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# Characterization and Quantification of Free and Esterified 9- and 13-Hydroxyoctadecadienoic Acids (HODE) in Barley, Germinating Barley, and Finished Malt

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The analysis of (*R*)-9- and (*S*)-9-hydroxy-10*E*,12*Z*-octadecadienoic acid as well as (*R*)-13- and (*S*)-13-hydroxy-9*Z*,11*E*-octadecadienoic acid (HODE) as free acids, esterified in triacylglycerols (storage lipids), and esterified in polar lipids (phospholipids, glycolipids, etc.) in barley, germinating barley, and finished malt was performed using [13-$^{18}O_1$]-(*S*)-13-HODE isotope dilution assays with GC-MS and straight- and chiral-phase HPLC. 9- and 13- HODE occur approximately racemically in barley, indicating an autoxidation. The enantiomeric excesses increase to 78% *S* for free 9-HODE and to 58% *S* for free 13-HODE in germinating barley as a result of lipoxygenase-2 (LOX-2) catalysis, but free HODEs are at low concentration. More than 90% of HODEs in barley and malt are esterified. In the storage lipids of green malt 53 mg/kg 9-HODE and 147 mg/kg 13-HODE were detected. This ratio of 30:70 reflects the regioselectivity of the LOX-2 enzyme in malt. In the polar lipids 45 mg/kg 9-HODE and 44 mg/kg 13-HODE were characterized. The latter indicate a hitherto unknown 9-lipoxygenase activity with polar lipids as substrates. During kilning the contents of most HODEs decreased significantly due to chemical and enzymatic degradation, whereas polar-esterified (*R*)-13-HODE increased (43%) in the finished malt.

**KEYWORDS: Lipoxygenase; barley; green malt; malt; analysis; HODE; polar lipids; enantiomers**

## INTRODUCTION

Lipoxygenases (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyze the enantioselective insertion of molecular oxygen into a (1*Z*,4*Z*)-pentadiene system of polyunsaturated fatty acids (PUFA) and conjugated *cis*,*trans*-diene hydroperoxides result.

Multiple isoenzymes of LOX have been described for many plant species, such as soybean, cucumber, tomato, potato, and rice (*1*−4). These LOX enzymes transform linoleic acid into (*S*)-9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid [(*S*)-9-HPODE] and (*S*)-13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid [(*S*)-13-HPODE], respectively.

The dioxygenation of linoleic and linolenic acid in plants is catalyzed by lipoxygenases, and resulting hydroperoxides are metabolized by further enzymes. Peroxygenases and reductases catalyze the synthesis of hydroxyoctadecadienoic acids (HODEs) or hydroxyoctadecatrienoic acids (HOTEs) (*5*), whereas the activity of a divinyl ether synthase leads to colnele(n)ic and etherole(n)ic acids (*6*−*8*). The signaling compound jasmonic acid originates from (*S*)-13-HPOTE by the activity of an allene oxide synthase (*9*). Hydroperoxide lyases catalyze the formation of aldehydes and ω-oxo acids (*10*), whereas epoxy alcohol synthases and epoxy alcohol hydrolases transform HPODE into trihydroxyoctadecenoates (*11*).

A lipoxygenase in barley was first reported by Franke and Freshe in 1953 (*12*), and subsequently the enzyme was purified and characterized from ungerminated barley (*13*−*15*). However, these investigations gave attention only to LOX-1, which is expressed in ungerminated barley. Later, the existence of a second isoenzyme (LOX-2), which generally appears to develop only after germination, was documented (*16*−18). The soybean and tomato lipoxygenases convert linoleic and linolenic acid regioselective into (*S*)-13-hydroperoxides and (*S*)-9-hydroperoxides, whereas the two barley isoenzymes are less regioselective. LOX-1 catalyzes the dioxygenation of linoleic acid into (*S*)-9-HPODE and (*S*)-13-HPODE in a ratio of ∼80:20, whereas 13-HPODE is the major product formed by LOX-2 (ratio 9-:13-HPODE = 30:70) (*19*−21).

All lipoxygenases characterized in plants catalyze the formation of (*S*)-hydroperoxides, including LOX from barley (*21*, 22). In contrast, several species of aquatic invertebrates catalyze the enzymatic oxygenation of their polyunsaturated fatty acids with (*R*)-specificity (*23*−25), and (12*R*)-LOX has been found in mammalian tissue (*26*). Furthermore, soybean lipoxygenase is able to oxygenate (9*Z*,12*E*)-9,12-octadecadienoic acid (*cis*,*trans*-linoleic acid) into (*R*)-13-HPODE. The rate of the reaction for the *cis*,*trans*-linoleic acid isomer is small (3%) compared to that for the isomeric *cis*,*cis*-substrate but certainly significant (*27*).

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In addition to free linoleic acid glycerol-esterified PUFAs are substrates for barley LOXs (*21*, *22*). The facts that germinating barley LOX-1 and LOX-2 formed both regioisomers of HPODE after incubation with linoleic acid and that they differ in spatial and temporal expression in barley embryos (*16*, 28) suggest that each isoenzyme plays different roles during germination. 13-LOX is required in the biosynthesis of jasmonates. The jasmonates are known to influence a wide variety of physiological processes in plants (*29*). In oilseed plants 13-LOX may play a crucial role as the first step of lipid mobilization to initiate $\beta$-oxidation (*30*, 31).

An increase of 9-lipoxygenase activity in response to infection and wounding has been reported for several plant−pathogen systems (*3*, 32). However, all physiological functions of the lipoxygenase pathway are not completely understood.

In contrast to lipoxygenase catalysis, autoxidation forms equal amounts of racemic 9- and 13-HPODE.

To get more information about the LOX activity in the different lipid classes during malting, the contents of the regio- and stereoisomers of the free, triacylglycerol-esterified, and polar-esterified hydroxyoctadecadienoic acids (HODE) in barley, germinating barley, and finished malt were characterized.

## MATERIALS AND METHODS

**Chemicals.** All chemicals and solvents were purchased from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Steinheim, Germany), Merck VWR International (Darmstadt, Germany), or Roth (Karlsruhe). They were of analytical grade, HPLC grade, or otherwise purified before use, if necessary. The soybean lipoxygenase was obtained from Fluka. $^{18}O_2$ gas was purchased from Campro Scientific (Miamisburg, OH).

**Plant Material.** Barley (*Hordeum vulgare* var. *Krona*) was germinated in a pilot malthouse. After 4 h of steeping, the degree of steeping increased to 45% in 48 h. The seeds were germinated at 14 °C and 96% air humidity. After 6 days of germination, samples were kilned for 18 h at 55 °C following 5 h at 85 °C.

**Lipid Extraction and Sample Workup.** After milling of the grains (35−40 g), the nonpolar fraction of barley and malt was isolated by extraction in a Soxhlet apparatus using *n*-pentane. After 16 h, the IS [[13-$^{18}O_1$]-(*S*)-13-HODE, dissolved in EA] was added to the pentane solution in the following amounts: for free HODE, 100 $\mu$g (barley, finished malt) and 1 mg (green malt); for triacylglycerol-esterified HODE, 1,5 mg (barley, finished malt) and 3 mg (green malt). No IS was added to samples that were subjected to HPLC analysis. The pentane solution was taken to dryness. To analyze the free HODE, the residue was esterified by treatment with diazomethane in diethyl ether/methanol 9:1 (v+v). To quantify the nonpolar-esterified HODE, the residue was dissolved in 20 mL of MeOH, and 15 mL of 40% KOH/MeOH was added. The mixture was stirred for 30 min under nitrogen at 60 °C, followed by the addition of 50 mL of water. The solution was acidified to pH 3 with 2 M phosphoric acid and extracted with ethyl acetate (2 × 50 mL). The organic layer was washed with brine until neutral reaction and dried over $Na_2SO_4$. The solvent was evaporated, and the residue was treated with diazomethane in diethyl ether/methanol 9:1 (v+v).

After removal of the nonpolar lipids from milled barley or malt by Soxhlet extraction, the solid residue was placed in a round-bottom flask and 1.5 mg of [13-$^{18}O_1$]-(*S*)-13-HODE was added as IS. The filter cake, which contains the polar lipids, was treated with KOH/MeOH according to the pentane fraction under nitrogen. After hydrolysis of the polar lipid fraction, the fatty acids were esterified by treatment with diazomethane. The methyl hydroxyoctadecadienoates (HODE-Me) were purified on a silica gel column [20 g of silica gel 60 (0.063−0.200 mm); solvent, PE/EA, 100 mL of 19+1 (v+v), 100 mL of 18+2, 100 mL of 15+5]. The HODE-Me eluted in the PE/EA 15+5 fraction.

**Gas Chromatography (GC)−Mass Spectrometry (MS).** HODE-Me were hydrogenated in $H_2$-satured diethyl ether with 15 mg of platinum(IV) oxide hydrate as catalyst. The solution was stirred at 0 °C for 90 min, filtered, and taken to dryness. The hydroxyoctadecanoic acid methyl esters ( ∼400 $\mu$g) were derivatized by treatment with pyridine (400 $\mu$L) and BSTFA (40 $\mu$L) at 80 °C for 30 min. Subsequently, the hydroxy fatty acid methyl esters were analyzed as trimethylsilyl ($Me_3Si$) ether derivatives.

For GC-MS investigations an HP1 column (cross-linked dimethyl−polysiloxane, 50 m × 0.2 mm internal diameter, 0.11 $\mu$m film, Hewlett-Packard; temperature program, 1 min at 80 °C, then 10 °C/min to 280 °C) installed in a GC HP 5890 coupled to a mass spectrometer (EI-MS ionization energy = 70 eV) was used. The mass spectra were recorded on an MSD HP 5970. The data were processed with HP ChemStation [version A.01.03. (1988)]. Carrier gas was He (5.6) at 75 kPa.

**UV Spectroscopy.** The UV spectra were recorded on a Uvikon spectrophotometer 922 (Kontron Instruments).

**High-Performance Liquid Chromatography (HPLC).** HPLC was performed on a Merck HPLC L-7100 with a quaternary solvent gradient system, which was coupled to a UV detector L-7400. The data were recorded and analyzed on a Merck Integrator D-7500. The regioisomers 9-HODE-Me and 13-HODE-Me were separated and isolated on a normal-phase semipreparative column Merck Hibar RT 250−10 Si60 (5 $\mu$m); the mobile phase was hexane/diethyl ether (70:30) with a flow rate of 3 mL/min. The eluate was monitored at 235 nm. Enantiomeric pairs of the respective HODE-Me were separated with a Chiralcel OD-H HPLC column (250 × 4.6 mm i.d., 5 $\mu$m; J. T. Baker) using hexane/2-propanol (98:2) as mobile phase at a flow rate of 1 mL/min according to the method of Martini et al. (*33*).

**(*S*)-13-Hydroxy-9*Z*,11*E*-octadecadienoic Acid.** To a solution of 280 mg (1 mmol) of linoleic acid in 250 mL of 0.1 M borate buffer (pH 9.0) at 4 °C was added soybean lipoxygenase (20 mg, 9.4 units/mg; Aldrich, Steinheim, Germany) in 5 mL of the buffer. Every 2 min, $O_2$ was bubbled for 20 s through the solution. After 10 min, further soybean lipoxygenase (20 mg) was added. The mixture was stirred and gassed with $O_2$ for another 20 min. The suspension was acidified to pH 4 using 2 M $H_3PO_4$ and extracted with $Et_2O$ (3 × 150 mL), and the combined organic phases were dried over $Na_2SO_4$ and evaporated. The residue containing the hydroperoxy acid was directly reduced with $NaBH_4$ (50 mg) in $Et_2O$ (30 mL) for 5 h at 4 °C. The crude product was purified on a silica gel column [20 g of silica gel 60 (0.063−0.200 mm); solvent 250 mL of PE/EA/acetic acid, 75+25+1].

**[13-$^{18}O_1$]-(*S*)-13-Hydroxy-9*Z*,11*E*-octadecadienoic Acid.** The synthesis of 13-$^{18}O_1$-labeled (*S*)-13-HODE was performed utilizing $^{18}O_2$ (95.4 atom % $^{18}O$, Campro Scientific), linoleic acid, and soybean lipoxygenase. One hundred and fifty milliliters of 0.1 M borate buffer (pH 9.0) was degassed and $N_2$-flushed in a closed three-neck round-bottom flask 10 times. Ten milliliters of $N_2$-saturated buffer with 20 mg of soybean lipoxygenase and 200 mg (0.71 mmol) of linoleic acid dissolved in 10 mL of KOH (1.2%, $N_2$-saturated) were added. Fifty milliliters of $^{18}O_2$ gas was added, and the mixture was stirred under $^{18}O_2/N_2$ atmosphere at 4 °C. After 3 h, the incubation was stopped by acidification with 2 M $H_3PO_4$. Extraction, reduction, and purification were performed according to the synthesis of unlabeled 13-HODE. The ratio of $^{18}O/^{16}O$ (*S*)-13-HODE was 91:9 (GC-MS), and there was $^{18}O$-isotope depletion of the hydroxyl group during the analytical procedure. This result is in agreement with the literature (*34*−36).

**(*S*)-9-Hydroxy-10*E*,12*Z*-octadecadienoic Acid.** Synthesis of (*S*)-9-HODE was performed according to the method of Matthew et al. (*2*).

**(*R*,*S*)-13-Hydroxy-9*Z*,11*E*-octadecadienoic Acid.** Under $N_2$ and at 0 °C 2.61 g of powdered $MnO_2$ (30 mmol) was added to a solution of (*S*)-13-HODE (285 mg, 0.96 mmol) in 12 mL of petroleum ether and stirred for 3 h. The $MnO_2$ was removed by vacuum filtration, and the solvent was evaporated: 153 mg of 13-keto-9*Z*,11*E*-octadecadieonic acid (KODE) (55%). The reaction was controlled by UV spectroscopy [$\lambda_{max}$ 234 nm (HODE) and 280 nm (KODE)].

$NaBH_4$ (83 mg, 2.18 mmol) was dissolved in 5 mL of 0.4 M $CeCl_3$/MeOH, and 13-KODE (153 mg, 0.52 mmol) was added. The solution was stirred for 5 min, followed by hydrolysis with 1 N HCl and extraction with diethyl ether. The organic phases were combined, washed with NaCl solution until neutral reaction, and dried with $Na_2SO_4$. The product was controlled by UV spectroscopy and GC-MS.

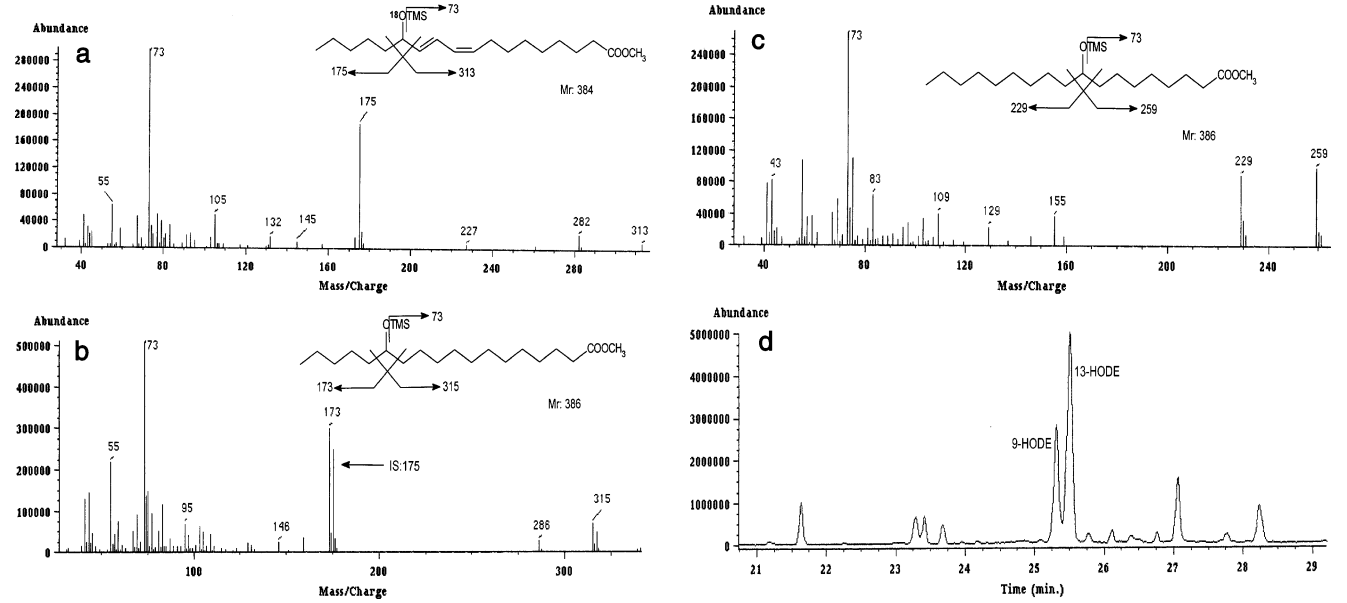


**Figure 1.** (**a**) Low-resolution EI-MS of [13-$^{18}O_1$]-13-HODE isotopic standard (IS). Sample was methylated and silylated before GC-MS analysis. (**b**) Low-resolution EI-MS of 13-HODE in malt with [13-$^{18}O_1$]-13-HODE isotopic standard (IS). Samples were methylated, hydrogenated, and silylated before GC-MS analysis. (**c**) Low-resolution EI-MS of 9-HODE in malt. Samples were methylated, hydrogenated, and silylated before GC-MS analysis. (**d**) Total ion chromatogram (GC-EI/MS) of 9-HODE and 13-HODE in green malt. Samples were methylated, hydrogenated, and silylated before GC-MS analysis

**(*R,S*)-9-Hydroxy-10*E*,12*Z*-octadecadienoic Acid.** The synthesis of (*R,S*)-9-HODE was performed starting with (*S*)-9-HODE according to the regioisomer (*R,S*)-13-HODE.

**Quantification.** All quantitative data presented (mg/kg and ee) are an average of a duplicate analysis. The standard deviations of the analytical methods were calculated by analyzing a reference malt 6 times. For GC-MS analysis the standard deviations were as follows: 12% (free), 9% (triacylglycerol), and 11% (polar) for 9-HODE and 7% (free), 5% (triacylglycerol), and 6% (polar) for 13-HODE. Standard deviations of the chiral HPLC analysis were 2% for free and triacylglycerol-esterified HODEs and 3% for polar -esterified HODEs.

## RESULTS

**GC-MS Analysis.** An analytical method for the characterization of free, triacylglycerol-esterified, and polar-esterified monohydroxyoctadecadienoic acids (HODE) was developed, and data from barley and malt are presented.

The analysis of HODE was performed by gas chromatography−electron impact mass spectrometry (GC-EI/MS) with isotope dilution assays utilizing [13-$^{18}O_1$]-(*S*)-13-hydroxy-9*Z*,-11*E*-octadecadienoic acid as standard. The EI-MS fragment ions (α-cleavage) of 13-HODE (as methylated, hydrogenated, and silylated derivative) *m*/*z* 173 and 174 (analyte, unlabeled) in comparison to *m*/*z* 175 and 176 (standard, labeled) (**Figure 1a**,**b**) were used for quantification. The mass spectrum of 9-HODE (as methylated, hydrogenated, and silylated derivative) is shown in **Figure 1c**. The MS is in agreement with that shown by Yang et al. (*19*). The amount of 9-HODE was determined relative to 13-HODE by GC-EI/MS using the total ion current (**Figure 1d**) and by GC-FID. This method was confirmed by mixing and analyzing the pure isomers. The classification of HODE into free, triacylglycerol-esterified, and polar-esterified lipids was performed as follows: Free HODE were analyzed by direct methylation of the pentane extract with diazomethane, triacylglycerol HODE after basic hydrolysis of the pentane extract (in sum with free HODE). The HODE esterified in the polar lipids were released by treatment of the solid residue of the Soxhlet extraction with KOH/MeOH and analyzed accordingly.

**HPLC Analysis.** The regioisomers 9- and 13-HODE were separated as methyl esters on semipreparative SP-HPLC, and the purified regioisomers were subjected to a chiral HPLC analysis (**Figures 2**). HODE standards were synthesized utilizing lipoxygenase from tomato [(*S*)-9-HODE] or soybean [(*S*)-13-HODE], and the respective (*R*)-enantiomers were obtained as racemates by oxidation ($MnO_2$) and reduction ($NaBH_4$) of the HODEs.

**9-HODE.** Investigations of free and esterified HODEs demonstrated the low amounts of free HODEs in barley and malt (**Table 1**). Only 4 mg/kg of 9-HODE (5% of total 9-HODE lipids) occurs in barley as free acids and increases in green malt up to 15% (18 mg/kg). During kilning the amount of free 9-HODE decreases, and in the finished malt <2% (1 mg/kg) was analyzed. In barley and malt ∼50% of the 9-HODE was characterized in the storage lipids (triacylglycerides) and ∼40% as polar-esterified 9-HODE.

In barley, enantiomers of 9-HODE occur nearly racemically. Only a small excess of (*R*)-9-HODE was detected in all three lipid classes. During germination the concentration of 9-HODE increases and is accompanied by a significant alteration of its enantiomeric ratio in green malt. The enantiomeric excess of (*S*)-9-HODE in 6-day-old green malt was determined as follows: ee (%) = 78(*S*) for free 9-HODE, ee (%) = 14(*S*) for triacylglycerol-esterified 9-HODE, and ee (%) = 16(*S*) for polar-esterified 9-HODE. After kilning, the finished malt was analyzed and a slight excess of (*R*)-9-HODE [ee (%) = 6(*R*)] was found in the esterified lipids. However, the small amount of free 9-HODE showed an excess of the (*S*)-configuration [ee (%) = 16(*S*)] in finished malt (**Table 1**). The concentrations of the HODE enantiomers are shown in **Figure 3**.

**13-HODE.** According to 9-HODE, the 13-HODE isomer was analyzed by GC-MS and chiral HPLC (**Table 1**; **Figure 3**). In barley and malt, the concentration of free 13-HODE was low. This result was comparable to the low content of the free acid of 9-HODE. The highest concentration of 13-HODE was detected in the storage lipids. The total concentration of 13-HODE in all lipid classes increased during germination from 88 mg/kg in barley to 234 mg/kg in the green malt. After kilning, 119 mg/kg was detected in the finished malt. In all lipid classes of barley, green malt, and finished malt 13-HODE was analyzed

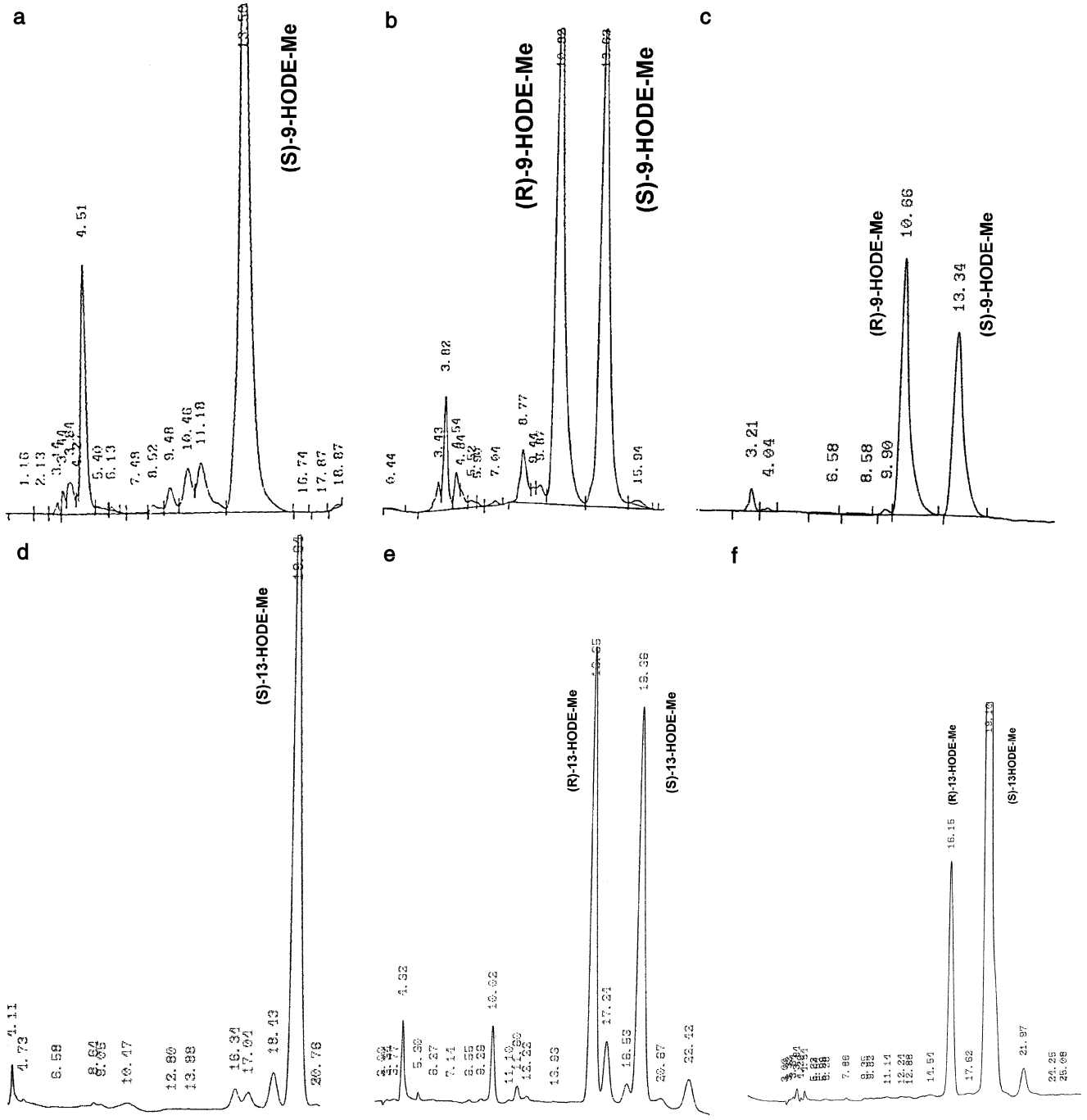


**Figure 2.** (**a**) HPLC chromatogram of (*S*)-9-HODE-Me on Chiracel OD-H; detection at 235 nm. (**b**) Separation of (*R*,*S*)-9-HODE-Me on Chiracel OD-H; detection at 235 nm. (**c**) Separation of (*R*,*S*)-9-HODE-Me from barley on Chiracel OD-H; detection at 235 nm. (**d**) HPLC chromatogram of (*S*)-13-HODE-Me on Chiracel OD-H; detection at 235 nm. (**e**) Separation of (*R*,*S*)-13-HODE-Me on Chiracel OD-H; detection at 235 nm. (**f**) Separation of (*R*,*S*)-13-HODE-Me from green malt on Chiracel OD-H; detection at 235 nm.

with an excess of (*S*)-configuration. The enantiomeric excess of the (*S*)-13-enantiomer was highest in green malt, which is in accordance with the (*S*)-9-HODE enantiomer. However, in the polar lipids the concentration of (*R*)-13-HODE increased from 14 mg/kg in green malt to 20 mg/kg in finished malt (**Figure 3c**).

**Total HODE.** In green malt the total HODE concentration rises to 350 mg/kg, whereby 192 mg/kg of HODE isomers was formed through the germination process (**Table 2**). The ratio of 9-:13-HODE regioisomers was found to be 26:74, which reflects the regioselectivity of malt LOX-2. However, (*R*)-HODE was analyzed with an increase of 42 mg/kg and a ratio of 17:83 for the 9- and 13-HODE regioisomers.

## DISCUSSION

9- and 13-HPODE are formed by enzymatic and chemical peroxidation of linoleic acid or its esters. The resulting hydroperoxides are very reactive and are transformed, for example, to hydroxides by peroxidases, peroxygenases, or other reductions in plants. Therefore, the amount of HODEs should reflect the generation of linoleic acid hydroperoxides.

**Barley.** The monohydroxy acids occur nearly racemically [(*R*):(*S*)) ≅ 50:50] in ungerminated barley. The small excess of the (*R*)-9-HODE enantiomer indicates an enzymatic degradation of (*S*)-9-H(P)ODE, because the formation of the (*S*)-enantiomer is catalyzed by LOX activity and autoxidation only forms racemic mixtures.

**Table 1.** Free and Triacylglycerol- and Polar-Esterified HODE in Barley, Green Malt, and Finished Malt (Dry Matter) and Enantiomeric Ratios of HODE Isomers

| | 9-HODE | | 13-HODE | |
|---|---|---|---|---|
| | mg/kg | *S:R* (%) | mg/kg | *S:R* (%) |
| barley | | | | |
| free | 4 | 48:52 | 3 | 53:47 |
| triacyl | 36 | 47:53 | 60 | 53:47 |
| polar | 30 | 46:54 | 25 | 52:48 |
| total | 70 | 47:53 | 88 | 53:47 |
| 6-day green malt | | | | |
| free | 18 | 89:11 | 43 | 79:21 |
| triacyl | 53 | 57:43 | 147 | 64:36 |
| polar | 45 | 58:42 | 44 | 68:32 |
| total | 116 | 62:38 | 234 | 68:32 |
| finished malt | | | | |
| free | 1 | 58:42 | 2 | 53:47 |
| triacyl | 40 | 47:53 | 67 | 60:40 |
| polar | 30 | 47:53 | 50 | 60:40 |
| total | 71 | 47:53 | 119 | 60:40 |

The analysis of 13-HODE in ungerminated barley demonstrated an excess of the (*S*)-enantiomer in all lipid classes. Although numerous investigations have not shown the existence of LOX-2 in sound barley, the formation of 13-hydroperoxides cannot be the result of an autoxidation exclusively. The barley LOX-1 enzyme catalyzes the oxygenation of linoleic acid into 9- and 13-hydroperoxides in a distinct ratio of 88:12, which explains the excess of (*S*)-13-HODE in barley. However, in consideration of the high amount of (*R*)-9-HODE and a minor excess of the (*S*)-13-HODE isomer, autoxidation is most likely involved in the formation of H(P)ODEs in ungerminated barley.

**Germination.** Upon germination a second lipoxygenase isoenzyme (LOX-2) is reported to develop (*16*−*18*).

The 6-day green malt showed a significant increase of the amounts of the free (*S*)-9- and (*S*)-13-HODE enantiomers (**Table 3a**). The concentrations of (*S*)-9-HODE rose from 2 to 16 mg/kg and those of (*S*)-13-HODE from <2 to 34 mg/kg (**Figure 3a**). These increases of 14 mg/kg [(*S*)-9-HODE] and 32 mg/kg [(*S*)-13-HODE] are in accordance with the LOX-2 catalysis, which forms 9- and 13-hydroperoxides in a ratio of 30:70.

Characterization of triacylglycerol-esterified HODE in 6-day green malt showed an increases of the (*S*)-enantiomer of 13 mg/kg for 9-HODE and 62 mg/kg for 13-HODE (**Figure 3b**; **Table 3b**). This ratio of 17:83 reflects the regioselectivity of LOX-2 catalysis. These data suggest the oxygenation of free and triacylglycerol-esterified linoleic acid during germination only by LOX-2 activity. However, the formation of H(P)ODEs in germinating barley seems to be more complex because the amounts of (*S*)- as well as (*R*)-enantiomers increase significantly. From barley to 6-day green malt the amounts of (*R*)-9-HODE showed an increase of 4 mg/kg and that of (*R*)-13-HODE of 25 mg/kg, which cannot be caused only by autoxidation. A possible explanation is the simultaneous degradation of autoxidation-derived (*R*)-9-HODE via $\beta$-oxidation. The $\beta$-oxidation of fatty acids in plants leads to (*S*)-3-hydroxyacyl-CoA as intermediate (*37*, 38), possessing the same absolute configuration as (*R*)-9-HODE and (*S*)-13-HODE, which therefore fit into this degradative pathway. In addition, a trans double bond in 13-HODE (C-11) is present, and enoyl-CoA isomerase can act directly without a need of other isomerases (*39*). (*S*)-13-Hydroxy-9*Z*,-11*E*-octadecadienoic acid is described as the main product of lipoxygenase-catalyzed oxygenation of storage lipids in cucumber and soybean, indicating the lipid mobilization during germination (*1, 30, 31*).

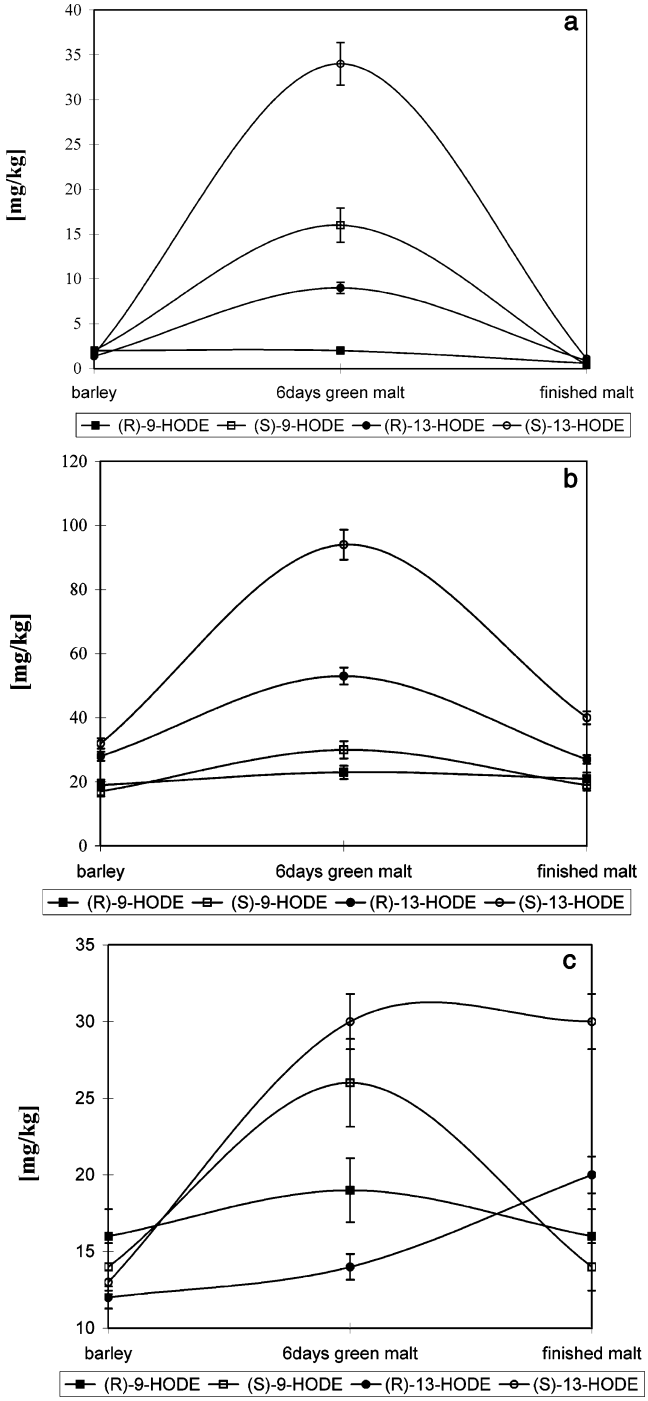


**Figure 3.** Concentrations of (**a**) free HODE isomers, (**b**) triacylglycerol-esterified HODE isomers, and (**c**) polar-esterified HODE isomers in barley, green malt, and finished malt (dry matter) in milligrams per kilogram.

**Table 2.** Total Amount (All Three Lipid Classes in Sum) of HODE Regioisomers in Green Malt and Increase during Germination

| | HODE concentration (mg/kg) | | | ratio (9-:13-) |
|---|---|---|---|---|
| | Σ HODE | 9-HODE | 13-HODE | (%) |
| total (analyzed in 6-day green malt) | 350 | 116 | 234 | 33:67 |
| effect of germination | +192 | +46 | +146 | 24:76 |
| (*S*)-HODE | +150 | +39 | +111 | 26:74 |
| (*R*)-HODE | +42 | +7 | +35 | 17:83 |

The polar-esterified HODEs in barley and malt were quantified by an indirect method, analyzing the saponified residue of

**Table 3.** Amount of HODE Isomers in Green Malt and Increase of HODE Enantiomers during Germination

| | HODE concentration (mg/kg) | | | ratio (9-:13-) |
|---|---|---|---|---|
| | Σ HODE | 9-HODE | 13-HODE | (%) |
| | (**a**) Free HODE | | | |
| total (analyzed in 6-day green malt) | 61 | 18 | 43 | 30:70 |
| effect of germination | +54 | +14 | +40 | 26:74 |
| (*S*)-HODE | +46 | +14 | +32 | 30:70 |
| (*R*)-HODE | +8 | 0 | +8 | 0:100 |
| | (**b**) TG-Esterified HODE | | | |
| total (analyzed in 6-day green malt) | 200 | 53 | 147 | 27:73 |
| effect of germination | +104 | +17 | +87 | 16:84 |
| (*S*)-HODE | +75 | +13 | +62 | 17:83 |
| (*R*)-HODE | +29 | +4 | +25 | 14:86 |
| | (**c**) Polar-Esterified HODE | | | |
| total (analyzed in 6-day green malt) | 89 | 45 | 44 | 51:49 |
| effect of germination | +34 | +15 | +19 | 44:56 |
| (*S*)-HODE | +29 | +12 | +17 | 41:59 |
| (*R*)-HODE | +5 | +3 | +2 | 60:40 |

**Table 4.** Amount of HODE Enantiomers in Green Malt and Finished Malt and Effect of Kilning

| | HODE concentration (mg/kg) | | change during |
|---|---|---|---|
| | green malt | finished malt | kilning (%) |
| | (**a**) Free HODE | | |
| (*S*)-9-HODE | 16 | 0,6 | –96 |
| (*R*)-9-HODE | 2 | 0,4 | –80 |
| (*S*)-13-HODE | 34 | 1 | –97 |
| (*R*)-13-HODE | 9 | 1 | –89 |
| Σ HODE | 61 | 3 | –95 |
| | (**b**) TG-Esterified HODE | | |
| (*S*)-9-HODE | 30 | 19 | –37 |
| (*R*)-9-HODE | 23 | 21 | –9 |
| (*S*)-13-HODE | 94 | 40 | –57 |
| (*R*)-13-HODE | 53 | 27 | –49 |
| Σ HODE | 200 | 107 | –47 |
| | (**c**) Polar-Esterified HODE | | |
| (*S*)-9-HODE | 26 | 14 | –46 |
| (*R*)-9-HODE | 19 | 16 | –16 |
| (*S*)-13-HODE | 30 | 30 | ± 0 |
| (*R*)-13-HODE | 14 | 20 | +43 |
| Σ HODE | 89 | 80 | –10 |

the Soxhlet extraction. Therefore, the class of the polar lipids may contain phospholipids, glycolipids, and other polar, water soluble lipids that are of high interest in the brewing process.

The amount of polar-esterified HODE isomers in barley was found in the same range, and this fact could be caused by autoxidation (**Table 1**). During germination the (*R*)-enantiomers of 9- and 13- HODE increased marginally (both ∼2 mg/kg), whereas the (*S*)-enantiomers increased significantly in a ratio of 40:60 (9-:13-HODE) catalyzed by LOX activity (**Figure 3c**; **Table 3c**). This result is not in accordance with the reported regioselectivity of LOX-2 and the presented data in triacylglycerols; a hitherto unknown (*S*)-9-lipoxygenase activity in germinating barley is postulated with polar lipids as substrates.

**Kilning.** Quantification of free and triacylglycerol-esterified HODEs in the finished malt demonstrated the chemical and enzymatical degradation of monohydroxy fatty acids (**Table 4a**,**b**; **Figure 3a**,**b**). In sum, the total concentration of 9-HODE and 13-HODE decreased in the nonpolar lipid classes (free and triacylglycerol) during kilning from 71 to 41 mg/kg (9-HODE) and from 190 to 69 mg/kg (13-HODE). The activity of the two LOX isoenzymes decreased during kilning, and only 1−3% of the total LOX activity developed remained in the cured malt (*40*). The decrease of HODEs could be caused by chemical degradation due to the higher temperatures in the drying process. The lowering of the content of (*S*)-13-HODE might be a result of a $\beta$-oxidative metabolism. In comparison, (*S*)-9-HODE could be degraded by other downstream enzymes in the LOX pathway such as the hydroperoxide lyase (from HPODE) or epoxy alcohol synthase into reactive (*S*)-9-hydroxy-12,13-epoxy-10*E*-octadecenoic acid. The hydroxy epoxy acids are hydrolyzed, for example, into trihydroxyoctadecenoic acids (*41*).

The amount of (*R*)-13-HODE in the storage lipids rose from 28 mg/kg in barley to 53 mg/kg in the 6-day green malt and decreased to 27 mg/kg after kilning, whereas the concentration of (*R*)-9-HODE changed only slightly during malting, which is evidence that not just autoxidation causes the high amounts of (*R*)-configured HODEs.

In contrast to the storage lipids, the amount of (*R*)-13-HODE in the polar lipids increased during kilning, whereas the (*S*)-13-HODE content was retained (**Table 4c**). The concentration of polar- and triacylglycerol-esterified (*R*)- and (*S*)-9-HODE changed similarly. The accumulation of (*R*)-13-HODE in finished malt was not expected because all other HODEs decreased during kilning (**Figure 3c**). A 9*Z*,12*E*-linoleic acid was reported to end up in the (*R*)-13-HPODE enantiomer by LOX catalysis in soybean (*27*), but this linoleic acid isomer has not been detected in polar lipids in malt so far.

## ABBREVIATIONS USED

BSTFA, *N*,*O*-bis(trimethylsilyltrifluoroacetamide); EA, ethyl acetate; ee, enantiomeric excess; GC-MS, gas chromatography−mass spectrometry; GC-EI/MS, gas chromatography−electron impact mass spectrometry; H(P)OTE, hydro(pero)xyoctadecatrienoic acid; 9-H(P)ODE, 9-hydro(pero)xy-10*E*,12*Z*-octadecadienoic acid; 13-H(P)ODE, 13-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid; IS, isotopic standard; Me, methyl ester; (SP)-HPLC, (straight phase) high-performance liquid chromatography; 13-KODE, 13-keto-9*Z*,11*E*-octadecadienoic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acids; PE, petroleum ether.

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