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Decrease of Aged Beer Aroma by the Reducing Activity of Brewing Yeast

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The flavor profile of beer is subject to changes during storage. Since, possibly, yeast has an influence on flavor stability, the aim of this study was to examine if there is a direct impact of brewing yeast on aged aroma. This was achieved by refermentation of aged beers. It was shown that several aged aroma notes, such as cardboard, ribes, Maillard and Madeira, were removed almost entirely by brewing yeast, independently of the yeast or the beer type. This was explained by the reduction of aldehydes, mainly (*E*)-2-nonenal, Strecker aldehydes, 5-hydroxymethylfurfural and diacetyl, to their corresponding alcohols. Furthermore, it became evident that the reducing capacity of brewing yeast is high, but that yeast strain and compound specific residual concentrations remained in the refermented beer independently of the initial concentration. Finally, it appeared that aldehydes were not only reduced but also formed during refermentation.

KEYWORDS: Beer; flavor; flavor stability; carbonyl compounds; reducing activity; yeast

INTRODUCTION

Several quality aspects of beer are subject to change during storage. Alteration of the flavor profile in particular is of great concern to brewers since flavor is considered as the main quality parameter. Moreover, a commercial beer should be consistent and satisfy the expectations of the consumer at all times. However, despite extensive research, understanding and controlling flavor stability is still very difficult (*1*) and the flavor changes appear to be much more complex than originally thought (*2*).

Carbonyl compounds seem to be of great importance in the appearance of aged aroma. (*E*)-2-Nonenal (T2N), giving cardboard aroma, has long been regarded as the molecule responsible for beer staling (*1*,*3*). On the other hand, several reports observed no significant increase of T2N during beer aging (*4*,*5*). When considering other linear aldehydes, acetaldehyde, (*E*,*E*)-2,4-decadienal (TT24DD) and (*E*,*E*)-2,4-hexadienal (*2*, *4*) were already reported to play a role. Additionally, 6 Strecker aldehydes are formed during aging (*6*, *7*): 2-methylpropanal (2-MP), 2-methylbutanal (2-MB), 3-methylbutanal (3-MB), benzaldehyde, phenyl acetaldehyde (PheAc) and methional. Especially methional (cooked potato-like) (*8*, *9*) and PheAc (honey-like) (*9*, *10*) are considered to contribute to stale aroma as assessed by AEDA, but 3-MB (malty) and to a lesser extent 2-MP, were also suggested to contribute (*2*). Furthermore, furan derivatives such as furfural, 5-hydroxymethylfurfural (5-HMF) (bready, caramel), 5-methylfurfural, 2-acetylfuran, and 2-acetyl-5-methylfuran are formed during beer aging (*11*, *12*). However, Meilgaard (*13*) and Saison et al. (*2*) showed that they have high thresholds (THs) and their contribution might rather be limited. Nevertheless, furfural and 5-HMF have been used regularly as indicators, since a close correlation is found with sensory scores for flavor staling (*12*,*14*). Next, β-damascenone (β-DS) (rhubarb, red fruit) has been reported regularly as an important contributor to stale aroma (*2*, *8*, *15*). Other ketones, such as methyl isobutyl ketone and 3-methylbutan-2-one, also increase during aging (*11*, *16*), but their actual contribution to aged flavor has not been reported. Furthermore, diacetyl (buttery) and 2,3-pentanedione can be formed during aging, and diacetyl may even surpass its flavor threshold (*2*, *6*). On the other hand, next to carbonyls, the concentration of several other compounds increases during aging. The concentration of 2-furfuryl ethyl ether (2-FEE) (solvent-like) easily surpasses its TH during beer aging (*2*, *11*, *17*). Besides, lactones such as γ-hexalactone and γ-nonalactone (peach, fruity) are also formed during aging (*18*) and the latter was already considered relevant for aged aroma (*8*). Dimethyltrisulfide was also related to aged beer aroma (*19*) and AEDA revealed the possible relevance of 3-methyl-3-mercaptobutyl formate (ribes-like) (*20*). Finally, volatile esters can either be formed or hydrolyzed. Ethyl esters, such as ethyl 2-methylbutyrate (Et2MB), ethyl 3-methylbutyrate (Et3MB), ethyl nicotinate and ethyl pyruvate, are synthesized during aging (*1*, *7*, *11*, *12*, *21*,) and the formation of Et2MB and Et3MB has already been linked to the development of winy aromas (*21*). Isoamyl acetate (IAA) on the other hand, giving a banana-like aroma, can be hydrolyzed, and consequently, the fruity character of beer diminishes (*22*). Many flavor compounds are thus involved in beer aging, and more research on flavor changes and the responsible compounds is still necessary.

The relevance of carbonyl compounds has been supported by Hashimoto and Bamforth, who added hydroxylamine and semicarbazide respectively to aged beer (*23*, *24*). These carbonyl-binding

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compounds could strip the main part of the oxidized aroma from beer in moments. However, Bamforth argues that oxidized character is only one aspect of staleness (*23*). Of course, the agents used in these studies cannot be used commercially in beer. On the other hand, perhaps the most powerful carbonyl-affecting agent involved in brewing is yeast. First, yeast can produce sulfite as an intermediate of the biosynthesis of sulfurous amino acids (*25*). Not only is sulfite a powerful antioxidant but it can also combine with flavor active carbonyl compounds to form flavorless sulfite−carbonyl complexes (*25*). Second, yeast is capable of reducing carbonyls to their equivalent alcohols (*23*, *26*, *27*) during fermentation because of the need to maintain a stable redox balance within the cell. These reducing reactions are mediated by a complex system of reducing enzymes which reconvert NAD(P)H to $NAD(P)^+$ (*28*). Consequently, a fresh beer is created after yeast activity, and in order to get a better insight, it is interesting to study the direct effect of yeast on aged beer flavor.

Bottle refermentation, also known as bottle conditioning or bottle krausening, involves an extra fermentation process in the bottle by adding fermentable carbohydrates and yeast in the bottle. The resulting beers are appreciated for their characteristic organoleptic evolution and the visual aspect of the yeast sediment in the bottle. Additionally, it is hypothesized that yeast offers a natural protection against oxygen. Indeed, it is proposed that yeast can act as an oxygen scavenger, making beer less sensitive to oxidation (*29*). Derdelinckx et al. (*30*) assumed that carbonyl compounds would undergo the same transformation as during the main fermentation, which they supposed to be biochemical reduction by alcohol dehydrogenase. Moreover, Van Opstaele et al. (*31*) showed that bottle refermentation gave rise to beers with prolonged flavor stability in comparison with nonrefermented samples and this even appeared to vary between yeast strains. The precise impact of yeast on flavor stability during refermentation is however unclear.

The purpose of this work was to study the effect of brewing yeast on the aged flavor of beer. In this way, a better insight can be gained in the compounds causing aged beer flavor and in the activity of yeast on these components. This was performed by aging, followed by refermentation of the aged beers. In this way, the focus can be specifically on typical aged beer characteristics and only volatiles relevant to aged aroma are considered. Therefore, after aging and subsequent refermentation, sensory analysis of the beers was performed and it was aimed to explain these results by analyzing the responsible compounds. Furthermore, the precise impact of yeast on aged aroma compounds during refermentation was studied and especially the reducing activity of brewing yeast seemed of great interest.

MATERIALS AND METHODS

Chemicals, Yeasts and Beers. All chemicals were purchased from Sigma (St. Louis, MO) with the highest purity available: acetaldehyde, hexanal, (*E*)-2-nonenal, (*E*,*E*)-2,4-decadienal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, benzaldehyde, phenyl acetaldehyde, methional, furfural, 5-hydroxymethylfurfural, 5-methylfurfural, acetylfuran, diacetyl, methyl isobutyl ketone, ethyl 2-methylbutyrate, ethyl 3-methylbutyrate, ethyl nicotinate, ethyl pyruvate, β-damascenone, γ-nonalactone and isoamyl acetate. The furanic ether 2-furfuryl ethyl ether with a purity of 95% was purchased from Narchem Corp. (Chicago, IL).

The experiments were carried out with industrial bottom (*Saccharomyces pastorianus*) and top fermenting (*Saccharomyces cerevisiae*) brewing yeast strains (bottom fermenting, BF-DS01, BF-DS02, BF-DS03; top fermenting, TF-DS04, TF-DS05 (DS05), TF-DS06, TF-DS07, TF-DS08 (DS08), TF-DS09, TF-DS10, TF-DS11, TF-DS12 and TF-DS13) from the collection of the Centre for Malting and Brewing Science (KULeuven, Leuven, Belgium).

Two lager and three ale beers were used and some of their main fresh characteristics (original extract (°P), alcohol percentage (v/v %), color (EBC) and bitterness (EBU)) are presented: lager A (11.7 °P, 5.1%, 6.5 EBC, 26 EBU), lager B (11.8 °P, 5.3%, 6.9 EBC, 22.6 EBU), 2 amber beers, namely, ale A (19.7 °P, 8.7%, 38 EBC, 18 EBU) and ale C (16.4 °P, 7.6%, 13.6 EBC, 16.9 EBU), and a blond beer: ale B (20.2 °P, 9.5%, 11.2 EBC, 38.0 EBU). In the presented experiments, either fresh beer was used or beer that was aged in the dark at a specific temperature−time interval. Lager A, lager B, ale A and ale B were placed at 20 °C for 1 year, ale C at 30 °C for 3 months and for the last experiment, lager A was also aged at 60 °C for 4 days.

Propagation and Refermentation Conditions. For yeast propagation, single yeast colonies were taken from stock plates and inoculated into 8 mL of 12 °P all-malt wort (in triplicate). After incubation at 25 °C for 24 h, the suspensions were transferred to 250 mL of wort (12°P) in 500 mL Erlenmeyer flasks and incubated at 20 °C for 20 h at 150 rpm. The concentration of the yeast slurry was determined by flow cytometry (YeastCyte, BioDETECT AS, Oslo, Norway). Afterward, yeast cells were centrifuged (3000 rpm, 3 min, 4 °C) and washed with cold, sterile, physiological water (0.7% (w/w) NaCl). Yeast cells were harvested at the end of the exponential phase in order to obtain yeast with a good and similar physiological condition.

Subsequent refermentations were started by adding 1 mL of a yeast suspension (250 mio cells/mL) and 1.25 mL of a sucrose solution (60% (w/w)) to 250 mL of beer, which corresponded with 1 mio cells/mL and 3 g/L sucrose in the bottle. Before closure of the bottles, air was driven out by overfoaming. Afterward, the bottles were stored at 20 °C for 3 weeks.

Extract Analysis. The extract and the alcohol measurements were analyzed with a density meter (DMA 4500) and an Alcolyzer plus (Anton Paar, Graz, Austria).

Total Sulfite Analysis. Total sulfite content was measured using the *p*-rosaniline method as recommended by the American Society of Brewing Chemists (Methods of Analysis, eighth rev. ed., St. Paul, Minnesota, 1992). Analyses were performed in triplicate.

Quantification of Volatile Aroma Compounds. Esters and higher alcohols were analyzed with a calibrated Autosystem XL gas chromatograph (GC) (HS40; Perkin-Elmer, Norwalk, CT) coupled with flame ionization detection (FID). The GC was equipped with a Chrompack-Wax 52 CB column (length, 50 m; 0.32 mm i.d.; layer thickness, 1.2 μm; Varian, Palo Alto, CA). Filtered beer (5 mL) was transferred to vials and subsequently heated for 16 min at 60 °C in the headspace (HS) autosampler before injection (needle temperature: 70 °C). Helium was used as the carrier gas. The oven temperature was kept at 50 °C for 7.5 min, increased to 110 at 25 °C/min and held at that temperature for 3.5 min. The FID temperature was kept constant at 250 °C. Analyses were carried out in duplicate and results were analyzed with Perkin-Elmer Turbochrom Navigator.

Carbonyl compounds and other staling compounds were measured with HS solid-phase microextraction (SPME) coupled to gas chromatography and mass spectrometry (MS). Three procedures were used that were previously described by Saison et al. (*32*, *33*). Carbonyls were determined with on-fiber ((*E*)-2-nonenal, (*E*,*E*)-2,4-decadienal, 2-methylpropanal and 2- and 3-methylbutanal) and in-solution (other carbonyls) derivatization using pentafluorobenzyl hydroxylamine (PFBHA) as derivatization agent. The other compounds were measured after extraction of underivatized compounds.

A 10 min preincubation extraction was applied for the three procedures at the respective temperatures. Subsequent SPME extraction was different for the three procedures. On-fiber derivatization was performed by first loading a PDMS-DVB fiber with PFBHA by exposure of the fiber to the headspace of a PFBHA solution (1 mg/mL, 10 min, 45 °C, 250 rpm). Afterward, the fiber was exposed to the HS of a vial containing 10 mL of beer, 50 μL of an ethanol solution with 100 mg/L *p*-fluorobenzaldehyde as internal standard (IS), and 3.5 g of NaCl (30 min, 45 °C, 250 rpm). In-solution derivatization was done by exposing a PDMS-DVB fiber to the HS of a vial containing 10 mL of beer, 50 μL of an ethanol solution with 100 mg/L *p*-fluorobenzaldehyde as IS, and 0.375 mL of a PFBHA solution (20 g/L) (40 min, 60 °C, 250 rpm). The underivatized compounds were measured following extraction with a DVB-CAR-PDMS fiber of the HS of a vial containing 10 mL beer, 50 μL of an ethanol solution with 200 mg/L 2-heptanol and 100 mg/L guaiacol, both used as IS, and 3.5 g of NaCl (30 min, 40 °C, 250 rpm).

Table 1. Concentrations and Flavor or Odor Threshold (TH) as Determined by Saison et al. (*2*) in Beer of a Selection of Staling Compounds in a Fresh Lager Beer, the Same Beer after Aging (1 Year at 20°C) and the Same Beer after Aging and Subsequent Refermentation with Yeast DS08 (Aged + RF)[a]

				1 year 20 °C		
	TH	CV (%)	fresh	aged	aged + RF	RI
acetaldehyde	1114	2.2	588	2426	1084	
hexanal	88	7.6	1.2	3.1	0.9	1449
(*E*)-2-nonenal	0.03	9.6	0.018	0.058	0.078	1813
(*E,E*)-2,4-decadiënal	0.11	11.2	0.006	0.024	0.040	1895
2-methylpropanal	86[b]	5.9	11	71	13	1216
2-methylbutanal	45	4.4	2.9	10.6	3.8	1306
3-methylbutanal	56[b]	4.5	8.8	25.9	8.9	1316
benzaldehyde	515	8.7	1.0	5.9	2.2	1681
phenylacetaldehyde	105	8.2	17	63	29	1727
methional	4.2	11.8	2.00	4.94	1.82	1588
furfural	15157[b]	7.3	39	579	8	1498
5-hydroxymethyl-furfural	35304[b]	9.6	3250	9107	3536	1468
5-methylfurfural	1174	6.3	0.22	3.45	0.20	1603
acetylfuran	513	7.0	6.9	28.0	33.3	1556
diacetyl	17	5.7	6.0	33.2	1.3	
methyl isobutyl ketone	2560[b]	2.8	3.6	14.5	14.6	1349
ethyl 2-methylbutyrate	27	3.2	0.41	1.12	1.15	853
ethyl 3-methylbutyrate	91	3.5	0.70	2.56	2.46	849
ethyl nicotinate	4555[b]	12.3	3.6	44.2	0.2	1228
ethyl pyruvate	22525	4.3	94	152	28	1457
2-furfuryl ethyl ether	11[b]	3.6	1.6	30.9	30.2	901
β-damascenone	203[b]	2.3	123	227	226	1396
γ-nonalactone	607[b]	7.7	30	35	45	1374
isoamyl acetate	510	5.9	356	216	343	

[a] Concentrations are given in μg/L. Additionally, the coefficient of variation (CV) of the presented samples is given for each compound and Kovats retention indices (RI) are given for the compounds that were analyzed with HS SPME GC−MS. The RI of carbonyl compounds are those of the corresponding derivative that was used for quantification. [b] Odor threshold.

GC−MS analysis was carried out using a Trace GC Ultra coupled to a dual stage quadrupole MS, consisting of a curved, small quadrupole as prefilter and a normal quadrupole (both from Thermo, Austin, TX). A Rtx-5SilMS column (60 m × 0.25 mm i.d.) with a film thickness of 1 μm was used. The GC was equipped with a split−splitless injector which was held at 250 °C. Compounds were analyzed following 2 min desorption and splitless injection. During the GC run, a constant flow rate (1.5 mL/min) of the carrier gas (helium) was maintained. The GC program for both derivatization procedures was the same and started at 60 °C for 2 min, then increased in 4 steps: 60 to 165 at 50 °C/min; 165 to 200 at 2 °C/min, 200 to 260 at 4 °C/min and 260 to 290 at 5 °C/min and was held at 290 °C for 6 min. The GC program for the underivatized compounds started at 30 °C, the oven temperature was raised in 3 steps after 2 min: 30 to 70 at 10 °C/min followed by 1 min at 70 °C; 70 to 190 at 4 °C/min and 220 to 270 at 25 °C/min and was finally held at 270 °C for 6 min. The mass spectra were obtained by electron impact (EI) ionization at 70 eV, and the ion volume and the transfer line were held at 250 and 290 °C respectively. The detector was set in TIC mode from m/z 35 to 400. The results were analyzed using Xcalibur software (Thermo, Austin, TX). Analyses were performed in triplicate and quantification was performed by using the area ratios (area of the compound divided by area of the IS) and according to the standard addition method in order to avoid matrix effects, as described by Saison et al. (*33*).

Identification of Volatiles. Identification of volatiles was performed by comparing the retention indices and GC−MS spectra of the obtained peaks with those of chemical standards. Additionally, Kovats retention indices were determined (presented in **Table 1**) and were compared with those of the NIST database. Mostly, derivatization gives rise to the formation of 2 derivatives for the cis- and the trans-configuration. Retention times were similar, and consequently, retention indices were also very close to each other. Therefore, only the retention index of the compound used for quantification is shown.

Sensory Analysis. Sensory tests were carried out with a trained panel of a minimum of 8 members. Aged beers and the corresponding refermented beers were always presented together in black glasses. Besides an evaluation of some fresh flavor aspects and the general aging character, stale aroma was also evaluated for 9 aspects (cardboard, metal, solvent, old hops, ribes, Maillard (caramel, burnt, bread, butter), stale-sulfury, acetaldehyde (green apple) and Madeira) by giving a score from 0 to 8. A score of 0 meant that the particular aroma aspect was not present, while a score of 8 indicated that this aspect was extremely strong. Finally, a global appreciation score was given on a scale from 1 to 9.

RESULTS AND DISCUSSION

Sensory Evaluation of Aged and Refermented Beers. The effect of yeast on stale beer flavor was studied by aging and subsequent refermentation of beers. First, 2 lager (lager A and B) and 2 ale beers (ale A and B) were aged naturally (20 °C) for 1 year and were afterward refermented with the top fermenting *Saccharomyces cerevisiae* strain DS08 for 3 weeks at 20 °C. Thereafter, the aged and refermented beers were tasted by an expert tasting panel and results of the most important aged aromas are presented in **Figure 1**. After aging, stale aromas were clearly present in the 2 lager beers. The flavor of both aged beers differed, but relatively high intensities were found for all the presented stale aroma characteristics. The aged aroma of the ale beers differed from the aged lagers. A limited cardboard aroma was observed and stale-solvent, Maillard-like aromas were especially apparent. After refermentation of the aged beers, a significant decrease ($p < 0.001$) of the aged flavor was observed in all beers (decrease of the average aging score from 4.7 to 1.2; 5.9 to 2.6; 3.8 to 1.8 and 3.4 to 1.9 for lager beers A and B and ale beers A and B respectively). Thus, the typical aged aroma mainly disappeared. Especially cardboard, ribes, Maillard and Madeira aroma notes diminished greatly in all beers. These results suggest that compounds responsible for the mentioned aromas can be removed or masked during the refermentation process, independent of the beer type. On the other hand, the stale-solvent aroma was hardly influenced by refermentation and the stale-sulfury aroma only diminished slightly. Finally, bitterness hardly changed and a slight increase of fruity and acetaldehyde-like (sour apple) aromas was observed after refermentation (results not shown). This typical acetaldehyde-like aroma is well-known at the first stage of the refermentation of beer (*30*).

To confirm the obtained results, an additional experiment was carried out and an ale beer (ale C) was aged at 30 °C for 3 months. Aged aromas were definitely present after aging, and this aged beer was refermented with 3 bottom and 10 top fermenting brewing yeast strains. The obtained beers were tasted, and the aging intensity scores of the beers are presented in **Figure 2**. All yeasts, either bottom or top fermenting, were clearly able to diminish the aged aroma significantly ($p < 0.01$), and the same aged aromas as previously mentioned disappeared almost completely. This resulted in similar aging scores for all beers after refermentation. The overall score on the other hand varied more, depending on the yeast strain. However, these differences seemed to be determined by variations in aromas produced by yeast such as sulfury, acetaldehyde-like and fruity, rather than by differences in the decrease of aged aromas.

It can thus be stated that yeast can diminish aged beer aroma, more specifically cardboard, ribes, Maillard-like and Madeira aromas, during refermentation, independent of the yeast or the beer type.

Evolution of Stale Aroma Compounds. Sensory results indicated a clear decrease of several aged aromas during refermentation. However, the molecular basis of these aged aromas is not entirely clear. Therefore, in order to explain this aroma evolution, it is interesting to study those compounds that increase during aging and decrease during refermentation. The evolution of a wide

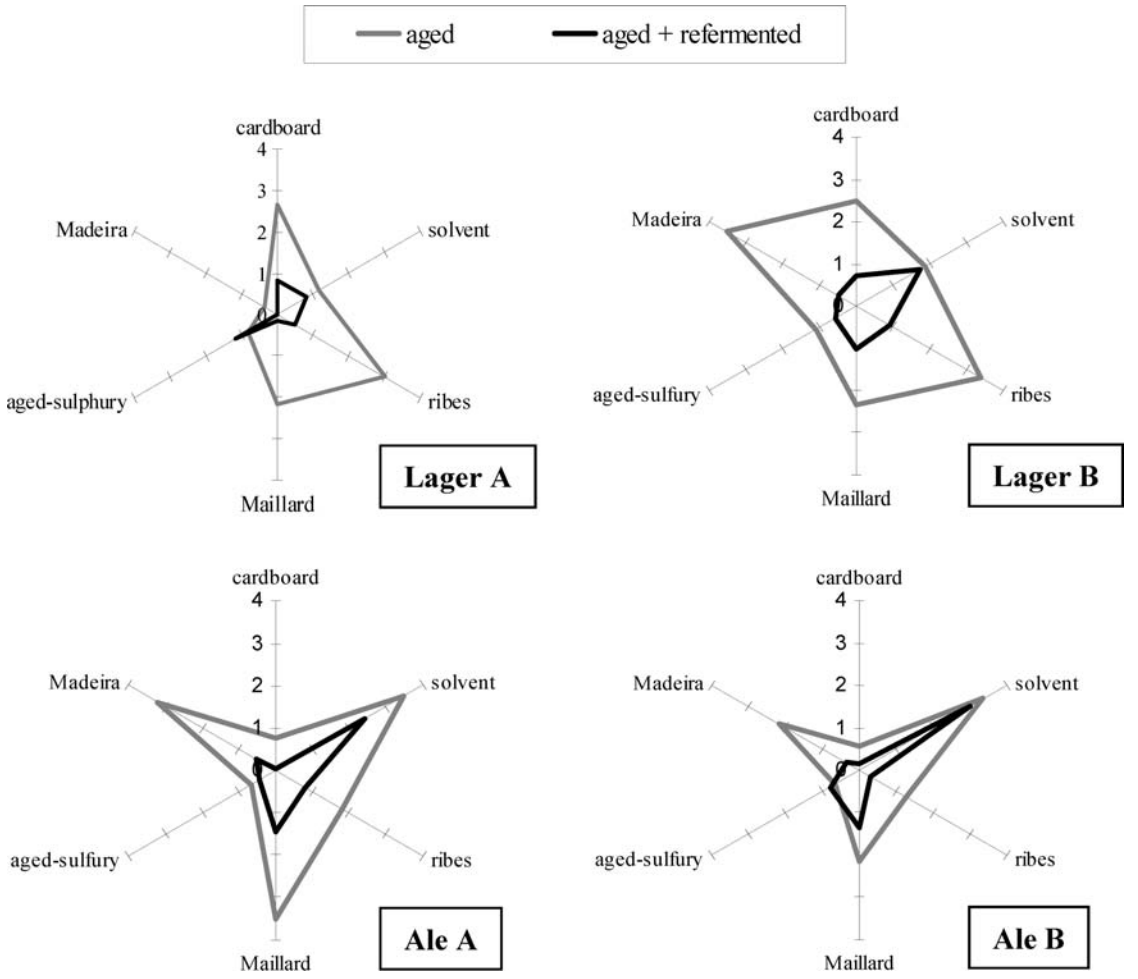

Figure 1. Sensory results of aged and subsequently refermented beers. Specific aged flavor notes are presented in spider plots of lager beers A and B and ale beers A and B. These were aged for 1 year at 20 °C and refermented afterward with DS08.

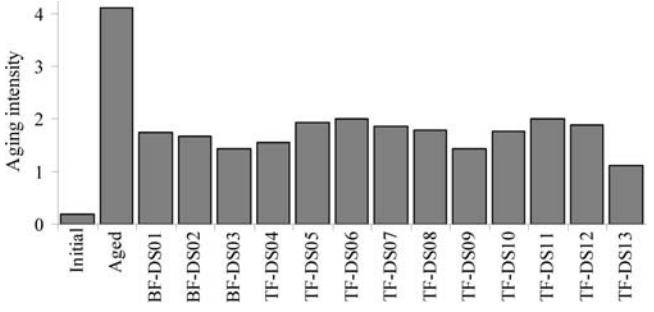

Figure 2. The overall aging intensities are presented of an ale beer (ale C), which was aged for 3 months at 30 °C and subsequently refermented with 3 bottom fermenting yeasts (BF-DS01−BF-DS03) and 10 top fermenting yeasts (TF-DS04−TF-DS13).

range of components that were previously linked to aged beer aroma was monitored during aging and subsequent refermentation of lager A (**Table 1**). These analytical data correspond with the sensory results of lager A that are shown in **Figure 1**. Apart from the concentrations, also the corresponding THs are presented. The thresholds are either flavor or odor THs that were determined in the same lager beer (lager A) in a previous study (*2*). Finally, in **Table 1**, coefficients of variation (CV) are given for each compound. The presented values correspond with the highest CV that was found for this compound in the different beers.

During aging, a substantial increase of acetaldehyde was observed, and after refermentation, the concentration was lowered again. Thus, a similar evolution as aged aroma perception was observed. Furthermore, the TH is exceeded during aging. During aging, hexanal increased negligibly and TT24DD moderately, but T2N increased greatly as compared to its TH. After refermentation, the hexanal concentration decreased, while T2N and TT24DD concentrations increased slightly. In previous studies, especially T2N has been assigned an important contribution to cardboard aroma (*2*, *34*−*36*). However, others did not observe an increase of T2N with aged aroma (*4*, *5*). Here, T2N and TT24DD did not follow the same evolution as the cardboard aroma during refermentation.

Furthermore, in lager A, a clear decrease of Maillard-like aromas was observed during refermentation (**Figure 1**). It was already shown that these kinds of aromas can be introduced in beer by Strecker aldehydes (*2*, *10*). Here, the Strecker aldehydes 2-MP, 3-MB, PheAc and methional increased to concentrations quite close to the TH after aging and clearly decreased during refermentation. The concentration of 2-MB did not approach the TH as close after aging, but also decreased during refermentation. Additionally, Maillard-like aromas, described as caramel, burnt, bread and butter, can be introduced by Maillard reaction products, such as 5-HMF and diacetyl (*2*, *34*). Accordingly, these compounds increased and decreased during aging and refermentation respectively. Although the concentration of furfural and 5-methylfurfural increased about 10−15-fold during aging and decreased to an even greater extent during

refermentation, their concentrations stayed far below their threshold.

Other aroma compounds, such as the ethyl esters of nicotinate and pyruvate, evolved similarly to aged aroma, but they did not approach their THs by far. Again other compounds, such as acetylfuran, methyl isobutyl ketone, Et2MB, Et3MB and γ-nonalactone increased during aging and increased further on during refermentation. Yeast can thus not influence the concentration of these compounds during refermentation. Besides, concentrations stayed well below the corresponding TH. The concentration of 2-FEE on the other hand was 1−3-fold the TH after aging and was not influenced by yeast during refermentation. Since the solvent aroma evolved similarly, these results are in accordance with the important role that is ascribed to 2-FEE for explaining this aroma (*2*, *37*). Another compound that showed a similar evolution and that exceeded its TH was β-damascenone. Finally, during refermentation, an increase of isoamyl acetate (IAA) was observed, which may also explain a lower aged aroma perception due to masking effects (*2*).

Of the analyzed compounds, only aldehydes, and one diketone, increased and decreased during aging and subsequent refermentation. This is in accordance with their presumed important role in aged aroma (especially cardboard, ribes, Maillard and Madeira aromas). In the end, other compounds than the analyzed compounds will probably also contribute to aged aroma. In particular those which increase with aged aroma during beer aging, and decrease afterward during refermentation.

Refermentation and Reducing Activity. The decrease of aldehyde and diacetyl concentrations appeared quite large, and therefore, additional refermentations of an aged lager beer (lager A, no addition) and the same beer but after addition of 2 different amounts of a carbonyl compound solution (lager A, with extra compounds A and B (EC-A and EC-B)) were performed with two yeast strains. Results are presented in **Table 2**. The concentrations of all compounds ended up at similar, compound-related residual values, regardless of the initial concentration. Yeast was thus able to decrease the concentrations of the presented compounds to the same level, even at high start concentrations. These results indicate that yeast reducing activity is very high. Even the concentration of T2N, which increased slightly after refermentation of the beer without addition, decreased to a similar residual concentration when higher initial concentrations were present.

Next to the large concentration decreases, it was remarkable that aldehydes were not removed entirely. Additionally, it appeared that observed residual concentrations were compound specific. Indeed, it can be questioned why for example 2-MP ended up at concentrations around 5 μg/L, while residual concentrations of 3-MB and PheAc with start concentrations of more or less the same magnitude were about 3.5 and 40 μg/L respectively after refermentation with DS05. These results suggest that residual concentrations are not ascribable to failure of yeast activity, but might rather be caused by interactions with the beer medium. Similar results for 2-MB, 3-MB and methional were obtained by Perpète et al. (*38*, *39*) during cold contact fermentations (0 °C). Moreover, they observed that, although a higher pitching rate led to a faster reduction of 3-MB, the same final concentration was reached. However, when a higher temperature (28 °C) and the same pitching rate was applied, a lower end concentration was reached (*39*). This might be attributable to interactions of aldehydes with the beer medium, making them unavailable for enzymatic reduction. On the other hand, it might be that a temperature increase renders the yeast cell membrane less rigid and thus more permeable for aldehydes. Perpète et al. showed that interactions with flavanoids may play a role at low temperatures (*38*). Applied concentrations (100 mg/L catechin) were however quite large as compared to those found in the studied lager beer (about 20 mg/L flavanoids), and it is not clear whether aldehyde−flavanoid complexes will be as stable at the higher temperatures applied here. Additionally, it was already shown that not all aldehydes (e.g., pentanal) could be bound in stable flavanoid complexes (*38*). However, other interactions, such as those with sulfite to form carbonyl−sulfite adducts (*25*, *38*) and with amino compounds to form imines (*39*−*41*), might also protect aldehydes from removal by yeast. Furthermore, addition of heat-inactivated yeast in a cold contact fermentation could not explain the observed aldehyde decreases and viable yeast is thus necessary (*39*). Finally, from **Table 2**, it appears that the observed residual concentrations were not only compound specific but also yeast strain dependent. This is quite remarkable, since it suggests that residual concentrations might be only partly explained or not be explained by interactions with the beer medium. Therefore, the removal of aldehydes by yeast was studied in more detail.

Table 2. Concentrations of Several Carbonyl Compounds in an Aged Lager Beer (1 Year 20 °C), the Aged Beer after Addition of Extra Carbonyl Compounds and the Corresponding Beers after Refermentation with Yeast Strains DS05 and DS08[a]

		concn (μg/L)		
compd	sample	initial	RF DS05	RF DS08
hexanal	no add.	4	3.0	4.9
	EC-A	10	1.5	1.0
	EC-B	33	1.5	2.9
T2N	no add.	0.02	0.04	0.03
	EC-A	0.06	0.03	0.01
	EC-B	0.22	0.04	0.03
2-MP	no add.	65	4.8	3.3
	EC-A	88	5.4	2.5
	EC-B	184	5.0	4.6
2-MB	no add.	10	1.4	1.1
	EC-A	37	1.7	0.8
	EC-B	160	1.8	1.4
3-MB	no add.	20	3.4	2.3
	EC-A	101	3.9	1.5
	EC-B	369	3.8	2.6
Meth	no add.	2.0	1.7	0.8
	EC-A	3.8	1.0	0.4
	EC-B	9.6	1.1	1.1
PheAc	no add.	25	31.5	12.0
	EC-A	44	43.4	12.3
	EC-B	117	47.9	17.5
furfural	no add.	503	12.1	4.9
	EC-A	809	15.6	3.6
	EC-B	1771	17.2	7.7
5-MF	no add.	8	1.5	0.4
	EC-A	20	2.4	0.5
	EC-B	71	4.2	1.2
diacetyl	no add.	36	1.2	1.2
	EC-A	45	1.1	1.1
	EC-B	82	1.3	1.2

[a] No add., no addition; EC-A and EC-B, after addition of extra carbonyl compounds; compd, compound; RF, refermented; T2N, (*E*)-2-nonenal; 2-MP, 2-methylpropanal; 2-MB, 2-methylbutanal; 3-MB, 3-methylbutanal; Meth, methional; PheAc, phenyl acetaldehyde; 5-MF, 5-methylfurfural.

It was proposed that reducing activities of yeast during the refermentation process can explain the observed decrease of aldehydes and, accordingly, of aged aroma. However, since refermentation conditions are theoretically advantageous for sulfite production (i.e., low threonine and methionine content and little growth) (*42*), another explanatory factor might be the aggregation of aldehydes in carbonyl−sulfite adducts. The amount of

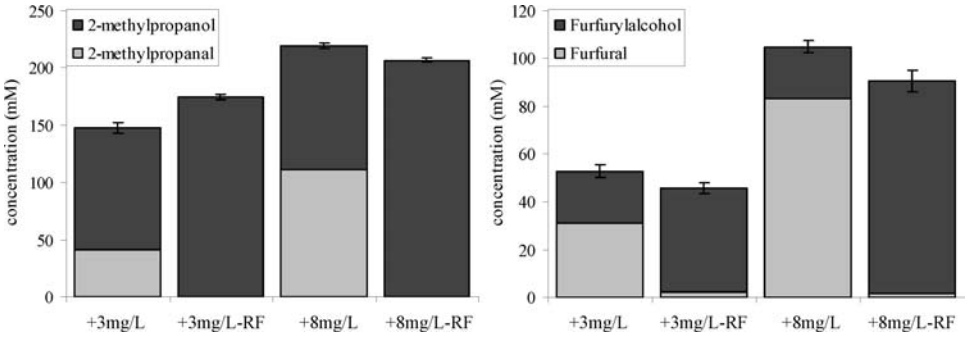

Figure 3. Concentrations of aldehydes and the corresponding alcohol in μM in a fresh beer after addition of an amount of the specific aldehyde before (+3 mg/L and +8 mg/L) and after refermentation (+3 mg/L-RF and +8 mg/L-RF) with yeast DS08.

energy on the other hand, is limited (0.3 °P). Measurement of total sulfite concentrations after refermentation revealed that the latter factor was decisive. Indeed, sulfite concentrations were determined after the previously mentioned experiment of the ale beer (ale C) with 13 different yeast strains (results not shown). None of the top fermenting strains produced a considerable sulfite amount, while two out of three of the tested bottom fermenting yeasts produced 1−1.5 mg/L sulfite. However, since aged aroma and carbonyls decreased in all refermented beers, it can be concluded that sulfite most probably was not responsible for the observed results.

The assumption that the yeast reducing power was responsible was tested by adding 3 and 8 mg/L of 2-MP (corresponding with 42 and 111 μM) to a lager beer (lager A) and the same amounts of furfural (corresponding with 31 and 83 μM) to other bottles of the same beer. These beers were refermented with DS08 and residual concentrations of the aldehydes and the corresponding alcohols were measured. The aldehyde addition was this large in order to be able to measure a difference in the concentration of the corresponding alcohols which were already present in high amounts. Furfural and 2-MP were removed almost entirely during refermentation as presented in **Figure 3** (concentrations were expressed as μM to compensate for the difference in molecular weight between aldehyde and alcohol). The onset beer already contained 8 mg/L 2-methylpropanol (106 μM) and 2 mg/L furfuryl alcohol (21 μM). During refermentation of the same lager beer without extra added compounds, about 0.8 and 0.2 mg/L (11 and 2 μM) of the respective compounds were formed. On the other hand, refermentation of the beers with added compounds resulted in much higher concentrations and it seemed that the entire amount of the added aldehyde was converted in the corresponding alcohol. This confirms the assumption that the reducing activity of yeast is responsible for the observed results and that this activity is high.

Since Peppard and Halsey's work, it is known that yeast reducing enzymes can convert carbonyl compounds to their corresponding alcohols during fermentation (*26*) in order to maintain a stable redox balance in the cell and for biosynthetic, catabolic and detoxication processes. Since then, there has been considerable speculation concerning the different enzymatic systems involved in aldehyde reduction and it has already been shown repeatedly that a large heterogeneity of carbonyl reducing enzymes exists in yeast (*28*, *43*, *44*). Among these, the best known is the NADH-dependent alcohol dehydrogenase (ADH) (*28*). Next to ADH, the aldo-ketoreductase family, containing enzymes with broad substrate specificity, seems to be of great importance for the reduction of many aliphatic and aromatic aldehydes and ketones (*44*, *45*). In general, it can be stated that *Saccharomyces cerevisiae* contains a complex system of reducing enzymes with overlapping substrate specificities and lots of research still has to be done to fully understand this reducing activity.

Next to differences of reductase activities between yeast strains, the yeast strain and compound specific residual concentrations observed in **Table 2** might be linked to the ability of carbonyls to enter the yeast cell and consequently to membrane permeability. This is supported by the observation of Debourg et al. (*43*) that reduction rates of carbonyls are clearly compound dependent as measured in vivo. However, comparing the reduction of carbonyls by whole cells and cell free extracts suggested that membrane permeability was not a determining factor since no difference was observed (*43*).

Evolution of Aroma Compounds during Refermentation. Finally, in order to get a better understanding of the observed results, the evolution of the selected carbonyl compounds during refermentation with two yeasts (DS05 and DS08) of an aged (5 days at 60 °C) and a fresh lager beer (lager A) was monitored. Both yeasts gave very comparable results and those of DS08 are presented in **Figure 4**. A steady decrease of the extract was observed until the final extract was reached after about 14 days. As reported previously (*30*), acetaldehyde was produced in high amounts in the glycolytic pathway early in the refermentation process, but was reduced afterward to concentrations close to those in the initial beer. On the other hand, hexanal was reduced immediately (results not shown). The concentration of T2N increased and decreased in the fresh and the aged beer respectively. Despite the ability of yeast to reduce considerable amounts of T2N (see **Table 2**), it appears that T2N can also be formed or released during refermentation. This increase was already observed by Vesely et al. (*46*) during fermentation. They suggested that this might be the result of the release from imine bindings. However, since this is probably due to a pH shift, and the pH hardly changed during refermentation, this might not be applicable here and other factors might be responsible. Furthermore, all Strecker aldehydes behaved more or less the same, which was also observed previously in normal (*46*) and cold contact fermentations (*39*). Strecker aldehydes can be formed from the oxoacid pool in yeast by decarboxylation. The oxoacids, in turn, can be formed either catabolically from exogenous amino acids (Ehrlich pathway) or anabolically from the main carbon source (Genevois pathway) (*28*, *47*). During refermentation, aldehydes were probably mainly formed from the anabolic pathway since beer only contains very low amounts of the corresponding amino acids (*46*). Then again, the formation appeared limited, which is probably attributable to the limited growth. In the end, the concentrations were more or less the same in the refermented fresh or aged beer. Next, furan aldehydes were reduced at different rates. While especially furfural, but also 5-methylfurfural, were reduced quickly, it seemed that, relatively seen, the reduction of 5-HMF was slower. On the other hand, the concentration of 5-HMF in the

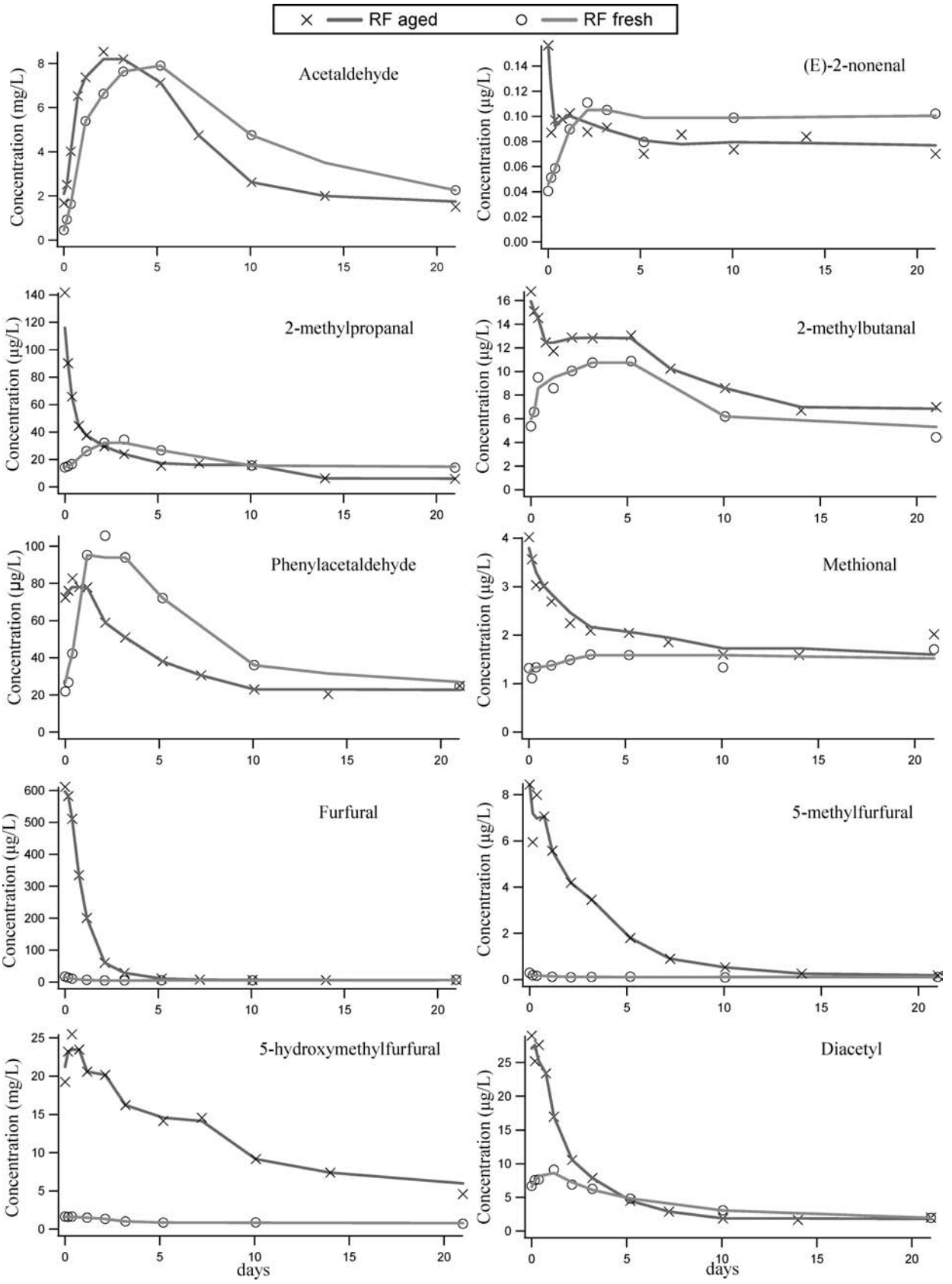

Figure 4. Concentration evolution of several aldehydes during refermentation of a thermally aged (4 days at 60 °C) and a fresh lager beer with yeast DS08.

aged beer was very high, and here, it might be that the reduction capacity of yeast was too limited to reduce the whole amount of 5-HMF during the refermentation process. Finally, since hardly any valine is present in finished beer, valine was synthesized in the beginning of the refermentation process and accordingly, diacetyl was formed. However, diacetyl formation was small as a consequence of the limited yeast growth and was hardly noticeable in the concentration evolution of the aged beer.

Altogether, it might be that reduction activity is linked to the presence of fermentable extract, since it seems that hardly any reduction occurred after the extract was exhausted (after 14 days). This seems logical, since extract conversion requires maintaining a stable redox balance and additionally, fewer yeast cells will be in solution due to sedimentation in the bottle. This might lead to the assumption that residual concentrations of several compounds are closely linked to their formation during refermentation. On the other hand, except for acetaldehyde, no formation of any compound was observed after 3 days and the concentration increase was sometimes not noticeable in the curves that started from higher initial concentrations (i.e., in the aged beer). Additionally, except for 5-HMF, residual concentrations were similar in the fresh and the aged refermented beer.

In the end, it can be concluded that after refermentation of aged beers, a significant improvement of the overall aroma was observed independent of the beer type or the yeast strain used. In these beers, several aged aromas disappeared almost entirely as a result of yeast activity. Especially cardboard, ribes, Maillard and Madeira aromas diminished greatly. The observed results were supported by the decrease of aldehydes, mainly (*E*)-2-nonenal, Strecker aldehydes, 5-hydroxymethylfurfural and diacetyl, because of the ability of yeast to reduce these compounds to their corresponding alcohols. Additionally, other aldehydes that were not yet linked to aging and that can be reduced by yeast are possibly interesting to explain aged aroma aspects. Additionally, stale-solvent aromas probably caused by the appearance of 2-furfuryl ethyl ether, and possibly other ethers, were hardly influenced by yeast. Furthermore, despite the limited amount of fermentable extract, the yeast reducing capacity appeared large, although it was noticed that yeast could not remove aldehydes entirely. Indeed, residual concentrations remained in the beer after refermentation, independent of the initial concentration. These residual concentrations appeared to depend on the aldehyde and the yeast strain used. This could probably be attributed to interactions with the beer medium, although it was also suggested that yeast strain specific characteristics may be responsible. Finally, it appeared that, during refermentation, not only does reduction take place but several compounds, like acetaldehyde, diacetyl and Strecker aldehydes, could also be formed. However, these observations could probably not explain the remaining concentrations of the aldehydes, since refermentation of fresh or aged beers resulted in approximately the same levels.

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