# Annexe 4

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# RETENTION BY PROTEINS AS SOURCE OF CARDBOARD OFF-FLAVOR IN AGED BEERS

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## Summary

Oxidation is usually recognized as the major cause of development of a stale flavor in beer. However, no significant difference in trans-2-nonenal concentration has been observed between oxygen-receiving and oxygen-free beers after aging. Although bottled $^{18}O_2$ did cause dramatic deterioration of sulfites, polyphenols, and isohumulones, it was not incorporated into the carbonyl fraction, indicating that the cardboard flavor in beer is not due to lipid oxidation. As shown by adding deuterated nonenal to the pitching wort, nonanol oxidation and sulfitic adduct degradation were also inefficient pathways of trans-2-nonenal synthesis.

All these data lead us to propose a non-oxidative mechanism for the production of alcenals in bottled beer. Trans-2-nonenal is synthesized by linoleic acid oxidation (LOX and autoxidation) through mashing and boiling. However, the free trans-2-nonenal level decreases due to retention by wort amino acids and proteins. The so-obtained imine protects trans-2-nonenal from yeast reduction but can release it at lower pH during aging. The nonenal potential of the wort turned out to be a good indicator of beer flavor staling. UV spectroscopy enabled us to visualize the chemical bond which is broken during such oxygen-free experiments and to determine experimental conditions (pH, temperature, additives) that destabilize trans-2-nonenal precursors in wort. Addition of $SO_2$ after wort filtration was very helpful : it reduced both linoleic acid autoxidation and the nonenal potential rise while the wort was boiling.

## Introduction

Oxidative changes occurring in packaged beer constitute one of the most serious problems in brewing. Although the mechanisms of beer staling have not been fully elucidated, oxidation is recognized as the major cause of the development of a stale flavor in beer. Oxygen in the headspace is consumed during storage of packaged beer and the more air in the headspace, the more the bottled beer deteriorates (Narziss, 1986). Apart from air exclusion, no practical solution has been found to date. An oxygen-free headspace, moreover, does not always effectively prevent the appearance of a cardboard flavor in aged beers. Gribsy et al. (1974) have shown that samples stored with increased $O_2$ level did develop a more pronounced oxidized character but the chief flavor change was to the sweet, caramelized note which is quite different from the cardboard character usually associated with beer staling. It is well known that the major contributor to this stale flavor is trans-2-nonenal (Jamieson and van Gheluwe, 1970; Wang and Siebert, 1974), which can be formed by oxidation of linoleic acid (Tressl et al., 1979).

In the present work, we have sought to clarify the issue of the impact of oxygen in the headspace of bottled beer. Trans-2-nonenal was quantified in oxygen-receiving and oxygen-free beers after aging. Using oxygen 18, we also determined the amount of carbonyls issued

from lipid oxidation in the bottled beer. In some experiments, deuterated trans-2-nonenal was added to the pitching wort to see if nonanol oxidation or sulfitic adduct degradation could explain synthesis of the alcenal.

The influence of the oxygen level during wort mashing was also assessed. As the measured nonenal potential proved a good indicator of beer flavor staling, UV spectroscopy was used to find which experimental conditions destabilize trans-2-nonenal precursors in wort. *In vivo* experiments confirmed that $SO_2$ can reduce both lipid autoxidation and the nonenal potential rise while the wort is boiling.

**Why a non-oxidative pathway ?**

96 ppm $^{18}O_2$ was injected into the bottle headspace of a low-sulfite (2 ppm) commercial lager beer (initial oxygen level below 0.1 ppm). After 5 days at 40°C (accelerated aging) or 3 months at 20°C (natural aging), trans-2-nonenal was extracted by vacuum distillation and C18/water/dichloromethane partitioning. Despite the large amount of oxygen injected into the headspace, GC-MS revealed no significant difference in trans-2-nonenal concentration between oxygen-receiving and oxygen-free samples (table I).

***Table I.*** *Trans-2-nonenal contents measured in an industrial beer after accelerated (5 days at 40°C) or natural (3 months at room temperature) aging with and without injection of oxygen 18 (96 ppm) into the headspace before storage (Collin et al., 1997)*

| | Trans-2-nonenal content (ppb) | | | |
|---|---|---|---|---|
| Fresh beer | 0.09<br>0.09 | | **0.09** | |
| | Beer **with** injection of $^{18}O$, before aging | | Beer **without** injection of $^{18}O$, before aging | |
| Beer after an Accelerated aging | 0.27<br>0.31 | **0.29** | 0.31<br>0.39 | **0.35** |
| Beer after a natural aging (3 months) | 0.21<br>0.24 | **0.23** | 0.20<br>0.22 | **0.21** |

In all cases, the trans-2-nonenal level increased from 0.1 ppb in fresh beer to 0.2-0.3 ppb in aged beer, whatever the oxygen level is. From this experiment, we can conclude that the cardboard flavor is not produced by an oxidative pathway.

The alcenal dichloromethane extract, also containing nonenoic acid and 3-hydroxynonanal, the two major degradation products of trans-2-nonenal (Noël et al., 1995), was then analyzed by proton bombardment after transfer from dichloromethane to isooctane. This experiment differs from the work of Owades and Jakovac (1966) who derivatized their carbonyls by 2,4 dinitrophenylhydrazine. As shown in table II, very low amounts of $^{18}O$ were measured in our extracts (exceeding the natural frequency of $^{18}O$ by only 0.025 and 0.017 atoms per hundred oxygen atoms). Assuming that the extracted carbonyls and related flavoring compounds (average molecular weight: 140) represent a maximum concentration of 5 ppb in the initial beer sample, it appears from our calculations that carbonyls having incorporated $^{18}O$ represent no more than 1 ppt. This incorporation level is very close to the sensitivity threshold of our method, and well below the 0.2 ppb of trans-2-nonenal that appear through aging. All our experiments thus confirm that the cardboard flavor is not due to the

oxidation of lipids in the final product.

*Table II. Proton bombardment analysis of carbonyl extracts issued from beers aged in presence of oxygen headspace (Lermusieau et al., 1998)*

| | cyclotron signal | $^{18}O$ content | µg of $^{18}O$ incorporated in 250 ml of beer | ppb of carbonyl compounds having bound an $^{18}O$ atom |
|---|---|---|---|---|
| **Beer after an accelerated aging** | | | 0.00004 | 0.001 |
| with 84 ppm $^{16}O_2$ | 33.71 ± 5.45 | 0.200 % | | |
| with 96 ppm $^{18}O_2$ | 37.98 ± 4.76 | 0.225 % | | |
| **Beer after a 3 months natural aging** | | | 0.00003 | 0.001 |
| with 84 ppm $^{16}O_2$ | 57.77 ± 1.35 | 0.200 % | | |
| with 96 ppm $^{18}O_2$ | 62.69 ± 6.41 | 0.217 % | | |

Moreover, 10 ppb of $C_4D_9$-$C_2H_4$-$C_2H_2$-CHO deuterated nonenal added at the beginning of the fermentation failed to yield deuterated nonenal in the aged beer (concentration of labeled nonenal below 0.03 ppb), suggesting that neither nonanol oxidation nor sulfitic adduct degradation can occur in the bottled beer (Lermusieau et al., 1998).

Although bottled oxygen does not cause trans-2-nonenal synthesis by lipid oxidation, brewers should nevertheless continue to avoid any trace of oxygen in bottled beer, because headspace oxygen does cause dramatic chemical deterioration of other organoleptically active fractions.

## Precursors upstream from the process

We propose that trans-2-nonenal is synthesized by oxidation before fermentation but protected from yeast reduction by binding to amino acids and proteins. Previous data (Noël and Collin, 1995) show that this kind of complex is the major degradation product of trans-2-nonenal during mashing and boiling. In the nonenal potential experiment (Drost et al., 1990), moreover, free nonenal is released from this complex (50%), suggesting that this mechanism is realistic at the pH of the beer (see figure 1).

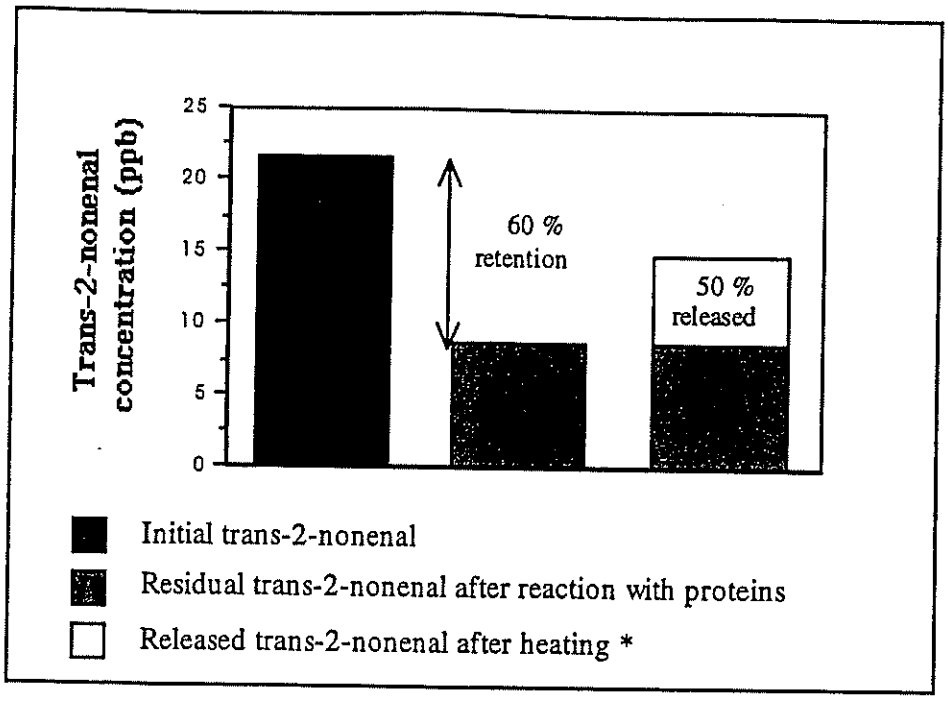


***Figure 1.*** *Trans-2-nonenal concentration before and after the heating of a solution of trans-2-nonenal (21.4 ppb) and malt albumins (886 ppm BSAeq) for 25 minutes at 50°C (Lermusieau et al., 1998)*

** trans-2-nonenal which is released after the Drost experiment (2 hours at 100°C under argon, pH 4) (Drost et al., 1990)*

As shown in table III, we logically detect higher nonenal potentials when oxidation occurs during mashing (higher LOX activity) or when the hot break is insufficiently eliminated (slight nonenal potential decrease). Moreover, the nonenal potential of the wort is clearly related to staling of the flavor of the corresponding beers, confirming that flavor stability is not related to beer packaging but to wort preparation.

## UV spectroscopy, a way to determine the stability of Schiff bases under various conditions

As measuring the nonenal potential is proposed as a means of quantifying the amount of bound nonenal in the wort and hence to assess the future cardboard flavor in beer, we have tried to determine how various parameters affect the stability of the alcenal/nitrogen compound bond. UV absorbance at 290 nm enabled us to visualize such Schiff bases under various conditions (figure 2).

***Table III.*** *Relation between nonenal potential of worts obtained under various experimental conditions, and the flavor stability of the corresponding beers (Lermusieau et al., 1998)*

| | Nonenal potential before boiling (ppb) | Nonenal potential before fermentation (ppb) | Trans-2-nonenal after accelerated aging (ppb) | Trans-2-nonenal after 3 months of natural aging (ppb) |
|---|---|---|---|---|
| Wort prepared with $CO_2$[a] | 0.3 | 1.4 | 0.22 | 0.27 |
| Wort prepared with high level of oxygen[b]; good hot break[c] | **3.9** | 3.3 | 0.40 | 0.98 |
| Wort prepared with high level of oxygen[b]; bad hot break[c] | **4.5** | **5.1** | 0.65 | 2.69 |

a. 4 L $CO_2$ bubbled for the first 15 minutes of mashing (57 L deoxygenated water and 18.2 kg deoxygenated flour)
b. 4 L $O_2$ bubbled for the first 15 minutes of mashing (57 L deoxygenated water and 18.2 kg deoxygenated flour)
c. amount and aspect

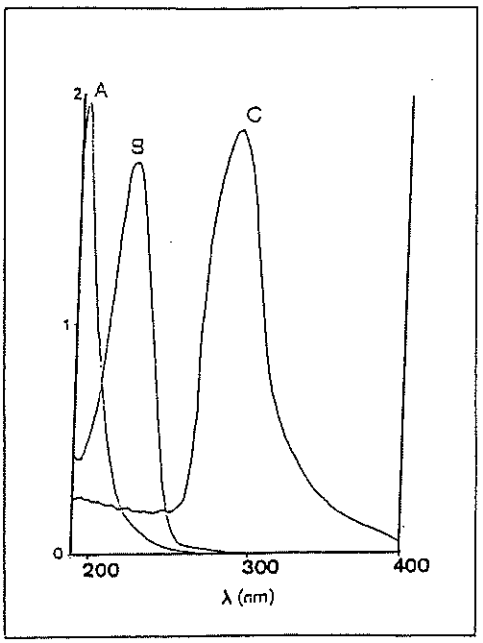


***Figure 2.*** *UV spectrum of lysine (A), trans-2-nonenal (B) and the trans-2-nonenal/lysine Schiff base issued from the reaction at 100°C and pH 5.4 for 30 minutes (C) (Lermusieau et al., 1998)*

Our results (figure 3) clearly indicate that temperature is a parameter increasing imine synthesis. Figure 4 shows that pH is another factor influencing the absorbance at 290 nm : the higher the pH, the higher the Schiff base concentration.

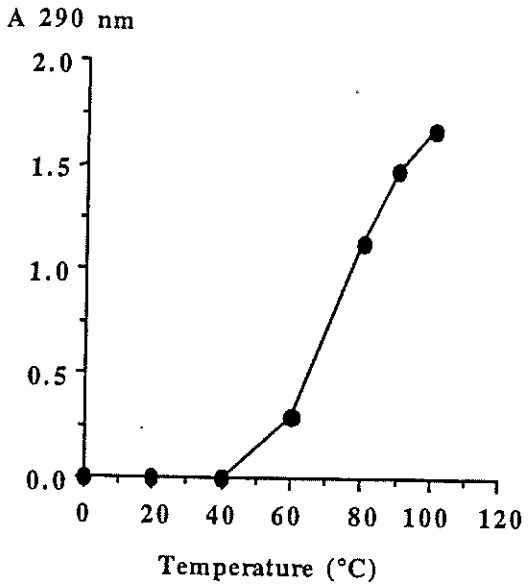


**Figure 3.** Influence of temperature (30 min. heating, pH 5.4) on the Schiff base lysine/trans-2-nonenal formation

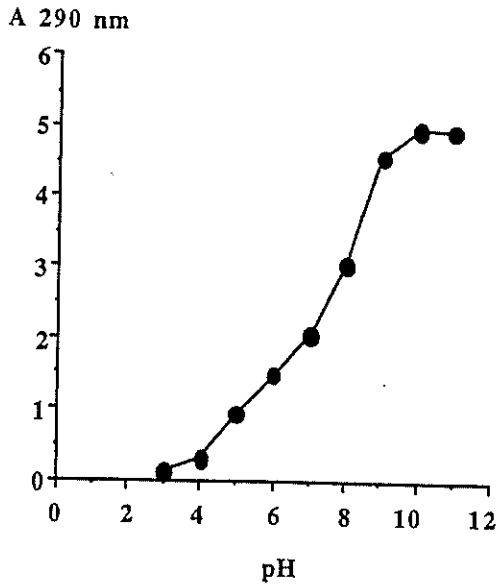


**Figure 4.** Influence of pH (heating at 100°C for 30 min.) on the Schiff base lysine/trans-2- nonenal formation

Most interesting was the effect of sulfites, since they suppressed formation of 50% of the C=N bonds at 100°C (pH 5.4). This led us to try to decrease the amount of nonenal precursors by adding 50 ppm $SO_2$ after wort filtration. As $SO_2$ addition also reduces lipid oxidation as the wort boils, very low nonenal potentials were measured in the final wort. Very good stability also characterized the beer obtained in this way (see table IV).

*Table IV. Nonenal potential concentration in the pitching wort and trans-2-nonenal content in the aged beer (5 days, 40°C) in relation with sulfite concentration (Lermusieau et al., 1998)*

| | Nonenal potential in the pitching wort *(ppb)* pH 5.4 | Trans-2-nonenal in aged beer *(ppb)* | | $SO_2$ in the pitching wort *(ppm)* | | $SO_2$ in fresh beer *(ppm)* | | $SO_2$ in aged beer *(ppm)* | |
|---|---|---|---|---|---|---|---|---|---|
| | | Free pH 4.3 | Total pH 9 | Free pH 5.4 | Total pH 9 | Free pH 4.3 | Total pH 9 | Free pH 4.3 | Total pH 9 |
| Blanco (+10 ppm $SO_2$ in the fresh beer) | 5.1 | 0.31 | 0.59 | 0.0 | 0.0 | 0.8 (+10.0) | 1.0 (+10.0) | 0.3 | 3.6 |
| Adding 50 ppm $SO_2$ after wort filtration | 3.5 | 0.18 | 0.21 | 6.0 | 10.0 | 1.8 | 6.3 | 1.5 | 4.4 |

## Conclusion

In conclusion, a non-oxidative degradation product of trans-2-nonenal is proposed to be the major precursor of the cardboard flavor in aged beers. Although bottled oxygen does cause considerable deterioration of sulfites, polyphenols, and isohumulones, it doesn't increase the trans-2-nonenal level because free radical reactions cannot oxidize lipids or nonanol in beer. Correlations previously described by Kaneda et al. (1995) and Uchida et al. (1996) between the reduction power of the final beer and staling may be due to the fact that the higher the level of antioxidants in beer, the lower the rate of linoleic acid oxidation during boiling. Due to their Schiff base structure, nonenal precursors can be destabilized at low pH or with sulfites. Addition of amino acids to beers, as recommended by Grisby et al. (1974), should logically delay alcenal release. The mechanism described here for nonenal synthesis through aging most probably also concerns other aldehydes and ketones produced in the final beer, such as 5-hydroxymethylfurfural, furfural (Madigan et al., 1998), or •- damascenone (Thedy et al., 1997).

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## References

Collin, S., Noël, S., Bonte, S., Metais, N., Bodart, E., Peladan, F., and Dupire, S. Utilisation d'$^{18}O_2$ pour évaluer l'impact du phénomène d'oxydation durant le brassage et le stockage de la bière. *Eur. Brew. Conv. Proc. Congr. 26th, Maastricht,* Oxford Univ. Press Inc., New York. 535-544, 1997.

Drost, B.W., Van den Berg, R., Freijee, F.J.M., van der Velde, E.G., and Hollemans, M. Flavor stability. *J. Am. Soc. Brew. Chem.* 48:124-131, 1990.

Grigsby, J. H., Palamand, S. R., and Hardwick, W.A. Further studies on the staling of beer. *Proc. Am. Soc. Brew. Chem.* 64-69,1974.

Jamieson, A.M., and Van Gheluwe, J.E.A. Identification of a compound responsible for cardboard flavour in beer. *Proc. Am. Soc. Brew. Chem.* 192-197, 1970.

Kaneda, H., Kobayashi, N., Furusho, S., Sahara, H., and Koshino, S. Reducing activity and flavor stability of beer. *MBAA Tech. Quart.* 32:90-94, 1995.

Lermusieau, G., Noël, S., Liégeois, C., and Collin S. A non-oxidative pathway for the synthesis of trans-2-nonenal. *J. Am. Soc. Brew. Chem. submitted,* 1998.

Madigan, D., Perez, A., and Clements, M. Furanic aldehyde analysis by HPLC as a device to determine heat-induced flavour damage to beer. *ASBC Newsletter* 58:15, 1998.

Narziss, L. Centenary review Technological factors of flavour stability. *J. Inst. Brew.* 92:346-353, 1986.

Noël, S., and Collin, S. Trans-2-nonenal degradation products during mashing. *Eur. Brew. Conv. Proc. Congr. 25th, Brussels*, Oxford Univ. Press Inc., New York. 483-490, 1995.

Owades, J.L., and Jakovac, J. Study of beer oxidation with $^{18}O$. *Proc. Am. Soc. Brew. Chem.* 180-183, 1966.

Thedy, V., Bunoni, M., and Tate, F. Identificazione di un composto carbonilico in birre invecchiate che presentano staling flavour: ricerca del damascenone. *Birre e Malto* 67:9-24, 1997.

Tressl, R., Bahri, D., and Silwar, R. Formation of aldehydes by oxidation of lipids and their importance as "off-flavour" components in beer. *Eur. Brew. Conv. Proc. Congr. 17th, Berlin*, DSW, The Netherlands. 27-43, 1979.

Uchida, M., Suga, S., and Ono, M. Improvement for oxidative flavor stability of beer - Rapid prediction method for beer flavor stability by electron spin resonance spectroscopy. *J. Am. Soc. Brew. Chem.* 54:205-211, 1996.

Wang, P.S., and Sierbert, K.J. Determination of trans-2-nonenal in beer. *MBAA Tech. Quart.* 11:110-117, 1974.