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Oxidative Degradation of Lipids during Mashing

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Although hardly any polyunsaturated fatty acids (PUFAs) are present in the endproduct, the ingredients used for the production of beer contain a high concentration of PUFAs, such as linolic and linolenic acid. These compounds are readily oxidized, resulting in the formation of lipid-derived products that reduce the taste and quality of beer enormously. During mashing relatively high amounts of PUFAs are exposed to atmospheric oxygen at a relatively high temperature. This makes mashing a critical step in the brewing process with regard to the formation of lipid-derived off-taste products. F_1 phytoprostane (PPF_1) changes in antioxidant capacity and monohydroxy fatty acids (OH-FAs) were used as markers for the detection of oxidative damage to fatty acids during mashing. The pattern of OH-FA formation indicates that enzymatic oxidation of PUFAs is more important than nonenzymatic oxidation during the mashing process. Nevertheless, substantial nonenzymatic radical formation is evident from the increase of specific OH-FAs and PPF_1s. It was found that a low oxygen tension reduces oxidative damage and gives a high antioxidant capacity of the mashing mixture. This indicates that mashing should be done under low oxygen pressure.

KEYWORDS: Markers; oxidative stress; nonenzymatic oxidation; monohydroxy fatty acids; phytoprostanes; TEAC; PUFA

INTRODUCTION

Beer is a complex mixture of various compounds, introduced to it by the ingredients hop, malt (i.e., germinated barley), water, and yeast. In the ingredients, used for the production of beer, as well as in the intermediate products of the first steps of the brewing process, a high concentration of polyunsaturated fatty acids (PUFAs) is present (*1*). However, beer itself contains hardly any PUFAs because they have been assimilated by the brewing yeast during the fermentation process (*1*).

The quality of beer is negatively influenced by oxidation. For example, off-taste products are formed by the oxidation of lipids and other beer components. The most vulnerable step, with regard to lipid oxidation, in the production of beer is the mashing. This process involves increasing the temperature of the mash, that is, a mixture of malt and water, to the optimum temperature for the desired enzymes, and maintaining a rest (i.e., holding for a period of time) at that temperature. Besides the elevated temperatures during the mashing, the mixture is exposed to atmospheric oxygen, which promotes oxidative damage. These conditions in combination with the presence of a high concentration of easily oxidized products, such as PUFAs (*2*), make mashing a critical step in the brewing process.

The most abundant PUFAs in the ingredients of mash are linoleic acid (C18:2) and linolenic acid (C18:3) (*1*). The latter PUFA is the most sensitive to oxidation, due to its three double bonds. Oxidation of these PUFAs will result in the formation of hydroperoxides. These hydroperoxides are reactive and degrade easily, forming monohydroxy fatty acids (OH-FAs), phytoprostanes, and other products, such as carbonyls, aldehydes, and ketones (**Figure 1**).

Apart from autoxidation, OH-FAs can also be formed by photo-oxidation (*3*) and enzymatic oxidation. It is possible to differentiate between these routes of formation because enzymatic conversions solely give 9(*S*)-products (*4*) and 13(*S*)-products, due to the specificity of the lipoxygenases (LOX) (*5*−*7*). Barley is reported to contain two LOX enzymes. LOX-1, mainly present in ungerminated barley (*8*), is responsible for the production of 9-products, whereas the 13-product is formed by the other isoenzyme, LOX-2 (*9*), that appears during germination (*8*). Although most of the LOX activity present in the germinating barley is lost during the kilning or drying process, in the resulting brewing malt both LOX-1 and LOX-2 activity can still be measured (*10*).

The nonenzymatic processes produce racemic compounds, giving all isomers (9-, 12-, 13-, and 16-OH-FAs) in almost the same yield (*4*). This makes 12- and 16-OH-FA formation

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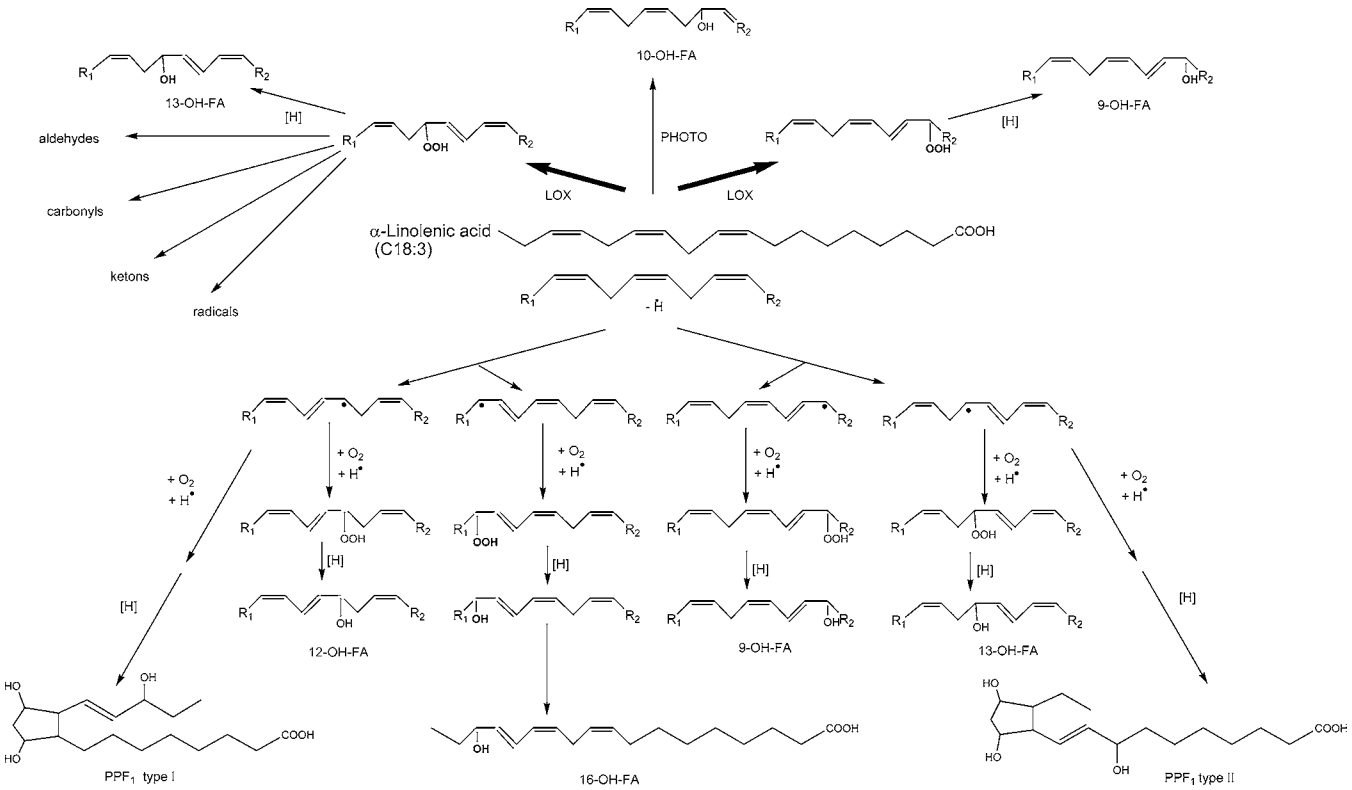

Figure 1. Oxidative degradation of linolenic acid. The products 9- and 13-OH-FA are formed during enzymatic oxidation. The 9-, 12-, 13-, and 16-OH-FAs are formed during nonenzymatic oxidation. Photo-oxidation results in 10-OH-FAs and 15-OH-FAs (not shown). Hydroperoxides also give rise to the formation of other products, such as aldehydes, carbonyls, and ketones, and to the formation of radicals.

specific for nonenzymatic oxidation (*11*). 10- and 15-OH-FAs are selectively used as markers of photo-oxidation (*12*).

F_1-phytoprostanes (PPF_1s) are cyclic oxylipins that are chemically stable endproducts of the autoxidation of linolenic acid (*13*). These plant-derived compounds are equivalent to the mammalian isoprostanes, but differ in the length of the carbon chain (18 carbons versus 20 carbons) and in the number of double bonds (PPF_1s contain one fewer double bond) (*14*).

Hydroperoxides can also directly degrade into low molecular weight compounds, such as carbonyls, ketones, and acids (*15*). These products can cause a stale, cardboard (*trans*-2-nonenal), or papery off-flavor (*1*, *2*, *15*, *16*). LOX-1 was found to have a major role in the production of *trans*-2-nonenal (*17*, *18*).

It is known that during mashing antioxidants, such as tannins and anthocyanogens, are released from the barley and are dissolved into the extract solution (*1*). The total antioxidant capacity of these compounds, which protect the mash against nonenzymatic oxidation, can be measured with the Trolox equivalent antioxidant capacity (TEAC) assay.

The aim of this study is to evaluate the role of oxidative lipid degradation during the most critical step in the brewing process, that is, the mashing. To achieve this, several parameters, such as the OH-FAs, PPF_1s, and changes in the TEAC, were measured during the mashing process. It has been reported that OH-FAs are mainly formed during mashing (*19*). The measurements will be done in both oxygen-rich and oxygen-poor environments to evaluate the role of oxygen on oxidative stress during the mashing process.

MATERIALS AND METHODS

Chemicals. Butylated hydroxytoluene (BHT), triphenylphosphine (TPP), (trimethylsilyl)diazomethane, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Aldrich Chemical Co. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce, Rockford, IL. Potassium persulfate (dipotassium peroxdisulfate) and trichloroacetic acid (TCA) were obtained from Merck, and 15-hydroxyeicosatetraenoic acid was from Cayman Chemicals, Ann Arbor, MI. All other chemicals were of analytical grade purity.

Preparation of the Mashing Samples. To assess the influence of oxygen, part of a batch of malt was milled under oxygen and another part was milled under argon. After milling, both malt parts were incubated with prewarmed water of 58 °C ($t = 0$ min) in a laboratory mash bath with a malt/water ratio of 1:3.5 (w/v). The oxygen-rich milled malt was mixed with aerated water, and the mixture was continuously exposed to oxygen (100% oxygen, 1 atm), whereas the oxygen-poor milled malt was mixed with twice deaerated water and was kept under argon (1 atm). A temperature program was started, raising the temperature in several steps and rests to 76 °C at 120 min.

At the time points $t = 0$, 5, 30, 60, and 120 min three 25 mL aliquots of each mixture were taken for measuring PPF_1s and OH-FAs. To two aliquots were added 125 μL of a 30 mg of BHT/mL solution and 10 mg of TPP. Subsequently these samples were centrifuged (5 min, 2000*g*, 4 °C), and the supernatant was frozen under liquid nitrogen. The third aliquot was directly frozen under liquid nitrogen without any additions. In this aliquot the TEAC was assessed. All samples were stored at −80 °C until analysis.

Monohydroxy Fatty Acid and Phytoprostanes. *Hydroxy Fatty Acid and Phytoprostane Extraction, Purification, and Hydrogenation (20).* Mash supernatant (5 mL) was transferred into a 50 mL Falcon tube. 15-Hydroxyeicosatetraenoic acid (15-HETE) and ^{18}O-labeled F_1-phytoprostanes were added as internal standards (100 ng of both for the analysis of free hydroxy fatty acids and free F_1-phytoprostanes). Saturated NaCl solution (10 mL), 1 M citric acid (0.5 mL), ethyl acetate [25 mL containing 0.005% (w/v) BHT as antioxidant], and TPP (50 mg) were added to the frozen sample. The mixture was homogenized for 3 min with a high-performance disperser (Ultra-Turrax T 25 at 24000 rpm, IKA-Werk). After centrifugation (10 min, 2000*g*), the ethyl acetate phase was removed and applied directly to an aminopropyl solid-phase extraction column (500 mg). The column was washed with 3 mL of chloroform/isopropanol, 2:1 (v/v), and OH-FAs were eluted with

6 mL of diethyl ether/acetic acid, 98:2 (v/v). Subsequently, PPF_1s were eluted with 6 mL of diethyl ether/methanol/acetic acid, 90:20:2 (v/v/v). Both samples were taken to dryness at 40 °C under a stream of nitrogen.

Fat Hydrogenation. PtO_2 hydrate catalyst (2−5 mg) was added to each sample. Samples were then suspended in 1 mL of methanol and stirred vigorously for 20 min under an atmosphere of hydrogen. The catalyst was filtered off by passing the suspensions through a pipet tip packed with glass fiber filter. The methanol solutions were taken to dryness and derivatized for GC-MS analysis.

Derivatization of Hydroxy Fatty Acids. The sample was methylated by adding 50 μL of methanol, 200 μL of diethyl ether, and 5 μL of (trimethylsilyl)diazomethane (2 M solution in hexane). After 5 min of incubation at room temperature, the sample was taken to dryness under a stream of nitrogen and suspended in 100 μL of chloroform. After the addition of 50 μL of BSTFA, the sample was heated at 40 °C for 1 h, taken to dryness, and reconstituted in 20 μL of hexane. An aliquot, 2 μL, was analyzed by GC-MS.

Derivatization of F_1-Phytoprostanes. The hydrogenated sample was reconstituted in 200 μL of chloroform and treated with 10 μL of PFB bromide and 10 μL of *N*,*N*-diethylisopropylamine at 40 °C for 1 h. The mixture was taken to dryness, reconstituted in 2 mL of chloroform, and applied to a silica solid-phase extraction column. The column was washed with 3 mL of diethyl ether. F_1-phytoprostane PFB esters were eluted with 6 mL of diethyl ether/methanol, 90:10 (v/v). The sample was taken to dryness, and TMS ether derivatives were prepared by the addition of 50 μL of BSTFA. After incubation at 40 °C for 1 h, the sample was dried under a stream of nitrogen and reconstituted in 20 μL of hexane.

GC-MS. Hydroxy fatty acids and phytoprostane derivatives were analyzed on a HP-5 column (30 m × 0.25 mm), with a linear He flow at 23 cm/s. For OH-FA analysis a column temperature step gradient of 225 °C at 20 °C/min, 225−275 °C at 5 °C/min, 275−300 °C at 20 °C/min, and 300 °C for 5 min was applied. The injector of the splitless mode was set at 280 °C. Data were collected in the EI mode. For analysis of F_1-phytoprostanes the column temperature was programmed from 175 to 285 °C at 30 °C/min and from 285 to 300 °C at 2.5 °C/min; data were collected in the NICI mode. Results are presented as the mean of at least two independent experiments. The error bars represent half of the range.

TEAC Assay. *Preparation of the ABTS• Solution.* A 7 mM solution of ABTS in Milli-Q was prepared, and ABTS• was formed after the addition of potassium persulfate to the mixture in a final concentration of 2.45 mM. After a 12−16 h incubation at room temperature, the stock solution was diluted with PBS until the desired concentration was reached (*21*).

TEAC Assay. The mashing samples were thawed at room temperature and centrifuged for 5 min at 3400 rpm (at 4 °C). The supernatant was used for the TEAC assay. To exclude the influence of light, the handling was done in reduced light.

To a fixed volume of 50 μL of supernatant was added ABTS• in a variable concentration. The concentration of ABTS• was varied from 0 to approximately 45 μM in several different incubations. After a 6 min incubation at 37 °C, the absorbance at 734 nm was spectrophotometrically determined. The concentration of ABTS• was calculated using a molar extinction coefficient of 1.5×10^4 M^{-1} (*21*). The reduction in ABTS• concentration is derived from the absorbance at 734 nm of the reference (containing only ABTS•) and the incubation containing the fixed volume of supernatant plus the same concentration of ABTS•.

The reduction in ABTS• concentration is plotted against the initial concentration of ABTS•. The curve is fitted according to the exponential function $y = C(1 - e^{-bx})$ using Sigma Plot (version 4.01) on a standard personal computer. In this formula y is the reduction in ABTS• concentration, x is the initial ABTS• concentration, and C is the maximal amount of ABTS• scavenged by the supernatant in the concentration/volume tested (*22*).

Statistical Methods. Results are expressed as means ± standard deviation. The nonparametric repeated-measures ANOVA of Friedman was applied to determine differences over time. In the case of significant differences over time, a post hoc test, described by Siegel and Castellan (*23*), was used to locate the differences in time (vs $t = 0$). Statistical significance was set at $P < 0.05$.

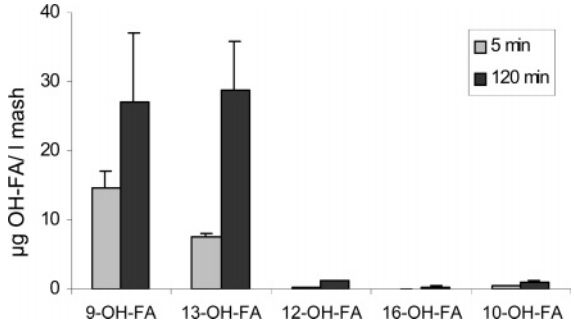

Figure 2. OH-FA concentrations in supernatants of mash, produced under high oxygen pressure, at 5 and 120 min. The nonenzymatically produced OH-FAs are represented by 12- and 16-OH-FAs; photo-oxidation yields 10-OH-FA. Enzymatic oxidation results solely in 9- and 13-OH-FAs.

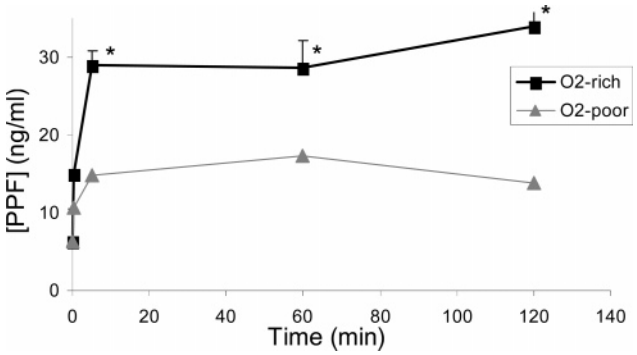

Figure 3. PPF1 concentration of both oxygen-poor and oxygen-rich mash in the course of time. ∗ indicates that the value is significantly different from the value at the start.

RESULTS

In a mash mixture, consisting of malt and water (1:3.5, w/w), exposed to high oxygen, OH-FAs were measured at $t = 5$ and 120 min. **Figure 2** shows that enzymatic OH-FA formation (reflected by 9- and 13-OH-FA formation) plays a more dominant role compared to the nonenzymatic autoxidation (reflected by 10, 12-, and 16-HOTE formation) through photo-oxidation and free radical catalyzed oxidation.

PPF_1s are chemically stable endproducts of nonenzymatic oxidation of linolenic acid (*14*). The presence of these compounds before the actual mashing (**Figure 3**) indicates that PPF_1s are already present in the malt. PPF_1 concentration increases in time. This indicates that nonenzymatic oxidation also occurs in the mashing process. Maximum PPF_1 formation takes place during the first 5 min of the mashing process. After this rapid increase in PPF_1 concentration, a plateau is reached. Oxygen pressure affects PPF_1 formation ($P < 0.05$). At 120 min the plateau with high oxygen (100% O_2, 1 atm) is twice as high as that with low oxygen pressure (100% Ar, 1 atm).

During mashing, the TEAC in both O_2-rich and O_2-poor mixture rises (**Figure 4**). A faster increase in TEAC is observed in the oxygen-rich mash. Nevertheless, the TEAC in the oxygen-poor mash is higher at the end of the mashing period ($P < 0.05$), indicating that the oxygen-poor mash has finally a higher antioxidant capacity than the oxygen-rich mash.

DISCUSSION

During mashing the easily oxidizable PUFAs in malt are subjected to high temperature and excess oxygen. This makes mashing a critical step in the brewing process.

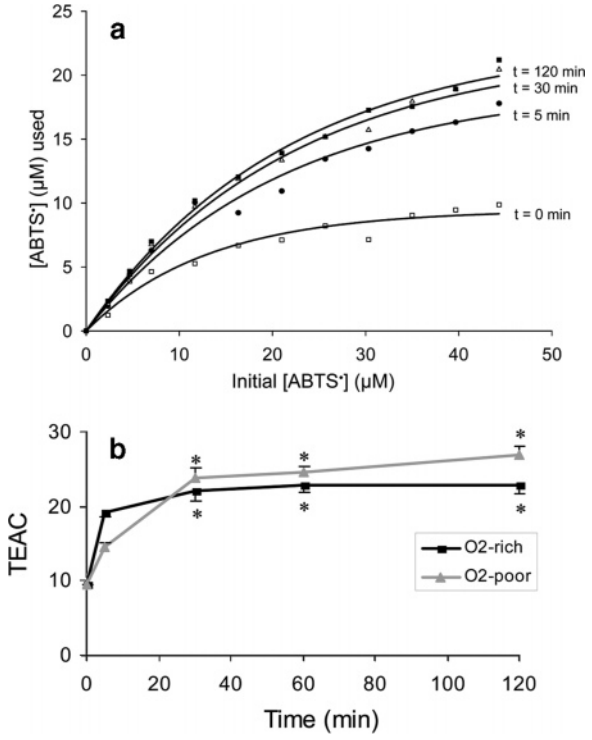

Figure 4. (**a**) Consumption of ABTS• by 50 μL of supernatant of mash at four different time points, produced under low oxygen tension, with a variable initial concentration of ABTS•. The data of the ABTS• consumption were fitted with an exponential function as described under Materials and Methods. A typical example of three independent experiments is shown. This approach gives the actual TEAC value (*22*). (**b**) TEAC values of low and high oxygen tension mash in time. $*$ indicates that the value is significantly different from the value at the start.

The results obtained from OH-FA and PPF_1 formation indicate that oxidative damage of lipids indeed takes place. The OH-FAs (**Figure 1**) are mainly formed by LOX-catalyzed oxidation, indicated by the formation of mainly 9- and 13-OH-FAs. It is possible that hydrolysis of esterified malt 9- and 13-OH-FAs (*24*) by the action of malt lipase also contributes to the increase in 9- and 13-OH-FAs.

The intermediate products in OH-FA formation are hydroperoxides, which easily generate radicals. These radicals might inflict damage to several targets, such as proteins. This indicates that the enzymatic oxidation of PUFAs to hydroperoxides will also give rise to nonenzymatic radical formation. Radical formation is also evident from the increase of OH-FAs and PPF_1s, markers of nonenzymatic hydroperoxide formation, in time during the mashing process.

It is expected that during an oxidative process, such as mashing, antioxidants are being consumed. Surprisingly, the total antioxidant capacity reflected by the TEAC increases in time. Possible explanations for this rise in TEAC include the following:

(1) Oxidation products may be formed that might have a higher TEAC value (*25*).

(2) Phenolic −OH groups that had been bound to a sugar may be released due to enzymes that are capable of splitting off sugars. In general, the aglycon is a better antioxidant than the sugar-containing compound, which leads to a higher TEAC value (*26*).

(3) Compounds may have a higher solubility due to the elevated temperature. During mashing, antioxidants, such as tannins and anthocyanogens, are released from the barley and are dissolved into the extract solution (*1*), which will lead to a higher TEAC.

(4) The denaturation of proteins may lead to protein unfolding, which allows previously inaccessible antioxidant amino acids to participate in the scavenging process (unpublished data).

(5) The reduced binding of antioxidants by protein denaturation results in less masking and a subsequently higher TEAC (*27*).

All of the above-mentioned processes might have a contribution to an increase of the TEAC, but it is unknown to what extent. The faster increase in TEAC in the oxygen-rich mash, compared to the oxygen-poor mash, can be explained by (i) the faster formation at high oxygen pressure of reaction products that have paradoxically a higher TEAC (*25*) and (ii) faster protein denaturation leading to less antioxidant protein binding and unfolding of proteins. The paradoxical rise in antioxidant capacity at this stage of the brewing process is advantageous because antioxidants prevent excessive oxidative damage during the mashing process.

The higher TEAC of the oxygen-poor mash at the end of the mashing period can be explained by a lower oxidative consumption of antioxidants compared to the oxygen-rich mixture. As expected, at high O_2 pressure PPF_1 formation is higher than at low O_2 pressure. This indicates that lipid oxidation is indeed higher at a higher oxygen tension, which is in line with the lower TEAC at the end of the mashing under high oxygen pressure.

In conclusion, enzymatic oxidation appears to be the main route of oxidation of the PUFA during the mashing. Hydroperoxides formed by lipoxygenases might give rise to the formation of free radicals that in turn are responsible for the observed nonenzymatic lipid oxidation yielding, among other products, PPF_1 and a series of OH-FA.

On the basis of these conclusions it can be hypothesized that PPF_1s might be used as quality markers of the mashing mixture. Possibly, these compounds can also be used as markers for oxidative damage during the production of beer, especially the mashing, because they are not degraded. No changes in PPF_1 concentration were detected during the storage of beer (unpublished results), indicating that during the oxidative processes that take place during the storage of beer no PPF_1s are being formed. This could have been anticipated because there are practically no PUFAs, that is, the precursors of PPF_1s, present in the beer (*28*, *29*).

Mashing under low oxygen tension results in less oxidative damage and a higher antioxidant capacity than mashing under high oxygen tension, as shown by the PPF_1 reduction. This indicates that mashing should be done under low oxygen pressure to minimize oxidative damage and preserve antioxidants.

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