double oxygenase activity since it is capable of converting its major reaction product of ARA oxygenation [15-H(*p*)ETE] to the double oxygenation products 8,15- and 5,15-diH(*p*)ETE [6,11]. ii) Similar to ALOX5 this enzyme exhibits a leukotriene synthase activity and thus, 14,15-LTA_4 hydrolysis products are formed [6]. iii) ALOX15 does also exhibit a pronounced lipohydroperoxidase activity (hydroperoxide isomerase) and this activity is responsible for the formation of the epoxy-hydroxy derivatives. The two dominant products, compound 3 and 4 (Fig. 2; Supplement, Figs. S4–5), which were tentatively identified as 8,15-diHEPE isomers on the basis of their fragmentation patterns, are likely to be formed *via* a similar mechanism as the 5,12-diHEPE isomers formed by ALOX5 (LTA_5 hydrolysis). Incubation under $^{18}O_2$ atmosphere (Supplement, Fig. S4A) suggests the presence of one ^{18}OH-group (*m/z* 335.2 ➔ 208.1), which is not located at C8 (*m/z* 335.2 ➔ 155.0) but at C15 (*m/z* 335.2 ➔ 179.1). When the incubation was carried out in $H_2{}^{18}O$ buffer (Supplement, Fig. S4B) an ^{18}OH-group was identified at C8 (*m/z* 335.2 ➔ 157.0). In contrast, the third 8,15-diHEPE isomer (compound 6) apparently originated from double lipoxygenation since incubations in $H_2{}^{18}O$ buffer indicate the insertion of two ^{16}O-atoms from the atmosphere for compound 6 (Supplement, Fig. S5). The fragmentation patterns of compounds 3 and 4 on the one hand and of compound 6 on the other do not overlap completely (Supplement, Fig. S2), which might be caused by different double bond configurations as a result of the formation routes. For another dominant product (compound 14) the fragmentation patterns

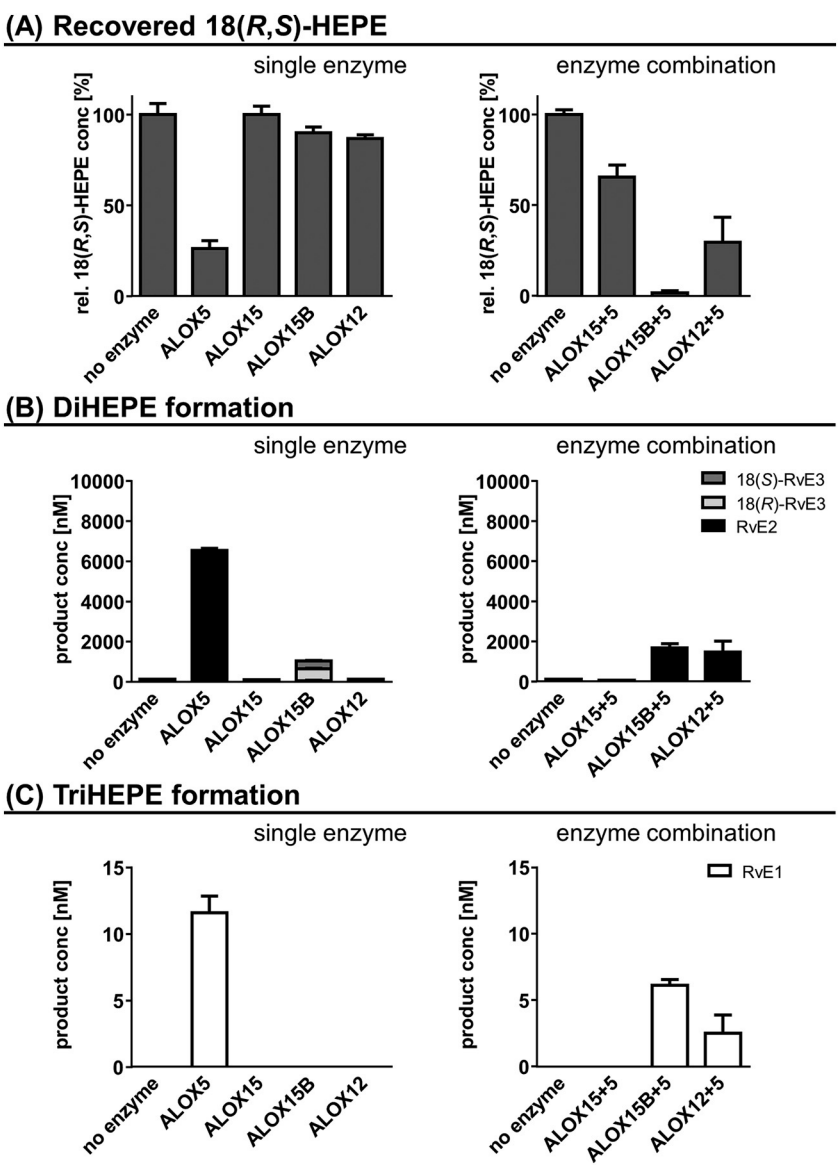

Fig. 5. Conversion of 18(*R*,*S*)-HEPE by recombinant human ALOX isoforms. Single and combined enzyme incubations of the different human ALOX isoforms were carried out with 18(*R*,*S*)-HEPE as described in Materials and methods (10 μM 18(*R*,*S*)-HEPE, 15 min, RT; n = 3) and remaining substrate (18(*R*,*S*)-HEPE) as well as established di- and triHEPEs that could be identified based on authentic standards were quantified by LC-MS/MS. (A) Recovered 18(*R*,*S*)-HEPE after the incubation period relative to the no enzyme incubation sample in %; (B + C) Assay concentration of EPA derived specialized pro-resolving mediators comprising (B) dihydroxy eicosapentaenoic acids (diHEPEs) and (C) trihydroxy eicosapentaenoic acids (triHEPEs).

suggested the structure of 14,15-diHEPE. This compound is formed *via* the double oxygenation pathway since two ^{18}OH-groups were incorporated when the reaction was carried out under $^{18}O_2$ atmosphere (*m/z* 337.2 ➔ 267.1). In contrast, two ^{16}OH-groups were incorporated when the reaction was performed in $H_2{}^{18}O$ buffer (*m/z* 333.2 ➔ 263.3) (Supplement, Fig. S4). The lipohydroperoxidase activity of ALOX15 is responsible for the formation of the epoxy-hydroxy compounds 17 and 20. The isomerization of hydroperoxy fatty acids to hydroxy-epoxy compounds was firstly described for the soybean LOX-1 leading to the formation of 11-hydroxy-12(13)-epoxy derivatives from 13-hydroperoxy octadecadienoic acid (13-HpODE) [37]. These compounds are formed *via* an intra-molecular rearrangement of the hydroperoxy oxygen atoms of ARA derived 12-HpETE and EPA-derived 12-HpEPE and were termed hepoxilin A3/B3 and hepoxilin A4/B4, respectively [33,38,39]. Indeed, incubations in $^{18}O_2$ gas indicate that compound 20 is formed *via* this reaction mechanism from 12-HpEPE (Supplement, Fig. S4). Hepoxilins are bioactive compounds as reviewed by Pace-Asciak [39] and for example involved in inflammatory processes, *e.g.* by affecting the chemotaxis of neutrophils. Moreover, first reports of EPA derived hepoxilin A4 and B4 indicated similar biological potency compared to ARA derived compounds [33]. In addition to two 10-OH-11(12)-EpETE isomers (compounds 20, 21), two 13-OH-14(15)-EpETE isomers (compounds 17, 18) were formed by ALOX15 and ALOX15B, which were also detected in incubations of porcine leukocytes with EPA [40] and might exert similar biological activities.

In the consecutive incubations with ALOX5 the clearly dominating products were compound 11 (for ALOX15 and ALOX15B) and compound 12 (for ALOX12). Both compounds might represent the double lipoxygenation products 5(*S*),15(*S*)-diHEPE (compound 11) and 5(*S*),12(*S*)-diHEPE (compound 12) formed by consecutive single oxygenation of the same molecule by the two ALOX isoforms. The consecutive action of two enzymes is of particular importance during the formation of SPMs, which may be formed *via* transcellular biosynthesis by 12- or 15-lipoxygenating ALOX and ALOX5 expressing cells [41]. In fact, highest levels of EPA derived LXA_5 were detected in combined incubation of ALOX15B with ALOX5 among several 5,6,15- and 5,14,15-triHEPE isomers as well as other triple oxygenated compounds derived from EPA (Fig. 1C; Supplement, Figs. S8–9). In order to mimic such transcellular biosynthetic mechanisms, we incubated the 12- and 15-lipoxygenating enzymes and ALOX5 in a consecutive manner. Additionally, simultaneous and inverse-order incubations were performed for the ALOX5/ALOX12 and ALOX5/ALOX15B combinations to explore whether the order of the oxygenation reaction impacts the product profile. Although the principle patterns of oxygenation products were

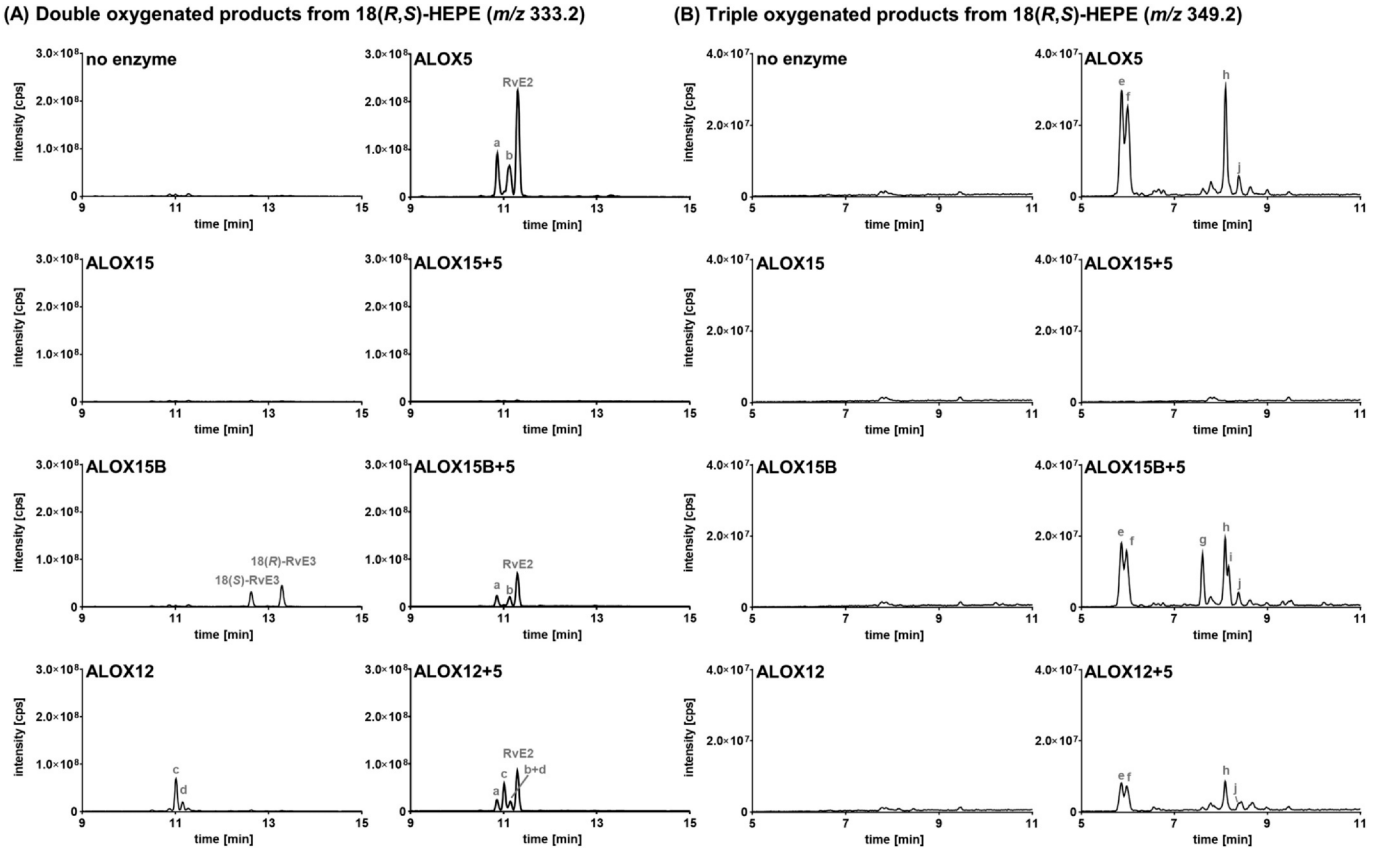

Fig. 6. Formation of double and triple oxygenation products from 18(*R*,*S*)-HEPE by recombinant human ALOX isoforms. 18(*R*,*S*)-HEPE was oxygenated by different human ALOX isoforms as described in Materials and methods (10 μM 18(*R*,*S*)-HEPE, 15 min, RT) and single and combined enzyme incubations were screened for formation of further double and triple oxygenation products from 18(*R*,*S*)-HEPE. (A) Total ion chromatograms (TIC) of product ion scans of *m/z* 333.2 (100–340 Da, CE ramp −18 to −26 V) representing the double oxygenated products; (B) TIC traces of product ion scans of *m/z* 349.2 (100–350 Da, CE ramp −18 to −26 V) representing the triple oxygenated products. Major products are labeled as compounds a-j and listed in Table 3.

very similar in the different incubation samples, we found that the relative shares of oxygenation products depended on the order of enzyme addition.

4.2. 18-HEPE is only formed in minor amounts by human ALOX isoforms

18-HEPE is an important substrate for the biosynthesis of the E-series resolvins but the *in vivo* source of this compound is still unclear. 18-HEPE derived dihydroxy fatty acids were detected in human plasma with and without dietary EPA supplementation [42]. However, the formation of SPMs, especially triple oxygenated EPA derivatives such as RvE1, is usually low in healthy subjects [43,44]. Since human ALOX15 is capable of catalyzing hydrogen abstraction from C10 and C13 of EPA [23] there was the possibility that this enzyme can also catalyze C16 hydrogen abstraction and thus, the formation of 18-HEPE. When we incubated human ALOX15 with EPA we found the formation of small amounts of 18-HEPE. This compound, which clearly exceeded the levels in no-enzyme control incubations (Fig. 4), only contributed about 1% to the sum of the mono-oxygenation products and analysis of its chirality indicated a preponderance of the 18(*S*)-enantiomer. However, the degree of chirality (*S*/*R*-ratio of 2.5:1) was lower than that of the major EPA oxygenation products (15(*S*)-HEPE/15(*R*)-HEPE > 10:1, data not shown). Taken together these data indicate that 18-HEPE can be formed from EPA by ALOX15 catalysis. However, this reaction is not very efficient and the stereochemistry is not tightly controlled by the enzyme.

4.3. 18(R,S)-HEPE is a substrate for human ALOX isoforms

When human ALOX5 oxygenated racemic 18-HEPE a number of di- and trihydroxylated EPA derivatives including RvE2 (5,18-diHEPE) were formed (Figs. 6A, 7). RvE2 formation by ALOX5 is rather straightforward since it simply requires C5 oxygenation of 18-HEPE [45]. In addition, similar amounts of other 5,18- and 8,18-diHEPE isomers were formed and these data indicate a low reaction specificity of the enzyme with this substrate. It should be noted that the substrate concentration (here 10 μM) might affect product formation and lower *in vivo* substrate concentrations might lead to different product pattern/product ratios. Previous studies have implicated ALOX15 in the biosynthesis of RvE3 (17,18-diHEPE) [21,46]. Under our experimental conditions we did not detect significant amounts of this metabolite (Fig. 6). These data do not necessarily mean that 17,18-diHEPE derivatives are not formed by this enzyme but when compared with ALOX15B much smaller amounts were detected. In fact, among the enzymes tested ALOX15B exhibited the highest RvE3 synthesizing capacity. Interestingly, in the combined ALOX15B + ALOX5 incubations RvE3 formation was completely abolished and a product pattern similar to the single ALOX5 incubation was observed. The most plausible explanation for this finding is that ALOX5 further oxygenates the RvE3 isomers leading to the formation of trihydroxylated EPA derivatives. In fact, significant amounts of such trihydroxy EPA derivatives were detected in the ALOX15B + ALOX5 incubation (Fig. 6B). Similar to ALOX15 human ALOX12 did not exhibit a major RvE3 synthesizing capacity (Fig. 6A). For this enzyme, as expected, 12,18-diHEPE isomers were identified as major 18(*R*,*S*)-HEPE oxygenation products (Fig. 7). In the ALOX12 + ALOX5 incubations (Fig. 6) a mixture of the reaction products identified for the single enzyme incubations was observed. Similar 18-HEPE derived dihydroxylated compounds were formed during human as well as murine leukocyte incubations and therefore

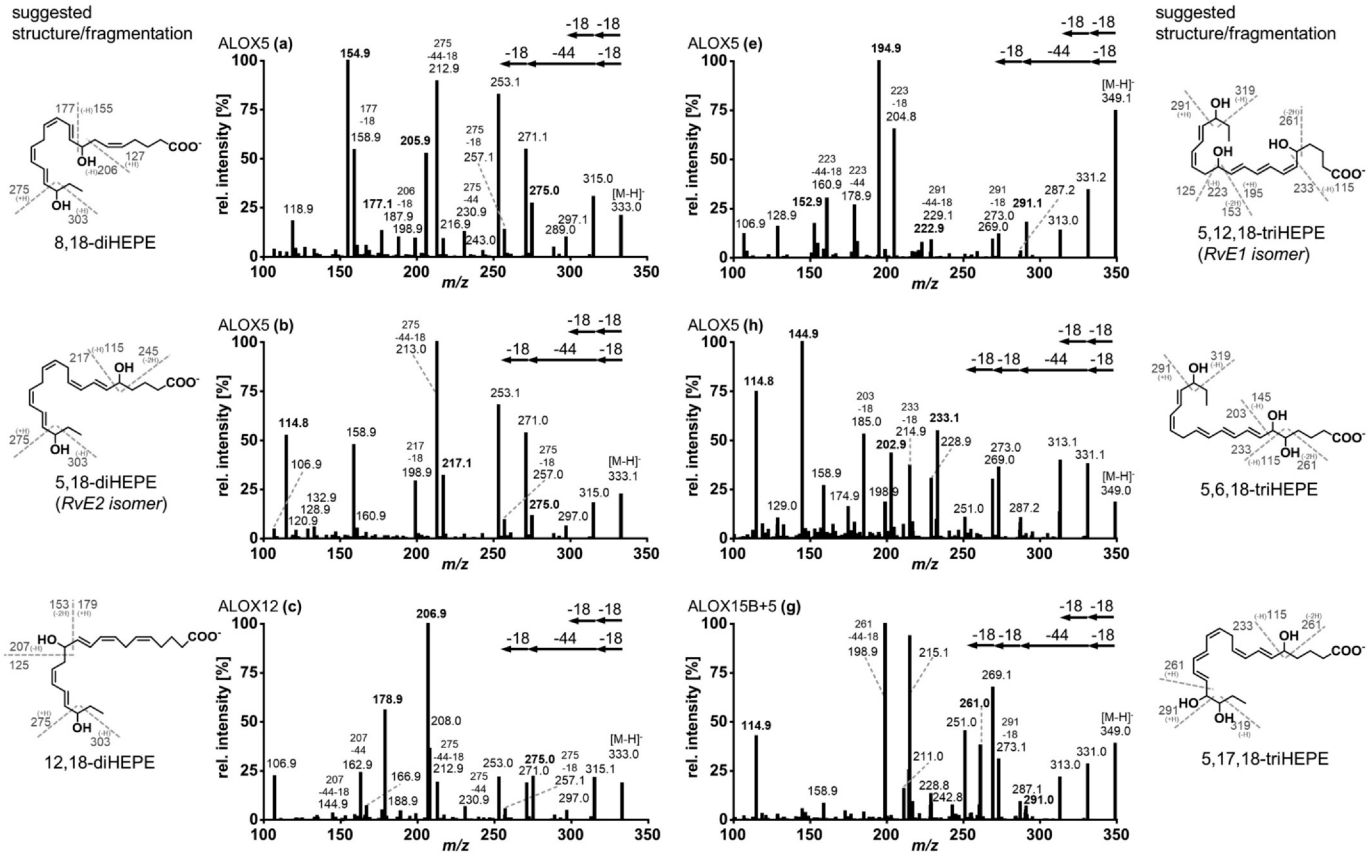

Fig. 7. Fragmentation patterns and suggested structures of representative 18(*R*,*S*)-HEPE oxygenation products formed by different recombinant human ALOX isoforms. Human ALOX isoforms were incubated with 18(*R*,*S*)-HEPE (10 μM, 15 min, RT) and the major double and triple oxygenation products shown in Fig. 6 (no reference compounds) were analyzed by mass spectrometry. Shown are collision induced dissociation product ion spectra of *m/z* 333.2 and *m/z* 349.2 (100–340 Da and 100–350 Da, CE ramp −18 to −26 V) alongside suggested structure and fragmentation sites for representative compounds formed during incubation of ALOX5 (compound a, b; e, h), ALOX12 (compound c) and ALOX15B + ALOX5 (compound g). Note that only one of several possible double bond configurations is shown.

Table 3
Tentative structures of 18(R,S)-HEPE derived double and triple oxygenated products formed by different recombinant human ALOX isoforms. Chemical structures for 18(R,S)- HEPE derived products (Fig. 6) were suggested based on their fragmentation patterns (Fig. 7, supplement Fig. S11-12). Compounds with similar fragmentation patterns and retention times were regarded as the same product. Marks (x) indicate enzyme incubations in which the respective compound was detected.

Compound	suggested positional isomer	ALOX						
		5	15	15+5	15B	15B+5	12	12+5
RvE2	5(S),18(R)-diHEPE	×				×		×
18(S)-RvE3	17(R),18(S)-diHEPE				×			
18(R)-RvE3	17(R),18(R)-diHEPE				×			
a	8,18-diHEPE	×				×		×
b	5,18-diHEPE	×				×		×
c	12,18-diHEPE						×	×
d							×	×
e	5,12,18-triHEPE	×				×		×
f		×				×		×
g	5,17,18-triHEPE`					×		
i						×		
h	5,6,18-triHEPE	×				×		×
j		×				×		×

attributed to the action of leukocyte 12/15-LOX [21]. Moreover, several 11,18- and two 17,18-diHEPE isomers were also formed *in vitro* by incubation of soybean LOX-1 with 18-HEPE [21]. Interestingly, under our experimental conditions, product patterns indicated ALOX5, ALOX12 and ALOX15B with the highest capability for 18-HEPE oxygenation rather than ALOX15. Furthermore, we observed distinct product profiles with 5,18- and 8,18-diHEPE isomers formed by ALOX5, 12,18-diHEPE by ALOX12 and 17,18-diHEPE by ALOX15B. This might have, on the one hand, implications for the *in vivo* synthesis of EPA derived double or triple oxygenated lipid mediators and the cell types capable of synthesizing these compounds. On the other hand, some of these compounds might exert biological activity, *e.g.* during inflammation and resolution. Isobe et al. demonstrated that administration of different 11,18-diHEPE isomers (which were not prominent in our incubations) had only little effect on PMNL infiltration in a murine model of zymosan-induced peritonitis [21]. However, other dihydroxylated EPA derivatives might be more active, for example, 8,18- and 12,18-diHEPE, which were also detected in human plasma and increased upon dietary EPA intake [42].

Trihydroxylated EPA derivatives were mainly detected in the incubations involving ALOX5. When ALOX5 was used as sole catalyst three major trihydroxylated EPA derivatives (compounds e, f, h) were observed. Resolvin E1 was only detected in trace amounts, which is consistent with findings by Tjonahen et al. using also recombinant ALOX5 [45] and can be explained by the requirement of enzymatic hydrolysis of the 5,6-epoxy-18-hydroxy intermediate to form the specific stereochemistry of RvE1, hence the involvement of LTA_4 hydrolase [47]. According to our mass spectral data, compounds e and f are 5,12,18-triHEPE derivatives (RvE1 isomers, *e.g.* 6-*trans*-5,12,18-triHEPE) [47]. The formation of 5,12,18-triHEPE most likely involves the formation of a 5,6-epoxide intermediate from 18-HEPE and its subsequent hydrolysis. It is also possible that the formation route involves C12 oxygenation and such catalytic activity has not been described for ALOX5 before. C12 oxygenation requires C10 hydrogen abstraction and

[+2] radical rearrangement. ALOX5 is capable of catalyzing C10 hydrogen abstraction. For [+2] radical rearrangement an inverse substrate alignment of the 5,18-diHEPE would be required. Compound h, which is the third dominant trihydroxylated EPA derivative formed by ALOX5 (Fig. 6B) was tentatively identified as 5,6,18-triHEPE on the basis of its fragmentation pattern (Fig. 7). Although we did not carry out more detailed mechanistic experiments, the biosynthetic route for this product likely involves the formation of 5,6-epoxy-18-hydroxy EPA (leukotriene synthase activity of ALOX5). The 5,6-epoxy-18-hydroxy intermediate then undergoes epoxide ring opening (hydrolysis) yielding the 5,6,18-triHEPE [47]. When ALOX15 was used as sole catalyst or when this enzyme was combined with ALOX5 no significant amounts of tri-OH EPA derivatives were observed. The molecular basis for this unexpected outcome has not been explored but two mechanistic scenarios might be discussed: 18-HEPE was almost completely recovered in the sole ALOX15 incubation and should have been available as substrate for ALOX5 (Fig. 5A). However, ALOX15 might interact with either 18-HEPE or ALOX5. If ALOX15 binds 18-HEPE non-covalently without its conversion to reaction products, it might not be available as substrate for ALOX5. Another scenario is the inhibition of ALOX5 by the presence of ALOX15 in the incubation sample. If this would be correct a physical interaction between ALOX15 and ALOX5 may take place in the incubation sample. Such an ALOX15-ALOX5 interaction has not been reported before but since the two enzymes form dimers [48,49] mixed dimer formation might be possible. More detailed experiments are needed to test such inhibitory mixed dimer formation. Similar to ALOX15, sole incubation of 18(*R*,*S*)-HEPE with ALOX15B and ALOX12 did not lead to the formation of major triple oxygenated EPA derivatives (Fig. 6B). However, in the combined ALOX15B + ALOX5 incubations two additional triple oxygenated EPA derivatives (compounds g and i) were detected. On the basis of their fragmentation patterns (Fig. 7) for both compounds the structure 5,17,18-triHEPE was suggested. Here again, we did not look in more detail into the mechanism of biosynthesis of these two compounds but the following scenario is highly probable: 18(*R*,*S*)-HEPE is firstly oxygenated by ALOX15B to 17,18-diHEPE (RvE3) and the formation of this compound was actually shown for ALOX15B (Fig. 6A). The reaction intermediate is subsequently oxygenated by ALOX5 at C5 yielding 5,17,18-triHEPE. If this reaction mechanism is true and the stereoselectivity of ALOX5 and ALOX15B catalyzed 18-HEPE oxygenation is the same as for EPA the tentative structure of the two compounds should be 5(*S*),17(*R*),18(*S*)- and 5(*S*),17(*R*),18(*R*)-triHEPE. The formation of these compounds from 18-HEPE (or its precursor EPA) by the consecutive action of human ALOX15B and ALOX5 has not been reported before. Since for RvE3 pro-resolving activities were described, *e.g.* decrease of chemotactic velocity of murine bone marrow PMNs [21], it might be possible that similar properties are exerted by these specific triHEPE compounds or other analogs.

The herein described lipoxygenase products may be formed *in vivo* by a single cell or *via* trans-cellular synthesis depending on the ALOX isoforms expressed in different cells. For example, blood platelets contain ALOX12 [50], while leukocytes, such as granulocytes and macrophages express ALOX5 [51]. Interactions between platelets and leukocytes have been described as formation pathway for lipoxins [15]. Consequently, the compounds formed in our ALOX12/ALOX5 combined incubations, *e.g.* 5,12-diHEPE, are likely products of this biosynthetic route as well. During the course of acute inflammation and inflammatory resolution, naïve monocytes differentiate to M1 (pro-inflammatory) or M2 (resolutory) macrophages that express different ALOX isoforms. For example, following differentiation of monocytes to M2 macrophages with CSF-1 (for 7 days) and interleukin 4 (for the last 2 days), these cells upregulate both ALOX5 and ALOX15B expression upon acute stimulation (16 h) of toll-like receptor 2 and 4 [52]. Therefore, in response to inflammatory stimuli M2 macrophages could be capable of synthesizing ALOX derived products that were detected in single and combined ALOX15B/ALOX5 incubations in the present study. Hence, the herein described double oxygenated products may be formed in human blood cells from their precursor fatty acid EPA present in membrane phospholipids. In order to substantiate this speculation, we screened plasma samples of Ca^{2+} ionophore stimulated human whole blood from EPA + DHA supplemented healthy humans from a previous study [53]. We detected double oxygenated compounds from EPA based on MRM transitions derived from the obtained fragment spectra in ALOX incubations indicating the formation of *e.g.* 5,15-diHEPE or 5,12-diHEPE in humans (Supplement, Fig. S14). Further experiments will be required to confirm the identity and formation pattern of these EPA derived compounds in human blood cells and investigate their biological relevance. It should be noted that based on a recent study by Norris et al. [54], the product 5(*S*),15(*S*)-diHEPE (compound 11) has been recently termed RvE4, which *e.g.* enhances macrophage efferocytosis, supporting the potential bioactivity of the ALOX products described herein.

In conclusion, ALOX5 and ALOX15 form complex product patterns of double oxygenated compounds from EPA *via* their double lipoxygenase, leukotriene synthase and lipohydroperoxidase activities. Complexity of formed products increases in combined incubations with ALOX5 simulating consecutive or trans-cellular biosynthesis. ALOX5, ALOX12 and ALOX15B effectively oxygenated 18-HEPE to double oxygenated compounds and several distinct triple oxygenated products were formed, mainly by ALOX5. Regarding the established role of structurally similar compounds such as lipoxins and resolvins, the described ALOX derived products from EPA may also show biologically potent effects. The herein presented, detailed analysis of ALOX secondary product patterns provides insight into the capability of each tested ALOX isoform (ALOX5, ALOX15, ALOX12, ALOX15B) to catalyze the formation of potentially bioactive double and triple oxygenated compounds. Further investigations will address their *in vivo* synthesis and biological functions.

CRediT authorship contribution statement

LK, WHS, HK and NHS designed experiments; KG, HK, LK, KR and MB carried out experiments and measurements; MJ and SFK provided 18(*R*,*S*)-HEPE; LK, MB, MR, WHS, HK and NHS discussed results; LK, HK and NHS wrote the manuscript. All authors approved the final manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

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Supplementary material

Human lipoxygenase isoforms form complex patterns of double and triple oxygenated compounds from eicosapentaenoic acid

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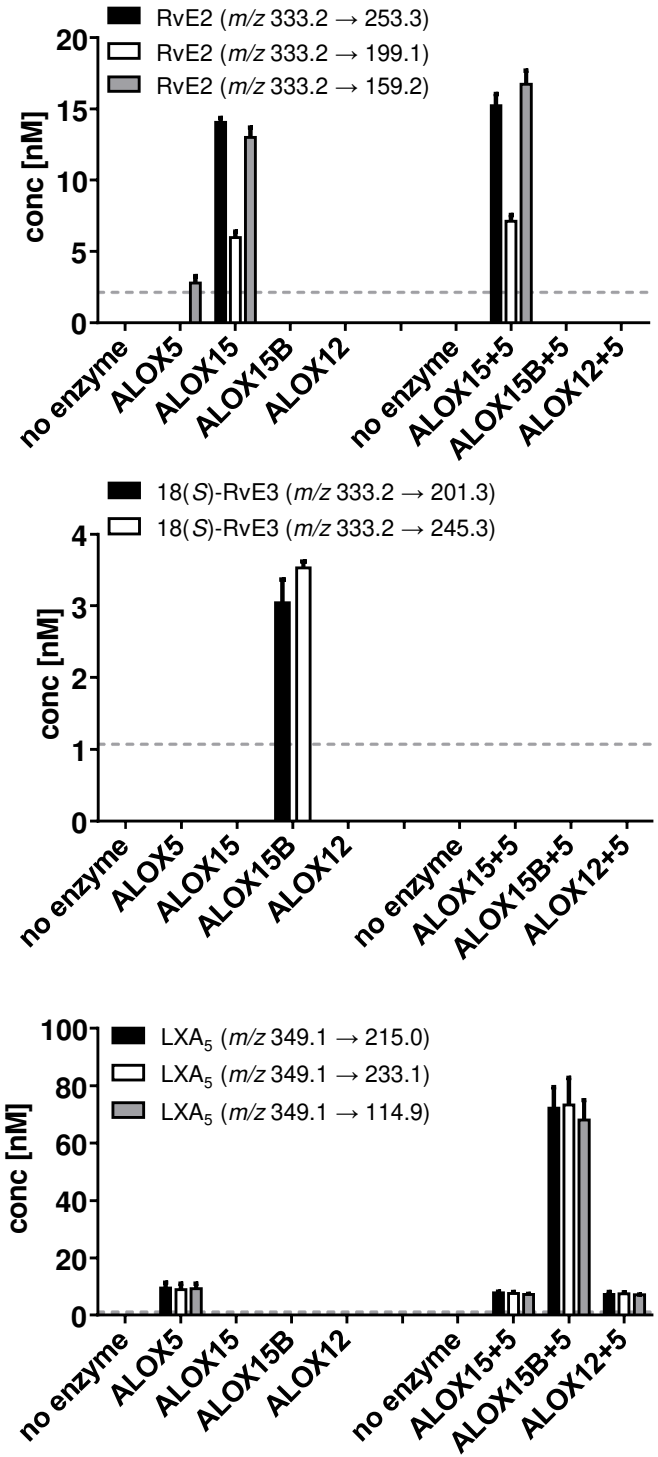
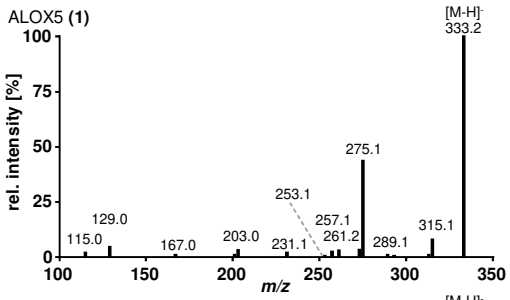
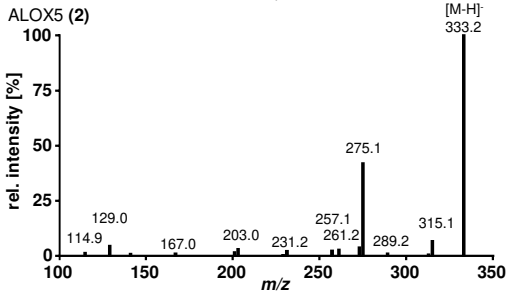


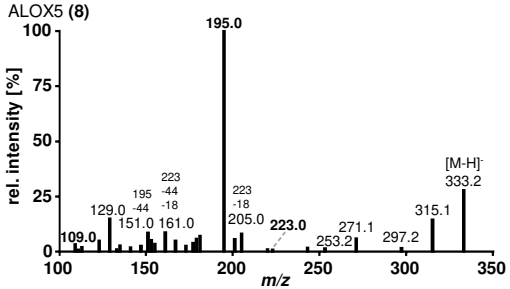
Fig. S1: Incubation of EPA with recombinant human ALOX isoforms.
Comparison of apparent concentrations quantified using different MRM transitions for di- and trihydroxy fatty acids formed during EPA incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM EPA, 15 min, RT). Shown are means ± SD (n=3). The lower limit of quantifcation (LLOQ) is indicated as dotted line.

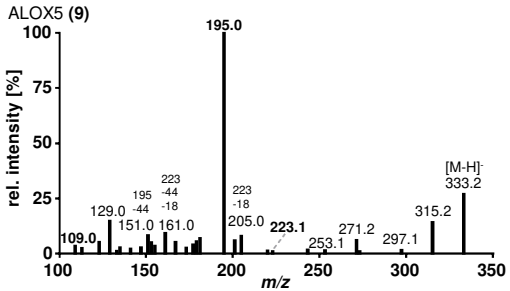
Fig. S2: Collision induced dissociation product ion spectra of ALOX products with *m/z* 333.2 formed during EPA incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM EPA, 15 min, RT).
Shown are product ion spectra of *m/z* 333.2 (100-350 Da, CE ramp -18 to -26 V) for products formed by (**A**) ALOX5, (**B**) ALOX15, (**C**) ALOX15B, (**D**) ALOX12 and in combined incubations of (**E**) ALOX15+ALOX5, (**F**) ALOX15B+ALOX5 and (**G**) ALOX12+ALOX5 alongside suggested structure and fragmentation. Note that only one of several possible double bond configurations is shown.

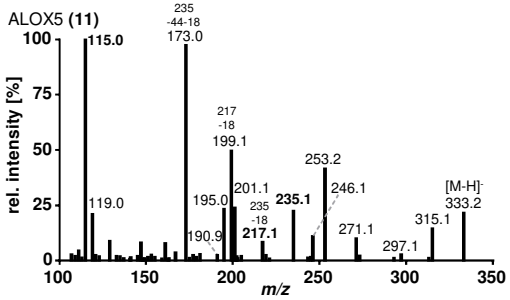
(A) ALOX5, *m/z* 333.2



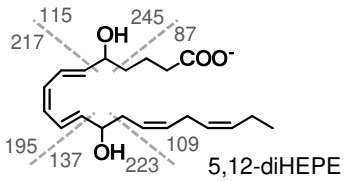


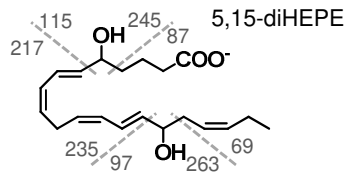


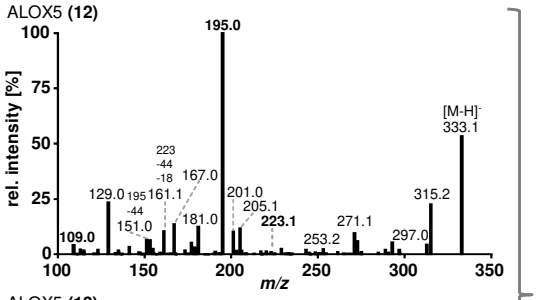


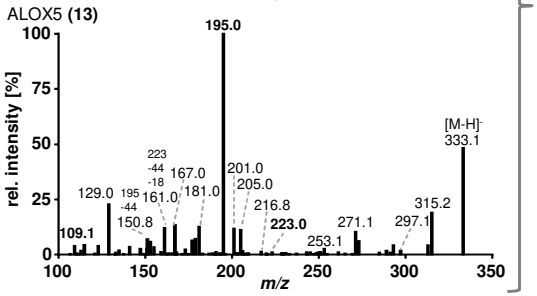


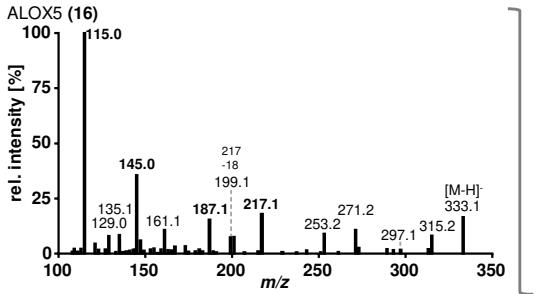
Suggested structure/fragmentation

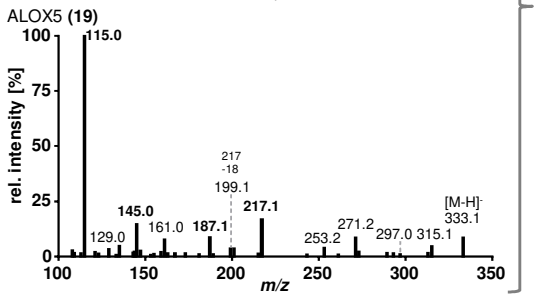












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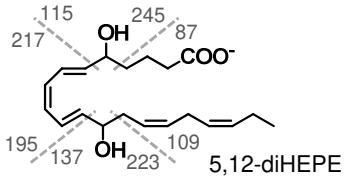


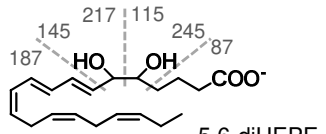
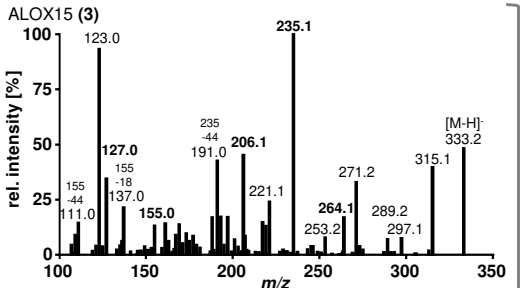
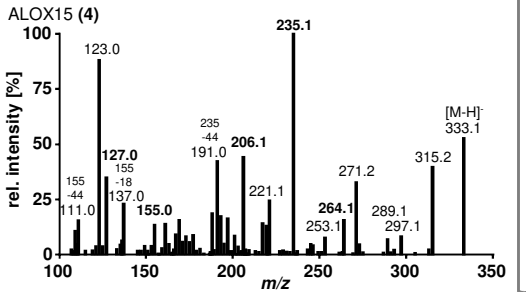


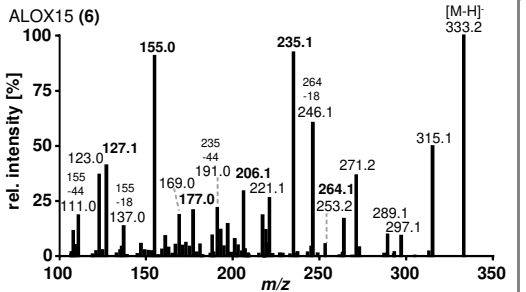
Fig. S2: *Continued.*

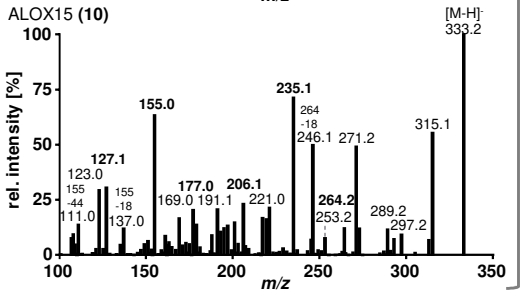
(B) ALOX15, *m/z* 333.2

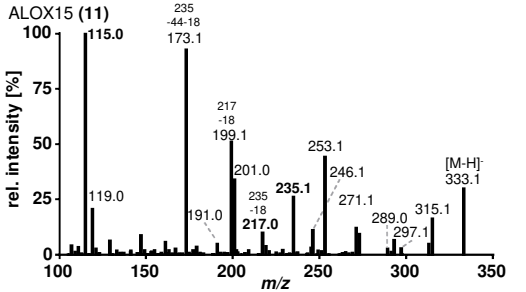
Suggested structure/fragmentation

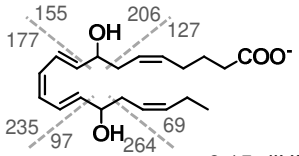




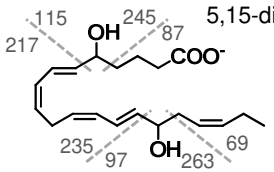






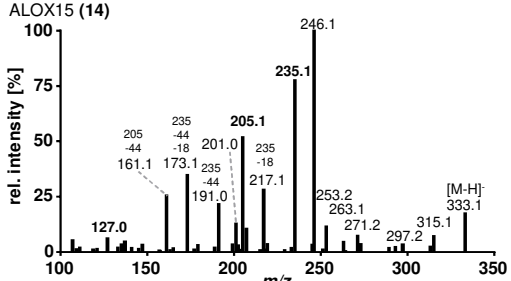


8,15-diHEPE



5,15-diHEPE

Suggested structure/fragmentation



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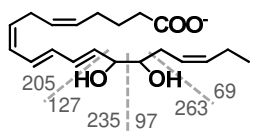
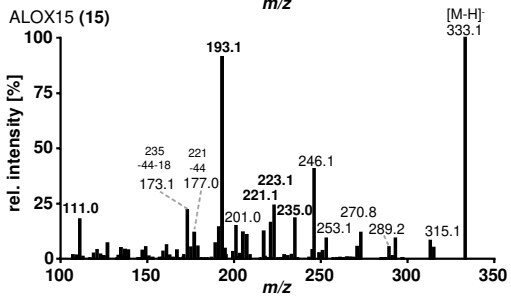
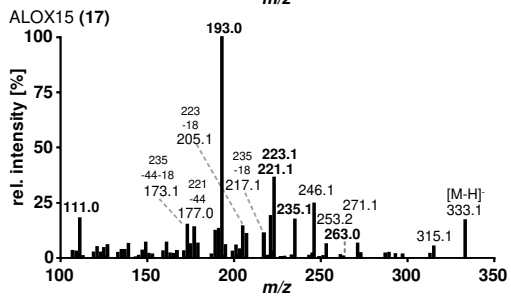
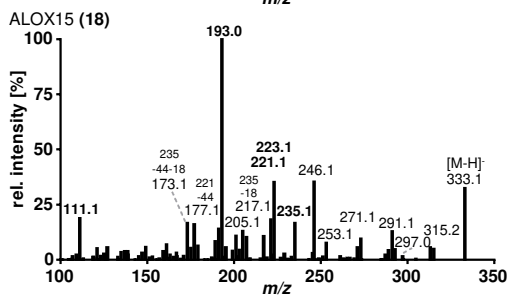
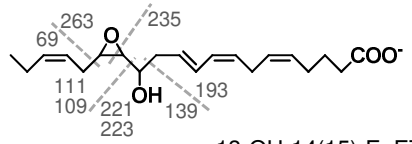


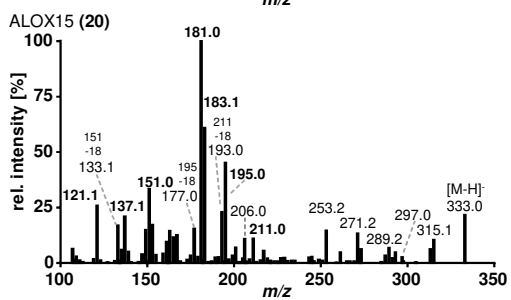


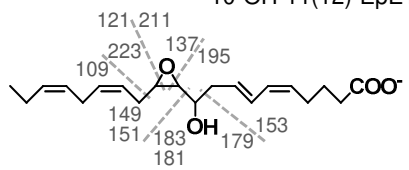






13-OH-14(15)-EpETE

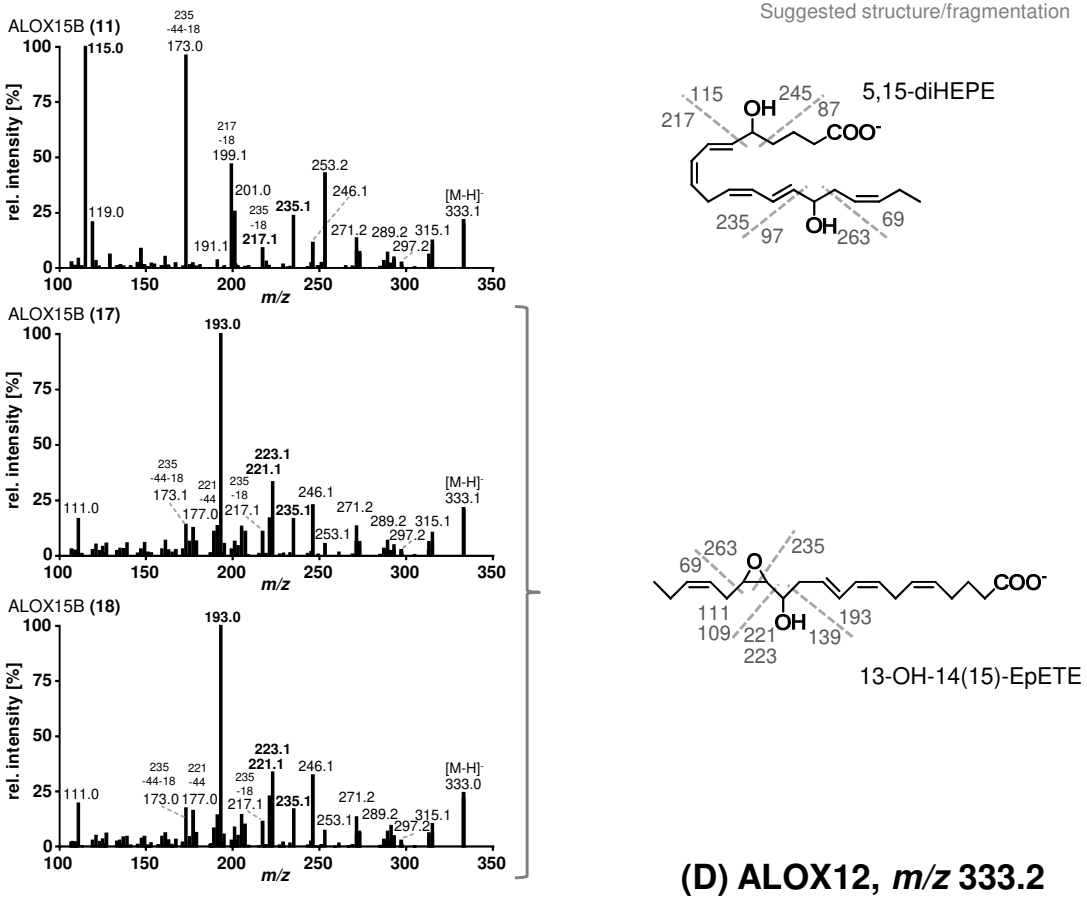




10-OH-11(12)-EpETE

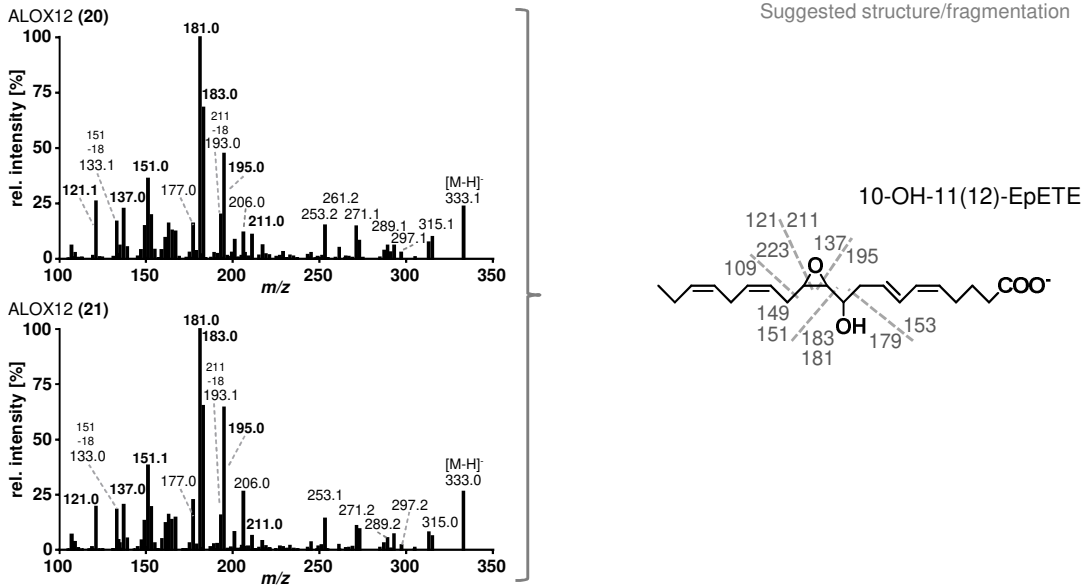
Fig. S2: *Continued.*

(C) ALOX15B, *m/z* 333.2



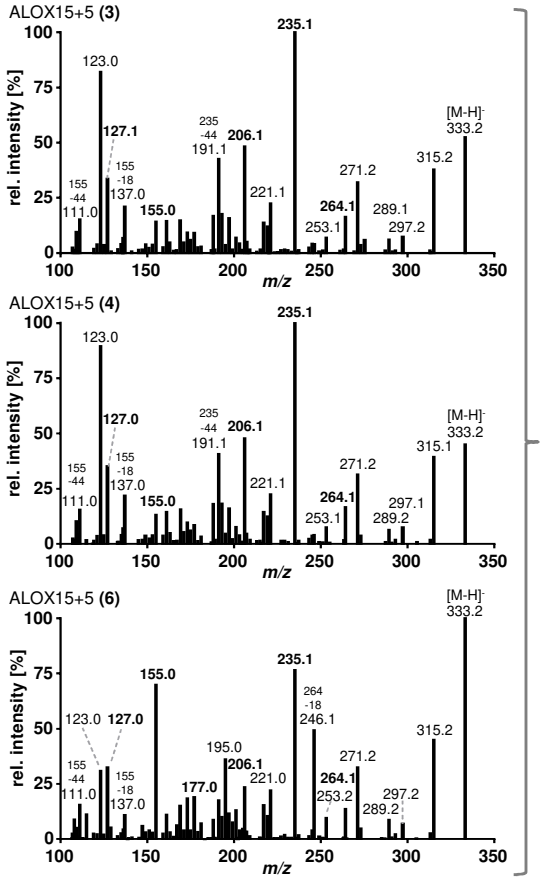
Suggested structure/fragmentation

(D) ALOX12, *m/z* 333.2

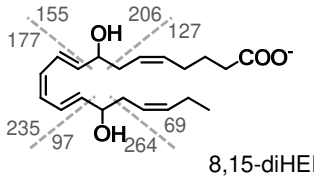


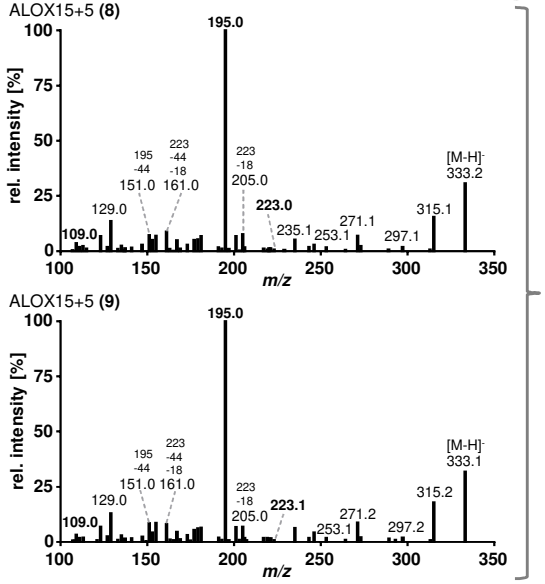
Suggested structure/fragmentation

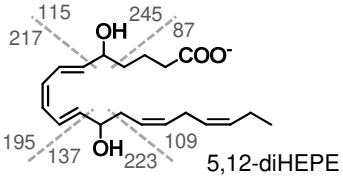
(E) ALOX15+ALOX5, *m/z* 333.2



Suggested structure/fragmentation







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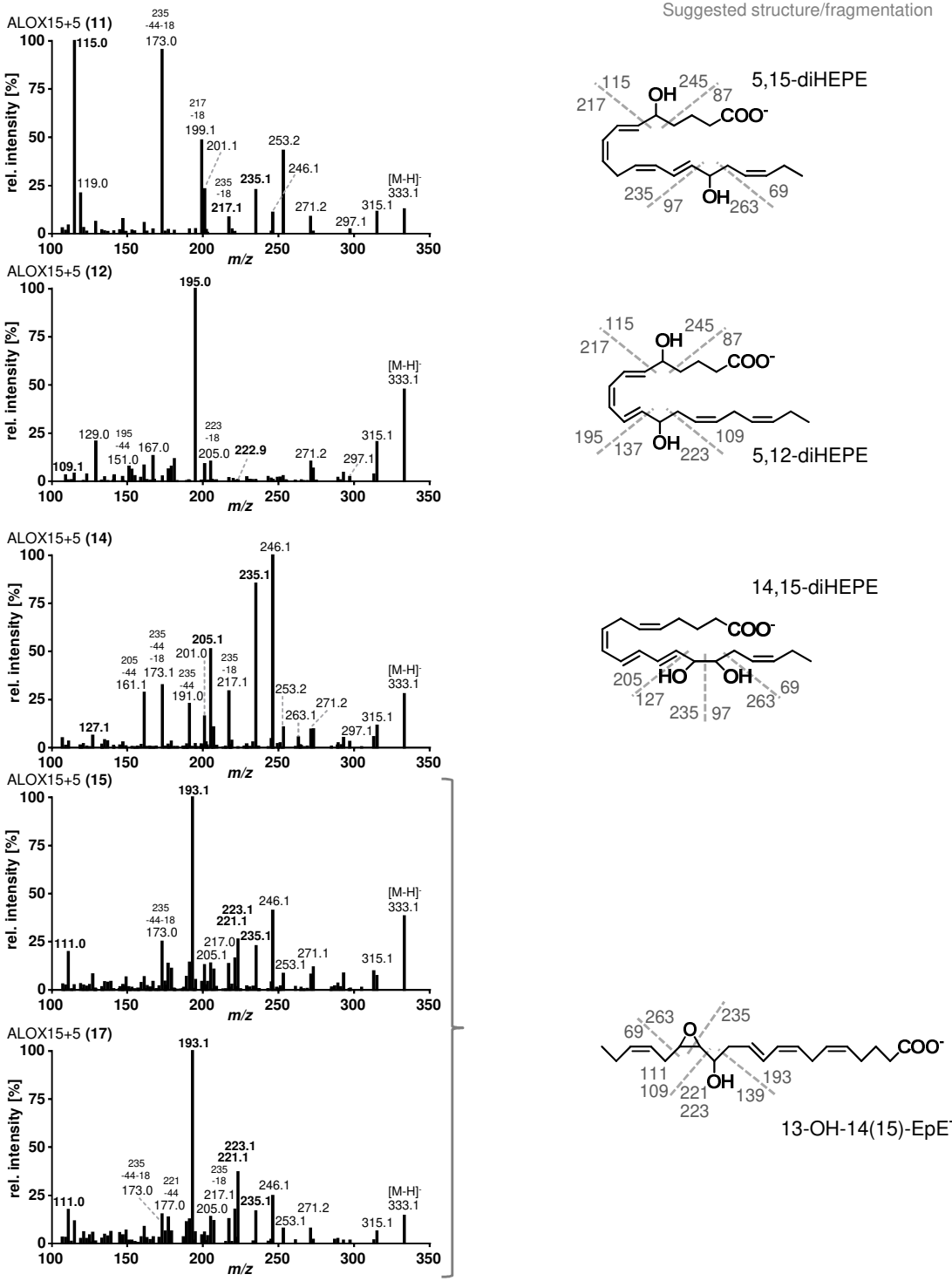
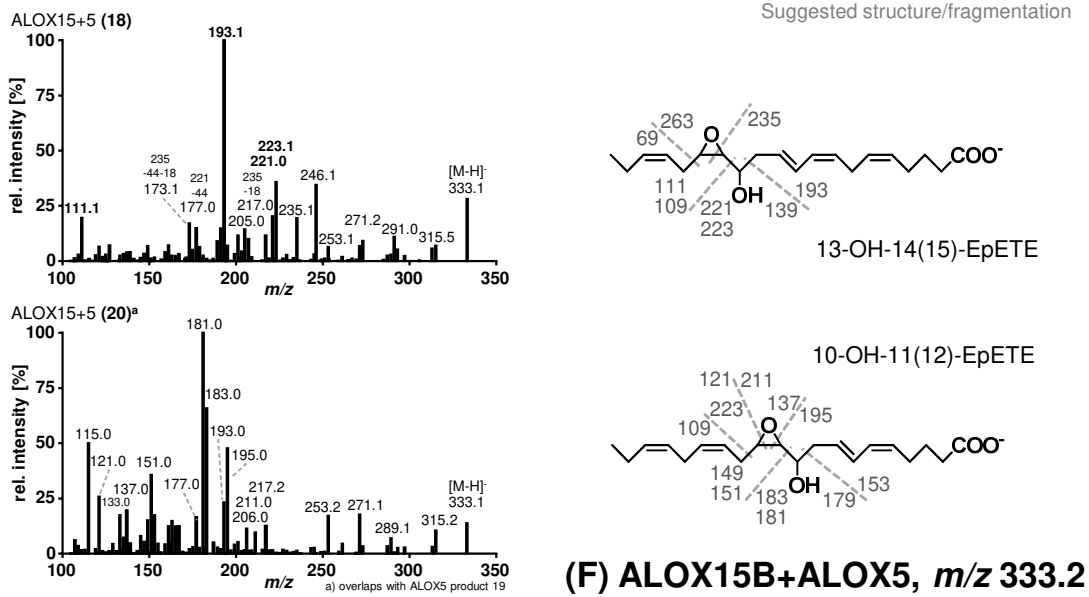
(E) ALOX15+ALOX5, *m/z* 333.2 *continued*



(E) ALOX15+ALOX5, *m/z* 333.2 *continued*



(F) ALOX15B+ALOX5, *m/z* 333.2

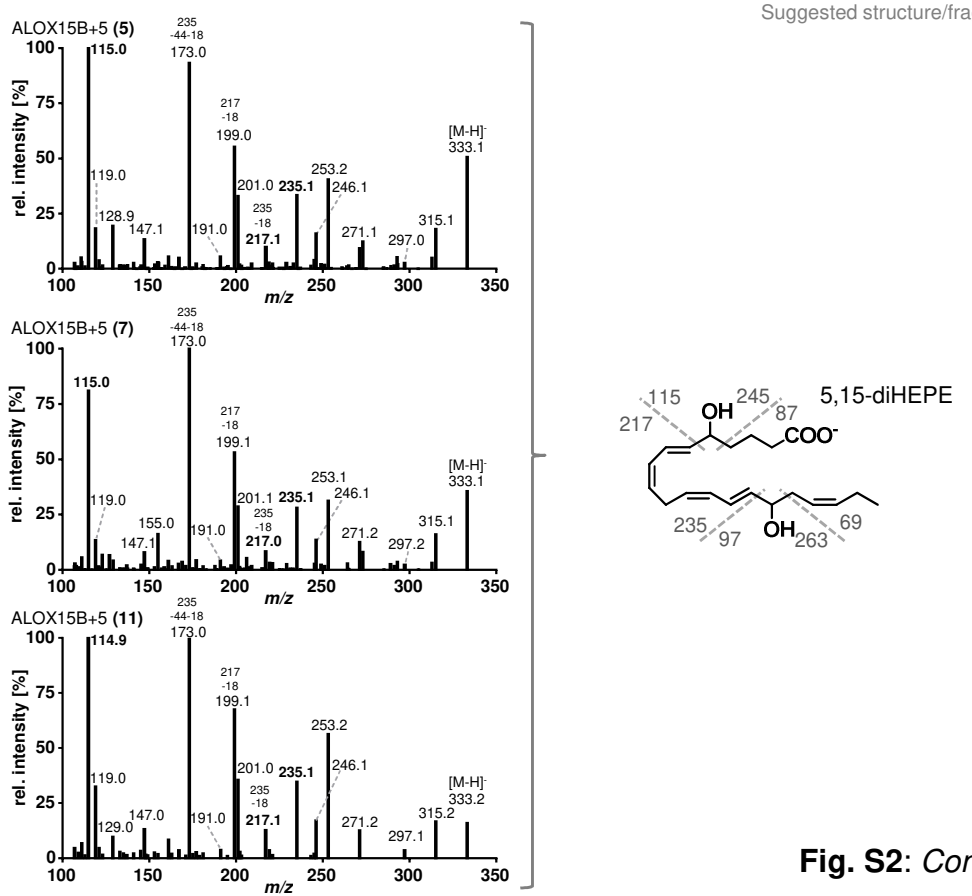
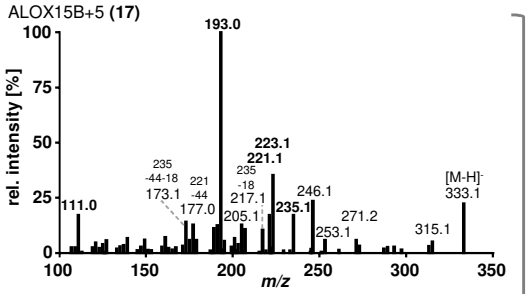
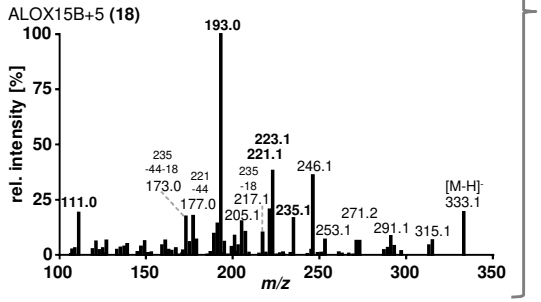


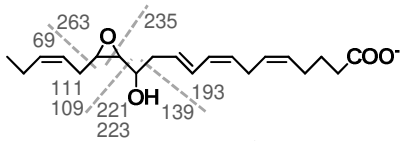
Fig. S2: *Continued.*

(F) ALOX15B+ALOX5, *m/z* 333.2 *continued*

Suggested structure/fragmentation



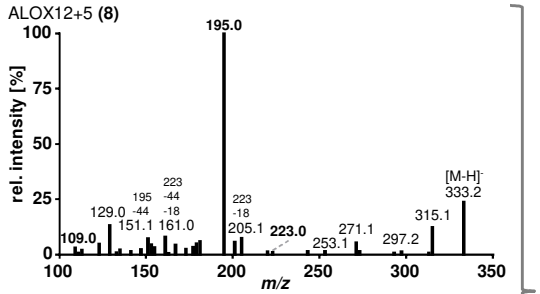


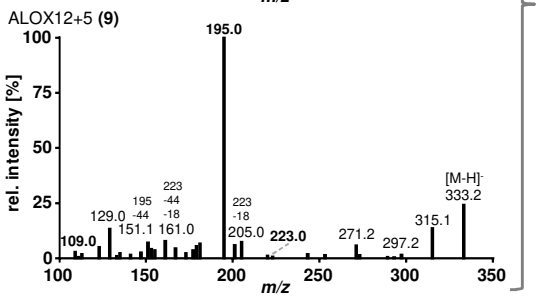


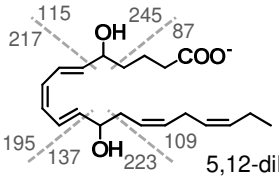
13-OH-14(15)-EpETE

(G) ALOX12+ALOX5, *m/z* 333.2

Suggested structure/fragmentation







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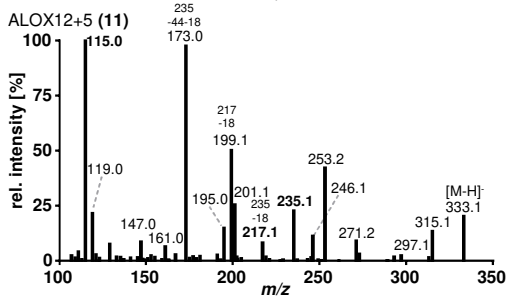
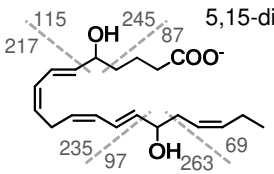
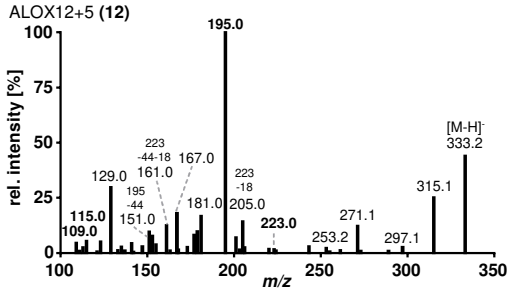


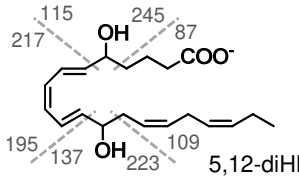


5,15-diHEPE

(G) ALOX12+ALOX5, *m/z* 333.2 *continued*

Suggested structure/fragmentation





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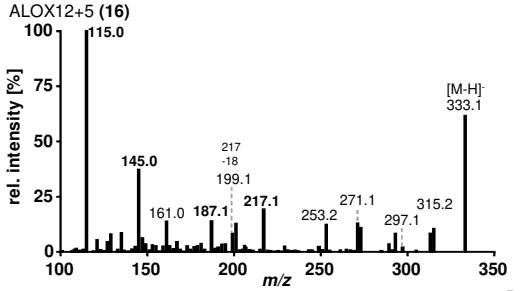

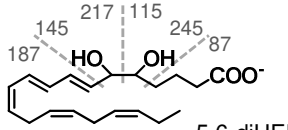

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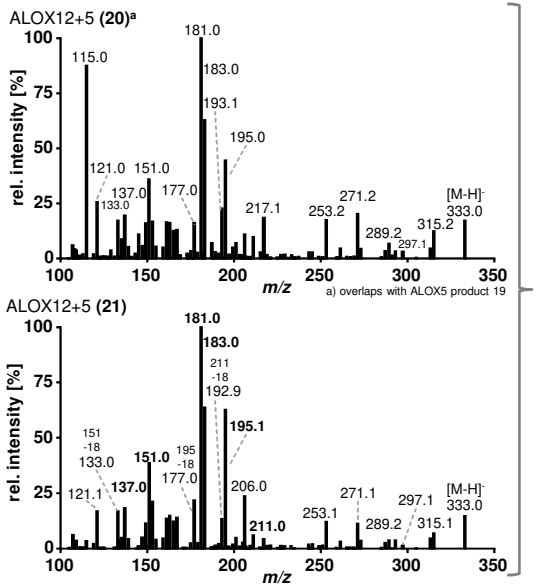
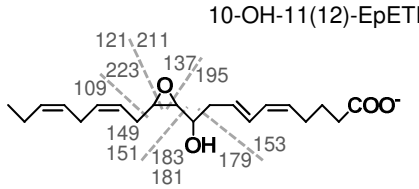




10-OH-11(12)-EpETE

Fig. S2: *Continued.*

Fig. S3: Formation of double oxygenated products during EPA incubation with recombinant human ALOX5 (10 µM EPA, 15 min, RT) in (**A**) $^{18}O_2$ atmosphere or (**B**) in $H_2{}^{18}O$ buffer. Shown are total ion chromatograms of product ion scans (55-340 Da, *m/z* 333.2, 335.2 and 337.2; CE ramp -18 to -26 V) as well as collision induced dissociation product ion spectra of exemplary products (as indicated in Fig. 2). Suggested stuctures and fragmentations are given considering incorporation of ^{18}O- and/or ^{16}O-atoms. Note that only one of several possible double bond configurations is shown.

Fig. S3: *Continued.*

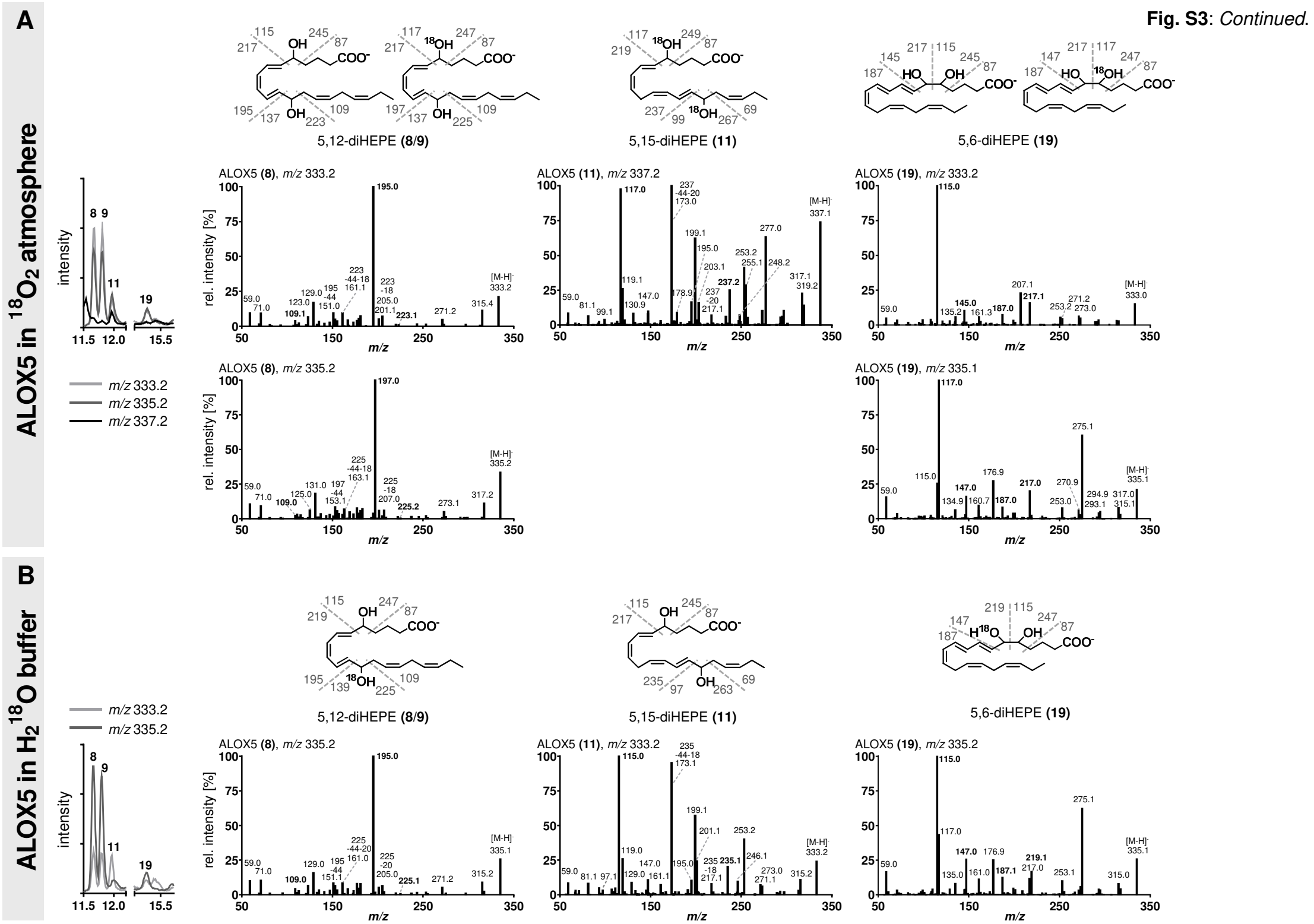
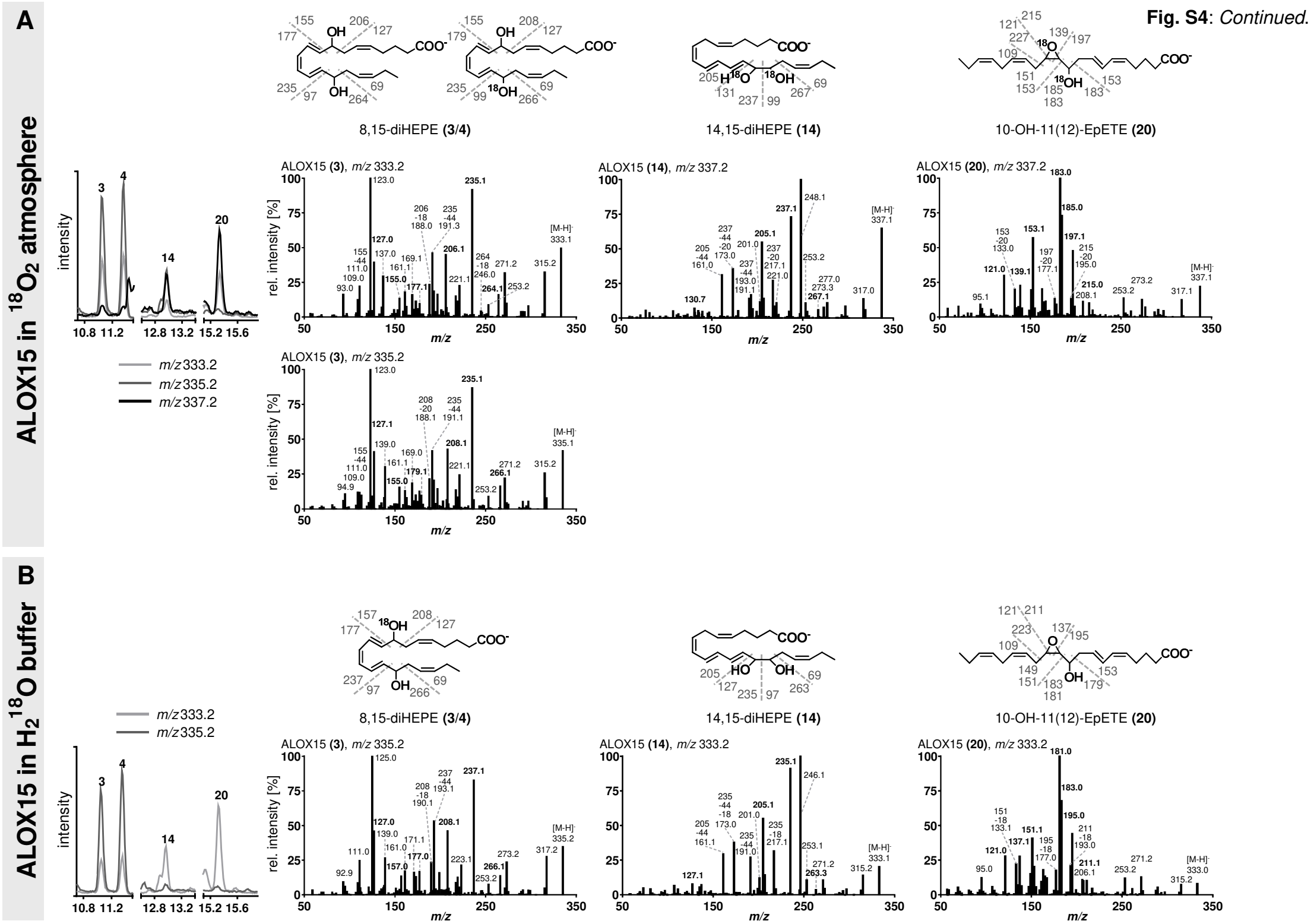


Fig. S4: Formation of double oxygenated products during EPA incubation with recombinant human ALOX15 (10 µM EPA, 15 min, RT) in (**A**) $^{18}O_2$ atmosphere or (**B**) in $H_2{}^{18}O$ buffer. Shown are total ion chromatograms of product ion scans (55-340 Da, *m/z* 333.2, 335.2 and 337.2; CE ramp -18 to -26 V) as well as collision induced dissociation product ion spectra of exemplary products (as indicated in Fig. 2). Suggested stuctures and fragmentations are given considering incorporation of ^{18}O- and/or ^{16}O-atoms. Note that only one of several possible double bond configurations is shown.

Fig. S4: *Continued.*



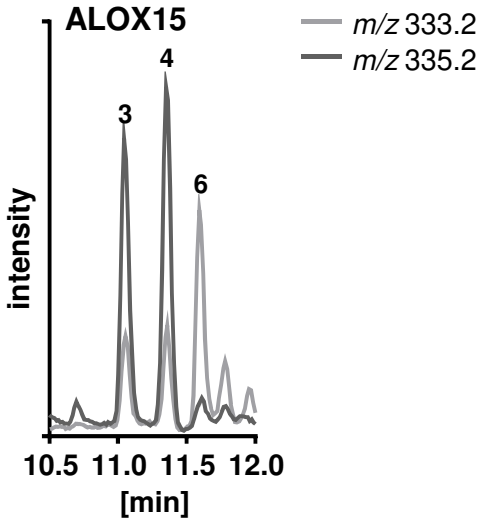


Fig. S5: Formation of compounds 3, 4 and 6 (8,15-diHEPE isomers) during EPA incubation with recombinant human ALOX15 (10 µM EPA, 15 min, RT) in $H_2{}^{18}O$ buffer. Shown are total ion chromatograms of product ion scans (55-340 Da, *m/z* 333.2 and 335.2; CE ramp -18 to -26 V). Prevailing detection of compounds 3/4 with *m/z* 335.2 in $H_2{}^{18}O$ buffer indicate formation *via* hydrolysis of an epoxide intermediate, while compound 6 with *m/z* 333.2 indicates insertion of two ^{16}O atoms by the ALOX enzyme.

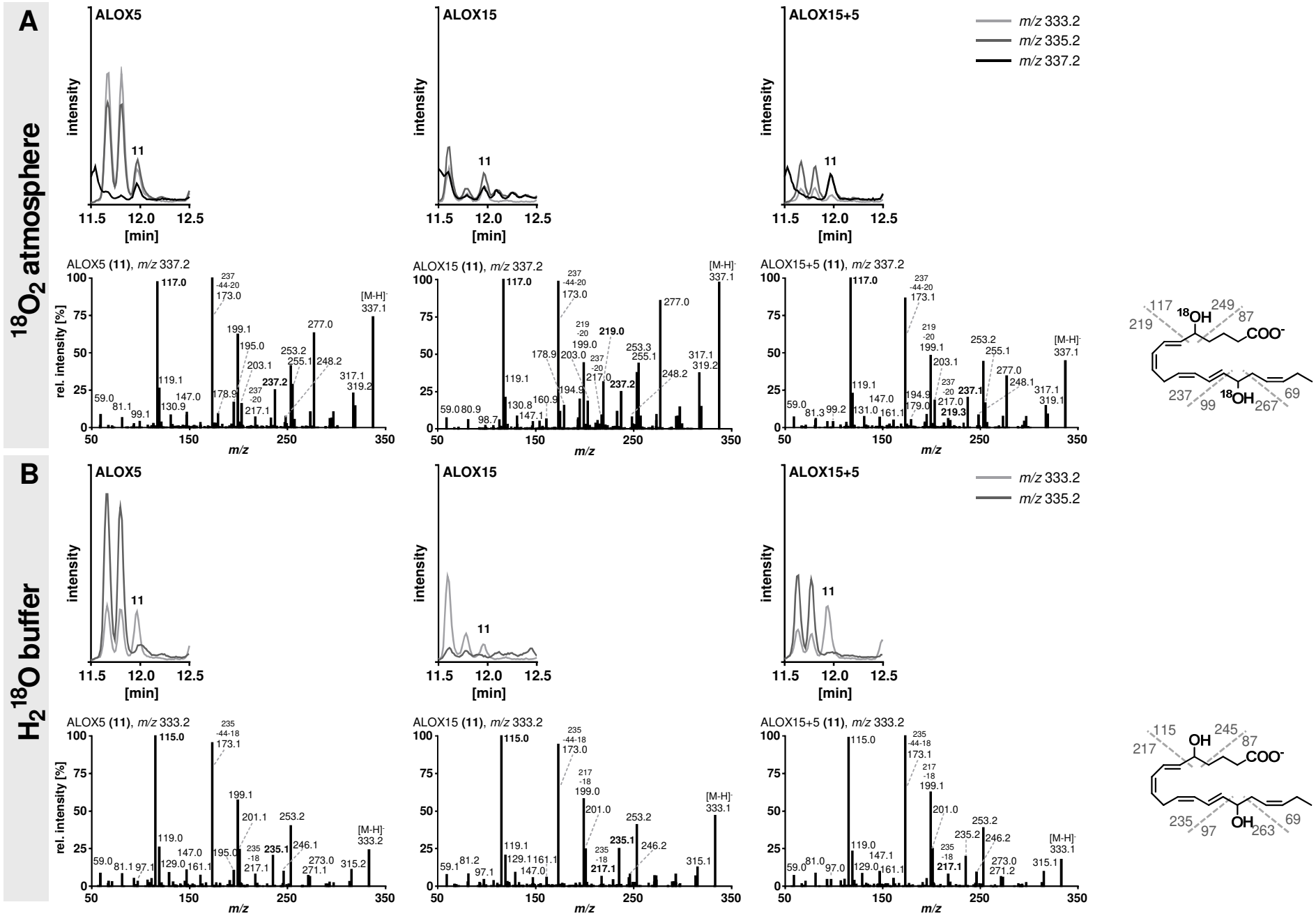


Fig. S6: Formation of compound 11 (5,15-diHEPE) during EPA incubation with recombinant human ALOX5 or ALOX15 or consecutive incubation of ALOX15+ALOX5 (10 µM EPA, 15 min, RT) (**A**) with $^{18}O_2$-gas or (**B**) in $H_2{}^{18}O$ buffer. Shown are total ion chromatograms of product ion scans (55-340 Da, *m/z* 333.2, 335.2, 337.2; CE ramp -18 to -26 V) as well as collision induced dissociation product ion spectra. Prevailing detection of compound 11 with *m/z* 333.2 in $H_2{}^{18}O$ buffer indicates insertion of two ^{16}O atoms by the ALOX enzyme instead of formation via hydrolysis of an epoxide intermediate. Consistently, in incubation samples with $^{18}O_2$-gas 5,15-diHEPE with two ^{18}O atoms (*m/z* 337.2) was detected.

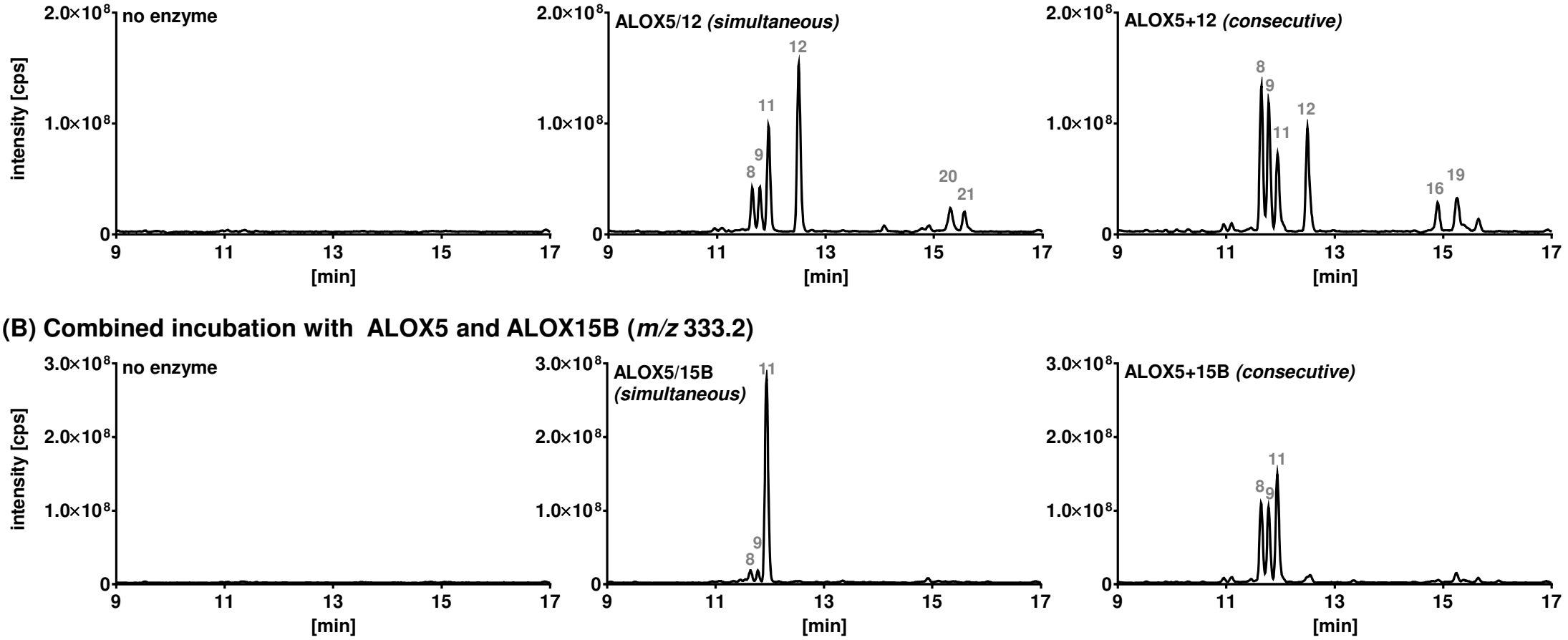


Fig. S7: Formation of double oxygenated products from EPA by recombinant human ALOX isoforms. Simultaneous and consecutive incubations were carried out as described in Materials and Methods (10 µM EPA, 15 min, RT), with the two enzymes added simultaneously or consecutively with ALOX5 added prior ALOX12 or ALOX15B to the incubation mixture. Shown are total ion chromatograms of product ion scans of *m/z* 333.2 (100-350 Da, CE ramp 18 to 26 V). Major double oxygenated products are labeled as indicated in Table 2.

Triple oxygenated products from EPA (*m/z* 349.2)

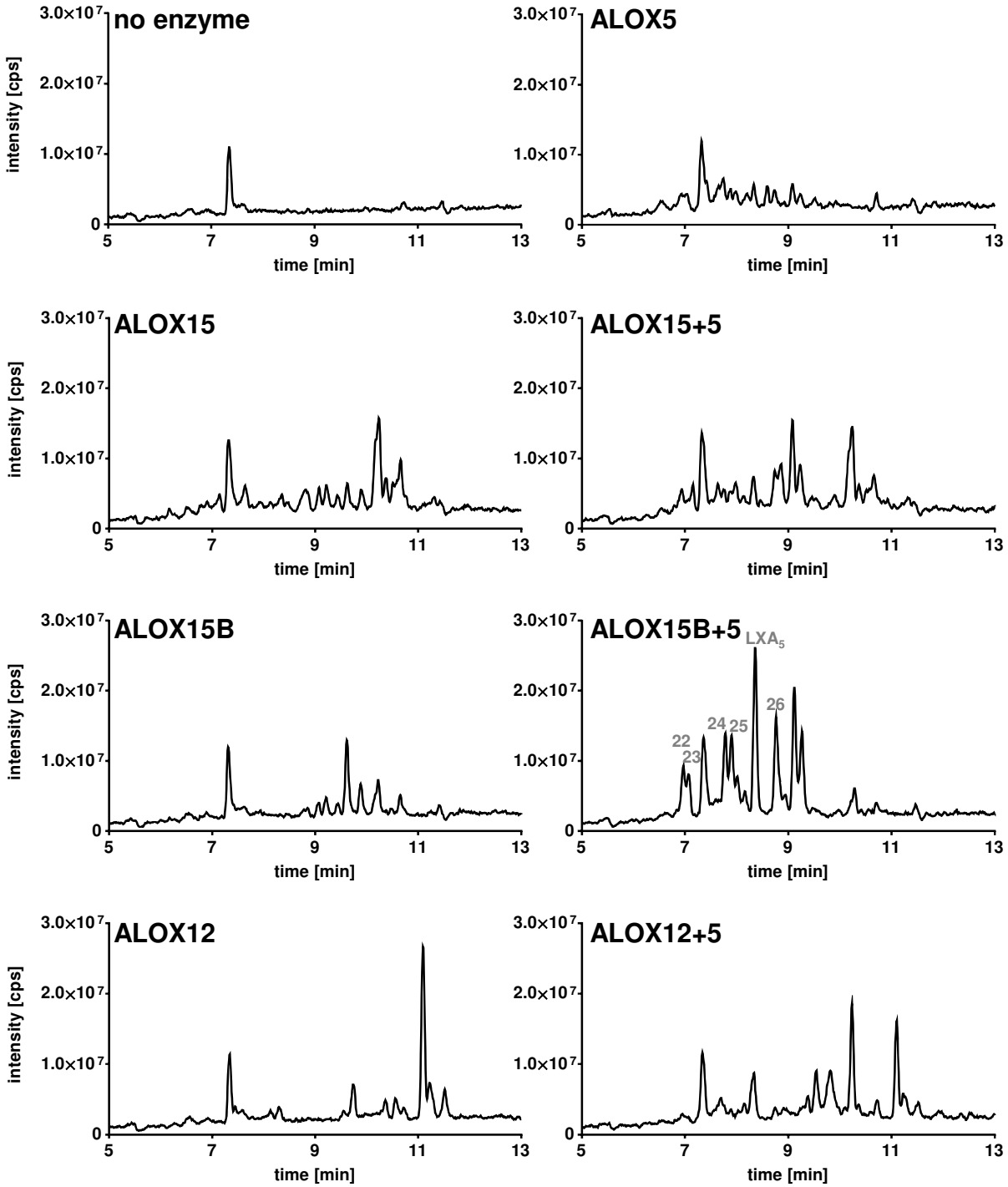
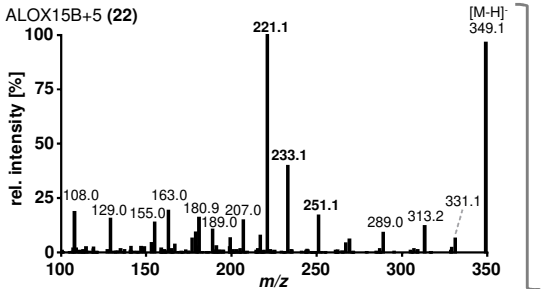
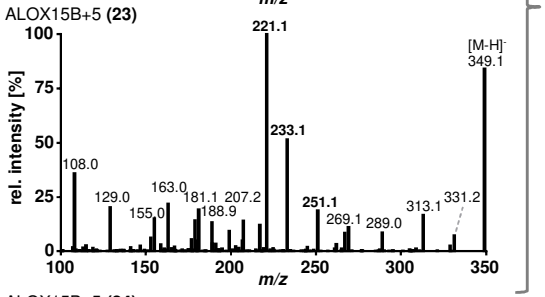


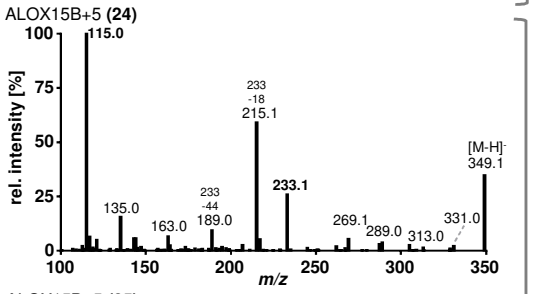
Fig. S8: Formation of triple oxygenated products during EPA incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM EPA, 15 min, RT). Shown are total ion chromatograms of product ion scans of *m/z* 349.2 (100-350 Da, CE ramp -18 to -26 V), dominant products are indicated as compounds 22-26.

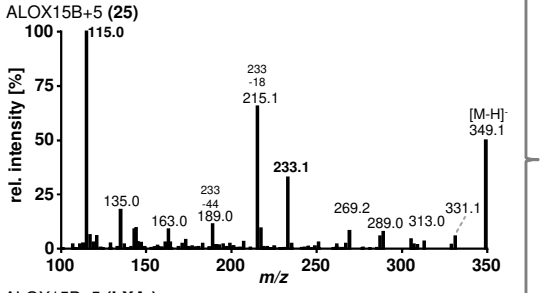
Fig. S9: Collision induced dissociation product ion spectra of ALOX products with *m/z* 349.2 formed during EPA incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM EPA, 15 min, RT).
Shown are product ion spectra of *m/z* 349.2 (100-350 Da, CE ramp -18 to -26 V) for products formed during combined incubation of ALOX15B+ALOX5 alongside suggested structure and fragmentation. Note that only one of several possible double bond configurations is shown.

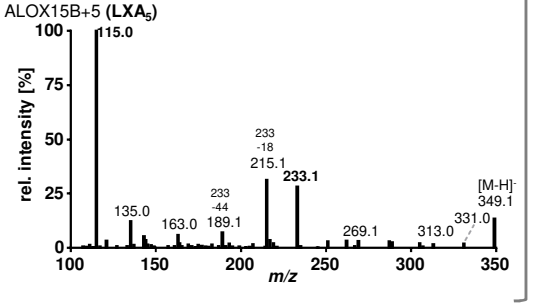
ALOX15B+ALOX5, *m/z* 349.2



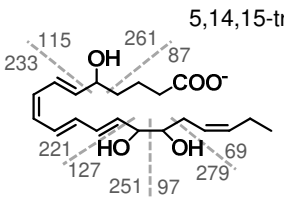


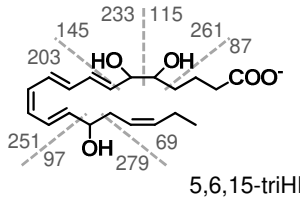




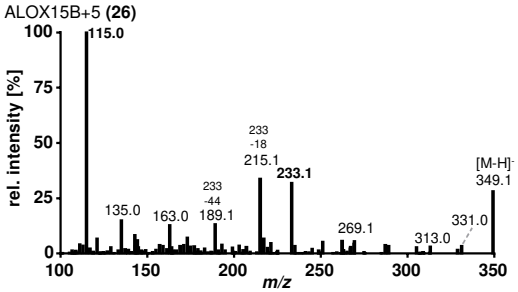


Suggested structure/fragmentation





ALOX15B+5 (**26**)



Suggested structure/fragmentation

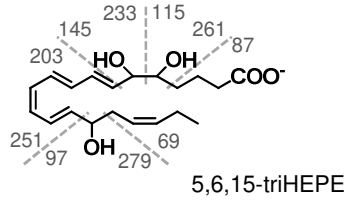
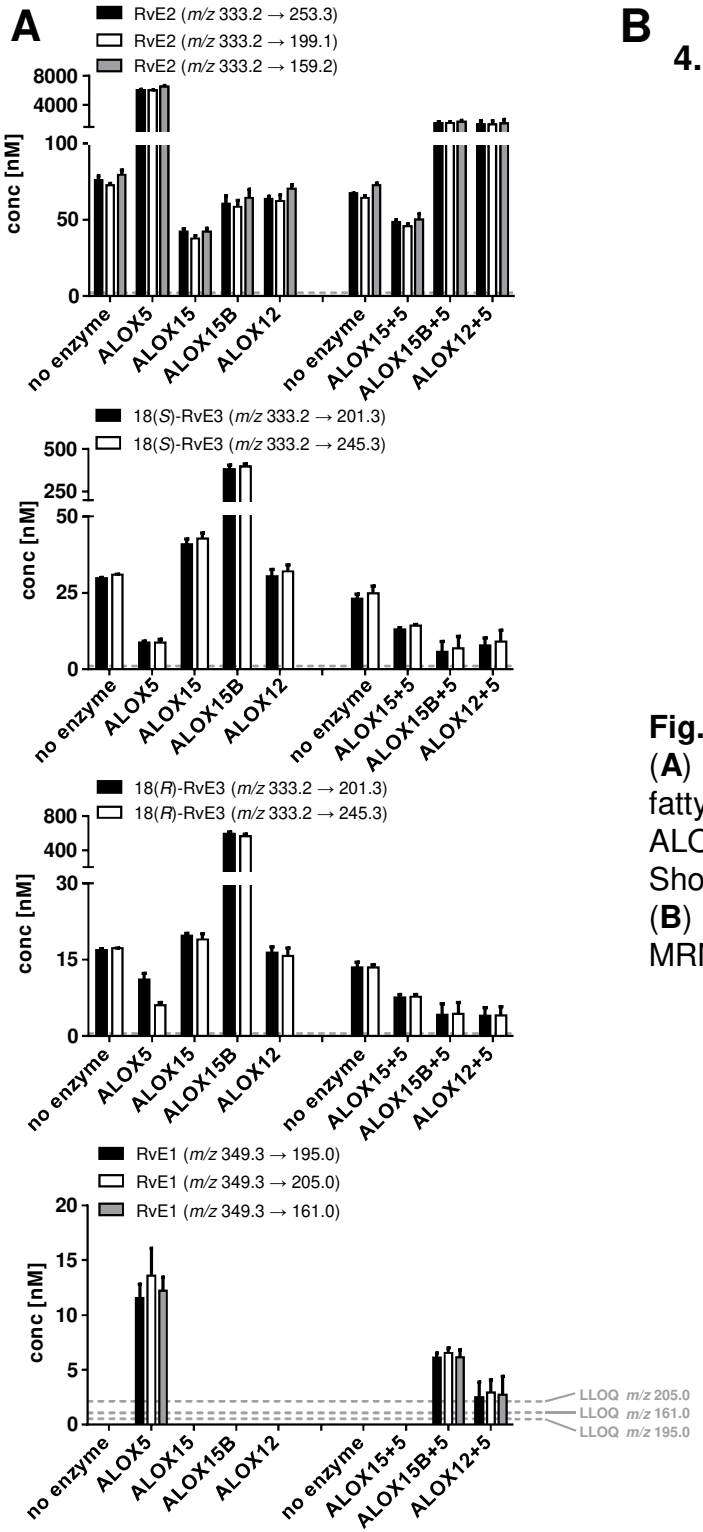


Fig. S9: *Continued.*



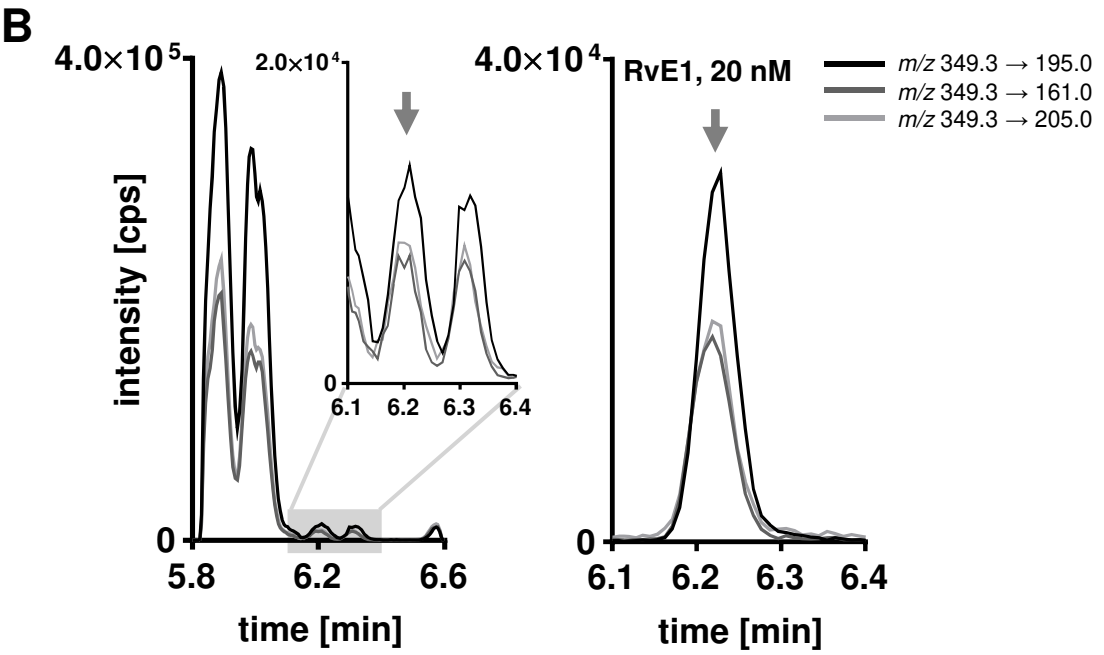
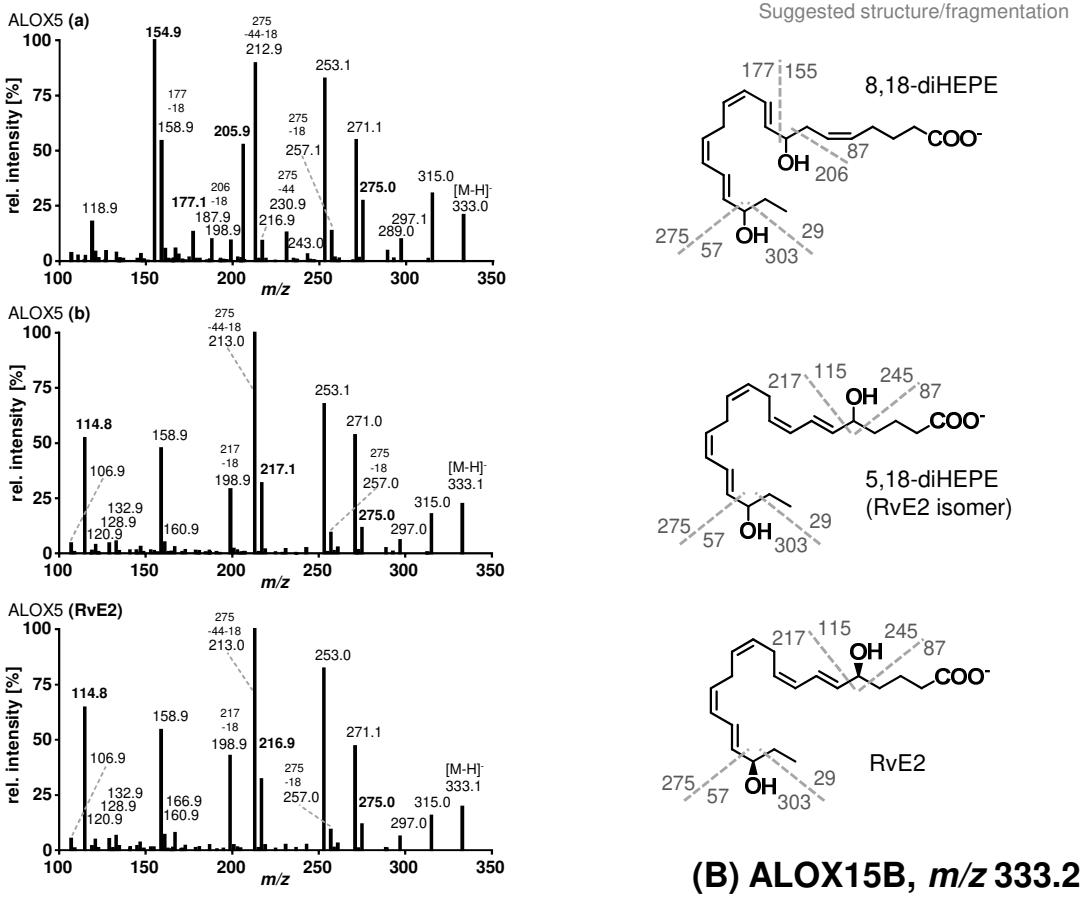


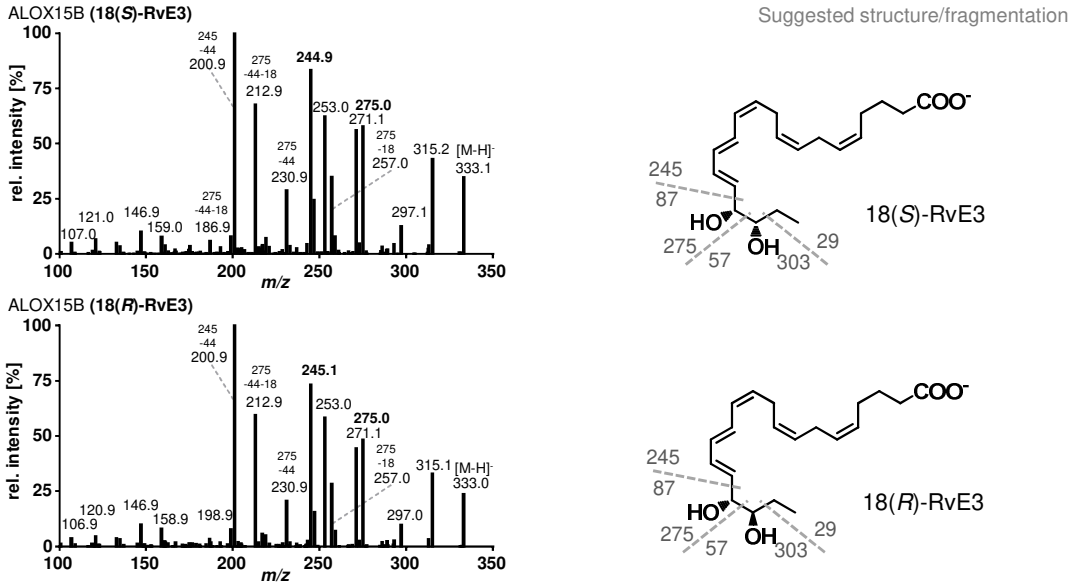
Fig. S10: Incubation of 18(*R*,*S*)-HEPE with recombinant human ALOX isoforms.
(**A**) Comparison of apparent concentrations quantified using different MRM transitions for di- and trihydroxy fatty acids formed during 18(*R*,*S*)-HEPE incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM 18(*R*,*S*)-HEPE, 15 min, RT). Shown are means ± SD (n=3). The lower limit of quantifcation (LLOQ) is indicated as dotted line.
(**B**) MRM transitions of RvE1 for an exemplary sample (ALOX5 incubation with 18(*R*,*S*)-HEPE) compared to MRM transitions of RvE1 standard (20 nM).

Fig. S11: Collision induced dissociation product ion spectra of ALOX products with *m/z* 333.2 formed during 18(*R*,*S*)-HEPE incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM 18(*R*,*S*)-HEPE, 15 min, RT).
Shown are product ion spectra of *m/z* 333.2 (100-340 Da, CE ramp -18 to -26 V) for products formed by (**A**) ALOX5, (**B**) ALOX15B, (**C**) ALOX12 and in combined incubations of (**D**) ALOX15B+ALOX5 and (**E**) ALOX12+ALOX5 alongside suggested structure and fragmentation. Note that only one of several possible double bond configurations is shown.

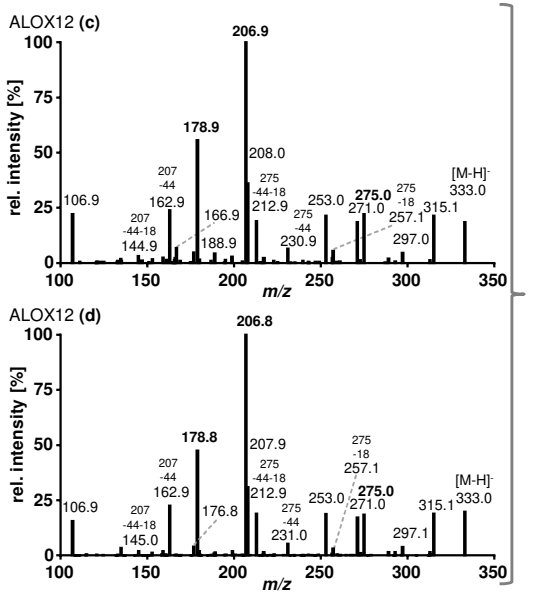
(A) ALOX5, *m/z* 333.2



(B) ALOX15B, *m/z* 333.2



(C) ALOX12, *m/z* 333.2



(D) ALOX15B+ALOX5, *m/z* 333.2

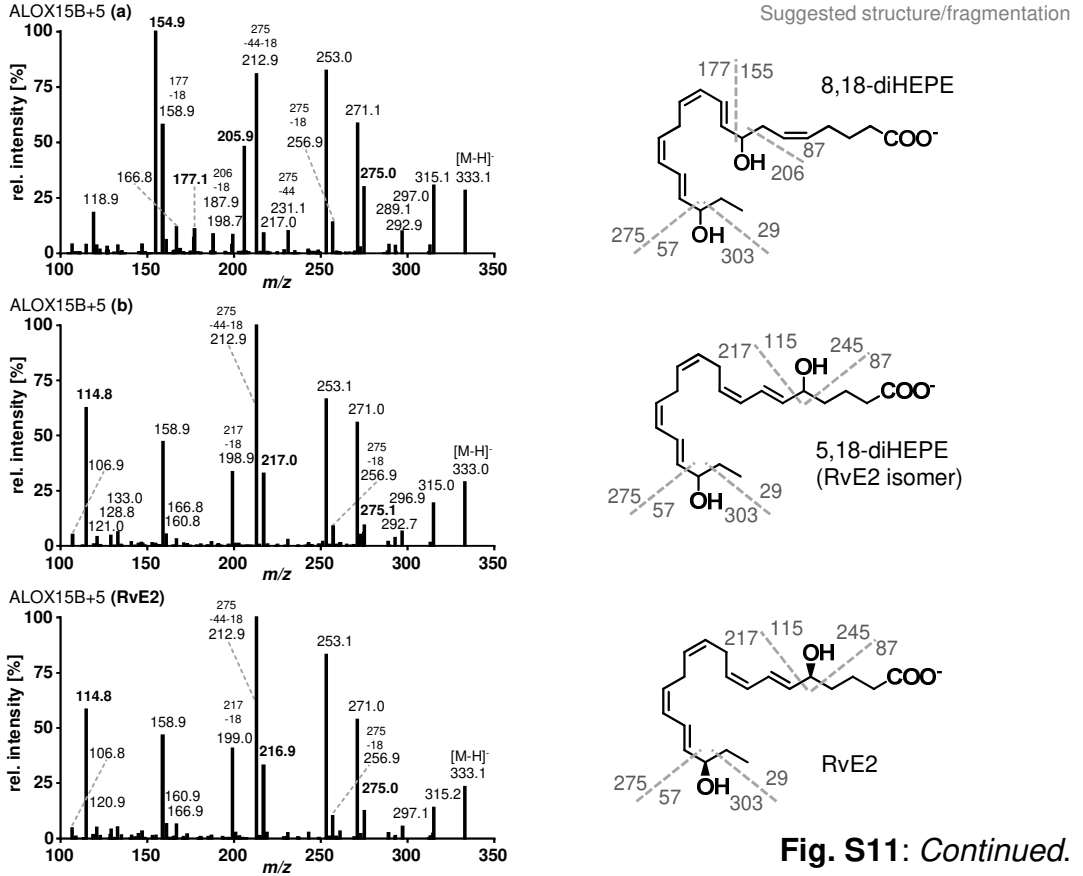


Fig. S11: *Continued.*

(E) ALOX12+ALOX5, *m/z* 333.2

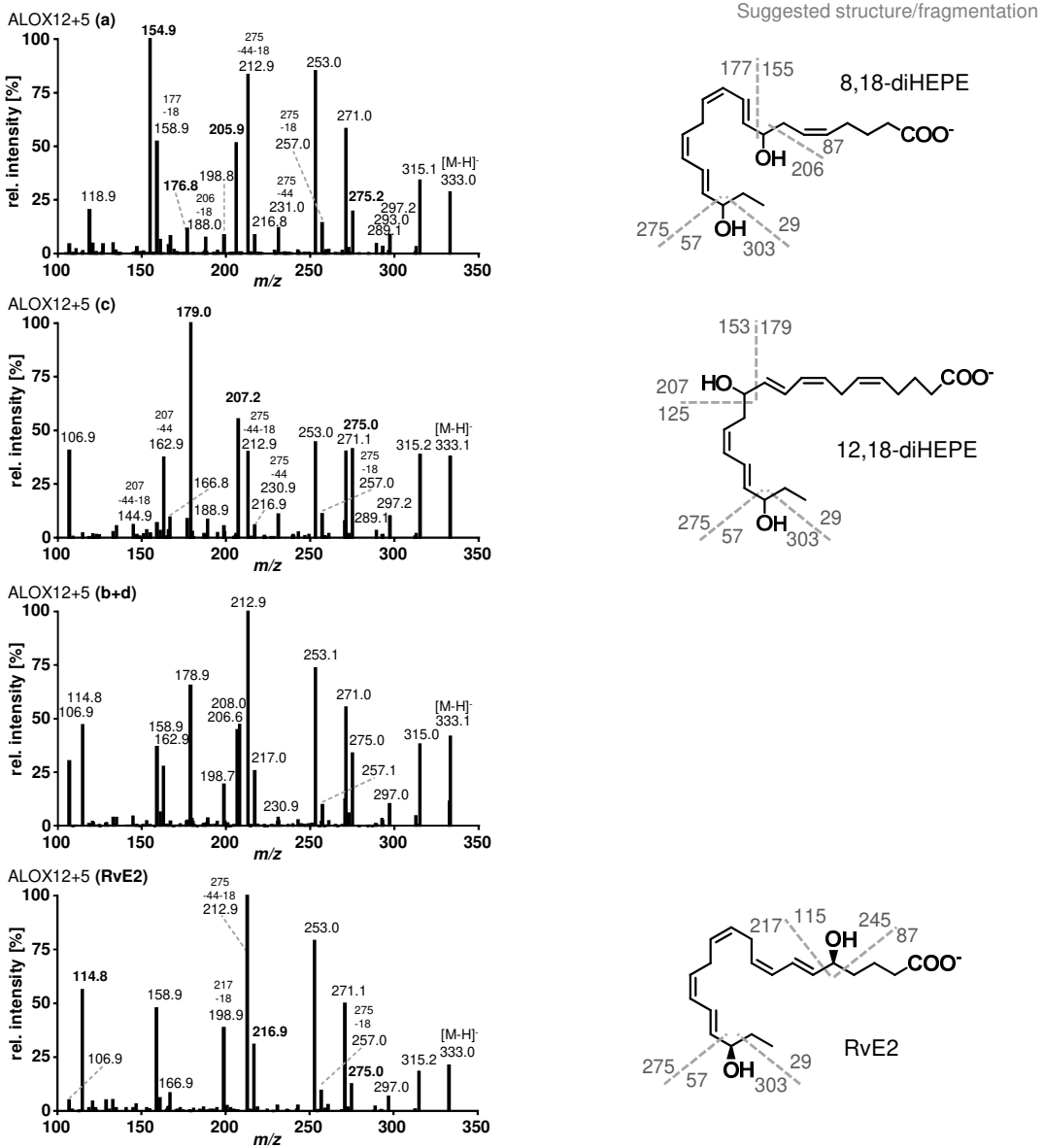
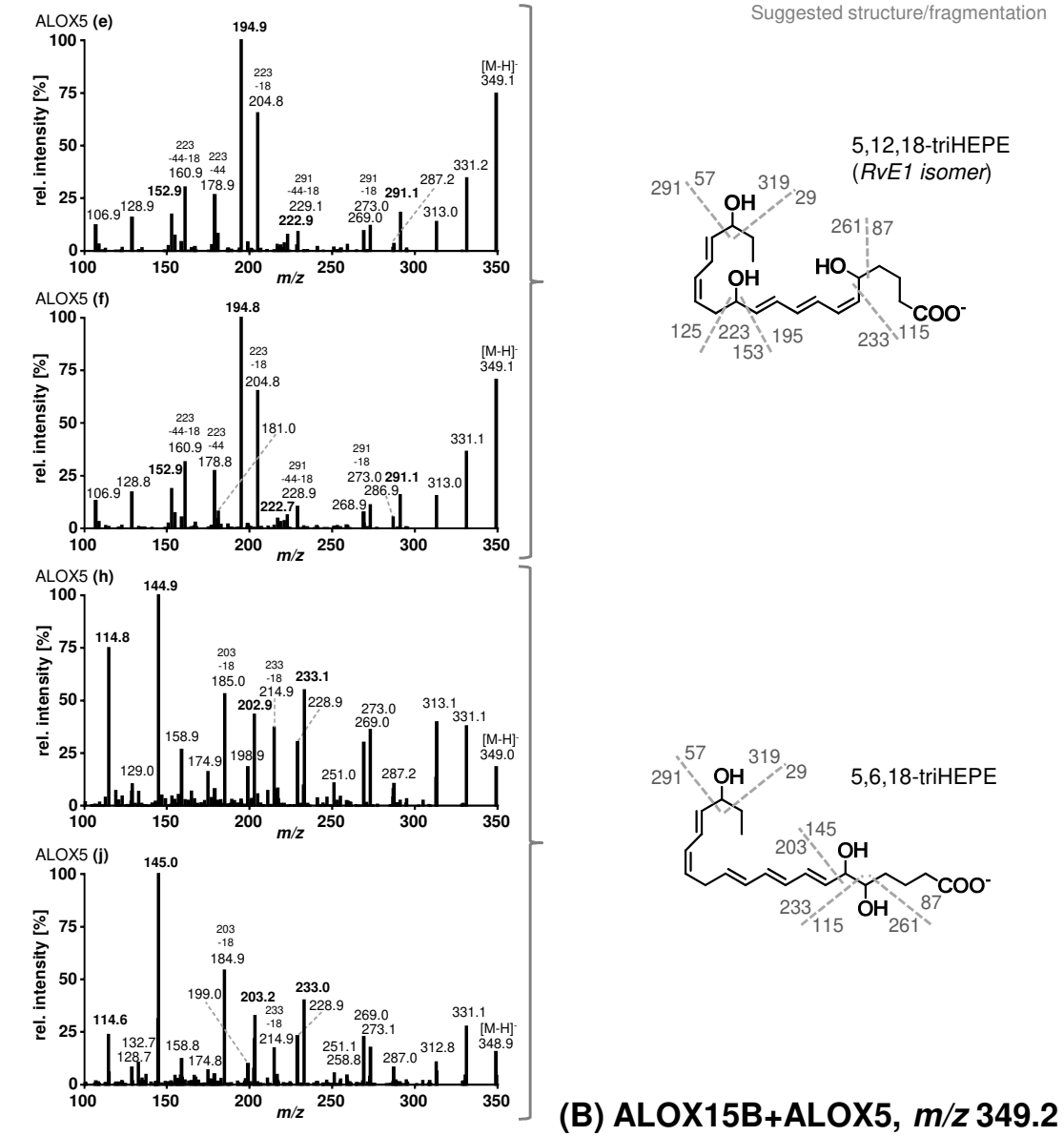


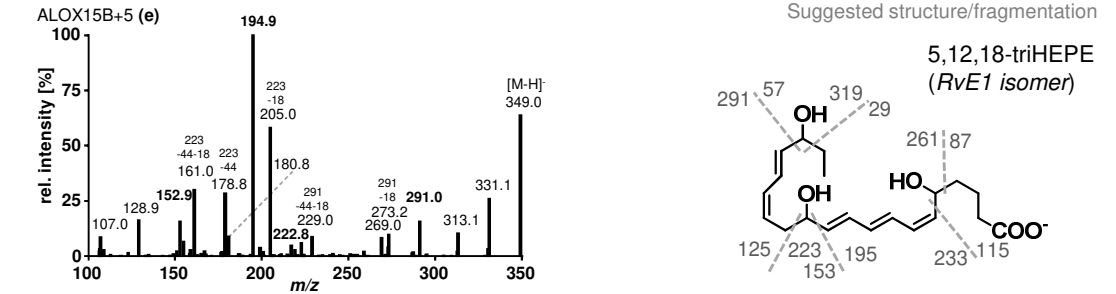
Fig. S11: *Continued.*

Fig. S12: Collision induced dissociation product ion spectra of ALOX products with *m/z* 349.2 formed during 18(*R*,*S*)-HEPE incubation with recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 alone and in combination with ALOX5 (10 µM 18(*R*,*S*)-HEPE, 15 min, RT).
Shown are product ion spectra of *m/z* 349.2 (100-350 Da, CE ramp -18 to -26 V) for products formed by (**A**) ALOX5 and in combined incubations of (**B**) ALOX15B+ALOX5 and (**C**) ALOX12+ALOX5 alongside suggested structure and fragmentation. Note that only one of several possible double bond configurations is shown.

(A) ALOX5, *m/z* 349.2



(B) ALOX15B+ALOX5, *m/z* 349.2



(B) ALOX15B+ALOX5, *m/z* 349.2 *continued*

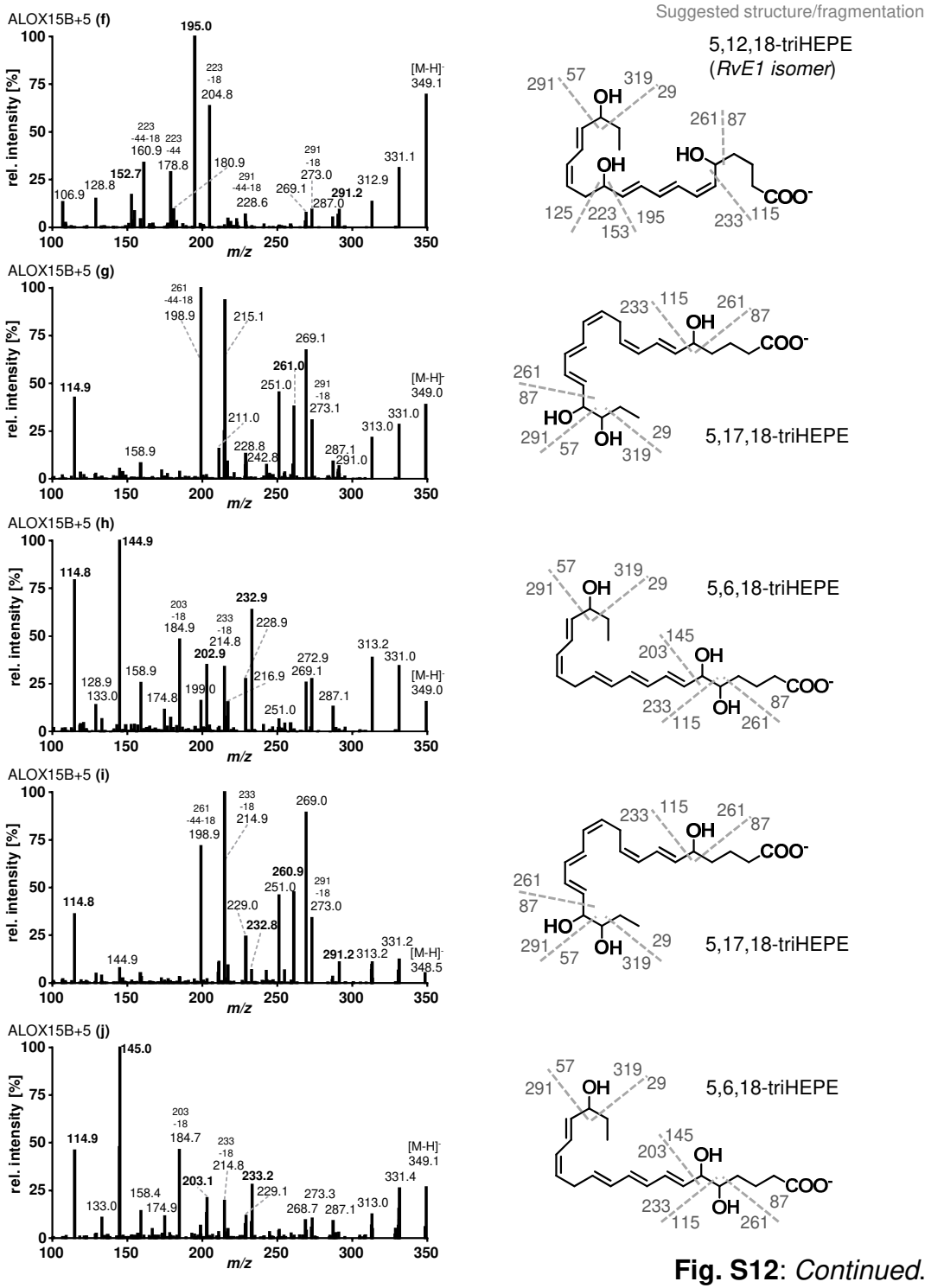
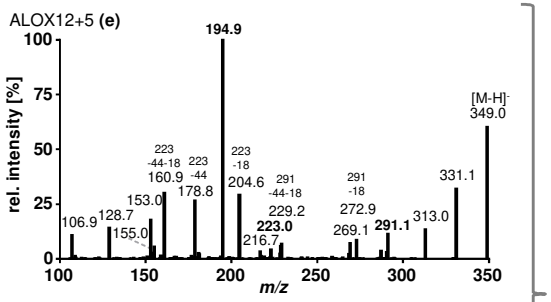
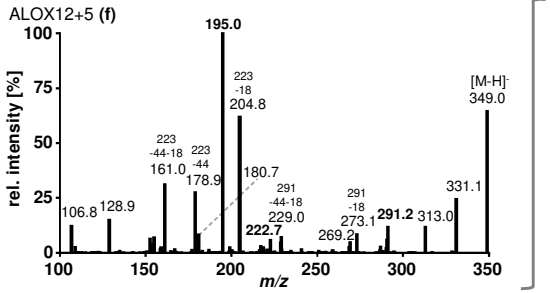
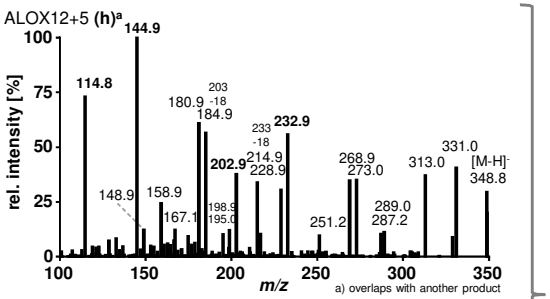


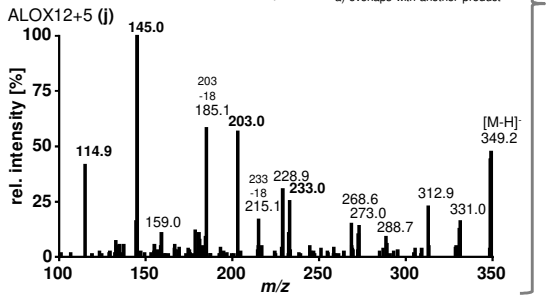
Fig. S12: *Continued.*

(C) ALOX12+ALOX5, *m/z* 349.2

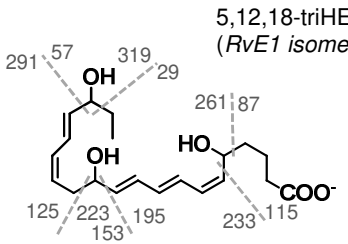








Suggested structure/fragmentation



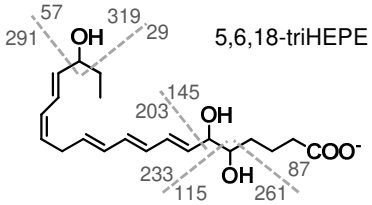


Fig. S12: *Continued.*

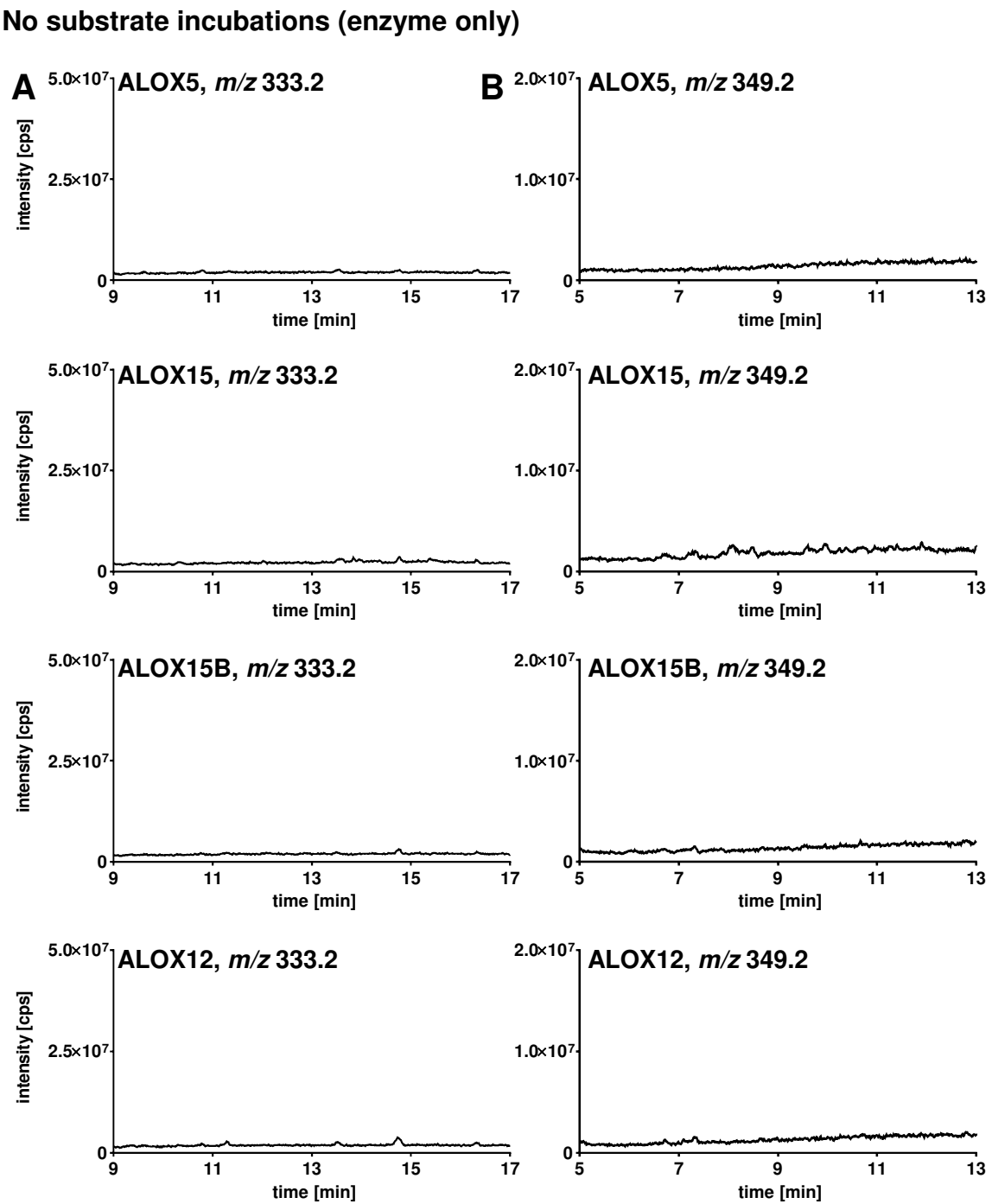


Fig. S13: No substrate (enzyme only) control incubations of recombinant human ALOX isoforms ALOX5, ALOX15, ALOX15B and ALOX12 (15 min, RT). Instead of fatty acid substrate solvent only (methanol) was added to the incubation mixture, samples were acidified and extracted twice with ethyl acetate. (**A**) Total ion chromatograms of product ion scans of *m/z* 333.2 (100-350 Da, CE ramp -18 to -26 V), (**B**) total ion chromatograms of product ion scans of *m/z* 349.2 (100-350 Da, CE ramp -18 to -26 V).

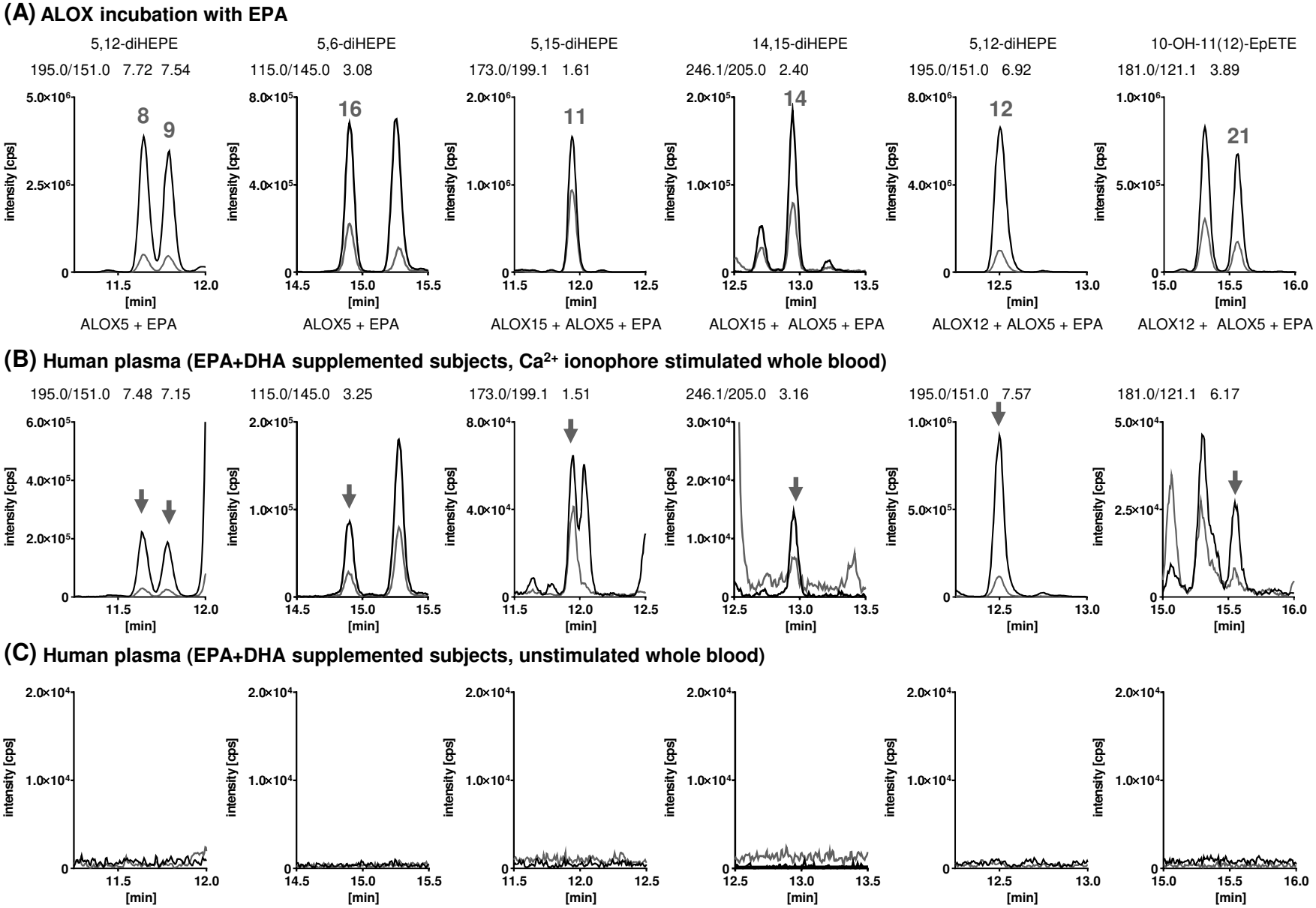


Fig. S14: Double oxygenated ALOX products in (**A**) enzyme incubations with EPA, (**B**) heparin plasma from Ca^{2+} ionophore (50 µM A23187) stimulated whole blood samples and (**C**) EDTA plasma from vehicle (0.1% DMSO) treated whole blood samples, both from subjects supplemented with EPA+DHA (Fischer *et al.*, J Lipid Res, 2014, 55(6):1150-64). Oxylipins were extracted by SPE after protein precipitation with methanol. Multiple reaction monitoring (MRM) transitions (CE -22 V, DP -80 V, CXP -9 V) were derived from fragment ion spectra of double oxygenated products in ALOX incubations with EPA: compound 8 and 9 (5,12-diHEPE) with *m/z* 333.2 → 195.0/151.0, compound 16 (5,6-diHEPE) with *m/z* 333.2 → 115.0/145.0, compound 11 (5,15-diHEPE) with *m/z* 333.2 → 173.0/199.1, compound 14 (14,15-diHEPE) with *m/z* 333.2 → 246.1/205.0, compound 12 (5,12-diHEPE) with *m/z* 333.2 → 195.0/151.0 and compound 21 (10-OH-11(12)-EpETE) with *m/z* 333.2 → 181.0/121.1. Human heparin plasma and EDTA plasma samples from six individuals were screened for these products. Two MRM transitions and their area ratio is shown for one representative female subject. Further experiments are needed to confirm the *in vivo* formation of these products in human blood.