

Polyfunctional Thiols in Fresh and Aged Belgian Special Beers: Fate of Hop *S*-Cysteine Conjugates

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ABSTRACT

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Odorant polyfunctional thiols such as 4-sulfanyl-4-methylpentan-2-one, 3-sulfanylhexan-1-ol (3SHol), and 3-sulfanyl-3-methylbutan-1-ol (3S3MBol) were shown to be strongly degraded in Belgian special beers after 1 year of aging. Yet, during the first 3 months, they also appeared to be synthesized in the bottle, even in the absence of yeast. Fresh filtered lager beer was spiked with nonvolatile *S*-cysteine conjugates of 3SHol, 3S3MBol, and 3-methylbut-2-en-1-thiol (MBT) before aging (1 to 3 months at 20°C or 5 days at 40 or 60°C). Thiol-specific *para*-hydroxymercuribenzoic acid extracts were analyzed by a GC-pulsed flame photometric detector, and HPLC-electrospray ionization/MS/MS was used to quantify the undegraded cysteinylated precursors. *S*-Cysteine conjugates were chemically degraded in the beer, releasing their corresponding thiols. The conversions were low (<1%) but could explain the trace amounts of 3SHol and 3S3MBol produced in beer during the first months of storage. On the other hand, chemical degradation of Cys-MBT in beer (although reaching up to 33%) proved not to be efficient enough to release significant amounts of the skunky off-flavor. In this case, photooxidative degradation of isohumulone most probably remains the major synthesis pathway. Complementary analyses are now needed to determine how other beer constituents, such as dicarbonyls, might participate in cysteine adduct degradation.

Keywords: Beer aging, Cysteine-*S*-conjugates, Flavor, Polyfunctional thiols

Polyfunctional thiols are sulfur compounds possessing a sulfhydryl group together with another chemical function (aldehyde, ketone, acid, ester, etc.). They are powerful odorant compounds that contribute to the flavor of many foods and beverages (24). In wine, 3-sulfanylhexan-1-ol and other thiols are generated from grape nonvolatile *S*-cysteine conjugates (21) or from the corresponding *S*-glutathione conjugates during alcoholic fermentation (3,14,16). Through the β -lyase activity of yeast, the carbon-sulfur bond of the *S*-cysteine conjugate is cleaved to release free thiol (26). The conversion rate of *S*-3-(1-Hydroxyhexyl)-cysteine (Cys-3SHol) by yeast, reported as below 1%, should contribute to only 3 to 7% of the total 3-sulfanylhexan-1-ol found in wine (18). Besides Cys-3SHol, *S*-3-(1-hydroxyhexyl)-glutathione has been found in grapes and wines (2) (conversion rate to free thiols assessed at 3 to 4.4% [4,15]).

In beer, volatile thiols are mainly provided by hops, in free or bound form. Discriminant thiol profiles have been found for different hop cultivars (5). Recent data show that many of the free polyfunctional thiols found in beer are produced during wort boiling and fermentation (5,6,9,10,11,17,19). After Gros et al (6) showed for the first time an *S*-cysteine conjugate of 3SHol (rhubarb, grapefruit) in Cascade hops (6), the occurrence of this cysteine adduct was confirmed in other varieties (Saaz, Nelson Sauvin, Tomahawk [7], Amarillo, Citra, Mosaic, and Hallertau Blanc [8]) by using an enzymatic assay in which apotryptophanase released the corresponding free thiols. Other interesting flavors, including 3-methylbut-2-en-1-thiol (MBT, skunky), 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone, blackcurrant), 3-sulfanylpentan-1-ol (catty, citrus), 3-sulfanyl-4-methylpentan-1-ol (3S4MPol, rhubarb, grapefruit), and 2-sulfanylethan-1-ol (2SEol, grilled) are also released from some cultivars (7,8).

Due to their high propensity to undergo oxidation, nucleophilic additions, or substitutions, thiols are unstable odorants in beer (24). 2-Sulfanylethyl acetate (2SE-A) and 3-sulfanylpropyl acetate (3SPr-A) (both with empyreumatic odors) have been shown to be strongly degraded during the first year of beer storage (22). Yet during the first 3 months, synthesis of 2SE-A and 3SPr-A can occur, most probably through chemical degradation of hop-derived precursors.

The aim of the present study was to investigate how other minor thiols might evolve through beer aging. Among them, 3SHol and 4S4M2Pone are usually found at much lower levels than 2SE-A, but because of their sub-ppb threshold values (55 and 1.5 ng/L, respectively [9]), they often contribute to hop flavors in beer. Another thiol worth following through aging is 3-sulfanyl-3-methylbutan-1-ol (3S3MBol, onion-like) with which, unfortunately, another compound produced after 3 months proved to co-elute on our polar column. An apolar column was therefore used to confirm some data. Last, fresh beers were spiked with *S*-cysteine conjugates of 3S3MBol (felinine), 3SHol, and MBT in order to assess whether their degradation might explain some increases in thiol concentration at the beginning of storage. In the spiked beers, levels of the remaining cysteinylated precursors were monitored by HPLC-electrospray ionization tandem mass spectrometry (ESI(+))/MS/MS and those of free thiols by GC-pulsed flame photometric detection (PFPD) during aging.

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EXPERIMENTAL

Chemicals

*p*HMB, Tris(hydroxymethyl)aminomethane, *N*-Boc-L-cysteine (98.5%), L-cysteine (97%), cyclohexyl isothiocyanate (98%), (*E*)-hexen-2-ol, 2SE-A, 3SPr-A, 3-sulfanylpropan-1-ol, and 3S3MBol were purchased from Sigma-Aldrich (Bornem, Belgium). 4-Methoxy-2-methyl-2-butanethiol, the internal standard (IST), was purchased from Endeavour (Northamptonshire, U.K.). 4S4M2Pone was purchased from Frutarom (Hartlepool, U.K.). 3SHol, and MBT were obtained from Oxford Chemicals (Oxford, U.K.). L-2-Amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (L-felinine) was provided by USBiological (Swampscott, MA). Dichloromethane was obtained from Romil (Cambridge, U.K.). A strongly basic Dowex resin 1 X 2, Cl⁻ form (Sigma-Aldrich, Bornem, Belgium) was stored in hydrogen chloride (0.1M). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany). Cesium carbonate, 1-chloro-3-methyl-2-butene (95%), and trifluoroacetic acid and sodium borohydride (98%) were purchased

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from Acros Organics (Geel, Belgium). Ethanol (99.9%), methanol, acetonitrile, sodium hydroxide, and hydrochloric acid (37%) were supplied by VWR (Belgium). 1-Sulfanylpentan-3-ol, and 3-sulfanylpentan-1-ol were produced by combinatorial synthesis according to Vermeulen et al (23). 3S4MPol was synthesized according to Gros et al (5).

Synthesis of Cysteinylated Precursors

S-3-(1-hydroxyhexyl)-cysteine. The compound S-3-(1-hydroxyhexyl)-cysteine (Cys-3SHol) was synthesized according to Gros et al (6): Michael addition of *N*-Boc-L-cysteine on (*E*)-hexen-2-al in acetonitrile in the presence of cesium carbonate; reduction of the obtained aldehyde with sodium borohydride in methanol; amine deprotection with trifluoroacetic acid.

S-(3-methyl-2-butenyl)-cysteine. S-(3-methyl-2-butenyl)-cysteine (Cys-MBT) was synthesized according to the method of Adsara et al (1): nucleophilic substitution of cysteine (1 eq) on 1-chloro-3-methyl-2-butene (1 eq) in ethanol, in the presence of NaOH (2 eq), at 0°C for 3 h. A white precipitate was formed during the reaction. After filtration, the product was washed with water followed by ethanol.

Beer Samples

Six top-fermentation Belgian beers (T1 to T6, Table I) were analyzed before and after storage (3, 6, 9, and 12 months) at 20°C in a dark room. (The concentrations of two sulfanylalkyl acetates have been reported previously for the same beers in another article [22]. They are beers S3 to S8). A commercial Belgian lager beer (Table I) was used in the spiking experiments.

Aging of a Lager Beer Spiked with Cysteine Adducts

A lager beer was spiked with Cys-3S3MBol (felinine, 5 mg/L), Cys-3SHol (7 mg/L), or Cys-MBT (15 mg/L) and stored in a dark room at 20°C for 3 months. Accelerated aging (5 days at 40 or 60°C) was also applied.

p-Hydroxymercuribenzoic Acid Extraction Procedure

Thiols were selectively extracted according to Vermeulen et al (25), as adapted from Tominaga et al (20). The IST was added at the first extraction step, at 0.67 g/L in beer.

Gas Chromatography Coupled with a Pulsed Flame Photometric Detector

Two microliters of each *p*HMB extract were analyzed 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 (600V, 250°C, 18-ms gate width, 6-ms gate delay, 3.45-Hz pulse frequency); the split vent was opened 1 min postinjection. Compounds were analyzed with a wall-coated open tubular apolar CP-Sil5-CB column (50 m × 0.32 mm i.d., 1.2 μm film thickness) or a polar free-fatty-acid phase (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 for the CP-Sil5-CB column and the FFAP column, 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 220°C (FFAP column) at 3°C/min.

The following general equation was used for compound A quantification (6): concentration (ng/L) = concentration (IST, ng/L) × (molecular weight [A])/molecular weight [IST] × (area [A]/area [IST]) × (molar response coefficient [IST]/molar response coefficient [A]) × (recovery factor [IST])/recovery factor [A].

For commercially available thiols, complete calibration curves relative to the IST were used. For commercially unavailable compounds (values carrying an IST superscript), the good equimolarity of the PFPD detector allowed us to set the IST-relative molar response coefficients at 1. The IST-relative recovery factor was set at 1 for all compounds (experimental values from 0.8 to 1.2, previously determined by standard addition) except for 2SEol (bad recovery at first dichloromethane extraction; approximate concentrations given by applying a ratio of 0.1 assessed by standard addition). The variation coefficients for thiols (extraction and analysis) were below 15%.

Gas Chromatography-Olfactometry

One microliter of *p*HMB extract was analyzed with a Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250°C; the split vent was opened 0.5 min postinjection. The carrier gas was nitrogen, and the pressure was set at 50 kPa (CP-Sil5-CB) or 30 kPa (FFAP). The oven temperature program and column were the same as described for GC-PFPD. In order to assess the olfactory potential of the extract, the column was connected to a gas chromatography-olfactometry port (Chrompack) maintained at 250°C. The effluent was diluted with a large volume of air (20 mL/min) prehumidified with an aqueous copper (II) sulfate solution.

Gas Chromatography Coupled to an Electronic Impact Mass Spectrometer

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 polar column (FFAP) and a splitless injector (220°C). One microliter of *p*HMB extract was injected. The split vent was opened 0.8 min postinjection. The carrier gas was helium, and the pressure was set at 45 kPa. The oven temperature program was the same as for GC-PFPD. Spectral recording was automatic throughout the separation (Xcalibur software was used).

HPLC-MS/MS Analysis of Cysteine Adducts

Reversed-phase (RP)-HPLC analyses were performed on a SpectraSystem (Finnigan Mat, San Jose, CA) equipped with an SCM1000 degasser, an AS3000 autosampler, and a P4000

TABLE I
Main Characteristics of the Investigated Beers

Type of beer	Bitterness (BU)	Ethanol (%)	pH	Color (°EBC)	Real extract (°P)	Original extract (°P)	Bottle-refermented beer ^a
Top-fermentation beers							
T1	15	6.5	4.2	12.5	4.6	14.4	
T2	21	7.9	4.5	16.5	5.1	16.8	+
T3	14	8.8	...	66.0	5.8	18.7	+
T4	29	8.1	4.4	14.5	4.2	16.3	+
T5	24	7.5	4.5	15.5	3.7	14.9	+
T6	29	6.6	4.1	26.0	3.2	13.3	+
Lager beer	21	5.3	4.2	6.6	3.8	11.8	

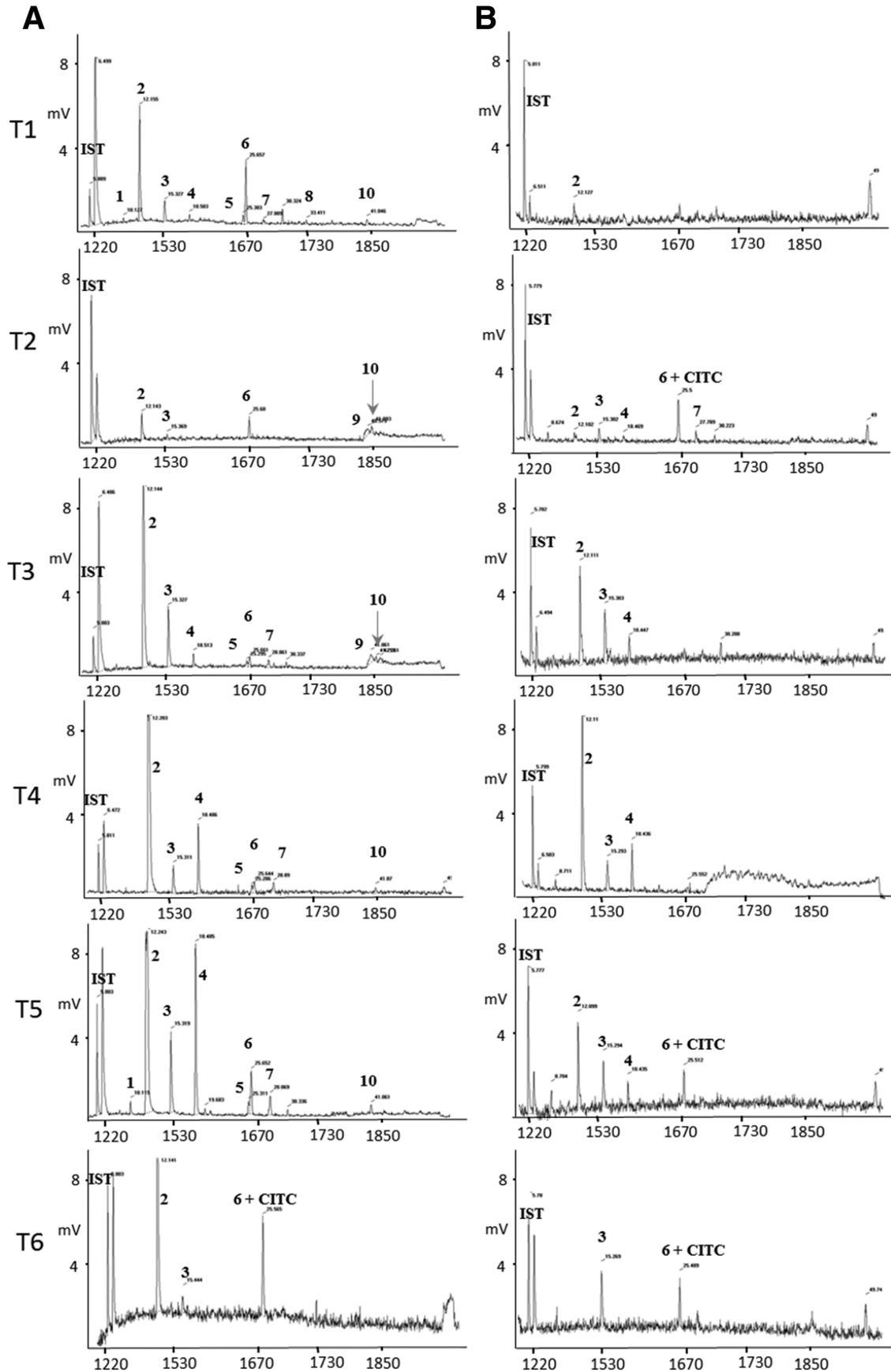


Fig. 1. Gas chromatographic–pulsed flame photometric detection chromatograms of *para*-hydroxymercuribenzoic acid extracts issued from (A) fresh, (B) 1-year-aged T1 to T6 beers (20°C). IST = internal standard, CITC = cyclohexyl isothiocyanate, T = top-fermented.

quaternary pump. The system was controlled with the Xcalibur software version 1.2. Five microliters of filtered beer sample was injected onto the Prevail C18 column (150 × 2.1 mm, 0.3 μm). The eluents were 0.1% aqueous formic acid (solvent A) and 0.1%

formic acid in acetonitrile (solvent B) at a flow rate of 0.2 mL/min. Gradient elution was as follows: from 95% A to 80% in 4 min, from 80 to 50% in 2 min, from 50 to 42% in 3 min, isocratic for 4 min, and return to the initial conditions for 15 min.

TABLE II
Polyfunctional Thiols in Fresh and 1-Year-Aged Special Beers^{a,b}

No.	RI		Substance	Acronym	Odor (GC-O)	Concentration ^c (ng/L)												
	FFAP	CP-Sil5				T1		T2		T3		T4		T5		T6		
						Fresh	1 year	Fresh	1 year	Fresh	1 year	Fresh	1 year	Fresh	1 year	Fresh	1 year	
1	1,381	915	4-Sulfanyl-4-methylpentan-2-one	4S4M2Pone	Catty, blackcurrant	86	-	-	-	-	-	-	-	-	94	-	-	-
2	1,454	880	2-Sulfanylethyl acetate	2SE-A	Burnt, grill	691	161	232	70	1,945	1,061	10,908	2,807	9,529	632	5,257	-	
3	1,538	722	2-Sulfanylethan-1-ol	2SEol	Grilled, gas	8,738	-	838	1,475	4,238	4,869	14,303	6,115	11,934	6,746	1,992	10,664	
4	1,579	1,000	3-Sulfanylpropyl acetate	3SPr-A	Grilled	+	+	-	+	186	226	2,551	603	2,119	219	-	-	
5	1,620	849	3-Sulfanylpropan-1-ol	3SProl	Potatoes, popcorn	-	-	-	-	32	-	126	-	72	-	-	-	
6	1,654	938	3-Sulfanyl-3-methylbutan-1-ol	3S3MBol	Sulfur, soup	573	-	108	55	59	-	155	+	522	27	93	42	
7	1,698	981	1-Sulfanyl-3-pentanol ^{IST}	1S3Pol	Stinging nettle, burnt	+	-	-	-	54	-	187	-	261	-	-	-	
8	1,760	1009	3-Sulfanylpentan-1-ol ^{IST}	3SPol	Grapefruit	-	-	+	-	-	-	-	-	-	-	-	-	
9	1,818	1208	3-Sulfanyl-4-methylpentan-1-ol ^{IST}	3S4MPol	Rhubarb, grapefruit	-	-	73	-	82	-	-	-	-	-	-	-	
10	1,858	1094	3-Sulfanylhexan-1-ol	3SHol	Grapefruit	91	-	50	-	109	-	190	-	128	-	-	-	

^a Quantified by pulsed flame photometric detection (PFPD). Assays in duplicate.

^b RI = retention index, T = top-fermented beer, FFAP = free fatty acid phase, GC-O = gas chromatography olfactometry.

^c - = not detected by PFPD; + = detected by PFPD under quantification limits (<20 ng/L).

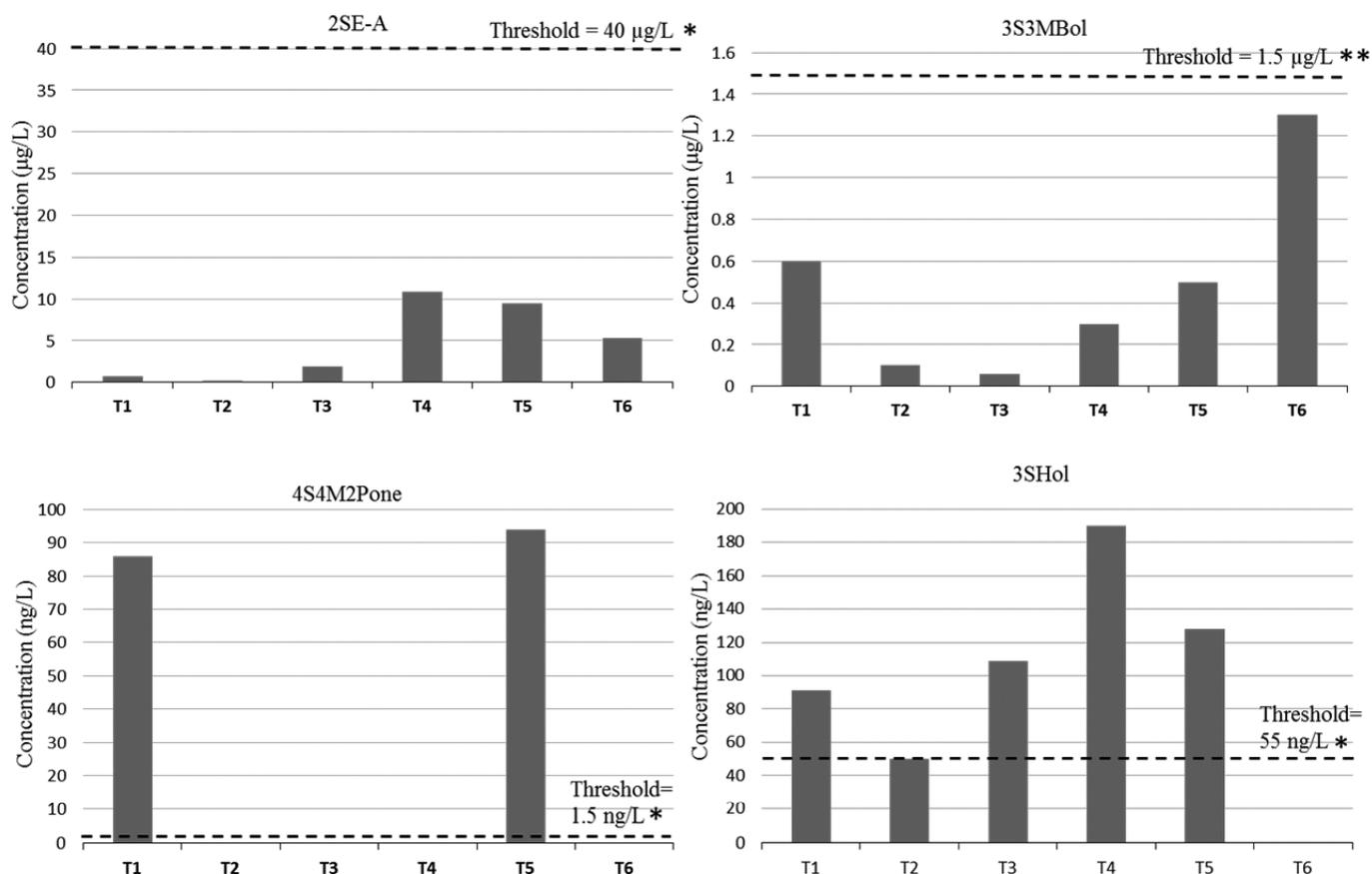


Fig. 2. Comparison of the odor thresholds determined in beer (*) or hydroalcoholic solution 12% (v/v) (**), with thiol concentrations found in fresh T1 to T6 beers. T = top fermented, 2SE-A = 2-sulfanylethyl acetate, 3S3MBol = 3-sulfanyl-3-methylbutan-1-ol, 4S4M2Pone = 4-sulfanyl-4-methylpentan-2-one, 3SHol = 3-sulfanylhexan-1-ol.

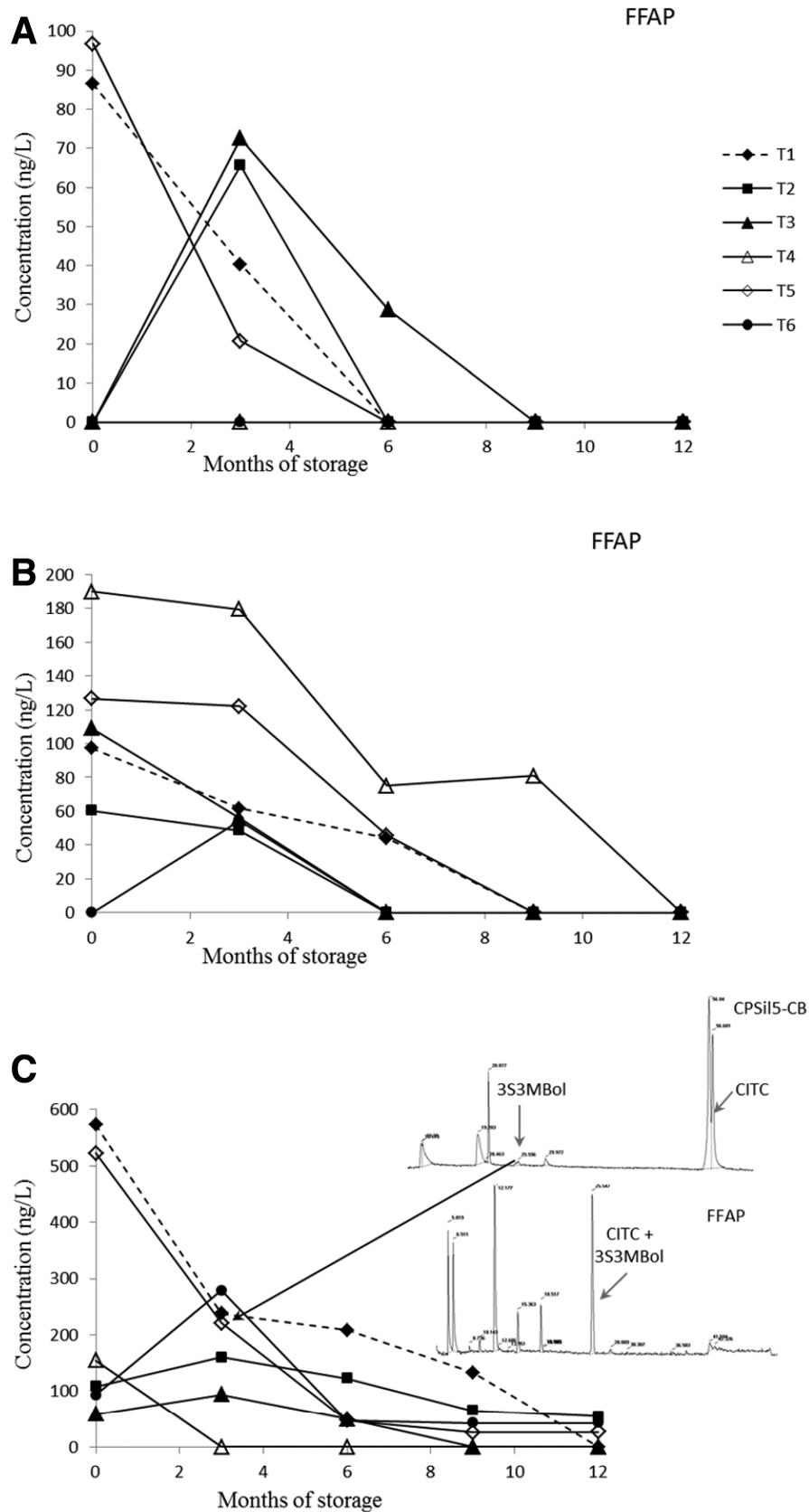


Fig. 3. Concentrations of (A) 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone), (B) 3-sulfanylhexan-1-ol (3SHol), and (C) 3-sulfanyl-3-methylbutan-1-ol (3S3MBol) during natural aging of top-fermented (T1) to T6 beers. Variation coefficient < 15%. For 3S3MBol, data from the CP-Si15-CB column were taken into account when coelution with cyclohexyl isothiocyanate (CITC) occurred on the free fatty acid phase (FFAP) column. Chromatograms are given for T5 after 3 months, on both columns.

Mass spectra were obtained with an LCQ mass spectrometer equipped with an ESI source (Thermo Fisher). The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary voltage, 4 V; capillary temperature, 300°C; and sheath gas, 20 psi. Standard solutions (0.5, 1, 2, 5, 7.5, 10, and 25 mg/L) of L-felinine, *S*-3-(1-hydroxyhexyl)-cysteine, and *S*-3-(3-methyl-2-butenyl)-cysteine were injected into the RP-HPLC-ESI(+)-MS/MS apparatus for calibration.

Conversion Ratio of *S*-Cysteine Conjugates

Conversion of cysteine adducts to free thiols was calculated as follows: molar conversion ratio (%) = concentration of released thiol (mol/L) \times (100/initial concentration of *S*-cysteine precursor [mol/L]).

RESULTS AND DISCUSSION

Beer polyfunctional thiols were selectively extracted by *p*HMB from six Belgian special beers (after 0, 3, 6, 9, and 12 months of storage) and quantified at the PFPD detector.

Polyfunctional Thiols in Fresh Belgian Special Beers

Due to the use of various hop varieties and different hopping procedures, the here-investigated fresh beers showed significantly different chromatograms (Fig. 1A). Antioxidant components (polyphenols, ascorbic acid, sulfites, etc) added either in the brewhouse or at bottling can also help to preserve thiols in some fresh beers. A total of 10 polyfunctional thiols were detected (Table II). All these thiols have been previously iden-

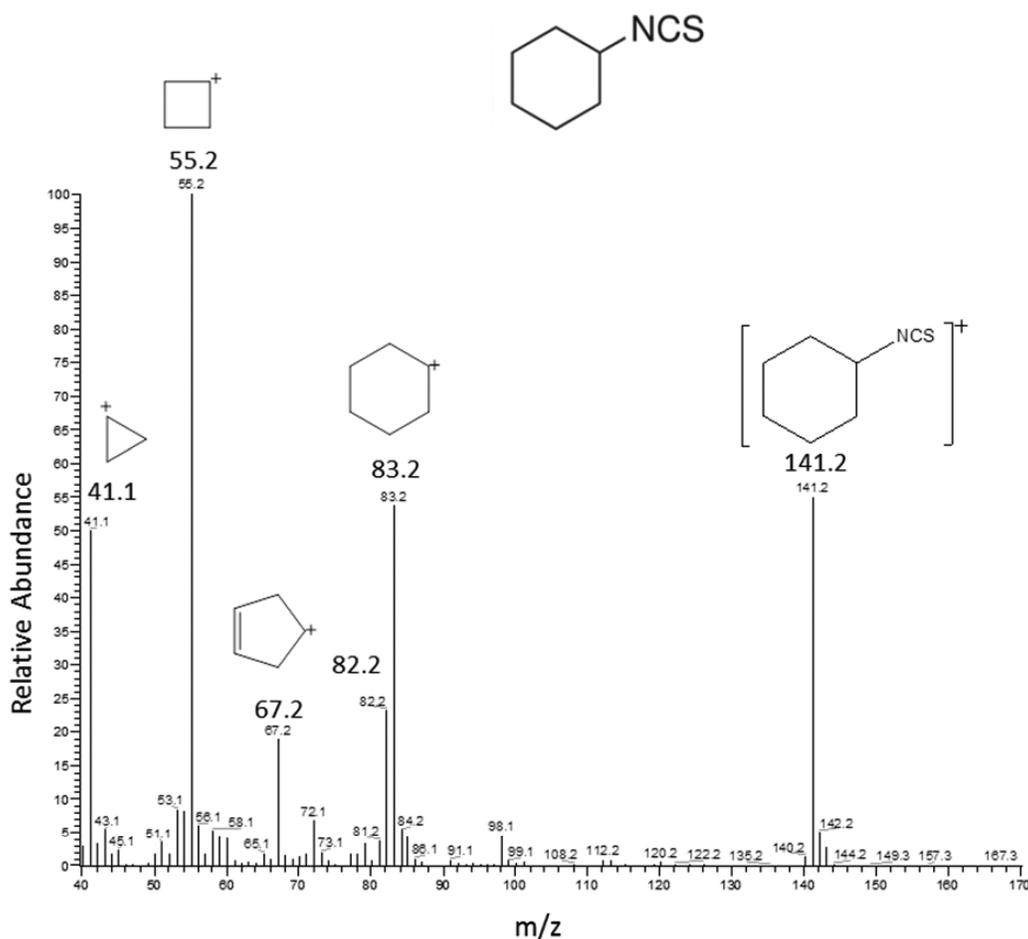


Fig. 4. Electronic impact mass spectrum of cyclohexyl isothiocyanate found in the *para*-hydroxymercuribenzoic acid extract of top-fermented (T) beer T3, aged for 3 months at 20°C.

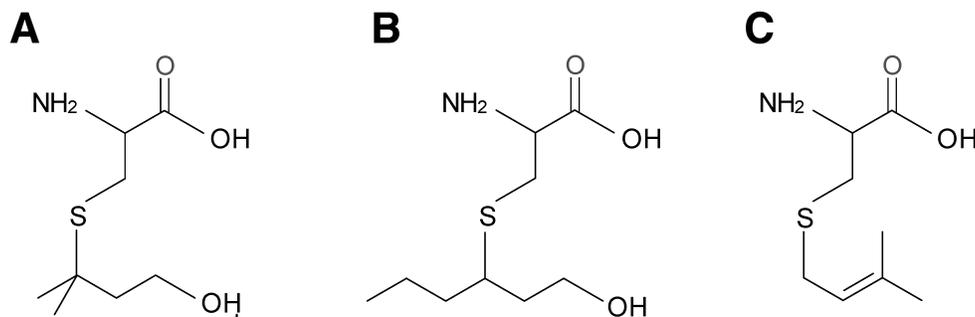


Fig. 5. Structures of (A) felinine (Cys-3S3MBol), (B) *S*-3-(1-hydroxyhexyl)-cysteine (Cys-3SHol), and (C) *S*-(3-methyl-2-butenyl)-cysteine (Cys-MBT).

tified in lager beers (5,6,10,19,25). 2SE-A (burnt, grilled) and 3S3MBol (sulfur, soup) were present in all fresh beers, 2SE-A always being the highest peak (232 to 10,908 ng/L). Yet their concentrations remained under the sensorial thresholds (Fig. 2).

On the other hand, as depicted in Figure 2, 3SHol (grapefruit) and 4S4M2Pone (blackcurrant) were often found above their sensory thresholds (55 and 15 ng/L, respectively [9]), despite their sub-ppb concentrations. 3S4MPol (rhubarb, grapefruit), previously proposed as a marker of some hop varieties (including Nelson Sauvin, Tomahawk, and Mosaic), was found only in T2 and T3 (concentrations of 73 and 82 ng/L^{IST}, respectively), also at levels above its odor threshold in beer (70 ng/L [19]).

Fate of Polyfunctional Thiols Through Beer Aging

The fate of beer thiols was investigated over 1 year of natural aging. The empyreumatic thiols 2SE-A and 3SPr-A are not discussed in this article, as they have been reported in another work (22). Although strongly degraded after 1 year, 2SE-A and 3SPr-A were revealed to be still being produced in most of the

lager and special commercial beers during the first 3 months of storage, even in the absence of yeast.

As depicted in Figures 1B and 3, all six beers here investigated had also lost the major part of their minor polyfunctional thiols after 1 year of storage (Table II). The three aroma-active thiols of fresh beer, 4S4M2Pone, 3SHol, and 3S4MPol (all with pleasant rhubarb/grapefruit/raspberry flavors) were completely absent after 1 year (in some samples, already after 6 months).

Moreover, as for 2SE-A and 3SPr-A (22), thiol synthesis could also occur during the first 3 months of storage. This was the case with 4S4M2Pone (especially in T2 and T3; 65 to 75 ng/L synthesized, Fig. 3A) and 3SHol (especially in T6; up to 60 ng/L synthesized, Fig. 3B). In the case of 3S3MBol (Fig. 3C), we could have concluded that synthesis occurred in almost all beers through aging, but mass spectrometry (Fig. 4) indicated that an FFAP coeluent, with a mass spectrum (and a retention index) identical to that of cyclohexyl isothiocyanate (CITC), was sometimes present, either in fresh beer (T6) or after 3 months of aging (T1 to T6). Fortunately, the CP-Sil5-CB column allowed us

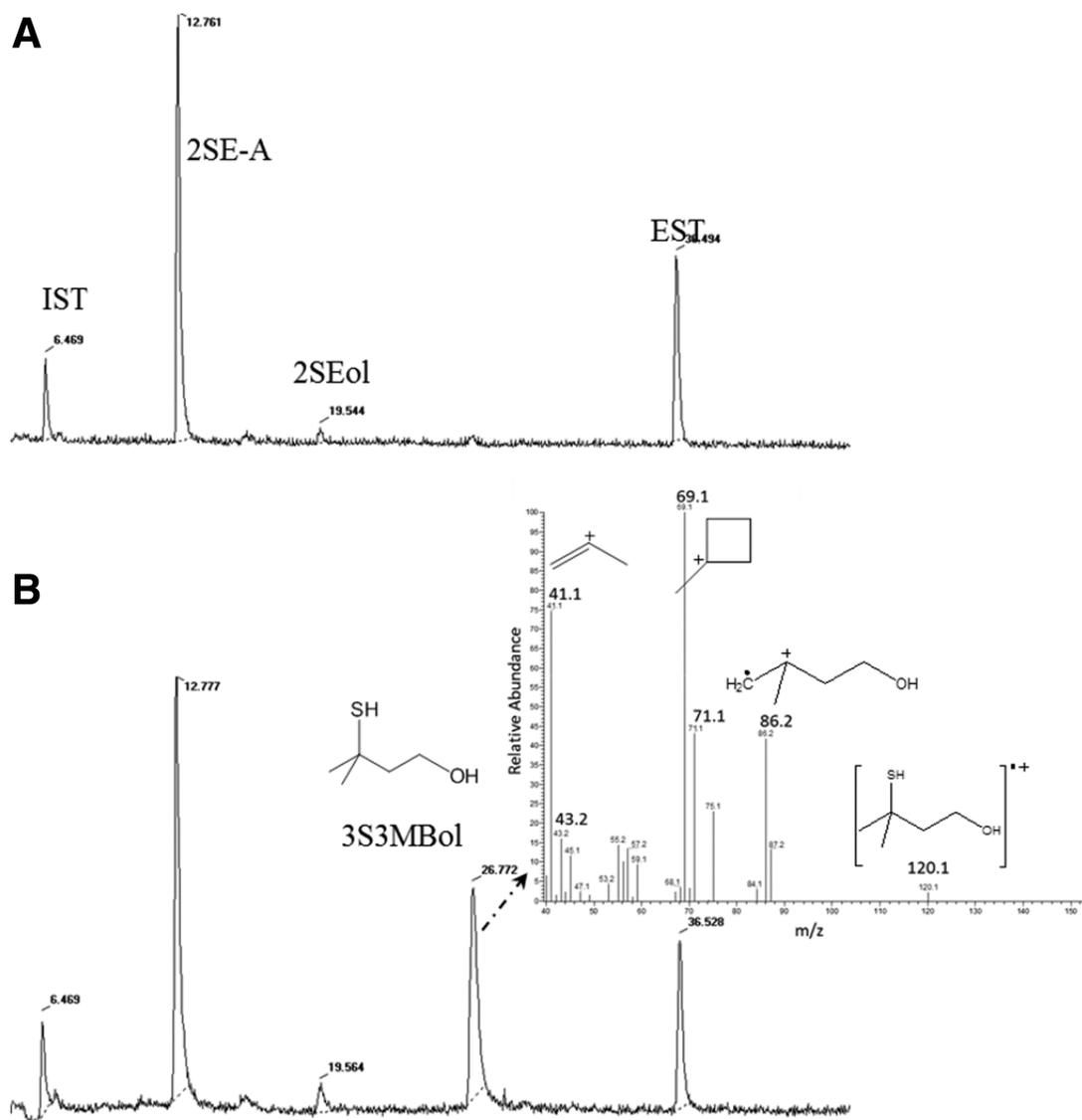


Fig. 6. Gas chromatographic-pulsed flame photometric detection chromatograms of *para*-hydroxymercuribenzoic extracts issued from an aged lager beer (60°C, 5 days) (A) unspiked, or (B) spiked before aging with 5 mg/L felinine. Experimental electrospray ionization mass spectrum at retention time = 26.8 min. IST = internal standard, 2SE-A = 2-sulfanylethyl acetate, 2SEol = 2-sulfanylethan-1-ol, EST = external standard, 3S3MBol = 3-sulfanyl-3-methylbutan-1-ol.

to differentiate the two compounds (retention index_{CP-Sil5-CB} = 1,215 and 944 for CITC and 3S3MBol, respectively, see both chromatograms in Fig. 3C). Finally, a synthesis of 175 ng/L 3S3MBol was found only in T6.

In conclusion, two distinct patterns were observed among our beers in the course of aging, as was the case for 2SE-A and 3SPr-A (22). Either the beer contained high thiol levels when it was fresh and, therefore, subsequent synthesis in the bottle remained insignificant.

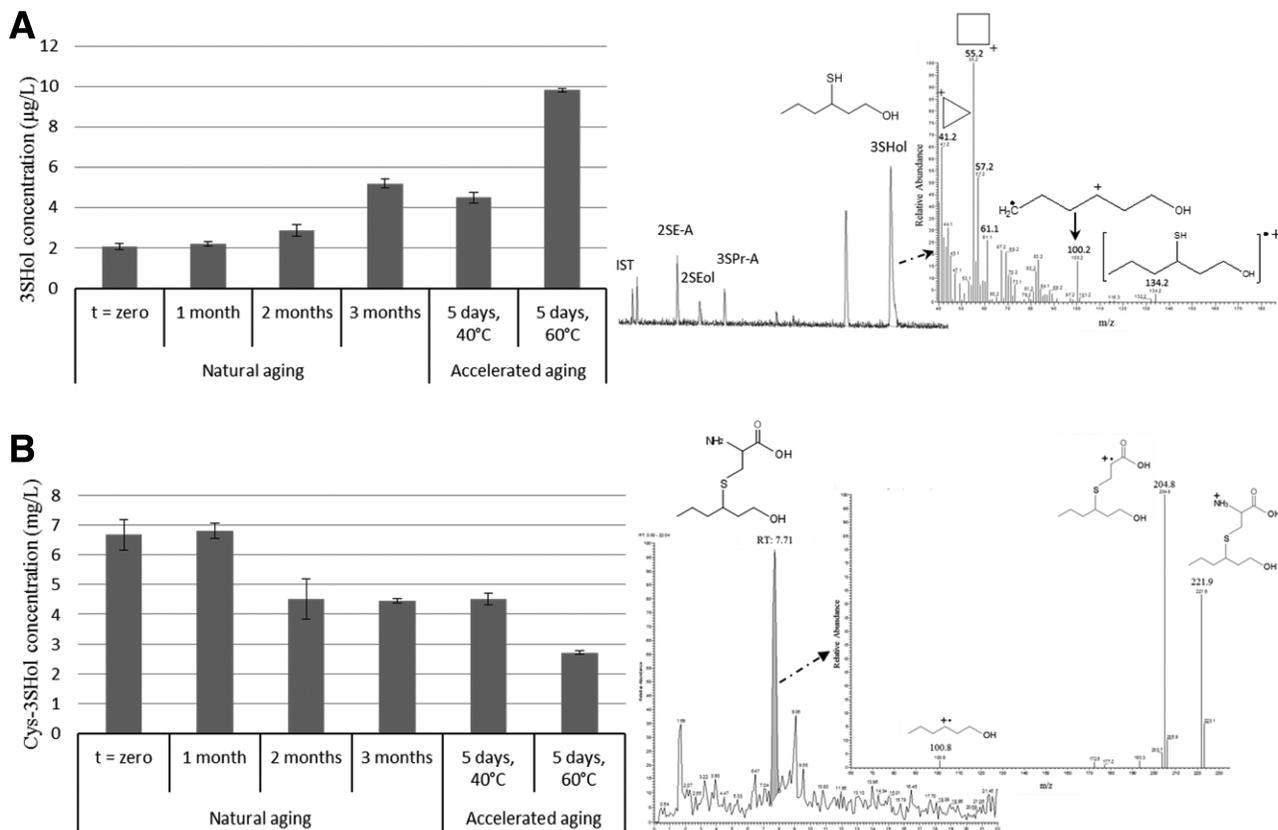


Fig. 7. Concentrations of (A) released 3-sulfanylhexan-1-ol (3SHol) and (B) remaining Cys-3SHol in a lager beer spiked with synthetic Cys-3SHol (7 mg/L) before aging. Gas chromatographic–pulsed flame photometric detection chromatogram of a *para*-hydroxymercuribenzoic extract (5 days at 60°C) with electrospray ionization experimental mass spectrum of 3SHol. Corresponding HPLC-electrospray ionization-MS/MS chromatogram showing the elution of Cys-3SHol at retention time (RT) = 7.7 min., and its $m/z + 1 = 222$. IST = internal standard, 2SE-A = 2-sulfanylethyl acetate, 2SEol = 2-sulfanylethanol-1-ol, 3SPr-A = 3-sulfanylpropyl acetate.

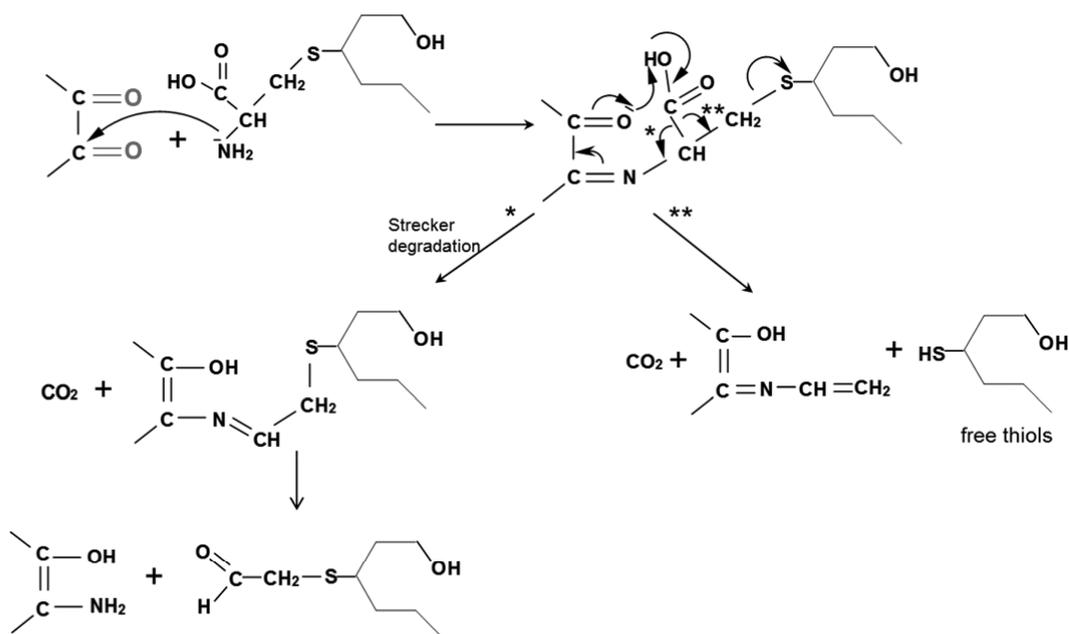


Fig. 8. Proposed cysteine adduct chemical degradation pathway involving beer dicarbonyls.

nificant as compared to loss through oxidation (e.g., T1, T5), or only traces were found in the beer before aging and thiol concentrations were found to increase during the first 3 months of storage (e.g., 4S4M2Pone in T2 and T3, 3SHol and 3S3MBol in T6).

As previously suggested for 2SE-A (22), our observations could result from the chemical hydrolysis of cysteine adducts present in fresh beer. To assess how such precursors might be degraded in the absence of yeast, one commercial (felinine) and two synthesized (Cys-3SHol and Cys-MBT) cysteine adducts (Fig. 5) were used to spike a filtered lager beer before aging.

Chemical Degradation of Cysteine Adducts through Beer Aging

Degradation of commercial Cys-3S3MBol (L-felinine). Felinine (5 mg/L) was first added (in duplicate) to a fresh lager beer in which no cyclohexyl isothiocyanate had been detected in preliminary aging assays. Strongly accelerated aging (60°C, 5 days) was carried out. As depicted in Figure 6, 2.9 g/L of 3S3MBol released from its added cysteinylated precursor was detected after 5 days at 60°C. This corresponds to 0.1% degradation of felinine in terms of molar ratio.

Degradation of synthetic Cys-3SHol. In a second experiment, Cys-3SHol was added to the same lager beer at 7 mg/L concentration. The beers were analyzed after 0, 1, 2, and 3 months at 20°C, and after 5 days at 40 or 60°C. Before pHMB extraction and PFPD quantification of free thiols (Fig. 7A), 1 mL of beer was sampled for HPLC-ESI(+)-MS/MS quantification of Cys-3SHol (Fig. 7B). All assays were conducted in duplicate.

After 1 month at room temperature, no release of 3SHol was evidenced. The degradation of Cys-3SHol was significant after both 2 and 3 months of storage at 20°C (31% loss) and after 5 days at 40 or 60°C (31 and 57% loss, respectively). In parallel with the degradation of its precursor, the level of free 3SHol was increased, reaching 5.2 g/L after 3 months at room temperature and up to 9.8 g/L after 5 days at 60°C. In a complex medium like beer, many coconstituents could participate in this chemical degradation. Among others, dicarbonyls could be involved via a modified Strecker pathway (Fig. 8).

The conversion ratios given in Table III (0.03 to 0.19%) show that the cysteine adducts were only slightly converted to free thiols. Such sub-ppb traces of detected odorants could, however, impact beer flavor. Low conversion rates have previously been reported in the presence of yeast, both for wine during ferment-

TABLE III
Conversion of S-3-(1-Hydroxyhexyl)-Cysteine (Cys-3SHol) During Beer Aging Under Different Conditions

Aging conditions	Degraded Cys-3SHol from 7 mg/L = 31.7 mol/L			Released 3SHol		Molar conversion ratio (%)
	mg/L	mol/L	Loss (%)	g/L	mol/L	
Natural, 20°C						
0 month	0	0	0	0	0	0
1 month	0	0	0	0.1	0	0
2 months	2.2	9.9	31	0.8	0.01	0.03
3 months	2.3	10.4	33	3.1	0.02	0.06
Accelerated						
5 days, 40°C	2.2	9.9	31	2.4	0.02	0.06
5 days, 60°C	4.0	18.1	57	7.7	0.06	0.19

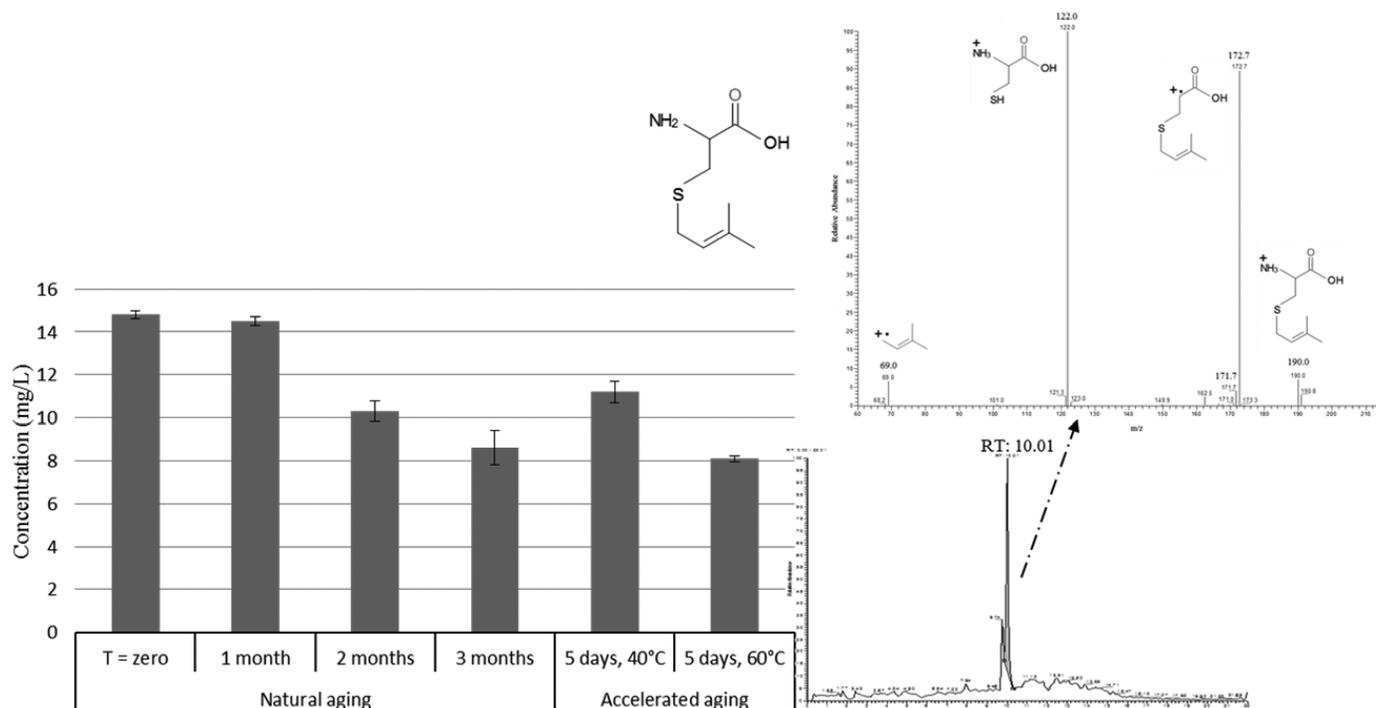


Fig. 9. Concentrations of remaining cysteine 3-methylbut-2-en-1-thiol (Cys-MBT) in a lager beer spiked with 15 mg/L synthetic Cys-MBT before aging. HPLC-electrospray ionization-MS/MS chromatogram obtained after 5 days at 60°C. The elution of Cys-MBT, shown at retention time (RT) = 10.0 min., and its corresponding mass spectrum ($m/z + 1 = 190$).

tation (0.3 to 4.5% [16], 70% of Cys-3SHol degraded after 60 h [12]) and in beer during bottle refermentation (0.09% [13]). On the other hand, 30% conversion was reached by Gros et al in enzymatic assays using the β -lyase activity of apotryptophanase (7). These authors, however, mentioned that the apotryptophanase lyase activity was strongly related to the substrate concentration (7).

Degradation of synthetic Cys-MBT. In a last experiment, synthesized Cys-MBT (15 mg/L) was added to lager beer before aging (Fig. 9). As previously observed for Cys-3SHol, a significant decrease in concentration was measured for Cys-MBT after 2 months at room temperature (29% degraded). Degradation reached 33% after 3 months at 20°C or 5 days at 60°C. Yet in contrast to 3SHol, no peak of free MBT was detected in the corresponding *p*HMB extracts at the PFPD detector. The high reactivity and volatility of MBT most probably explain our results.

In conclusion, *S*-cysteine conjugates can be chemically degraded in beer to release their corresponding thiols. The conversion ratios are low (<1%) but may explain the trace amounts of 3SHol and 3S3MBol produced in beer during the first months of storage, even in the absence of yeast. On the other hand, the chemical degradation of Cys-MBT in beer (although up to 33%) does not appear efficient enough to release significant amounts of the skunky off-flavor. In this case, photooxidative degradation of isohumulone most probably remains the major synthesis pathway. Complementary analyses are now needed to determine how other beer constituents, such as dicarbonyls, might participate in cysteine adduct degradation.

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