SPi | Journal Code: J I B | Article ID: 2 4 | Dispatch: 28.07.12 | CE: | No. of Pages: 7 | ME:

Research article

Institute of Brewing & Distilling

Received: 2 May 2012 Revised: 5 July 2012 Accepted: 6 July 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jib.24

Fate of 2-sulphanylethyl acetate and 3-sulphanylpropyl acetate through beer aging

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Sulphanylalkyl alcohols and their corresponding acetates were investigated in 14 commercial Belgian beers. Although it was the major peak at the pulsed-flame photometric detector, the empyreumatic 2-sulphanylethyl acetate was found at concentrations below its individual odour threshold, estimated at 40 μg/L (0–4 μg/L in most fresh beers, 5–12 μg/L in three fresh high-bitter beers). Both the Ehrlich pathway and hop constituents contribute to this content. In 11 of the investigated samples, synthesis of 2SE-A and 3-sulphanylpropyl acetate (roasted, burned) continued during the first three months of storage. Although below their individual thresholds, these compounds might interact by synergy with other aged flavours. As yeast was absent from most of the investigated bottles, chemical degradation of precursors is suspected. Copyright © 2012 The Institute of Brewing & Distilling

Keywords: beer aging; empyreumatic aroma; esters; hop; polyfunctional thiols; cysteine

Introduction

Although foods and beverages contain only trace levels of polyfunctional thiols, these molecules make a major sensorial contribution owing to their very low threshold values *(1)*. 3-Methylbut-2-en-1-thiol (MBT), responsible for the light-struck off-flavour, is generated in beer by a light-induced non-enzymatic reaction between cysteine and isohumulones, involving riboflavin *(2)*. Yet it can also be found in light-protected beers, where it might arise through nucleophilic hydrogen sulphide substitution on hop 3-methylbut-2-en-1-ol *(3,4)*. 2-Methylfuran-3-thiol, very well-known for the empyreumatic flavour it brings to coffee, has been found in lager beer, where it can be formed from a ribose Amadori product and cysteine *(4–6)*.

In fresh lager beers, two sulphanylalkyl alcohols have been identified: 3-sulphanyl-3-methylbutan-1-ol and 2-sulphanyl-3-methylbutan-1-ol (3- and 2S3MBol), both with an onion-like aroma *(4)*. 2S3MBol is found at higher concentrations in beers exhibiting a strong onion-like aroma defect *(7)*. As shown in model media, both are synthesized from 3-methylbut-2-en-1-ol and hydrogen sulphide, by electrophilic Markovnikov and radical anti-Markovnikov addition, respectively *(4,8)*. Gros *et al.* *(8)* have shown that these reactions also occur in beers through the presence of hydrogen sulphide excreted by yeast. In sensorial analyses performed on purified 2S3MBol, its typical freshly cut onion flavour appears very different from the 'onion soup' descriptor currently used to describe dimethyltrisulphide in beer *(8)*.

Many β-sulphanylalkyl ketones, alcohols and esters have been detected in beer. 4-Sulphanyl-4-methylpentan-2-one (4S4M2Pone) confers a box-tree fruity character to lager beer *(4,5)*. 3-Sulphanylhexan-1-ol (3SHol), 3-sulphanylhexyl acetate (3SH-A) and 1-sulphanylpentan-3-ol (1S3Pol) are also found in most lager beers *(4,9)*. 3-Sulphanyl-4-methylpentan-1-ol (3S4MPol) and 3-sulphanyl-4-methylpentyl acetate (3S4MP-A) have been evidenced in beer hopped with the Nelson Sauvin cultivar *(10)*, while in Tomahawk-hopped beers, Gros *et al.* *(11)* have shown that 3SHol, 3-sulphanyloctan-1-ol (3SOol), 4S4M2Pone, 3S4MPol and 3-sulphanyl-2-ethylpropyl acetate (3S2EPr-A) most probably exceed their threshold values.

Polyfunctional thiols exist in free form in hop pellets *(9–12)*, but are also formed during boiling and fermentation, and this suggests the presence of precursors in malt or hop *(9–11)*. In wine, the presence of cysteinylated and glutathionylated precursors has been shown *(13–15)*, and the same precursors are suspected in hop *(16)*. Another biogenetic pathway starting from α,β-unsaturated carbonyls has been proposed in wine *(17)*. Likewise, hydrogen sulphide excreted by brewing yeast might generate sulphanylalkyl aldehydes and ketones, with further reduction to sulphanylalkyl alcohols and esterification to sulphanylalkyl esters.

Thanks to combinatorial syntheses *(18)*, four other very interesting polyfunctional thiols have recently been identified in fresh lager beers: 2-sulphanylethan-1-ol (2SEol, grilled), 3-sulphanylpropan-1-ol (3SProl, potato-like), 2-sulphanylethyl acetate (2SE-A, burned) and 3-sulphanylpropyl acetate (3SPr-A, burned) *(4)*. In this case, the Ehrlich pathway of cysteine or homocysteine could be involved. Very few data are available concerning the occurrence of these compounds in different kinds of beer, and their fate through aging. Recent data indicate that the concentration of 3SPr-A and 2SE-A drops markedly in wine during bottle aging *(19)*. What about their fate in beer? Beer aging defects, especially linked to Strecker aldehydes *(20)*, *trans*-non-2-enal *(21)*, dimethyltrisulphide *(22)*, β-damascenone *(23)*, 4-vinylsyringol *(24)* and ethylfurfurylether *(25)*, have been extensively investigated, but very little is yet known about polyfunctional thiols and their

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possible synergy with other aged flavours *(10,26,27)*. To our knowledge, only 3-sulphanyl-3-methylbutyl formate, with a catty, ribes aroma, has been reported to increase upon accelerated aging *(28)*.

In the present work, sulphanylalkyl alcohols and their corresponding acetates were investigated in various lager and special beers, before and after storage. Specific thiol extraction [*p*-HMB *(29)*] and detection (GC-PFPD) procedures were used.

Materials and methods

Beer samples

T1 As depicted in Table 1, six commercial Belgian lager beers (L1–L6) and eight top-fermentation special beers (S1–S8) were investigated. All of them were stored for 12 months at 20 °C in a dark room and sampled every 3 months.

Chemicals

p-Hydroxymercuribenzoic acid (*p*-HMB), dodecane (99.9%), thiazole, 2-acetylthiophene, L-cysteine hydrochloride, 2-sulphanylethan-1-ol, 2-sulphanylethyl acetate, 3-sulphanylpropan-1-ol and 3-sulphanylpropyl acetate were purchased from Sigma-Aldrich (Bornem, Belgium). Dichloromethane was obtained from Romil (Cambridge, UK), glucose from Acros Organic (Geel, Belgium), saccharose from ISCAL (Frasnes-Lez-Buissenal, Belgium) and 4-methoxy-2-methylbutan-2-thiol from Oxford Chemicals (Oxford, UK). A strongly basic Dowex resin 1×2, Cl^- form (Sigma-Aldrich, Bornem, Belgium) was stored in hydrogen chloride (0.1 M). Anhydrous sodium sulphate was obtained from Merck (Darmstadt, Germany) and Tris(hydroxymethyl)aminomethane (Tris) from USB (Cleveland, OH, USA).

Top-fermentation in minimal model media spiked with cysteine

Culture media. The *Saccharomyces cerevisiae* strain INBR 268 (Université Catholique de Louvain, Louvain-la-Neuve, Belgium) was propagated in YPS medium (1% yeast extract, 0.5% peptone) at 28 °C on a rotary shaker. Yeast cells were collected in the exponential phase (24 h) by centrifugation. The supernatant was removed and the yeast was washed and pitched at 10^7 cells/mL in 230 mL minimal medium: glucose, 50 g/L; saccharose, 50 g/L; $MgCl_2$, 0.7 g/L; $CaCl_2$, 0.1 g/L; $(NH_4)_2SO_4$, 2 g/L; NaCl, 0.5 g/L; KH_2PO_4, 2 g/L; $FeCl_3$, 0.003 g/L; and asparagine, 1 g/L. The pH was adjusted to 5.2 with sodium hydroxide. After autoclaving, 10 mL/L vitamin solution (D-biotin, 0.004 g/L; thiamine hydrochloride, 0.1 g/L; nicotinic acid, 0.1 g/L; *p*-aminobenzoic acid sodium salt, 0.1 g/L; mesoinositol, 0.3 g/L) was added after sterilization by filtration *(26)*. Fermentations were carried out (with or without addition of 20 mg/L L-cysteine, in duplicate) in 500 mL flasks for 100 h at 20 °C without shaking.

Extraction procedure. After centrifugation, 230 mL samples spiked with 0.67 μg/L 4-methoxy-2-methylbutan-2-thiol (internal standard, IST; spiking of 230 μL of the 0.67 mg/L stock solution) were extracted with 50 mL dichloromethane. The organic phase was dried on anhydrous sodium sulphate. The sample was concentrated to 0.5 mL in a Kuderna–Danish apparatus (2-acetylthiophene as external standard – EST; spiking before concentration of 1 mL of the 0.20 mg/L stock solution). Gas chromatography–pulsed flame photometric detection (GC-PFPD) and gas chromatography–mass spectrometry (GC-MS) were performed as described below.

Bottom-fermentation of a wort spiked with 2SEol

An experimental wort was produced from pale malt (Malterie du Château, Beloeil, Belgium) in a 50 L-scale pilot plant (Coenco, Oostkamp, Belgium). The 15°P wort was diluted to 11°P before boiling. Supercritical CO_2 hop extract (Tomahawk; Yakimachief, Louvain-la-Neuve, Belgium; 60 mg/L) was added at the beginning of boiling (total time = 75 min, 12° Plato after boiling). Lager yeast (strain INBR 291, Université Catholique de Louvain, Louvain-la-Neuve, Belgium) was pitched at 15×10^6 cells/mL into the cooled oxygenated (8 mg/L) wort and 10 μg/L 2SEol was added. The fermentation temperature was maintained at 12–13 °C for 7 days. *p*-HMB extraction was applied to the fermented wort.

Table 1. Main characteristics of the investigated beers (EBC standard analyses) *(30)*

Type of beer	Bitterness (BU)	Ethanol (%)	Colour (°EBC)	Real extract (°P)	Original extract (°P)	Bottle-refermented beer
Lager beers						
L1	21	5.3	6.6	3.80	11.80	
L2	22	5.6	6.7	3.20	11.73	
L3	21	5.3	6.6	3.77	11.78	
L4	21	5.3	6.6	3.77	11.78	
L5	15	5.1	7.7	3.95	11.77	
L6	15	5.2	7.7	3.95	11.77	
Top-fermentation beers						
S1	17	6.7	15.5	5.61	15.55	
S2	10	5.2	—	3.97	11.80	
S3	15	6.5	12.5	4.62	14.41	
S4	21	7.9	16.5	5.10	16.83	+
S5	14	8.8	66.0	5.80	18.70	+
S6	29	8.1	14.5	4.16	16.28	+
S7	24	7.5	15.5	3.68	14.87	+
S8	29	6.6	26.0	3.22	13.31	+

p-HMB extraction procedure

Thiols were selectively extracted according to the protocol of Tominaga *et al*. *(29)*. A 500 mL aliquot of beer spiked with 0.67 μg/L 4-methoxy-2-methylbutan-2-thiol (IST; spiking of 500 μl of the 0.67 mg/L stock solution) was extracted with 200 mL dichloromethane. The mixture was allowed to clarify during 45 min, and a 30 min centrifugation at 3500 rpm was necessary before collecting the organic layer. The organic layer was extracted with 2 × 20 mL of *p*-hydroxymercuribenzoic acid (*p*-HMB) solution (360 mg *p*-HMB and 24.6 g Tris in 1 L Milli-Q water). The aqueous layers were loaded on a strongly basic anion-exchange column (Dowex resin) washed sequentially beforehand with 50 mL sodium hydroxide (2 M), 150 mL Milli-Q water, 50 mL hydrogen chloride (2 M) and 150 mL Milli-Q water. After loading, the resin was washed with 50 mL sodium acetate buffer (pH 6) and thiols were eluted with 60 mL cysteine solution (640 mg L-cysteine hydrochloride in 60 mL Milli-Q Water; pH 7.5). The eluate was extracted first with 4 mL and then with 3 mL dichloromethane. The extract was then dried with anhydrous sodium sulphate. The dried extract was first concentrated to 0.5 mL in a Kuderna–Danish (thiazole as external standard; spiking before concentration of 1 mL of the 0.20 mg/L stock solution) and to 70 μL in a Dufton apparatus. This final extract was stored at −80 °C for further analyses.

Gas chromatography hyphenated to sulphur-selective pulsed-flame photometric detection

Two microlitres of *p*-HMB extract were analysed on a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250 °C and connected to a ThermoFinnigan Trace PFPD detector (600 V, 250 °C, 18 ms gate width, 6 ms gate delay, 3.45 Hz pulse frequency); the split vent was opened 1 min post-injection. Compounds were analysed with a wall-coated open tubular apolar CP-Sil5-CB (50 m × 0.32 mm i.d., 1.2 μm film thickness) or with a polar FFAP capillary column (25 m × 0.32 mm i.d., 0.3 μm film thickness). The carrier gas was helium at a flow rate of 1.3 mL/min (pressure set at 90 or 45 kPa, respectively). The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C (CP-Sil5-CB column) or to 220 °C (FFAP column) at 3 °C/min. The variation coefficients for 2SE-A and 3SPr-A (extraction and analyses) were <15%. For their quantification, complete calibration curves relative to the IST were used. Quantitative data are not given for 2SEol and 3SProl (no significant increase or decrease through beer aging, higher variation coefficients owing to losses of the most polar compounds at the first dichloromethane extraction).

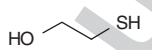
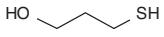
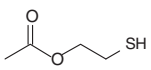
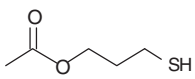
Gas chromatography hyphenated to mass spectrometry

Electronic impact mass spectra were recorded at 70 eV (full scan with a mass range from 40 to 380 *m/z*) on a ThermoFinnigan Trace MS simple quadrupole mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a low-bleed MS capillary column (CP-Sil5-CB) and a splitless injector (250 °C, split vent opened after 0.8 min). One microlitre of *p*-HMB extract was injected. The carrier gas was helium and pressure was set at 100 kPa. The oven temperature was as described for GC-PFPD. Spectral recording was automatic throughout separation (Xcalibur software was used). All compounds were identified by coincidence of GC retention indexes on two capillary columns (CP-Sil5-CB and FFAP) and by comparison of the mass spectra with those of standards.

Gas chromatography hyphenated to olfactometric detection (GC-O)

One microlitre of *p*-HMB extract was analysed with a Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250 °C; the split vent was opened 0.5 min post-injection. Compounds were analysed with the columns and temperature programs described for PFPD. The carrier gas was nitrogen and the pressure was set at 50 kPa (CP-Sil5-CB) or 30 kPa (FFAP). In order to assess the olfactory potential of the extract, the column was connected to a GC-O port (Chrompack) maintained at 250 °C. The effluent was diluted with a large volume of air (20 mL/min) pre-humidified with an aqueous copper (II) sulphate solution. All extracts were analysed immediately after extraction by three trained panellists. Aroma extract dilution analysis (AEDA) *(31)* was performed on *p*-HMB extracts by two

Table 2. Properties of 2SEol, 3SProl, 2SE-A and 3SPr-A

	Structure	Odour in GC-O	$IR_{CP\text{-}Sil5}$	IR_{FFAP}	Main *m/z* ions in GC-MS	Odour Threshold in beer (μg/L)
2SEol		Grilled, gas	722	1538	47, 60, 78	2000
3SProl		Potatoes	849	1620	57, 58, 45	400
2SE-A		Roasted, burned	880	1454	43, 60, 61	40
3SPr-A		Roasted, burned	989	1565	74, 43, 47	40

2SEol, 2-Sulphanylethan-1-ol; 3SProl, 3-sulphanylpropan-1-ol; 2SE-A, 2-sulphanylethyl acetate; 3SPr-A, 3-sulphanylpropyl acetate.

sniffers. The extracts were diluted stepwise (2-fold) with dichloromethane. Flavour dilution (FD) is defined as the highest dilution at which the compound can still be detected (FD = 2^n with $n+1$ = number of dilution applied on the extract until no odour was perceived). The precision of this AEDA is $n \pm 1$ (factor 2 between FD values).

Odour threshold determination *(26)*

The odour thresholds of 2SEol, 3SProl and their corresponding acetates were measured in beer. The fresh lager beer L3 was spiked with increasing concentrations of each tested compound. The samples were presented to eight panellists in six three-alternative forced choice tests *(32)* (covered glasses containing 40 mL of beer). The odour threshold was estimated as the smallest concentration at which 50% of the panellists were able to perceive a difference in odour between the spiked and non-spiked beers.

Results and discussion

Sulphanylalkyl alcohols (2SEol and 3SProl) and their corresponding acetates (2SE-A and 3SPr-A; structures given in Table 2) were studied in 14 commercial Belgian beers (in duplicate) after a thiol-specific extraction *(29)*. GC-PFPD, GC-MS and GC-O were applied to aroma extracts issued from fresh and aged samples. Odour thresholds were also determined by spiking the fresh L3 sample, devoid of the investigated compounds. T2

As in the case of other fusel alcohols and esters, commercial standards of sulphanylalkyl esters proved to be more quickly perceived by the panellists than the corresponding alcohols (Table 2). The individual odour threshold of each ester was found to be 40 μg/L (50 times and 10 times lower than that of 2SEol and 3SProl, respectively). At the sniffing port, only the empyreumatic 2SE-A was strongly perceived in beer *p*-HMB extracts (FD = 8–128; FD < 4 for 2SEol and 3SPr-A; 3SProl not detected at all).

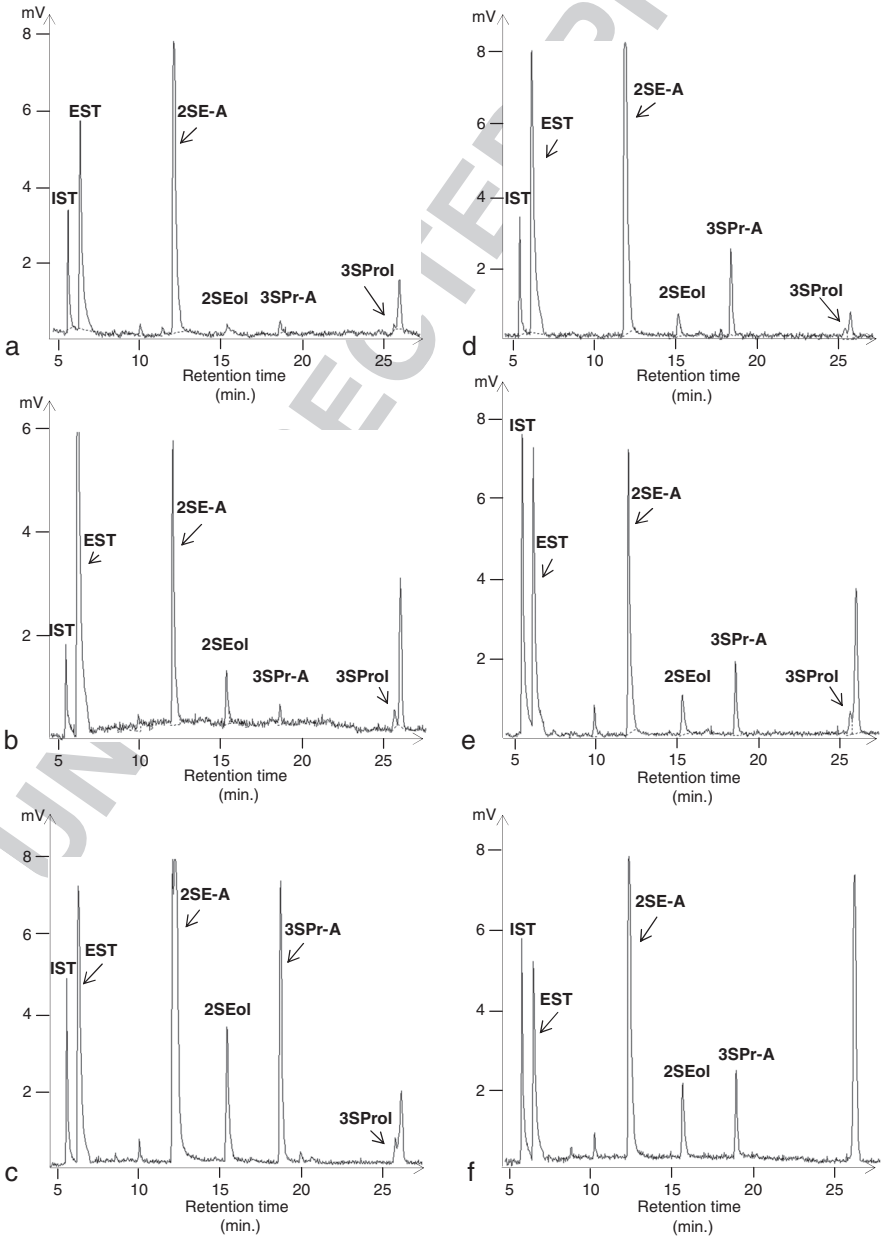


Figure 1. GC-PFPD chromatograms (FFAP column) of *p*-HMB extracts issued from the fresh (a–c) and 3-month-aged (d–f) beers L6 (a, d), S3 (b, e) and S7 (c, f).

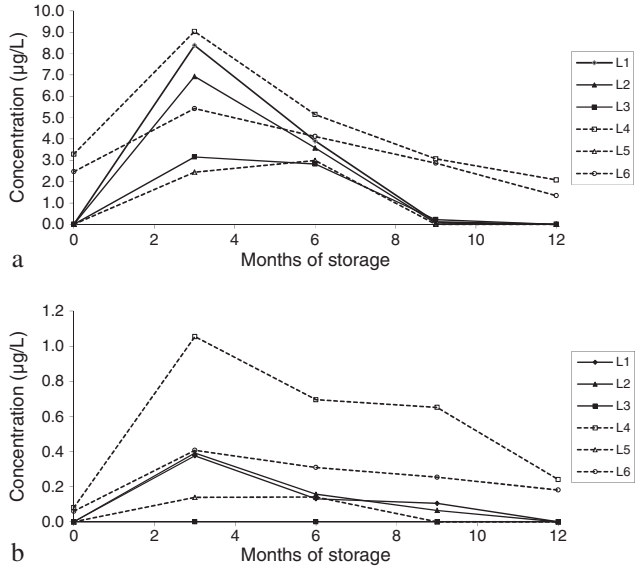


Figure 2. Concentrations of 2SE-A (a) and 3SPr-A (b) in lager beers (L1–L6) through aging (GC-PFPD quantifications).Variation coefficient <15%.

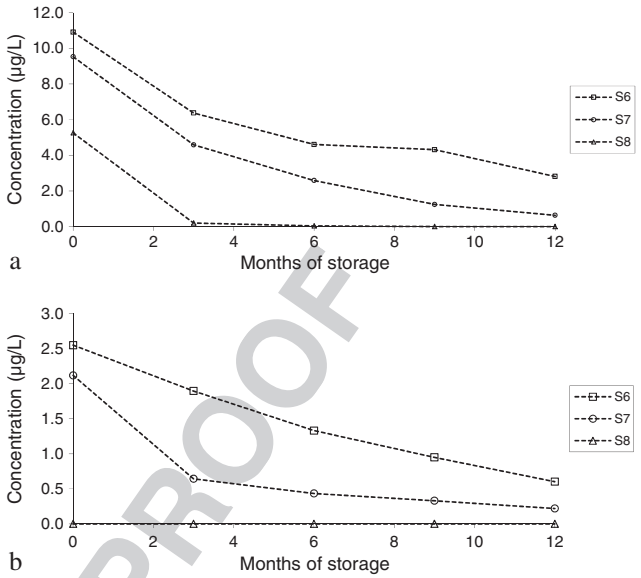


Figure 4. Concentrations of 2SE-A (a) and 3SPr-A (b) in high-bitter special beers (S6–S8) through aging (GC-PFPD quantifications).Variation coefficient <15%.

F1 As depicted in Fig. 1, 2SE-A also gave rise to the best-resolved PFPD peak in all beer extracts. Yet its concentration remained below its individual odour threshold in all fresh samples: <4 µg/L F2 in the five lager beers (Fig. 2a), <2 µg/L in most special beers F3F4 (Fig. 3a), and 5–12 µg/L in three more bitter beers (Fig. 4a). As in dry white wines *(19)*, but in contrast to Sauternes wines *(26)*, the 2SE-A/3SPr-A ratio was above one in all 14 beers (Figs 2–4a, b).

As suggested by Vermeulen *et al. (4)*, 2SE-A and 3SPr-A found in fresh beers might arise, respectively, through yeast Ehrlich F5 degradation of cysteine and homocysteine (Fig. 5a). This hypothesis is strengthened by the presence of both sulphanylalkyl acetates in non-hopped beers (up to 2 µg/L of 2SE-A) *(33)*. To confirm this origin, we spiked a synthetic medium with 20 mg/L cysteine before top-fermentation. As depicted in Fig. 5b, the results confirmed the efficiency of the Ehrlich pathway in primary fermentation. The rate of conversion observed here between cysteine and 2SE-A (0.43 mg/L detected; 2.2% of conversion) may explain why 2 µg/L 2SE-A was found by Gros *et al. (33)* in a non-hopped beer (0.1–1 mg/L cysteine in the brewing wort).

The efficiency of bioconversion between 2SEol and 2SE-A was confirmed by a second experiment in which a pitching wort was spiked with 10 µg/L 2SEol: the here-used lager yeast quickly converted 2SEol to 2SE-A (Fig. 5c) (no quantifiable peak of 2SEol, and 13 µg/L 2SE-A after fermentation; molar conversion rate = 84.7%).

2SEol, 2SE-A, 3SProl and 3SPr-A were identified in hop, in either free or bound forms *(11)*. For all hop varieties *(11)*, free 2SEol was found at the PFPD detector. The empyreumatic 2SE-A and 3SPr-A, and the broth/potato 3SProl, were also detectable at the sniffing port in all *p*-HMB hop extracts *(11)*. However, taking into account the dilution factor usually applied between hop and beer, this contribution should be relatively low compared with that of the Ehrlich pathway.

Although 2SE-A was present at levels below 4 µg/L in the six fresh lager beers, its synthesis was found to continue in the bottle during aging (Fig. 2a), its concentration reaching a maximum of 9 µg/L after three months. A similar pattern was observed for 3SPr-A, which reached 1 µg/L after the same period (Fig. 2b). Upon longer storage, levels of both 2SE-A and 3SPr-A gradually diminished, to less than 2.1 and 0.2 µg/L respectively after one year. Sauternes wines *(19)*, in contrast, show no initial increase in 2SE-A and 3SPr-A during storage, their concentrations decreasing to undetectable levels over a 2-year storage period *(26)*. As in filtered lager beers, no yeast survives in the bottle, and the Ehrlich pathway cannot explain our present results. For degrading hop cysteinylated or glutathionylated thiol adducts, the β-lyase yeast activity is probably also required. The reactions involved should be purely chemical *(13–15)*. Very recently, Starkenmann *et al. (34)* identified new types of thiol precursors in onion: *S*-alk(en)ylthiol-L-cysteine derivatives. If present in hop, the disulphide bridge of such compounds could probably be chemically reduced in a highly reducing medium such as beer (presence of polyphenols, ascorbic acid, sulphites, etc.).

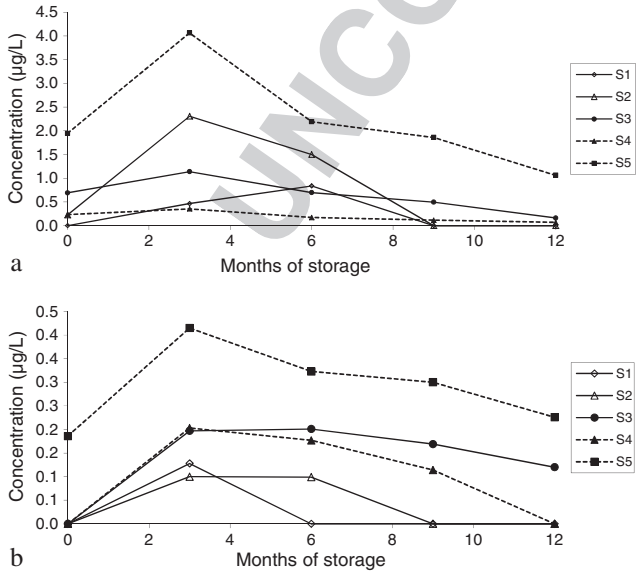


Figure 3. Concentrations of 2SE-A (a) and 3SPr-A (b) in special beers (S1–S5) through aging (GC-PFPD quantifications).Variation coefficient <15%.

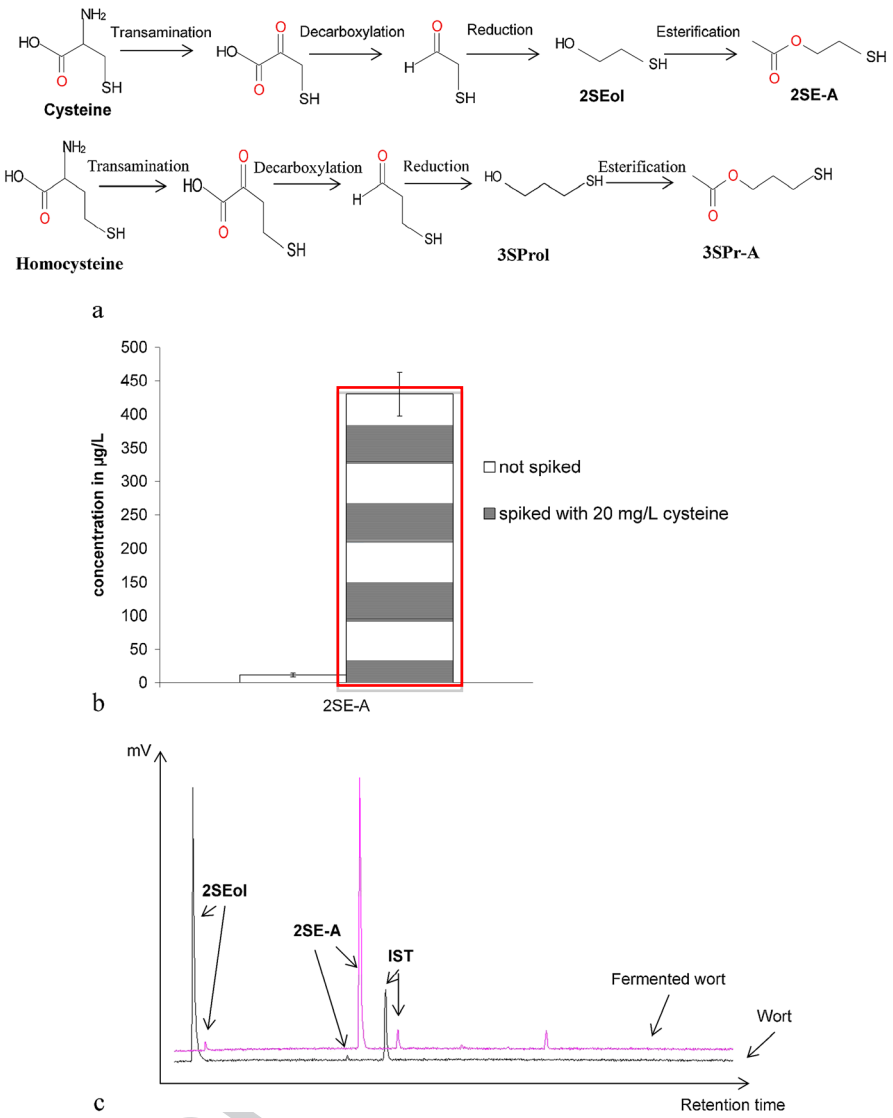


Figure 5. (a) Ehrlich pathways leading to 2SEol and 3SProl, and subsequent reductions and esterifications. (b) Concentrations of 2SE-A in fermented synthetic medium spiked or not with 20 mg/L cysteine. (c) GC-PFPD chromatograms (CP-Sil5-CB) of *p*-HMB extracts issued from a wort spiked with 2SEol before fermentation, and from the corresponding fermented wort (IST, internal standard).

In five special beers (S1–S5), the evolution patterns for 2SE-A and 3SPr-A were similar to those observed in lager beers (Fig. 3a and b). In S5, 2SE-A reached 4.1 µg/L after 3 months, while 3SPr-A peaked at 0.4 µg/L. As in the case of lager beers, hydrolysis of precursors probably occurred during the first months of storage, followed by oxidation. As S1, S2 and S3 were not bottle refermented, yeast activity can again be ruled out. In S4 and S5, an additional effect of yeast cannot be excluded.

The fate of sulphanylalkyl acetates in the three bitter beers characterized by higher amounts of 2SE-A before aging (S6–S8, 24–29°EBU, Table 1) is worth stressing (Fig. 4a and b). In the bottle, as in the case of Sauternes wines, sulphanylalkyl acetates levels dropped directly. It is probable in this case that the 2SE-A and 3SPr-A concentrations were so high in the fresh beers that, during aging, their oxidation outweighed their release from precursors.

In conclusion, up to 8 µg/L 2SE-A can be produced through beer aging. The possible synergy of this compound with other staling flavours should be investigated. Complementary analyses are also required to elucidate the chemical mechanisms releasing 2SE-A and 3SPr-A in the bottle, even in the absence of yeast. The impact of bottle refermentation should also be investigated.

Acknowledgement

T. T. H. Tran thanks the Government of Vietnam and J. Gros thanks the Inbev-Baillet Latour Foundation (Leuven, Belgium) for financial support.

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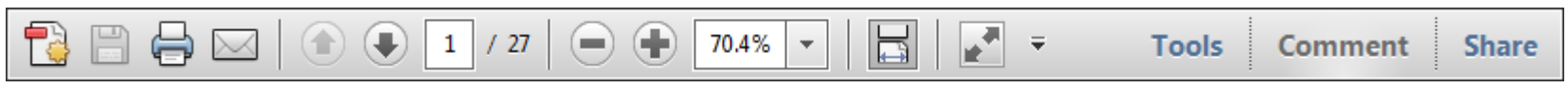

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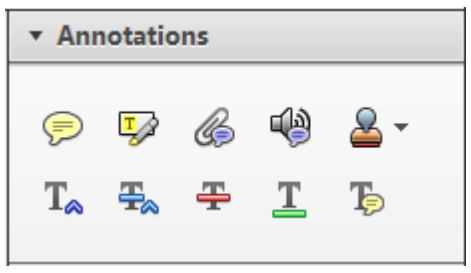

1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
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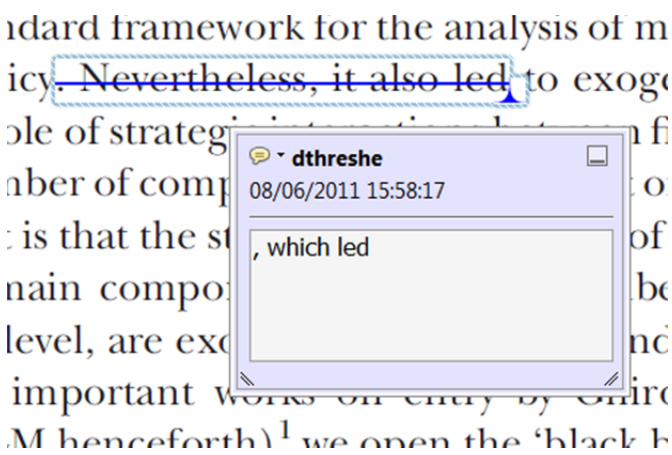

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Strikes a red line through text that is to be deleted.

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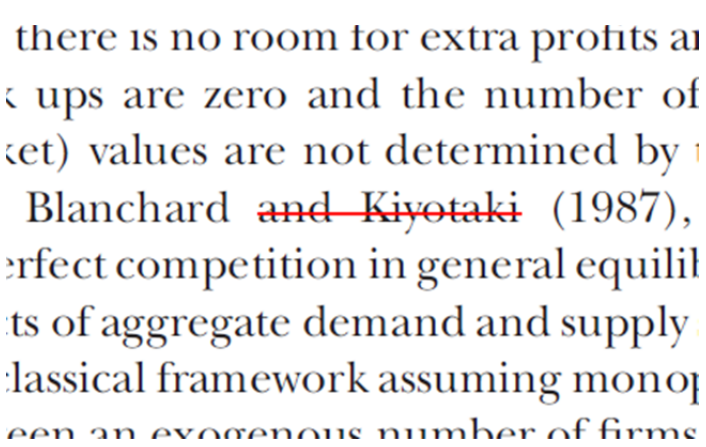
- Click on the Strikethrough (Del) icon in the Annotations section.



3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

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- Click on the Add note to text icon in the Annotations section.
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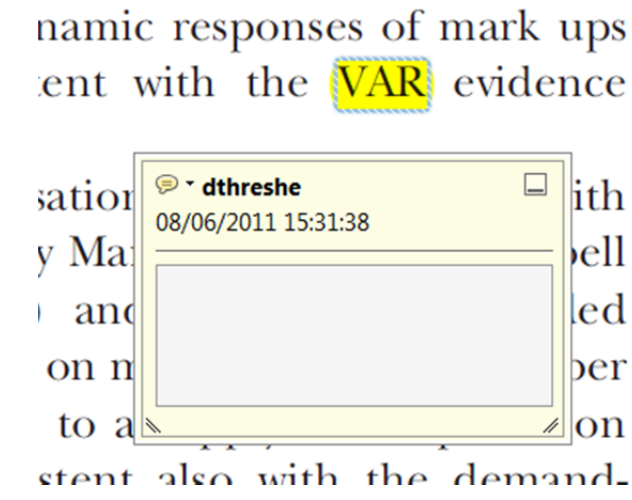

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Marks a point in the proof where a comment needs to be highlighted.

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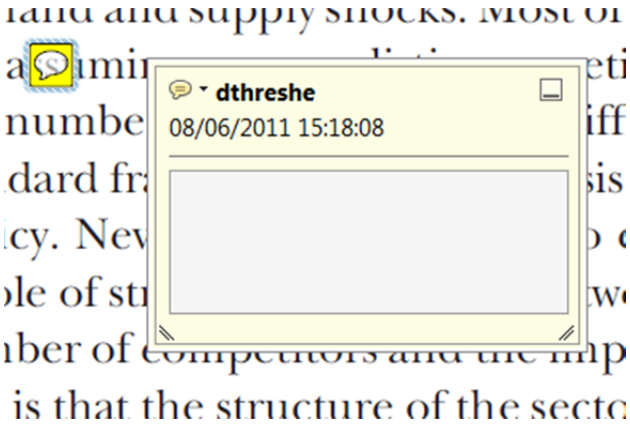

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5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

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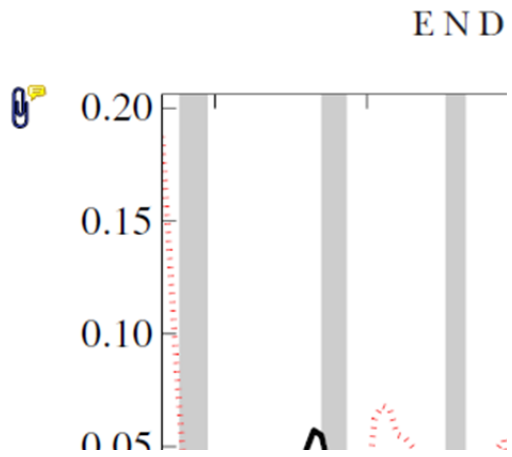

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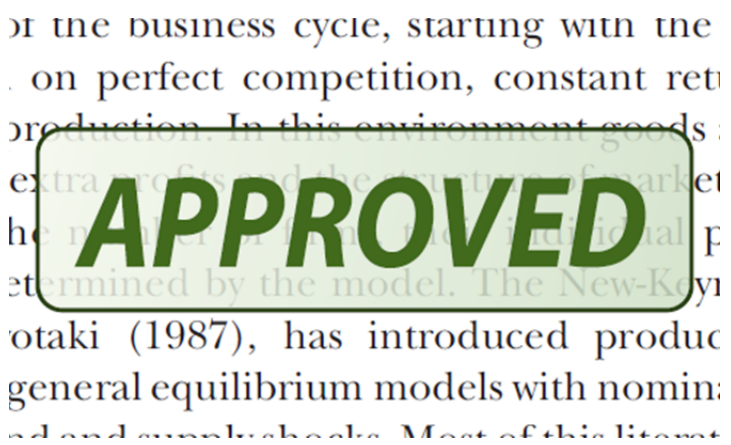

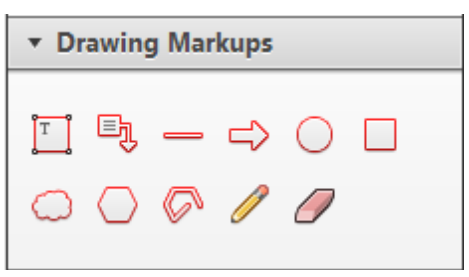

7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
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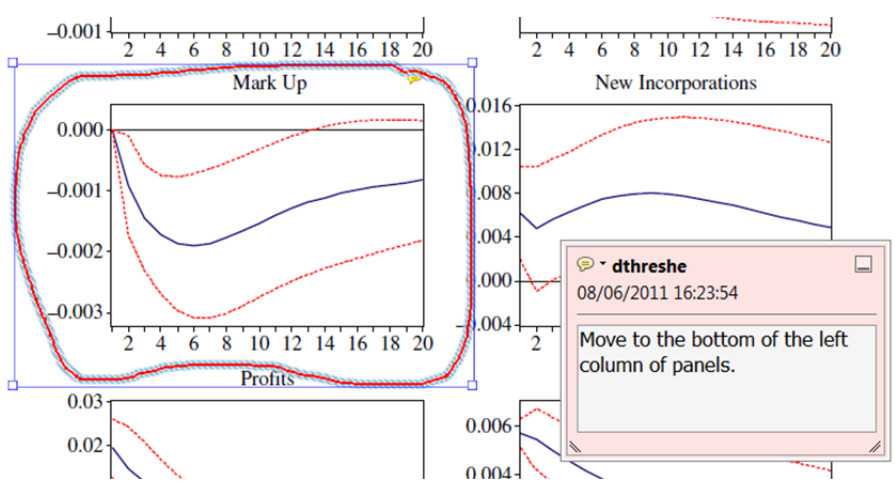

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