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First Evidence of the Production of Odorant Polyfunctional Thiols by Bottle Refermentation

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ABSTRACT

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Bottle refermentation, which confers effervescence and resistance against infection and oxidation to beers, has also long been known to affect the fruity character imparted by esters. Yet it is recognized to improve the flavor perception, first by reducing stale aldehydes (*trans*-2-nonenal, 3-methylthiopropionaldehyde, etc.) to low-odorant alcohols, and also by bringing new pleasant odors. In this work, the polyfunctional thiol contents of a beer subjected or not to bottle refermentation were compared. A trained panel detected a strong organoleptic impact of bottle refermentation. Specific *p*HMB thiol extraction was applied and the extracts were analyzed by GC-MS, GC-PFPD, and GC-olfactometry (AEDA). Many sulfanylalkylalcohols, sulfanylalkylacetates, and sulfanylalkylcarbonyls were shown to be produced during the refermentation process, especially after 3 weeks. Among them, the hoppy 1-sulfanyl-3-methyl-2-butene was still perceived at the sniffing port after diluting the extract by a factor of 32,768. The major thiol, 2-sulfanylethyl acetate, reached 10 µg/L. As shown by spiking deuterated cysteine before bottle refermentation, the Ehrlich pathway revealed still efficient in the bottle. Most of the other identified polyfunctional thiols shared a common beta-sulfanyl structure, which lead us to suspect that hop cysteine adducts might be hydrolyzed by yeast-derived lyases. The spiking of 5 and 10 mg/L of S-3-(1-hydroxyhexyl)cysteine confirmed the ability of yeast to release free thiols through bottle refermentation. Therefore, a better control of the refermentation process requires both an excellent control of yeast (Ehrlich pathway and β-lyase activity) and strict selection of the hop variety (level of cysteine adducts).

Keywords: Beer aroma, Bottle refermentation, Cysteine adducts, Polyfunctional thiols

RESUMEN

Refermentación en la botella, que confiere la efervescencia y la resistencia contra la infección y la oxidación a las cervezas, también ha sido durante mucho tiempo conocida por afectar el carácter afrutado impartido por ésteres. Sin embargo, se reconoce a mejorar la percepción del sabor, primero mediante la reducción de aldehídos rancios (*trans*-2-nonenal, 3-methylthiopropionaldehyde, etc.) a los alcoholes de bajo olor, y también por traer nuevos olores agradables. En este trabajo, el contenido de tiol polifuncional de una cerveza sometido o no a refermentación en la botella se compararon. Un panel de jueces entrenados detectaron un fuerte impacto organoléptico del refermentación en la botella. Específica *p*HMB extracción tiol se aplicó y los extractos fueron analizaron por GC-MS, GC-PFPD, y GC-olfatometría (AEDA). Muchos sulfanilalquiloalcohols, sulfanilalquiloacetatos y sulfanilalquilocarbonilos se muestra que se produjo durante el proceso de re-fermentación, especialmente después de 3 semanas. Entre ellos, el lúpulo 1-sulfanil-3-metil-2-buteno fue percibido todavía en el puerto de inhalación después de diluir el extracto por un factor de 32,768. El tiol importante, 2-sulfaniletil acetato, alcanzó 10 µg/L. Como se muestra por adición de cisteína deuterado antes de refermentación en la botella, la vía de Ehrlich revelado todavía eficiente en la botella. La mayoría de los otros identificados tioles polifuncionales compartían la misma beta-sulfanil estructura, lo que nos lleva a sospechar que los aductos cisteína del lúpulo podría ser hidrolizado por liasas derivados de levaduras. El adición de 5 y 10 mg/L de S-3-(1-hidroxihexil) cisteína confirmó la capacidad de la levadura para liberar los tioles libres a través de refermentación en la botella. Por lo tanto, un mejor control del proceso de re-fermentación requiere tanto un excelente control de la levadura (vía de Ehrlich y la actividad β-liasa) y estricta selección de la variedad de lúpulo (nivel de aductos de cisteína).

Palabras claves: Aroma de la cerveza, Refermentación en la botella, Aductos de cisteína, Tioles polifuncionales

INTRODUCTION

Bottle refermentation, which imparts effervescence and resistance against infection and oxidation to beer, is also known to improve its flavor profile and stability (4,25,26). Around 6×10^5 yeast cells/mL are usually pitched into the beer before bottling, in the presence of sugar ($2x - (0.3C + D)$ grams, taking into account that x g/L carbon dioxide will be produced; C = remaining fermenting sugars, and D = carbon dioxide from another source) (4). The beer is then kept in a room maintained at 20–28°C for 2–4 weeks, and this is sometimes followed by a short period of cold storage at 4°C to avoid hydrogen sulfide defects. The physiological condition and propagation medium of the pitching yeast seem to have an impact on the refermentation process and global aroma (4,26). The refermentation efficiency also depends on the type of sugar, the alcohol content of the beer, its bitterness level, and its ionic balance (4,28).

A too-long period of storage at 20°C may lead to yeast autolysis, with excretion of intracellular components including enzymes (1,2,5,17). Esterases can strongly affect the fruity character of beer. Some esters are hydrolyzed (isoamyl acetate, ethyl hexanoate, and ethyl octanoate), while others seem still to be produced (ethyl isovalerate, ethyl isobutyrate, and ethyl phenylacetate) (16,19). Yet bottle refermentation is recognized to improve the flavor perception, first by reducing stale aldehydes (*trans*-2-nonenal, 3-methylthiopropionaldehyde, 3-methylbutanal, 5-hydroxymethylfurfural) to low-odorant alcohols (19,20) and also by bringing new, pleasant odors. The available chemical data are too scant to explain why the global perception of the fresh refermented beer is so strongly improved.

Thiols and other sulfur compounds have a strong impact on the overall aroma of fermented beverages (18,29,31). Among brewing raw materials, some hop cultivars contain interesting free poly-

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functional thiols with delicate passion fruit/grapefruit/Sauvignon descriptors (7,22). These, however, are rarely transferred to the final beer, unless added at the very late stages of boiling (7,8,22). Primary fermentation is known to produce new polyfunctional thiols because yeast, by excreting hydrogen sulfide, can transform hop allylic alcohols into onion-like sulfanylalkylalcohols such as 2- and 3-sulfanyl-3-methylbutanol (6,11,31). Hydrogen sulfide can probably also add onto wort α,β-unsaturated carbonyls to generate various beta-sulfanylalkylcarbonyls, further converted to beta-sulfanylalkylalcohols and/or esters (31). As in the case of wine (15,18,23), another suspected route is the hydrolysis of hop cysteine adducts by yeast β-lyases (7,8). Very recently, a cysteine-S-conjugate (S-3-(1-hydroxyhexyl)cysteine) has been evidenced by HPLC-HRMS/MS in the cascade hop variety (8). A few thiols such as 2-sulfanylethanol, 3-sulfanylpropanol, and their corresponding acetates mainly arise through Ehrlich degradation of sulfur-containing amino acids (12,31). Very recently it was shown that dry hopping can still bring new polyfunctional thiols to beer (3).

No scientific paper currently describes how bottle refermentation affects the beer thiol profile. As yeast continues to excrete hydrogen sulfide into the bottle, radical, nucleophilic, and electrophilic additions onto allylic alcohols and unsaturated carbonyls might still occur. Additionally, residual hop cysteine-thiol adducts might be hydrolyzed by yeast enzymes. The aim of the present work was therefore to compare beers subjected or not to bottle refermentation. Specific extraction with *p*HMB (*p*-hydroxymercuribenzoic acid) was applied and the extracts were analyzed by GC-MS, GC-PFPD, and GC-olfactometry (AEDA). The organoleptic impact of bottle refermentation was also assessed by a panel of trained experts.

MATERIALS AND METHODS

Materials

Dodecane (99.9%), *L*-cysteine hydrochloride monohydrate, *p*-hydroxymercuribenzoic acid (*p*HMB), (*E*)-hexen-2-al, N-Boc-L-cysteine, and HCl (37%) were provided by Sigma-Aldrich (Bornem, Belgium). Tris(hydroxymethyl)aminomethane (TRIS) and sodium acetate were supplied respectively by USB (Cleveland, OH) and UCB (Brussels, Belgium). Sodium hydroxide and 99% sodium sulfate were supplied by Janssen (Geel, Belgium). Cesium carbonate, sodium borohydride, and trifluoroacetic were sourced from Acros Organics (Geel, Belgium). L-cysteine-d2 (98%) and L-homocysteine-d4 (98%) were provided by Cambridge Isotope (Andover, MA). Dichloromethane (99.9%) from Romil (Cambridge, U.K.) was distilled twice before use. Milli-Q water was used (Millipore, Bedford, MA). A strongly basic Dowex resin 1 X 2, Cl^- form was provided by Oxford Chemicals (Oxford, U.K.). Acetonitrile and methanol were obtained from VWR (Belgium). 3-Sulfanylpropan-1-ol (n°3), 2-sulfanylethyl acetate (n°4), 2-sulfanyl-4-methoxy-2-methylbutane (n°5), and 3-sulfanylpropyl acetate (n°10) were purchased from Sigma-Aldrich (Bornem, Belgium). 1-Sulfanyl-3-methyl-2-butene (n°2) was obtained from Oxford Chemicals (Oxford, U.K.). 4-Sulfanyl-4-methylpentan-2-one (n°6) was from Frutarom (Hartlepool, U.K.). 3-Sulfanylbutan-2-one (n°1), 1-sulfanylpentan-3-one (n°7), 1-sulfanylpentan-3-ol (n°9), 3-sulfanylhexan-1-ol (n°13), 4-sulfanylnonan-2-ol (n°17), 3-sulfanyloctan-1-ol (n°18), and 3-sulfanylnonan-1-ol (n°21) were produced by combinatorial synthesis according to (29). 3-Sulfanylbutyl acetate (n°12), 3-sulfanylpentyl acetate (n°14), 3-sulfanyl-2-methylpentyl acetate (n°15), and 3-sulfanyl-2-butylpropyl acetate (n°19) were also synthesized prior to the present work (30), as were 3-sulfanylpentanal (n°8) and 3-sulfanylhexanal (n°11) (27). 3-Sulfanyl-4-methylpentyl acetate (n°16) was produced according to (7).

Beer Samples

Two commercial unrefermented lager beers (A and B; differently hopped) were kindly supplied by a Belgian brewer.

Bottle Refermentation Process Without Spiking of Thiol Precursors

Bottle refermentation was applied to half of the commercial A samples (addition of 11 g/L saccharose and 600,000 cells/mL of yeast strain MUCL 34627 before storage in the dark for 1, 2, or 3 weeks at 27°C; A1, A2, and A3). After each week, samples were directly extracted and compared to the reference beer stored for 3 weeks at 27°C without refermentation (ARf). A refermentation without sugar (addition of yeast only under the same conditions as for A3, 27°C for 3 weeks) was also tested (A3WS).

Labeled Cysteine and Homocysteine Spiking Before Bottle Refermentation

20 mg/L of L-cysteine-d2 or L-homocysteine-d4 were added into the commercial lager beer B. After 3 weeks of refermentation at 27°C (addition of 11 g/L saccharose and 600,000 cells/mL of yeast strain MUCL 34627), each sample was extracted.

Synthesis and Spiking of S-3-(1-Hydroxyhexyl)cysteine

The synthesis was conducted as described by Gros et al. (8) (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). Zero, 5, and 10 mg/L of S-3-(1-hydroxyhexyl)cysteine were added into beer B. After 3 weeks of refermentation (same conditions as those described here above), each sample was extracted.

Extraction of Polyfunctional Thiols with *p*HMB

Beer (500 mL) and distilled CH_2Cl_2 (200 mL) were stirred for 30 min. After decantation (± 15 min), the lower phase and interfacial emulsion were centrifuged for 20 min at 10,000 rpm. The organic phase was then extracted once for 5 min and once for 10 min with 20 mL of *p*HMB solution (360 mg *p*HMB, 24.6 g Tris in 1 L of Millipore water) (23). The combined aqueous phases were loaded into a strongly basic anion exchanger column (Dowex 1WX2-100 resin) washed beforehand successively with 2 M NaOH, ultrapure water, and 2 M HCl. Then 50 mL sodium acetate buffer (0.1 M, pH 6) was poured on the resin to remove impurities. Next, volatile thiols were released by percolating a purified cysteine solution (640 mg of *L*-cysteine hydrochloride, monohydrated in 60 mL Millipore water—this solution was washed with 2 × 5 mL distilled CH_2Cl_2 before use). The eluate containing the volatile thiols was collected and extracted with 4 and then 3 mL of distilled CH_2Cl_2, under magnetic stirring (5 min). The organic phases were pooled, dried on anhydrous Na_2SO_4, and finally concentrated to 500 µL in a Kuderna-Danish (for GC-O) and to 70 µL in a Dufton system (for GC-PFPD), to be stored at –80°C before analysis. The internal standard (IST) was 2-sulfanyl-4-methoxy-2-methylbutane (added at the first extraction step, 0.67 µg/L in beer).

Sensorial Analyses

All samples were presented to 10 trained panelists in 500 mL ''Breughel'' glasses (Durobor, Belgium) covered with a glass top and containing 20 mL of beer per glass. Samples were assessed at room temperature in individual booths illuminated with red light. A triangular test was first applied to determine whether refermentation significantly modified the global aroma. Aroma and flavor attributes were quantified by 10 expert judges.

Gas Chromatography–Olfactometry (GC-O)

One microliter of *p*HMB extract (issued from the Kuderna-Danish flask) 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 post-injection. Compounds were analyzed with a wall-coated open tubular (WCOT) apolar CP-Sil5-CB (50 m × 0.32 mm i.d., 1.2 µm film thickness) and a polar FFAP (25 m × 0.32 mm i.d., 0.3 µm film thickness) capillary column. The carrier gas was nitrogen and the pressure was set at 60 kPa (CP-Sil5-CB) or 30 kPa (FFAP). The oven temperature was programmed to rise from 36°C to 85°C at 20°C/min, then to 145°C at 1°C/min, and finally to 250°C at 3°C/min and held for 30 min. 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) sulfate solution. The *p*HMB extracts were analyzed by two trained panelists immediately after extraction. Complete AEDA was performed by one operator, with the CP-Sil5-CB column. The extracts were diluted stepwise with dichloromethane (1+1 by volume). The dilution factor (FD) is defined as the highest dilution at which the compound could still be detected (FD = 2^n with n + 1 = number of dilutions applied to the extract until no detection by GC-O). The precision of this AEDA analysis is n ± 1 (factor 2 between FD values). Retention indices were determined by connecting the column to an FID maintained at 250°C.

Gas Chromatography Coupled to an Electronic Impact Mass Spectrometer (GC-MS)

Mass spectra (m/z = 40 to 380) were recorded at 70 eV on a ThermoFinnigan Trace MS mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector and an apolar CP-Sil 5 CB MS capillary column (50 m × 0.32 mm i.d., 1.2 µm film thickness). The carrier gas was helium and the pressure was set at 100 kPa. The oven temperature program was the same as that described for GC-O. Spectral recording was automatic throughout elution; Xcalibur software was used. The single-ion monitoring (SIM) mode was applied for the deuterated labeled media (m/z = 47/61 for 2SEA, 49/63 for 2SEA-d2, 61/74 for 3SPrA, and 65/78 for 3SPrA-d4).

Gas Chromatography Coupled to a Pulsed Flame Photometric Detector (GC-PFPD)

Two microliters of each *p*HMB extract (issued from the Dufton system) was analyzed on a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250°C and connected to the O.I. Analytical PFPD, model 5380. Injections were carried out in the splitless mode at 250°C, the split being turned on after 0.5 min. The carrier gas was helium at a pressure of 90 kPa. At the detector, the following parameters were selected: 250°C as the temperature, 600 V as the voltage, 18 ms as the gate width, 6 ms as the gate delay, 580 mV as the trigger level, and 3.70 Hz as the pulse frequency. The oven temperature program and the column were the same as described for GC-O.

Identifications

For all thiols detected by GC-MS, MS identifications were done by comparing the mass spectra obtained from each sample with those obtained with pure or synthesized compounds injected

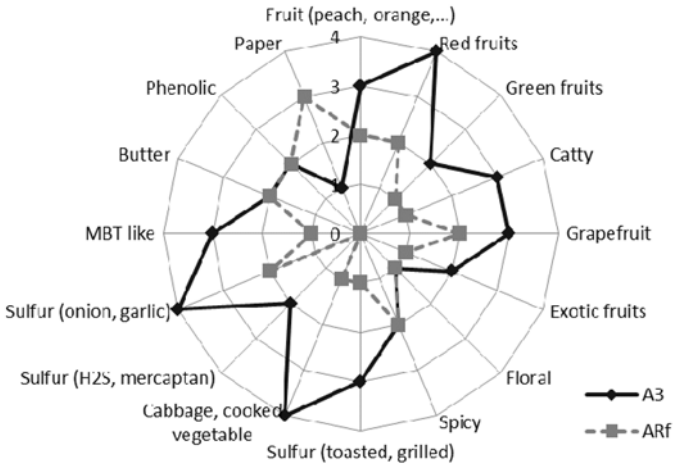

Fig. 1. Spider diagrams giving the intensity of 16 descriptors perceived by sensorial panelists in A3 (refermented for 3 weeks) and the reference ARf.

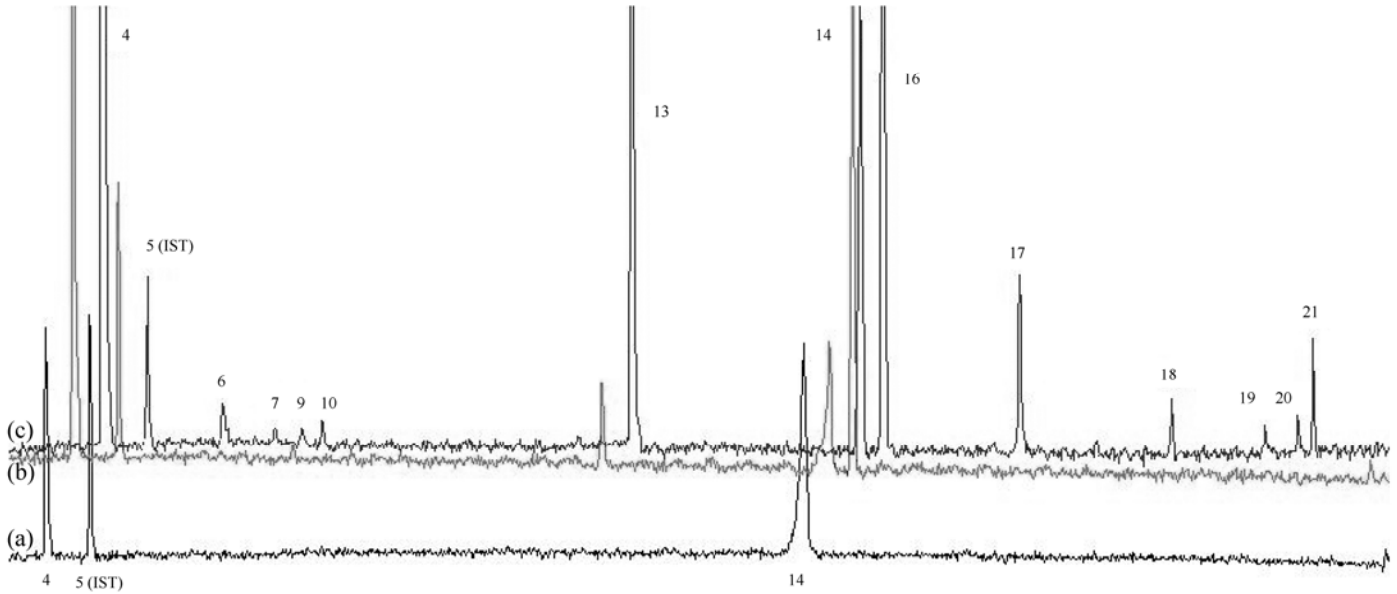

Fig. 2. PFPD chromatogram (CPSil5 column) of refermented and unrefermented beers. **(a)** ARf, beer without refermentation **(b)** A2, beer refermented for 2 weeks, **(c)** A3, beer refermented for 3 weeks.

TABLE I
Sulfanyl Compounds Produced Through Bottle Refermentation. PFPD Quantifications and Dilution Factors (FD)[a]

	RI			ARf		A1		A2		A3		A3WS			
PN (symbol)	CPSIL 5	FFAP	Compound[b]	conc. (µg/L)	FD	conc. (µg/L)	FD	conc. (µg/L)	FD	conc. (µg/L)	FD	conc. (µg/L)	FD	Odor	Identification (3 main m/z ions in parentheses)[c]
Sulfanylalkyls carrying a carbonyl function															
8 (3SPal)	961	1,492	3-sulfanylpentanal	ud[1]	0	ud[1]	ud	ud[1]	ud	ud[1]	8	ud[1]	4	sweet	I⁻
11 (3SHal)	1,011	1,544	3-sulfanylhexanal	ud[1]	16	ud[1]	16	ud[1]	16	ud[1]	64	ud[1]	64	floral	I⁻
1 (3S2Bone)	789	1,295	3-sulfanylbutan-2-one	ud[1]	4	ud[1]	8	ud[1]	32	ud[1]	32	ud[1]	16	coffee, burned, onion	I⁻
6 (4S4M2Pone)	919	1,383	4-sulfanyl-4-methylpentan-2-one	ud[3]	16	ud[3]	16	0.01[3]	64	0.21[1]	128	0.11[2]	64	catty	I⁺ (132, 75, 55)
7 (1S3Pone)	954	1,521	1-sulfanylpentan-3-one	ud[2]	4	ud[2]	4	ud[2]	32	0.11[1]	32	ud[2]	4	sewer, sulfur	I
Sulfanylalkyls carrying an alcohol function															
3 (3SProl)	851	1,657	3-sulfanylpropan-1-ol	ud[1]	*[d]	ud[1]	*	ud[1]	*	ud[1]	*	ud[1]	*	potatoes	I⁻
9 (1S3Pol)	981	1,718	1-sulfanylpentan-3-ol	ud[2]	16	ud[2]	16	ud[2]	16	0.11[1]	32	0.06[1]	32	green	I
13 (3SHol)	1,094	1,829	3-sulfanylhexan-1-ol	ud[4]	4	ud[4]	8	0.23[3]	16	3.96[1]	64	1.18[2]	32	sulfur, rhubarb	I⁺ (57, 41, 61)
17 (4S2Nol)	1,291	1,977	4-sulfanylnonan-2-ol	ud[3]	16	ud[3]	32	ud[3]	16	1.03[1]	32	0.10[2]	16	lemon, citrus fruit	I⁺ (47, 71, 41)
18 (3SOol)	1,310	2,031	3-sulfanyloctan-1-ol	ud[2]	0	ud[2]	0	ud[2]	0	0.24[1]	4	0.16[1]	2	sweet, cabbage	I⁺ (41, 55, 57)
21 (3SNol)	1,428	2,085	3-sulfanylnonan-1-ol	ud[2]	0	ud[2]	0	ud[2]	0	0.17[1]	8	0.13[1]	16	sweet, green	I⁺ (41, 55, 57)
Sulfanylalkyls carrying an acetate function															
4 (2SEA)	890	1,457	2-sulfanylethyl acetate	0.58[4]	0	0.62[4]	0	1.93[3]	0	9.53[1]	8	2.54[2]	4	grilled	I⁺ (43, 60, 61)
10 (3SPrA)	1,001	1,570	3-sulfanylpropyl acetate	ud[2]	4	ud[2]	8	0.03[2]	16	0.12[1]	32	ud[2]	4	onion, burned	I⁺ (74, 43, 47)
12 (3SBA)	1,039	1,511	3-sulfanylbutyl acetate	ud[1]	0	ud[1]	0	ud[1]	0	ud[1]	8	ud[1]	0	cabbage	I⁻
14 (3SPA)	1,130	1,612	3-sulfanylpentyl acetate	1.15[2]	4	0.38[3]	16	0.43[3]	32	3.06[1]	32	1.18[2]	16	flower	I⁺ (43, 73, 102)
15 (3S2MPA)	1,202	1,674	3-sulfanyl-2-methylpentyl acetate	ud[1]	0	ud[1]	0	ud[1]	0	ud[1]	4	ud[1]	0	peach, grapefruit	I⁻
16 (3S4MPA)	1,239	1,708	3-sulfanyl-4-methylpentyl acetate	ud[3]	16	ud[3]	16	1.51[2]	16	5.41[1]	32	1.74[2]	16	broth, sweet	I⁺ (83, 116, 73)
19 (3S2BPrA)	1,327	1,820	3-sulfanyl-2-butylpropyl acetate	ud[2]	0	ud[2]	0	ud[2]	0	0.14[1]	4	0.16[1]	4	lemon, rhubarb	I
Other sulfanyl compounds															
2 (MBT)	811	1,112	1-sulfanyl-3-methyl-2-butene	ud[1]	32	ud[1]	32	ud[1]	128	ud[1]	32,768	ud[1]	32,768	cannabis, hop	I⁻
5 (IST)	904	-	2-sulfanyl-4-methoxy-2-methylbutane	0.67[1]	2,048	0.67[1]	2,048	0.67[1]	2,048	0.67[1]	2,048	0.67[1]	2,048	catty	I⁺ (45, 85, 69)
20 (-)	1,364	1,824	unknown	ud[2]	0	ud[2]	0	ud[2]	0	0.46[1]	32	0.07[2]	4	fruity, pleasant	I

[a] Assay in duplicate. All samples that do not share a common number are significantly different ($P < 0.05$) according to Tukey's test (only applied on GC-PFPD quantification). ud: Undetected.
[b] Compound identified by coincidence with GC-PFPD retention indexes and odor descriptors of pure or synthesized compounds on two capillary columns (CPSil5-CB and FFAP).
[c] I⁺, additional confirmation by mass spectroscopy (full scan monitoring). I⁻, compounds identified only with odor descriptors of pure or synthesized compounds on two capillary columns (CPSil5-CB and FFAP).
[d] Coelution with another similar odor.

under the same conditions and/or present in the NIST library. The retention indices were determined by injection onto two capillary columns (CP-Sil5-CB and FFAP-CB) connected to the FID or the olfactometric detector (identification checked by co-injection). In the case of PFPD detections (interesting for traces giving no GC-MS peak), injection of thioesters allowed translation into the alkane-related decimal numeral system.

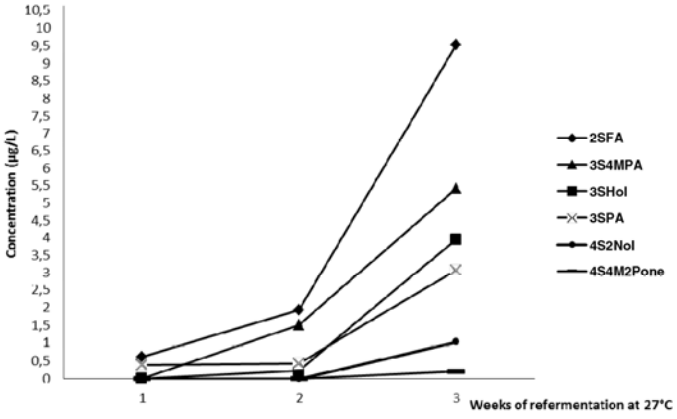

Fig. 3. Evolution of 2SEA, 3S4MPA, 3SHol, 3SPA, 4S2Nol, and 4S4M2Pone concentrations through bottle refermentation.

Quantifications

For commercially available thiols, complete calibration curves relative to the IST (internal standard) were used. For commercially unavailable thiols, quantifications are given in IST equivalents.

Statistical Analyses

Analyses were carried out in duplicate and comparison of means were performed by means of Tukey's test with SAS software version 9.2 (SAS Institute, INC., Carry, NC.) Values that do not share a common letter are significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

A commercial lager beer was subjected for 1 (A1), 2 (A2), or 3 weeks (A3) to bottle refermentation and compared to the reference (ARf) stored for 3 weeks at 27°C without yeast or added sugar. A refermentation without sugar (addition of yeast under the same conditions as for A3) was also tested (A3WS).

The efficiency of the refermentation was confirmed by a significant CO_2 increase (from 3.1 before refermentation to 5.9 g/L in A3). Ethanol increased by only 0.1% (v/v) over the same period (5.2% (v/v) in A3).

Sensorial comparisons were made between ARf and A3 between A3 and A3WS by a panel of trained expert judges. The triangular test enabled us to conclude to significant differences between ARf and A3 ($\alpha = 0.05$). On the other hand, only 30% of the tasters could perceive the difference between A3 and A3WS

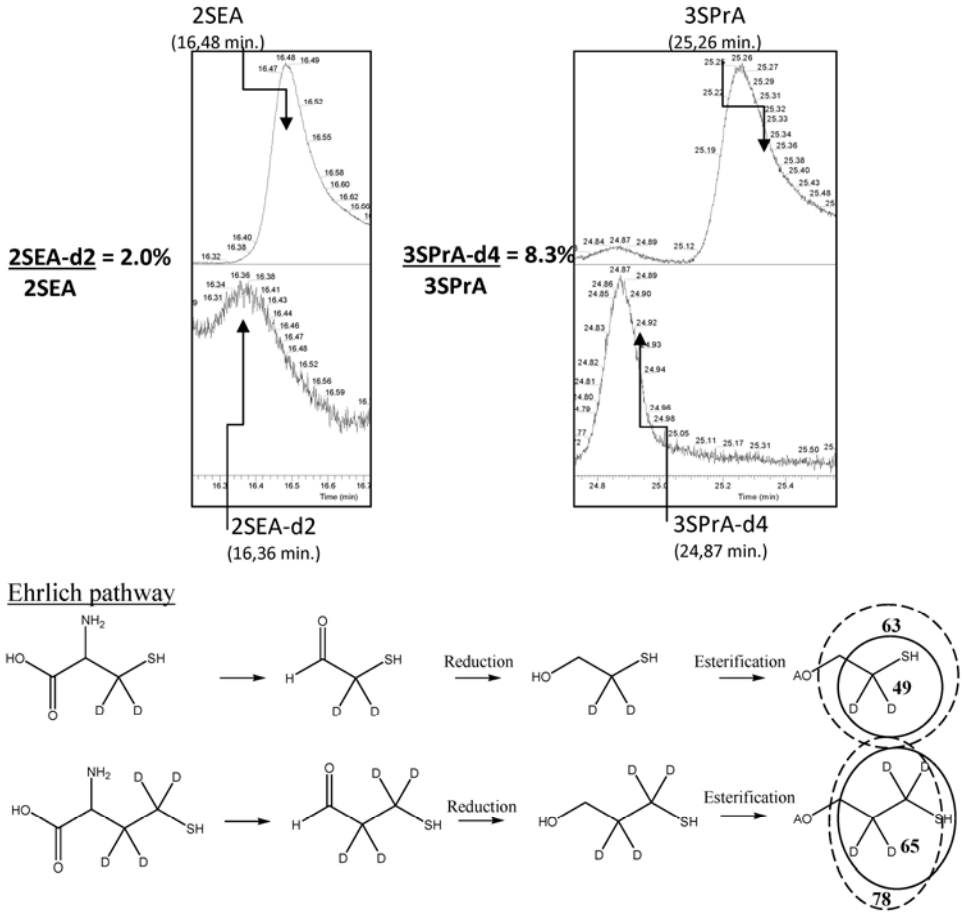

Fig. 4. Addition of 10 mg/L of L-cysteine-d2 or L-homocysteine-d4 before refermentation. SIM detection of 2SEA, 2SEA-d2, 3SPrA, and 3SPrA-d4 in beer extracts.

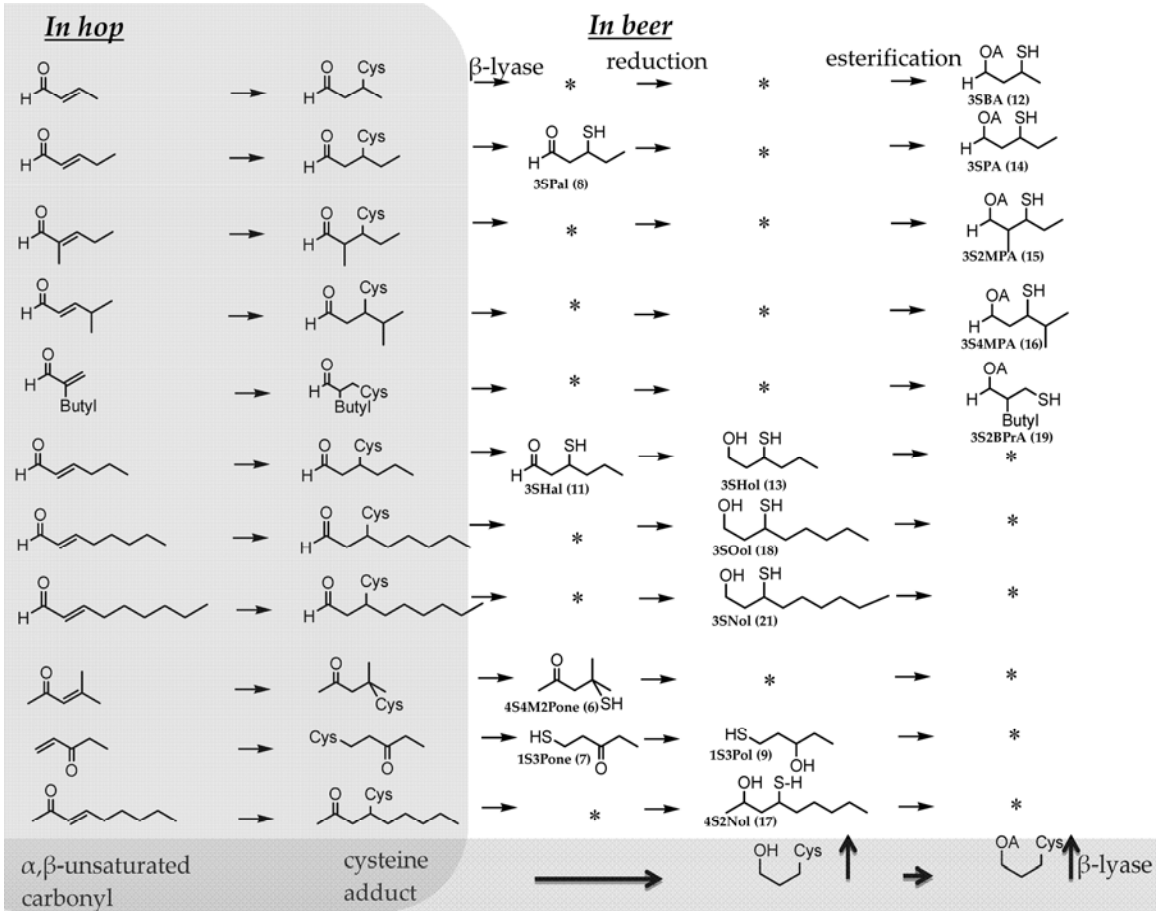

Fig. 5. Suspected cysteine adducts in hop, leading to free thiols in beer. (*) Not found in the bottle refermented beers.

(not significant). Sulfur, onion, and cabbage descriptors were strongly associated with A3 and A3WS. The refermented beer was also perceived as more fruity (red fruits) and less cardboard-like (Fig. 1).

Specific pHMB thiol extraction was applied to ARf, A1, A2, A3, and A3WS. The extracts were directly analyzed by gas chromatography coupled to a specific sulfur detector. As depicted in Table I and Fig. 2, GC-O (AEDA), GC-PFPD, and GC-MS enabled us to identify 19 thiols after 3 weeks (A3) of refermentation (these were absent or present at lower levels in the reference stored for the same period without yeast—ARf). Among them were found 5 sulfanylalkylcarbonyls, 6 sulfanylalkylalcohols, and 7 sulfanylalkylacetates. Most of them were only slightly detected after 2 weeks of refermentation, suggesting that yeast autolysis could facilitate their synthesis (Fig. 2).

Between ARf and A3, the most marked sensorial increase was observed for 1-sulfanyl-3-methyl-2-butene (n°2, MBT). MBT is known by brewers as responsible for the famous lightstruck skunky off-flavor of beers exposed to light. In this case, it comes from isohumulone photodegradation in the presence of riboflavin. Despite protection from light throughout the extraction, MBT emerged as the most odorant active volatile thiol at all stages of the refermentation process (FD = 32,768 in A3). In the absence of light, traces of MBT can be produced in the presence of hydrogen sulfide by nucleophilic substitution on 3-methyl-2-buten-1-ol (hop aglycone), leading to pleasant hoppy flavors (13,31). In hop, a similar reaction could occur with cysteine, leading to MBT-cysteine adducts.

Quantitatively, the strongest impact of refermentation was observed for 2-sulfanylethyl acetate (n°4, 2SEA), which reached up to 9.53 µg/L after refermentation (Table I and Fig. 3). Also worth stressing is the synthesis of the sweet 3-sulfanyl-4-methylpentyl acetate (n°16, 3S4MPA; 5 µg/L, FD = 32), the exotic/rhubarb-like 3-sulfanylhexanol (n°13, 3SHol; 4 µg/L, FD = 64), the flowery 3-sulfanylpentyl acetate (n°14, 3SPA; 3 µg/L, FD = 32), the fruity 4-sulfanylnonan-2-ol (n°17, 4S2Nol; 1 µg/L, FD = 32), and the box-tree-like 4-sulfanyl-4-methylpentan-2-one (n°6, 4S4M2Pone; 0.2 µg/L, FD = 128) (Table I and Fig. 3). After refermentation, the concentration of 3SHol was clearly above its threshold value, assessed at 55 ng/L in beer (9). In the same way, 0.2 µg/L of 4S4M2Pone could be significant in terms of its contribution to refermented beer aroma as its threshold is as low as 1.5 ng/L (10).

Surprisingly, sugar addition proved not to be required to produce some polyfunctional thiols, above the threshold values (e.g., 3SHol, 1 µg/L), although A3WS displayed lower concentrations than A3 (Table I).

2SEA (n°4) could arise from cysteine degradation. The efficiency through bottle refermentation of the Ehrlich pathway was confirmed by spiking 10 mg/L of L-cysteine-d2 and L-homocysteine-d4 into the bottles (Fig. 4). SIM mass spectrometry allowed us to detect labeled 2SEA and 3SPrA in the refermented spiked beers, already after 1 week.

Most of the other thiols described here-above showed the common beta-sulfanyl feature. As suggested by the recent results of Gros et al. (8), hops contain adducts issued from the addition of cysteine (or glutathione) onto α,β-unsaturated carbonyls (Fig. 5). The saturated carbonyls thus obtained could be further subjected to reduction and/or esterification, either in hop or beer. In order to assess the ability of yeast to hydrolyze cysteine adducts through bottle refermentation, S-3-(1-hydroxyhexyl)cysteine was synthe-

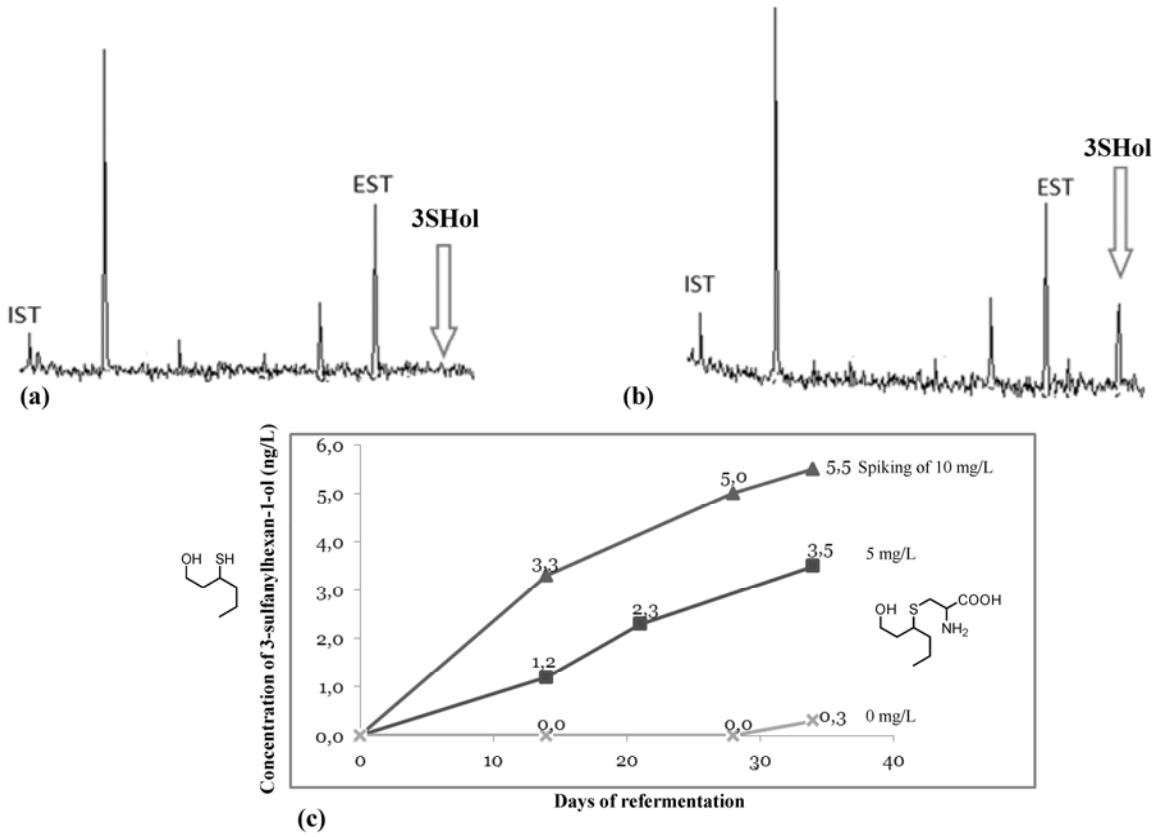

Fig. 6. PFPD detection and quantification of 3-sulfanylhexan-1-ol in refermented beers. Addition of 0 (a, c), 5 (c), or 10 (b, c) mg/L of S-3-(1-hydroxyhexyl)cysteine before bottle refermentation.

sized and spiked into the beer (Fig. 6). The spiking of 5 and 10 mg/L of S-3-(1-hydroxyhexyl)cysteine confirmed the ability of yeast to release free thiols in the bottle.

In conclusion, bottle refermentation has a strong sensorial impact by increasing the levels of many thiols, especially after 3 weeks. Better control of the refermentation process probably requires excellent control of yeast (Ehrlich pathway and β-lyase activity) and strict selection of the hop variety. Complementary experiments are in progress to determine the relative impact of each suspected formation pathway.

ABBREVIATIONS USED

*p*HMB	*p*-Hydroxymercuribenzoic acid
TRIS	Tris(hydroxymethyl)aminomethane
IST	Internal standard
EST	External standard
GC	Gas chromatography
GC-O	GC-Olfactometry
AEDA	Aroma Extract Dilution Analysis
GC-MS	GC-Mass Spectrometry
HPLC-HRMS	HPLC-High Resolution Mass Spectrometry
SIM	Single-Ion Monitoring
GC-PFPD	GC-Pulsed Flame Photometric Detector
ARf	Lager beer A, unrefermented, stored for 3 weeks
A1	Lager beer A, refermented for 1 week
A2	Lager beer A, refermented for 2 weeks
A3	Lager beer A, refermented for 3 weeks
A3WS	Lager beer A, refermented for 3 weeks without sugar
B	Lager beer B
FD	Flavor dilution factor
RI	Retention index

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