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A NON-OXIDATIVE PATHWAY FOR THE SYNTHESIS OF TRANS-2-NONENAL DURING BEER STORAGE AND TRANSPORT

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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 synthetized by linoleic acid oxidation (LOX and autoxidation) through mashing and boiling. However, the free trans-2-nonenal level decreases due to retention by wort aminoacids 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 autooxidation 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. Grisby 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 the synthesis of the alcenal. The impact of beer stabilization treatments (addition of polyvinyl-polypyrrolidone powder, potassium metabisulfite, ascorbic acid) was also investigated.

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 autooxidation and the nonenal potential rise while the wort is boiling.

WHY A NON-OXIDATIVE PATHWAY?

96ppm $^{18}O_2$ was injected into the bottle headspace of a low-sulfite (2ppm) commercial lager beer (initial oxygen level below 0.1ppm). 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 0.09		0.09	
	Beer with injection of $^{18}O_2$ before aging		Beer without injection of $^{18}O_2$ before aging	
Beer after an accelerated aging	0.27 0.31	0.29	0.31 0.39	0.35
Beer after a natural aging (3 months)	0.21 0.24	0.23	0.20 0.22	0.21

In all cases, the level increased from 0.1ppb in fresh beer to 0.2-0.3ppb in aged beer, whatever the oxygen level. 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 5ppb in the initial beer sample, it appears from our calculations that carbonyls having incorporated ^{18}O represent no more than 1ppt. 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).

WHAT ABOUT THE OXYGEN DAMAGE?

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.

Three fractions are particularly sensitive to oxidation: polyphenols, sulfites and isohumulones (Table III). Oxidation reactions revealed to be quite different in case of accelerated or natural aging.

It emerges from this study that many oxidation reactions can occur upon beer aging. The oxidation of polyphenols, sulfites and isohumulones most likely alters the organoleptic properties of beer. On the other hand, fatty acids appear to be protected from oxidation.

Table III. *Incorporation of oxygen 18 in beer extracted fractions, measured by proton bombardment analysis and isotopic mass spectroscopy (Collin et al., 1997)*

	Accelerated aging	Natural aging 3 months	Natural aging 9 months	Initial content
Oxidized polyphenols (Triol pathway)	6.5%	0.3%	0.6%	57.4 ppm
Oxidized sulfites	3.0%	3.5%	100%	2.1 ppm
Oxidized isohumulones	2.7%			26.8 ppm

INFLUENCE OF VARIOUS BEER STABILIZATION TREATMENTS

To evaluate the impact of usual beer treatments, we measured incorporation of oxygen 18 in KMS-, PVPP- and AA-treated beers subjected to accelerated aging.

As shown in Table IV, SO_2 strongly protected the polyphenol fraction (25-50 ppm) from oxidation (0.3% oxidized polyphenols when 13 ppm SO_2 was added versus 6.6% without treatment).
On the other hand, the PVPP treatment (see table V) increased the sensitivity of sulfites (16-20 ppm) to oxidation (12.5% of oxidized sulfites versus 7.9%).

Table IV. *Impact of the SO_2 treatment. Incorporation of oxygen 18, by proton bombardment analysis, in molyphenol and sulfate gractions extracted from beer after accelerated aging (5 days at 40°C) in the presence of 96 ppm oxygen (Collin et al., 1997)*

	Beer A (2.1 ppm SO_2)	Beer B (15 ppm SO_2)
Oxidized polyphenols (triols)	0.65%	0.3%
Oxidized sulfites	3.1 %	3.1%

Table V. *Impact of the PVPP treatment. Incorporation of oxygen 18, by proton bombardment analysis, in polyphenol and sulfate fractions extracted from beer after accelerated aging (5 days at 40°C) in the presence of 96 ppm oxygen (Collin et al., 1997)*

	Beer C (95 ppm polyphenols)	Beer D (50 ppm polyphenols)
Oxidized polyphenols (triols)	0.4%	0.4%
Oxidized sulfites	7.9 %	12.5%

Most interesting was the effect of ascorbic acid on KMS-treated beer (20 ppm SO_2, 95 ppm polyphenols; table VI). This well-known antioxidant increased oxidation of polyphenols and sulfites, probably due to regeneration of iron and copper ions causing synthesis of hydroxyl radical by the well-known Fenton reaction.

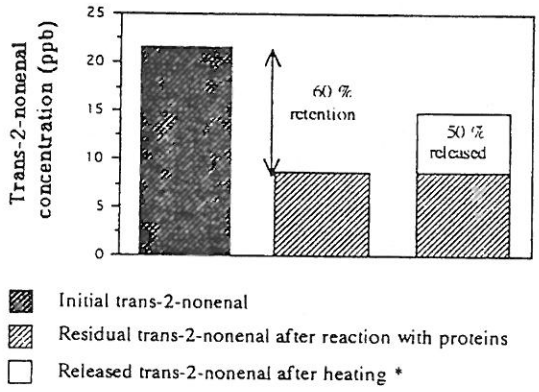
Table VI. *Impact of the ascorbic acid treatment. Incorporation of oxygen 18, by proton bombardment analysis, in polyphenol and sulfate fractions extracted from beer after accelerated aging (5 days at 40°C) in the presence of 96 ppm oxygen (Collin et al., 1997)*

	Beer E (0 ppm ascorbic acid)	Beer F (26 ppm ascorbic acid)
Oxidized polyphenols (triols)	0.4%	0.9%
Oxidized sulfites	7.9%	37.0%

PRECURSORS UPSTREAM FROM THE PROCESS

We propose that trans-2-nonenal is synthetized 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, 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 VII, 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.

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

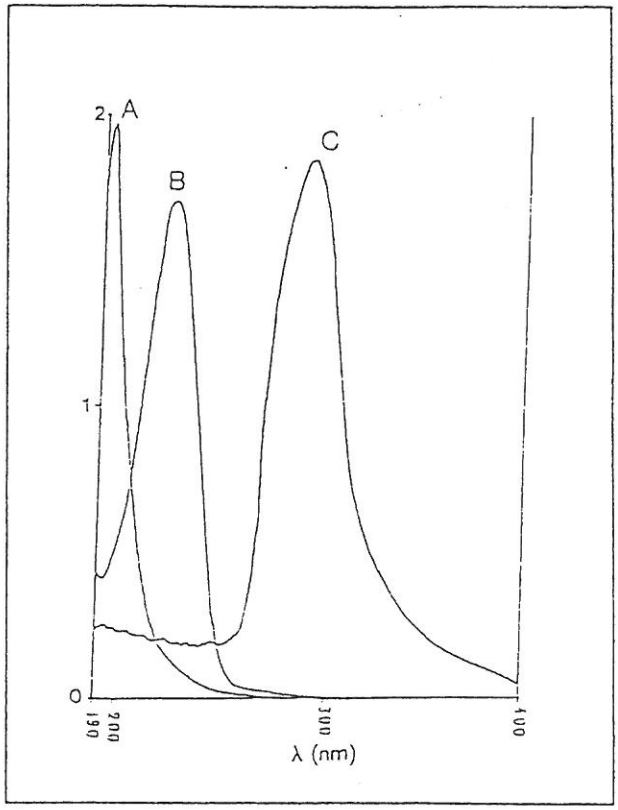
	Nonenal potential before boiling (ppb)	Nonenal potential before fermentation (ppb)	Trans-2-nonenal afteraccelerated aging (ppb)	Trans-2-nonenal after 3 months of natural aging (ppb)
Wort prepared with CO_2[a]	0.3	1.4	0.22	0.27
Wort prepared with high level of oxygen[b]; good hot break[c]	3.9	3.3	0.40	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

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 visualise such Schiff bases under various conditions (figure 2).

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 concentrations.

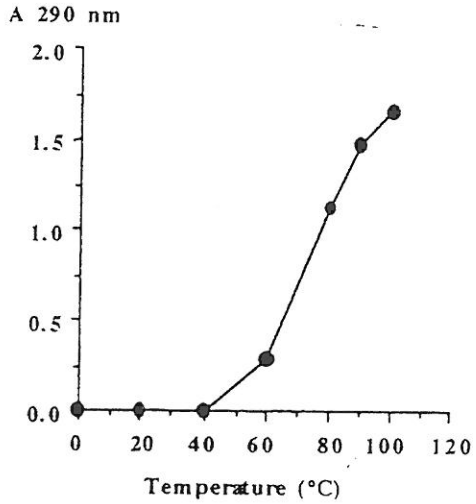


Figure 3. *Influence of temperature (30 min. heating, pH 5.4) on the Schiff base lysine/trans-2-nonenal formation*

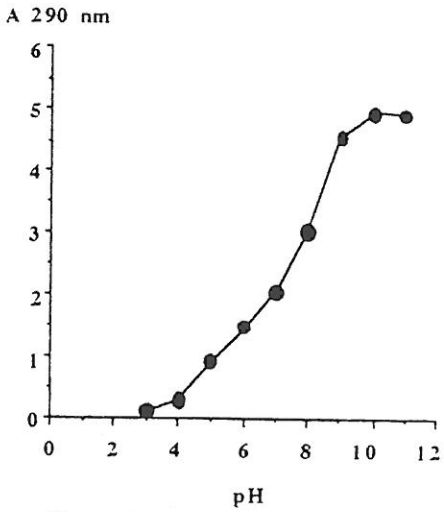


Figure 4. *Influence of pH (heating at 100°C for 30 min.) on the Schiff base lysine/trans-2-nonenal formation*

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 VIII).

Table VIII. *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)	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)	
	pH 5.4	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 by low pH or 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 β-damascenone (Thedy et al., 1997).

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