The use of Oxygen 18 in appraising the impact of oxidation process during beer storage

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To appraise the incidence of oxygen in bottled beer, the stable nonradioactive oxygen isotope $18O_2$ was injected into the headspace just before ageing and subsequently analysed by proton bombardment and isotopic mass spectroscopy of all the most interesting beer fractions. Although oxygen did cause considerable oxidation of sulphites, polyphenols, and isohumulones, it was not incorporated into the carbonyl fraction, indicating that the cardboard flavour in beer is not due to lipid oxidation but to wort preparation. Nonenal potential measurement was found to be a good indicator of beer flavour staling. The impact of beer stabilisation treatments (addition of polyvinyl pyrolidone powder, potassium metabisulphite, ascorbic acid) was also investigated.

Key Words: *Flavour, stability, beer, ageing, oxidation.*

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 recognised as the major cause of the development of a stale flavour 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[14]. 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 flavour in aged beers. Gribsy *et al.*[8] have shown that samples stored at increased O_2 levels did develop a more pronounced oxidised character but the chief flavour change was to the sweet, caramelised note which is quite different from the cardboardy character usually associated with beer staling. It is well known that the major contributor to this stale flavour is trans-2-nonenal,[9,21] which can be formed by oxidation of linoleic acid[19].

A few studies have dealt with chemical changes occurring when beer oxidises. The best known is the work of Owades and Jakovac[16]. These authors injected a nonradioactive oxygen isotope, oxygen 18, into the headspace of bottled beer. After 8 months of ageing, they analysed various fractions by proton bombardment in a Van de Graaf accelerator. The most significant result was the absence of oxygen 18 in the post-derivatization aqueous fraction, indicating that neither lipid oxidation nor polyphenol hydrogen removal had occurred in the bottle. On the other hand, high levels of oxygen 18 were found in the polyphenol (65% oxidised polyphenols) and 2,4-dinitrophenylhydrazone fractions (30%) and lower levels in isohumulones (5%). Unfortunately, no mechanism was proposed to explain these results.

More recently, Kaneda *et al.*[10,11] observed a good correlation between the presence of active oxygen species in beer and flavour staling. The endogenous antioxidant activity of beer, affected by each step in the brewing process from raw materials to packaging, would thus protect against ageing[12,13,20]. Drost *et al.*[5], on the other hand, concluded from their work that the lipoxygenase activity of malt is likely to be the main factor modulating the appearance of a cardboard flavour.

In the present work we have sought to clarify the issue of the impact of oxygen in the headspace of bottle beer. A comparatively high concentration(104 ppm) of oxygen 18 was injected before ageing and the most interesting beer fractions were analysed by proton bombardment in a cyclotron and by isotopic mass spectroscopy. This methodology was also used to compare various beer stabilisation treatments: treatment with polyvinyl polypyrrolidone powder (PVPP), potassium metabisulphite (KMS), or ascorbic acid (AA).

EXPERIMENTAL PROCEDURES

Beer

A Belgian commercial pasteurised lager beer bottled with a 2 ppm sulphite level was analysed initially.

Pilot productions were used to compare beer treatments (KMS, PVPP, AA).

Ageing of bottled beer in the presence of oxygen in the headspace

15 ml oxygen (isotope 16 or 18) was injected with a gas-tight syringe into the headspace of industrial beer through a silicone top (Vel N°4). The bottles were then Crown sealed and the beer aged at 40°C for 5 days in a dark room (accelerated ageing) or at room temperature for 3-9 months (natural ageing).

ANALYSIS OF BEER COMPOUNDS

Steam vacuum distillation (Fig. 1)

Beer carbonyl compounds were extracted by vacuum distillation based on the method of Currie *et al.*[3]. The beer

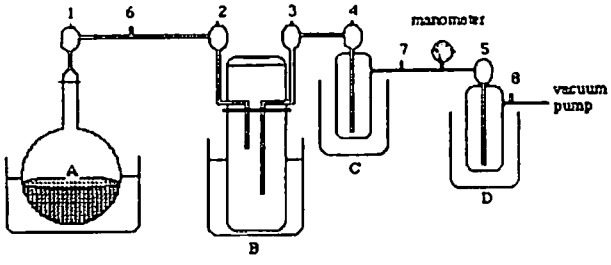

FIG. 1. Steam vacuum distillation equipment.

samples (1.5 litre) and sodium chloride (300g) were poured into flask A and heated in a 30°C water bath. The mixture was stirred at 250 rpm. Valves 2, 3, 4, and 5 were opened and valve 1 was closed. Vacuum was applied to the system (2-5 mm Hg). Traps B, C, and D were cooled with liquid nitrogen. Vacuum was applied to flask A by gradually opening valve 1. The samples were distilled at 30°C for one hour and at 35°C for 30 minutes. The volatile fraction was collected in cold trap B.

Solvent transfer

After thawing, the distillate (approximately 300-400 ml) was passed through a C18 Bond Elut column (500 mg) conditioned beforehand with methanol (40 ml) and dichloromethane (30ml). Nonpolar volatiles were eluted with 25 ml dichloromethane.

Concentration

The extract and 5 ml of external standard (nonane, 5 mg/litre in dichloromethane) were reduced by Kuderna-Danish evaporation to approximately 0.5ml. Dichloromethane extracts were analysed for the presence of trans-2-nonenal by gas chromatography.

Gas chromatography / Mass spectrometry

For gas chromatography, a Hewlett Packard Model 5890 gas chromatograph fitted with a 50 m x 0.32 mm, wall coated, open tubular (WCOT) apolar CP-SIL5 CB capillary column (film thickness: 1,2µM) was used. The carrier gas was helium at a flow rate of 1.3 ml/min. The oven temperature was programmed to rise from 30°C to 80°C at 20°C/min and then to 200°C at 2°C/min.

Splitless injections (2 µl) were carried out at an injector temperature of 250°C. The column was directly connected to a Hewlett Packard 5988 quadrupole mass spectrometer. Electron impact mass spectra were recorded at 70 eV. Spectral recording throughout elution was automatically performed with the HP59970C software. Trans-2-nonenal was detected and quantified using the single ion monitoring (SIM) mode (selected ions: 70, 81, 83, 85, 98, 140, 142). The detector response was calibrated with authentic standards. A recovery factor above 80% was measured for trans-2-nonenal with a variation coefficient of 3-5%

Quantification of incorporated oxygen 18 in various fractions

Polyphenol extraction

100ml HCl (1M), 300 ml H_2O, and 500 ml iso-octane were mixed with 100 ml degassed beer in a settled flask. After 5 minutes of settling, the organic phase was eliminated and the aqueous phase (300ml) was extracted three times with 300 ml ethyl acetate. The ethyl acetate extracts were evaporated with a Rotavapor at 30°C, then lyophilised, leaving the polyphenol fraction. Total polyphenols were quantified by measuring, according to De Clerk and Jerumanis[4], the intensity of the red colour produced by the reaction between polyphenols and ferric ions in a basic solution.

Sulphate extractions

100 ml degassed beer was acidified with 1ml of 6N HCl. The mixture was heated to 60°C, then 20 ml BaCl2 (10%) was added. The mixture was kept at room temperature for one hour before filtering through Millipore filter (S & S, 400714; 3µm; diam. 50mm). The residue was washed three times with hot water. The precipitates was placed for 24 h in an oven at 600°C. The sample was weighed (SW) and its sulphate content determined according to the following equation:

$$\text{Sulphate content (ppm)} = SW \times 4{,}115$$

Carbonyl compound extraction

The carbonyl compounds from five vacuum distillations (see above) were transferred after extraction and concentration to 50 µl isooctane, of which 30 µl was subjected to cyclotron of ^{18}O.

Isohumulone extraction

In a graduated cylinder, 25 ml degassed beer was acidified with 2.5 ml of 6N HCl.50 ml isooctane was added and the flask shaken for approximately 1 min. The mixture was allowed to stand for 15 min, then the organic phase was mixed with a same volume of acidified methanol (3.2 volumes 4N HCl plus 6.8 volumes methanol). The isooctane phase was then recovered, evaporated at 30°C in a Rotavapour and lyophilised for cyclotron analysis of ^{18}O. Isohumulones were quantified according to Rigby and Bethume[17].

Cyclotron analysis of oxygen 18

Cyclotron (Louvain-la-Neuve, Belgium) analysis of ^{18}O bombarding the samples with energetic protons, causing production of ^{18}F, a radioactive fluorine isotope[18]. The different beer fractions (minimum 50 mg or 30 µl) were placed in a sample case and sealed with tantalum foil. They were then irradiated for 30 minutes with a 7 MeV proton beam at 15 nA on target. The ^{18}F isotope produced decays with a half-life of 110 minutes and emits radiation which is easily measured with a gamma detector. Gamma emissions were measured every 20 minutes for 8 hours after irradiation. From the ^{18}F decay profile the computer directly determined the amount of ^{18}O present in the sample before bombardment. In what follows, all ^{18}O contents and changes in ^{18}O content are expressed as percentages of the total oxygen content of the sample, the latter being calculated on the basis of chemical composition of each fraction.

Isotopic mass spectroscopy

To determine the relative ^{18}O concentration in a water sample, 2 ml water was allowed to exchange oxygen atoms with added carbon dioxide for 24 h at 25°C[7]. A SMOW sample (Vienna Standard Mean Ocean Water, from International Atomic Energy – Vienna, Austria) was used as a reference. The CO_2 aliquots were analysed with a double collection mass spectrometer (Delta S Finnigan Mat, CNRS, Vernaison, France). The difference in ^{18}O concentration between the sample and the reference (expressed in per mil) was calculated on the basis of the intensities measured at molecular weights 44, 45, 46, and 47:

$$\partial^{18}O = 1000 \times \left\{ \frac{\frac{^{18}O}{^{16}O}\ (sample)}{\frac{^{18}O}{^{16}O}\ (SMOW)} - 1 \right\}$$

with $\frac{^{18}O}{^{16}O}\ (sample)$ = ratio between $46 + 47$ ($^{12}C^{16}O^{18}O + {}^{13}C^{16}O^{18}O$) and $44 + 45$ ($^{12}C^{16}O_2 + {}^{12}C^{16}O^{17}O + {}^{13}C^{16}O_2$)

and $\frac{^{18}O}{^{16}O}\ (SMOW) = 0.20052$

The following relation links the $^{18}O/^{16}O$ ratio measured in CO_2 with the corresponding ratio in water:

$$\frac{^{18}O}{^{16}O}\ (CO_2) = \alpha \times \frac{^{18}O}{^{16}O}\ (H_2O)$$

where $\alpha = 1.0409$ at 25°C 1,6

RESULTS AND DISCUSSIONS

104 ppm $^{18}O_2$ was injected into the bottle headspace of a low-sulphite lager beer. After 5 days at 40°C (accelerated ageing) or 3 months at 20°C (natural ageing), 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 differences in trans-2-nonenal concentration between oxygen-receiving and oxygen-free samples (Table I).

TABLE I. Trans-2-noneal contents measured in an anenal industrial beer after accelerated (5 days at 40°C) or natural (3 months at room temperature) ageing with and without injection of oxygen 18 (104 ppm) into the headspace before storage

Trans-2-nonenal content (ppb)	Beer with injection of $18O_2$ before ageing		Beer without injection of $18O_2$ before ageing	
Fresh beer	0.10 0.10	**0.10**	0.09 0.09	**0.09**
Beer after an accelerated ageing	0.27 0.31	**0.29**	0.31 0.39	**0.35**
Beer after a natural ageing (3 months)	0.21 0.24	**0.23**	0.20 0.22	**0.21**

TABLE II. Proton bombardment analysis of carbonyl extracts issued from beers aged in presence of oxygen in the headspace

	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 ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	33.71 ± 5.45 37.98 ± 4.76	0.200 % **0.225 %**	0.00004	**0.001**
Beer after a 3 month natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	57.77 ± 1.35 62.69 ± 6.41	0.200 % **0.217 %**	0.00003	**0.001**

In all cases, the level increased from 0.1 ppb in fresh beer to 0.2-0.3 ppb in aged beer, whatever the oxygen level. Furthermore, the single-ion-monitoring mass spectrum showed no increase in an ion with an m/z ratio of 85, indicating the absence of any hydroperoxide rearrangement after oxidation of linoleic acid by $^{18}O_2$.

The alcenal dichloromethane extract, also containing nonenoic acid and 3-hydroxynonanal, the two major degradation products of trans-2-nonenal[15], was then analysed by proton bombardment after transfer from dichloromethane to isooctane. Very low amounts of ^{18}O were measured in the extracts (exceeding the natural frequency of ^{18}O by only 0.025 and 0.017 atoms per hundred oxygen atoms) (Table II). Assuming that the extracted carbonyls and related flavouring compounds (average molecular weight: 140) represent a concentration of 5 ppb in the initial beer sample, it appears from these calculations that carbonyls having incorporated ^{18}O represent no more than 1 ppt. This incorporation level is very close to the sensitivity threshold of the method employed, and well below the 0.2 ppb of trans-2-nonenal that appear through ageing.

All these experiments thus confirm that the cardboard flavour is not due to the oxidation of lipids in the final product. Most probably, trans-2-nonenal is synthesised

TABLE III. Relation between nonal potential of worts obtained under various experimental conditions and the flavour stability of the correspondence beers

	Nonenal potential before fermentation (ppb)	Trans-2-nonenal after accelerated ageing (ppb)	Trans-2-nonenal after 3 months of natural ageing (ppb)
Sample I	1.37	0.22	0.27
Sample II	3.31	0.40	0.98
Sample III	5.10	0.65	2.69

before fermentation but protected from yeast reduction by linkage to amonoacids and proteins. Previous data[2,15] indicate that the nonenal potential measured before fermentation[5] gives a good idea of the extent of this protection. A relationship between the nonenal potential of worts under various experimental conditions and flavour staling of the corresponding beers was observed (Table III), again confirming that flavour stability is not related to beer packaging but to wort preparation.

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.

Polyphenols, in particular, are sensitive to oxidation, as shown by proton bombardment of the ethyl acetate extracts (Table IV). In this case the cyclotron signal was converted assuming that only one atom of ^{18}O could be added per molecule of polyphenol. A molecular weight of 580 was assumed. The increase in ^{18}O content measured after accelerated ageing was 0.538%, corresponding, in the beer, to 3.7 ppm polyphenols (6.48% of the polyphenol molecules) having incorporated one atom of ^{18}O during ageing. The increase measured after 3 and 9 months of natural ageing were respectively 0.026 and 0.051 atoms of ^{18}O per hundred oxygen atoms. This means that polyphenols oxidised to triols during 3 and 9 months of natural ageing represent respectively 0.18 and 0.35 ppm in the beer sample (i.e. 0.31 and 0.61% of the polyphenol molecules). Moreover, as shown by isotopic mass spectroscopy, a large number of polyphenol molecules are probably also oxidised to quinones. After accelerated ageing and 9 months of natural ageing respectively, this should lead to 3.97 and 10.17 mg ^{18}O in the water of each bottle of beer. These data differ significantly from the previously published results from Owades and Jakovac[16]. The low sensitivity of the Van de Graaf technique is thought to account for the absence of oxygen 18 in their water fraction.

TABLE V. Proton bombardment analysis of beer sulphate fractions after ageing in the presence of headspace oxygen

	Cyclotron signal	^{18}O content	μg of ^{18}O incorporated in 250 ml beer	ppm of sulphite oxidized to labelled sulphate
Beer after accelerated ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	274.88 ± 15.69 303.03 ± 3.27	0.2 % **0.22 %**	4.49	**0.064 ppm (3.05%)**
Beer after 3 months of natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	261.64 ± 2.26 292.32 ± 2.12	0.2 % **0.223 %**	5.16	**0.073 ppm (3.47%)**
Beer after 9 months of natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	225.5 ± 1.22 1005.6 ± 113.43	0.2 % **0.891 %**	155.1	**2.21 ppm (100%)**

TABLE IV. Proton bombardment analysis of beer polyphenol fractions and water ^{18}O analysis by isotopic mass spectrometry after ageing in the presence of headspace oxygen

Proton bombardment analysis of beer polyphenol fraction	Cyclotron signal	^{18}O content	μg of ^{18}O incorporated in 250 ml beer	ppm of labelled polyphenols (percentage of the total amount)
Beer after accelerated ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	142.85 527.42 ± 106.27	0.200 % **0.738 %**	28.84	**3.7 (6.48%)**
Beer after a 3 months natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	337.86 ± 36.98 382.53 ± 6.13	0.200 % **0.226 %**	1.39	**0.18 (0.31%)**
Beer after a 9 months natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	357.75 ± 4.67 449.42 ± 56.24	0.200 % **0.251 %**	2.73	**0.35 (0.61%)**

Water isotopic mass spectrometry	$^{18}O/^{16}O$ (%)	Labeled oxygen in water (mg/bottle)
Beer after accelerated ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	0.1988 0.2004	3.97
Beer after a 9 months natural ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	0.1989 0.2030	10.17

Sulphates produced by sulphite oxidation constitute another interesting fraction. Accelerated ageing caused an ^{18}O increase of 0.020 atoms per hundred over the natural level (0.2%), meaning that 0.064 ppm of the sulphite present in the initial beer sample (or 3.05%) was oxidised (Tabled V). After 3 or 9 months natural ageing, the measured incorporation was 0.023% or 0.691% over the natural ^{18}O level, representing 0.07 or 2.21 ppm (3.47 0r 100%). The favourable sulphite-maskings effect of aldehydes (which form complexes with sulphites) may thus be reduced in the presence of high amounts of oxygen.

The last fraction studied in this work contained isohumulones (Table VI). An ^{18}O increase of 0.545% over the natural content, indicates that 0.73 ppm isohumulones (2.72%) was oxidised during accelerated ageing. This should affect bitterness perception.

It emerges from this study that many oxidation reactions can occur upon beer ageing. The oxidation of

TABLE VI. Proton bombardment analysis of beer isohumulone fractions after ageing in the presence of headspace oxygen

	Cyclotron signal	^{18}O content	μg of ^{18}O incorporated in 250 ml beer	ppm of labeled isohumulones (percentage of the total amount)
Beer after accelerated ageing with 96 ppm $16O_2$ with 104 ppm $18O_2$	20.39 ± 1.81 75.96 ± 0.44	0.2 % 0.745 %	9.47	0.73 ppm (2.72%)

polyphenols, sulphites and isohumulones most likely alters the organoleptic properties of beer. On the other hand, fatty acids appear to be protected from oxidation.

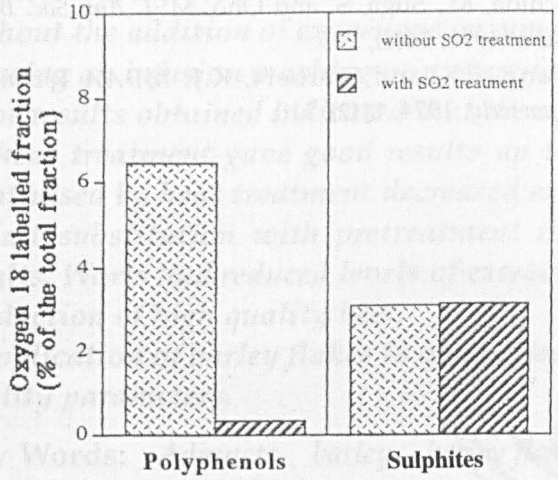

FIG. 2. Impact of SO_2 treatment.

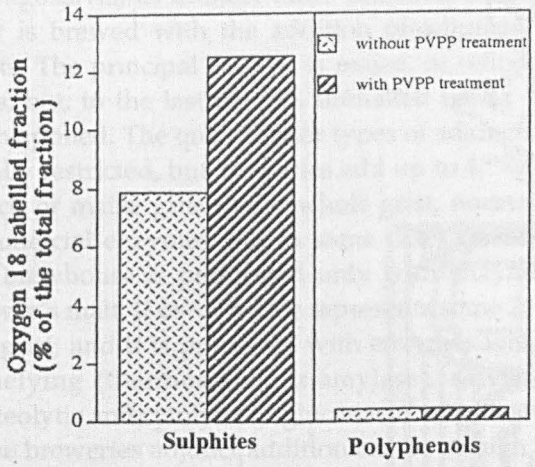

FIG. 3. Impact of PVPP treatment.

To evaluate the impact of usual beer treatments, the incorporation of oxygen 18 in KMS-, PVPP- and AA-treated beers subjected to accelerated ageing was measured. SO_2 strongly protected the polyphenol fraction from oxidation (0.3% oxidised polyphenols when 13 ppm SO_2 was added versus 6.5% without treatment) (Fig. 2).

PVPP treatment (see Fig. 3), as expected, decreased polyphenol concentration (50 ppm against 95 ppm), but it increased the sensitivity of sulphites to oxidation (12.5% of sulphites oxidised versus 7.9%).

Most interesting was the effect of ascorbic acid on KMS-treated beer (Fig. 4). This well-known antioxidant increased oxidation of polyphenols and sulphites, probably due to regeneration of iron and copper ions causing synthesis of hydroxyl radical by the well-known Fenton reaction.

As a conclusion, protonic bombardment and isotopic mass spectroscopy proved to be of great value in

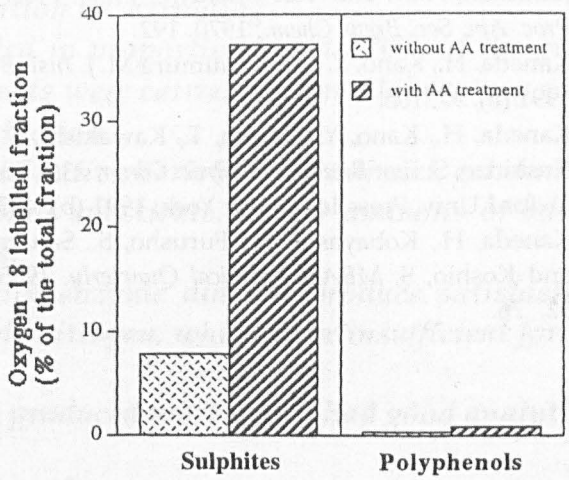

FIG. 4. Impact of the ascorbic acid (AA) treatment.

investigating molecules subjected to oxidation. In contrast to lipids, polyphenols, sulphites and isohumulones have been demonstrated to react with bottled oxygen. Their respective oxidation rates strongly depend on the stabilisation process which is used. Oxidation of linoleic acid only occurs before fermentation, leading to trans-2-nonenal which is, unfortunately, protected from yeast reduction by a linkage to amines. The nonenal potential measurement is proposed to quantify the amount of bounded nonenal in wort and hence, to assess the future cardboard flavour in beer.

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