

## Occurrence of Polyfunctional Thiols in Fresh Lager Beers

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Polyfunctional thiols are known to have a strong impact on the overall aroma of many fermented foods. Surprisingly, very little data is available on their occurrence in beer. A specific extraction with *p*-hydroxymercuribenzoic acid was performed on four different fresh light-protected lager beers. Gas chromatography–olfactometry, gas chromatography–mass spectrometry, and gas chromatography–pulsed-flame photometer detector analyses of the extracts revealed the presence of more than 10 polyfunctional thiols. All of them were absent from wort, suggesting a key role of the H<sub>2</sub>S excreted by yeasts. 3-Methyl-2-buten-1-thiol, 2-mercapto-3-methylbutanol, 3-mercapto-3-methylbutanol seem to be created from hop allylic alcohols via four different mechanisms: nucleophilic substitution, addition–elimination, and radical anti-Markovnikov or electrophilic Markovnikov additions. 1,4 Addition of hydrogen sulfide to wort  $\alpha,\beta$ -unsaturated aldehydes or ketones may explain the synthesis of 1-mercapto-3-pentanol, 3-mercaptohexanol, and 4-mercapto-4-methyl-2-pentanone through fermentation. Finally, 2-mercaptoethanol, 3-mercaptoopropanol, and their corresponding acetates may derive from Ehrlich degradation of sulfur amino acids, while 2-methyl-3-furanthiol should be logically issued from Maillard reactions.

**KEYWORDS:** Polyfunctional thiols; sulfur compounds; beer; hop; formation pathways

### INTRODUCTION

Beer is a very complex matrix in which more than 620 volatile compounds have already been identified (1). Among them, ethanol (1), 2-phenylethanol (2, 3), 3-methylbutanol (3), 2-methylbutanoic acid (1), 3-methylbutanoic acid (1), isoamyl acetate (4, 5), ethyl 2-methylpropanoate (1), ethyl butanoate (1, 3), ethyl 4-methylpentanoate (1), ethyl hexanoate (3), ethyl octanoate (1), acetaldehyde (1), (*E*)- $\beta$ -damascenone (1), 2'-*o*-aminoacetophenone (2), furaneol (1, 3), linalool (1, 4, 5), and 4-vinylguaicol (3) have emerged as key flavors. Thiols are also most likely strongly involved in the flavor of fresh beer, as demonstrated by Vermeulen et al. (6) simply by adding copper to samples. This methodology is usually used to assess the organoleptic role of thiols because copper is able to trap them specifically into odorless chemical complexes.

Surprisingly, in contrast to other yeast-fermented beverages such as wine, only six polyfunctional thiols have been mentioned in the brewing literature (7). Olsen et al. detected 2-mercapto-3-methylbutanol in a Finnish brand characterized by an intense “onion-like” off flavor and suggested that it likely came from hop (8). Hop isohumulone degradation is the main source of

the well-known lightstruck off flavor imparted by 3-methyl-2-buten-1-thiol (MBT). At higher concentrations, this compound exhibits a repulsive “skunky” odor (9), while at lower concentrations, it may be appreciated by consumers (10, 11). Another type of beer sensorial defect was reported by Cosser et al. in 1980. It was associated with 4-mercapto-4-methyl-2-pentanone inducing a strong and deleterious “ribes, catty” aroma. Its extraordinarily high concentration was due to a barley contamination with mesityl oxide, a constituent of the malthouse paint (12). Later, Schieberle found a similar odor in pale lager beer after aging (flavor dilution value of <1 in fresh beer and =32 in stored beer). In that case, 3-mercapto-3-methylbutyl-formate was incriminated (3). Finally, 2-methyl-3-furanthiol (4, 5) and 3-mercaptohexanol (6) are invariably present in fresh lager beers, although their amount and organoleptic impact seem to be quite limited.

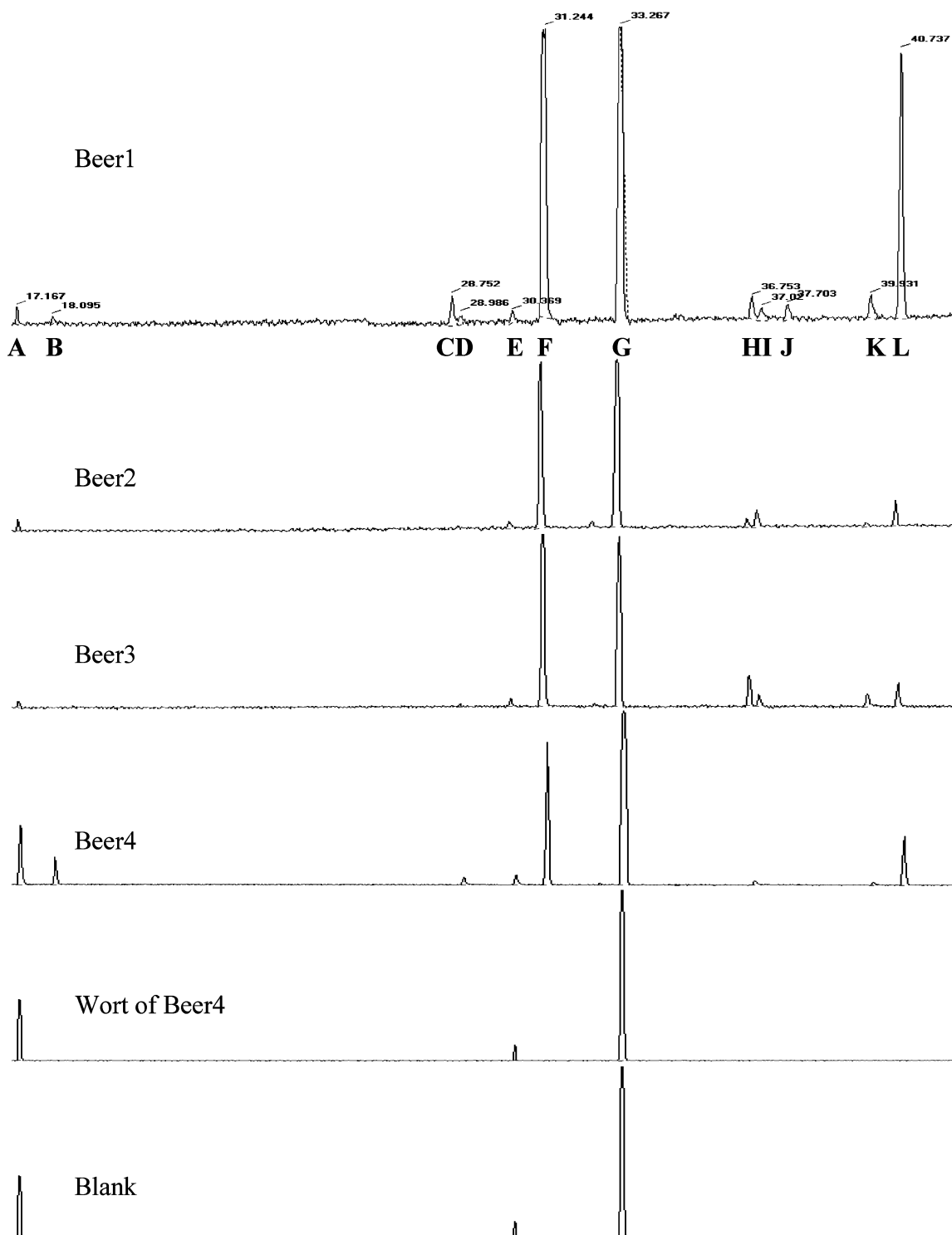
Because yeasts used in wine production play an important role in thiol formation (e.g., enzymatic transformation of *S*-cysteinylated molecules into thiols (13–15), excretion of H<sub>2</sub>S during fermentation), we were interested in knowing whether these sulfur compounds are also naturally present in beer. Therefore, we performed specific thiol extraction with *p*-hydroxymercuribenzoic acid (*p*HMB) (16) to isolate new potential aroma-impact compounds. Using our combinatorial library data (17–21) and various gas chromatographic detectors [human nose, pulsed-flame photometer detector (PFPD) (22, 23), and mass spectrometry (MS)], we identified thiols on the basis of their odor, their retention indices on CP-Sil 5 CB and FFAP capillary columns, as well as their mass spectrum when

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**Figure 1.** GC-PFPD chromatograms of *p*HMB extracts obtained from four different beers, a wort, and a blank (A, EST; B, 2-mercaptoethanol; C, 3-mercaptoopropanol; D, 2-methyl-3-furanthiol; E, unidentified impurity; F, 2-mercaptoethyl acetate; G, IST; H, 3-mercapto-3-methylbutanol; I, 1-mercapto-3-pentanone; J, 2-mercapto-3-methylbutanol; K, 1-mercapto-3-pentanone; L, 3-mercapto-propyl acetate).

possible. Hypothetical formation pathways were suggested and partially validated by experiments conducted in model media.

## MATERIALS AND METHODS

**Materials.** Hydrochloride L-cysteine monohydrated, *p*-hydroxymercuribenzoic acid, and 37% HCl were provided by Aldrich Chemicals (Bornem, Belgium). Tris(hydroxymethyl)aminomethane and sodium acetate were supplied by USB (Cleveland, OH) and UCB (Bruxelles, Belgium), respectively. NaOH and 99% Na<sub>2</sub>SO<sub>4</sub> were supplied by

Janssen (Geel, Belgium). Dichloromethane (99.9%) from Romil (Cambridge, U.K.) was distilled before use.

Four different commercial lager beers (beer 1 from France, beer 2 and beer 4 from Belgium, and beer 3 from Germany) were bought in a supermarket and stored for only a few days at 4 °C before extraction. The wort of beer 4 was purchased from the brewery.

**Extraction of Polyfunctional Thiols by *p*HMB (16).** Beer or wort (500 mL) and distilled CH<sub>2</sub>Cl<sub>2</sub> (200 mL) were stirred for 30 min. After decantation (±15 min), the lower phase and the interfacial emulsion were centrifuged for 20 min at 10 000 rpm. The organic phase was

**Table 1.** Polyfunctional Thiols Identified in Beer and Their Potential Formation Pathways<sup>a</sup>

molecule retention indexes odor	identified by GC (peak label on Figures 1 and 2, respectively) <sup>b</sup>	estimated concentration in beer given in IST equivalent (ppt) (mean of the standard deviations = 6.33%)				hypothetical formation pathway
		beer 1	beer 2	beer 3	beer 4	
3-methyl-2-buten-1-thiol $I_{CP-Sil\ 5\ CB} = 808$ , $I_{FFAP} = 1112$ skunky, beer, pungent, plastic	O	traces	traces	traces	traces	nucleophilic substitution of 3-methyl-2-buten-1-ol by H <sub>2</sub> S or the addition of H <sub>2</sub> S + H <sub>2</sub> O elimination on 2-methyl-3-buten-2-ol (Figures 3 and 4)
3-mercapto-3-methylbutanol $I_{CP-Sil\ 5\ CB} = 944$ , $I_{FFAP} = 1671$ broth, onion, sweat	O/PFPD-[H]/MS	115	36	82	354	electrophilic addition of H <sub>2</sub> S on the 3-methyl-2-buten-1-ol double bond (Figure 3)
2-mercapto-3-methylbutanol $I_{CP-Sil\ 5\ CB} = 964$ , $I_{FFAP} = 1656$ onion, groundnut, sulfur	O/PFPD-[J]/MS	76	traces	traces	traces	radical addition of H <sub>2</sub> S on the 3-methyl-2-buten-1-ol double bond (Figure 3)
4-mercapto-4-methyl-2-pentanone $I_{CP-Sil\ 5\ CB} = 915$ , $I_{FFAP} = 1382$ blackcurrant, catty	O	traces	traces	traces	traces	biolysis of cysteine conjugate (S-4-[4-methylpentan-2-one]-L-cysteine) or Michael addition of H <sub>2</sub> S on mesityl oxide (Figure 5)
1-mercapto-3-pentanone $I_{CP-Sil\ 5\ CB} = 947$ , $I_{FFAP} = 1517$ greenery	PFPD-[I] <sup>c</sup>	90	traces	179	128	biolysis of cysteine conjugate (S-1-[3-pentanone]-L-cysteine) or Michael addition of H <sub>2</sub> S on 1-penten-3-one (Figure 5)
1-mercapto-3-pentanol $I_{CP-Sil\ 5\ CB} = 981$ , $I_{FFAP} = 1698$ stinging nettle, burnt	O/PFPD-[K]/MS	153	20	29	157	reduction of the 1-mercapto-3-pentanol (Figure 5)
3-mercaptohexanol $I_{CP-Sil\ 5\ CB} = 1095$ , $I_{FFAP} = 1853$ rhubarb	O/PFPD	traces	traces	traces	traces	biolysis of cysteine conjugate (S-3-[hexan-1-ol]-L-cysteine) or Michael addition of H <sub>2</sub> S on (E)-2-hexenal followed by a reduction (Figure 5)
2-methyl-3-furanthiol $I_{CP-Sil\ 5\ CB} = 847$ , $I_{FFAP} = 1306$ broth, meaty	O/PFPD-[D]/MS	161	13	traces	traces	reaction between H <sub>2</sub> S and products arising from sugar thermal degradations
2-mercaptoethanol $I_{CP-Sil\ 5\ CB} = 717$ , $I_{FFAP} = 1501$ roasted, sewage	O/PFPD-[B]	32	184	traces	traces	enzymatic transformation of cysteine via the Ehrlich pathway followed by a bioreduction (Figure 6)
3-mercaptoopropanol $I_{CP-Sil\ 5\ CB} = 840$ , $I_{FFAP} = 1665$ roasted, broth, potato	O/PFPD-[C]	57	78	traces	16	enzymatic transformation of homocysteine via the Ehrlich pathway followed by a bioreduction
2-mercaptoethyl acetate $I_{CP-Sil\ 5\ CB} = 887$ , $I_{FFAP} = 1444$ roasted	O/PFPD-[F]/MS	2503	1236	2094	2412	esterification of the 2-mercaptoethanol (Figure 6)
3-mercapropyl acetate $I_{CP-Sil\ 5\ CB} = 992$ , $I_{FFAP} = 1565$ roasted	O/PFPD-[L]/MS	1622	407	240	240	esterification of the 3-mercaptoopropanol (Figure 6)

<sup>a</sup>  $I_{CP-Sil\ 5\ CB}$  or  $I_{FFAP}$  are respectively the retention index (RI) on the apolar CP-Sil 5 CB or polar FFAP capillary columns (see the Analytical Methods for more details).

<sup>b</sup> GC-O, identified by olfactometry on two different columns (CP-Sil 5 CB and FFAP) by a comparison of odor and RIs corresponding to commercial or synthetic standards (17–21); GC-MS, identified by mass spectrometry on a CP-Sil 5 CB MS capillary column by a comparison of RI and mass spectra (Figure 2) with commercial or synthetic standards (17–21); GC-PFPD, presence of sulfur confirmed in the suspected molecules. <sup>c</sup> Tentatively identified, considering the RI of the compound present in our extract and the RI of a synthetic standard on CP-Sil 5 CB.

then extracted by 2 × 20 mL of a *p*HMB solution (360 mg of *p*HMB and 24.6 g of Tris in 1 L of Millipore water) for 5 and 10 min, respectively. The combined aqueous phases were loaded into a strongly basic anion-exchanger column (Dowex 1WX2-100 resin from Aldrich Chemicals), washed beforehand by 2 M NaOH and 2 M HCl, and in between rinsed by ultrapure water. Then, 50 mL of 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 hydrochloride L-cysteine monohydrated in 60 mL of Millipore water; this solution was washed with 2 × 5 mL of distilled CH<sub>2</sub>Cl<sub>2</sub> before use). The eluate containing the volatile thiols was collected and extracted by 4 and then 3 mL of distilled CH<sub>2</sub>Cl<sub>2</sub> using magnetic stirring (5 min). The organic phases were pooled, dried on anhydrous Na<sub>2</sub>SO<sub>4</sub>, and finally concentrated under nitrogen to 70 μL to be stored at -80 °C before gas chromatography (GC) analysis. Note that 99.5% 4-methoxy-4-methyl-2-butanethiol (Oxford Chemicals), added at the first extraction step, and 99% thiazole (Aldrich Chemicals), added before the final concentration step, were used respectively as internal and external standards (IST and EST).

A blank was obtained by performing the extraction (adding of IST and EST included) without any matrix. This revealed the presence of only one impurity (peak E in Figure 1).

**Syntheses in Model Media.** First, a water/ethanol model medium (50 mL, 5% ethanol) was adjusted to pH 4.2 by a 0.2 M acetate buffer, saturated with a continuous flow of H<sub>2</sub>S, and spiked with catalytic traces of FeS. Then, 100 ppm of 3-methyl-2-buten-1-ol or 2-methyl-3-buten-2-ol was added. After 3 h, 5 mL of the reaction mixture was filtered and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 1 mL). The organic phases were concentrated under nitrogen to 70 μL.

**Analytical Methods.** Gas Chromatography Hyphenated to a Sniffing Port (GC-O) or to a Flame Ionization Detector (GC-FID). This was performed using a Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250 °C and opened after 0.5 min. Sulfur compounds were analyzed on a 50 m × 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-Sil 5 CB capillary column (film thickness of 1.2 μm) or a 25 m × 0.32 mm i.d., WCOT fused silica polar FFAP CB capillary column (film thickness of 0.3 μm). The oven temperature, initially set and maintained for 4 min at 40 °C, was then programmed to rise from 40 to 132 °C at 2 °C/min and then from 132 to 250 °C at 10 °C/min. The final temperature was held for 15 min. The eluent was sent to a GC-sniffing port (250 °C) and diluted with a large volume of humidified air (20 mL/min) to improve the transport of the effluent out of the funnel (24). Sensorial analyses (odor detection and description) were performed by at least two judges

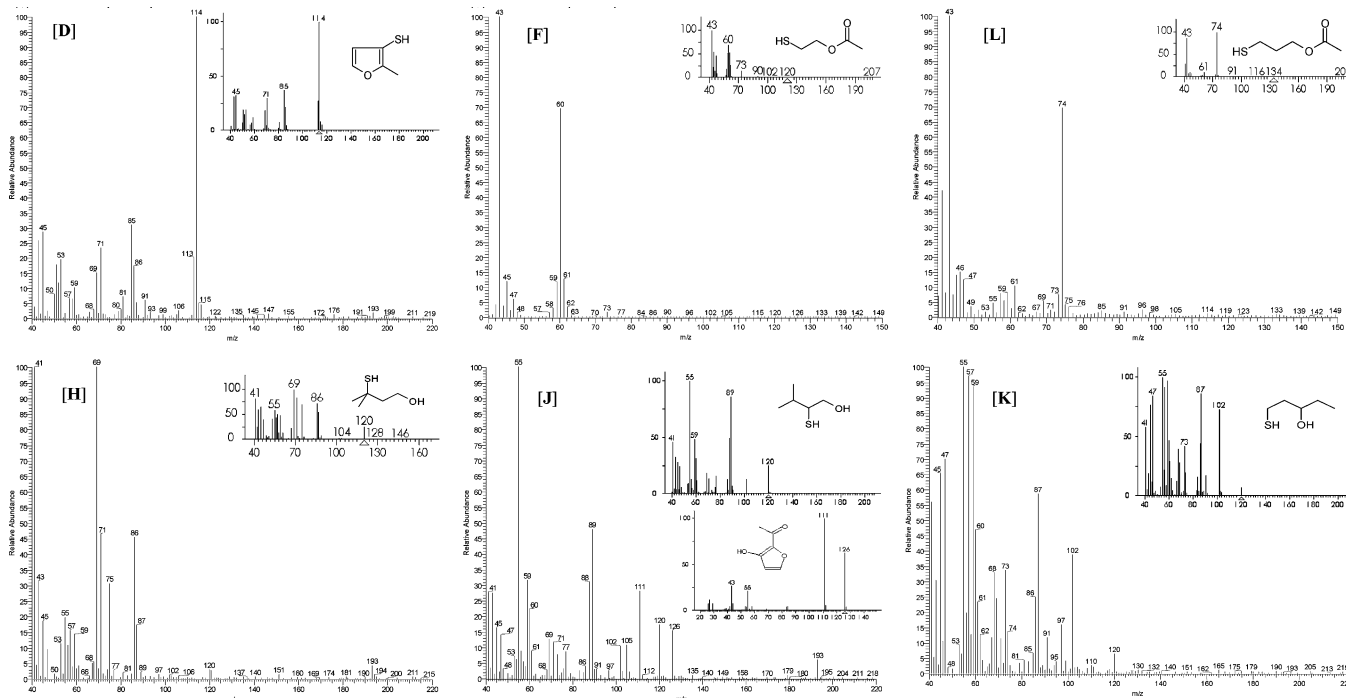


Figure 2. MS results observed for six thiols in the pHMB beer extracts in comparison with their synthesized analogues (little spectra).

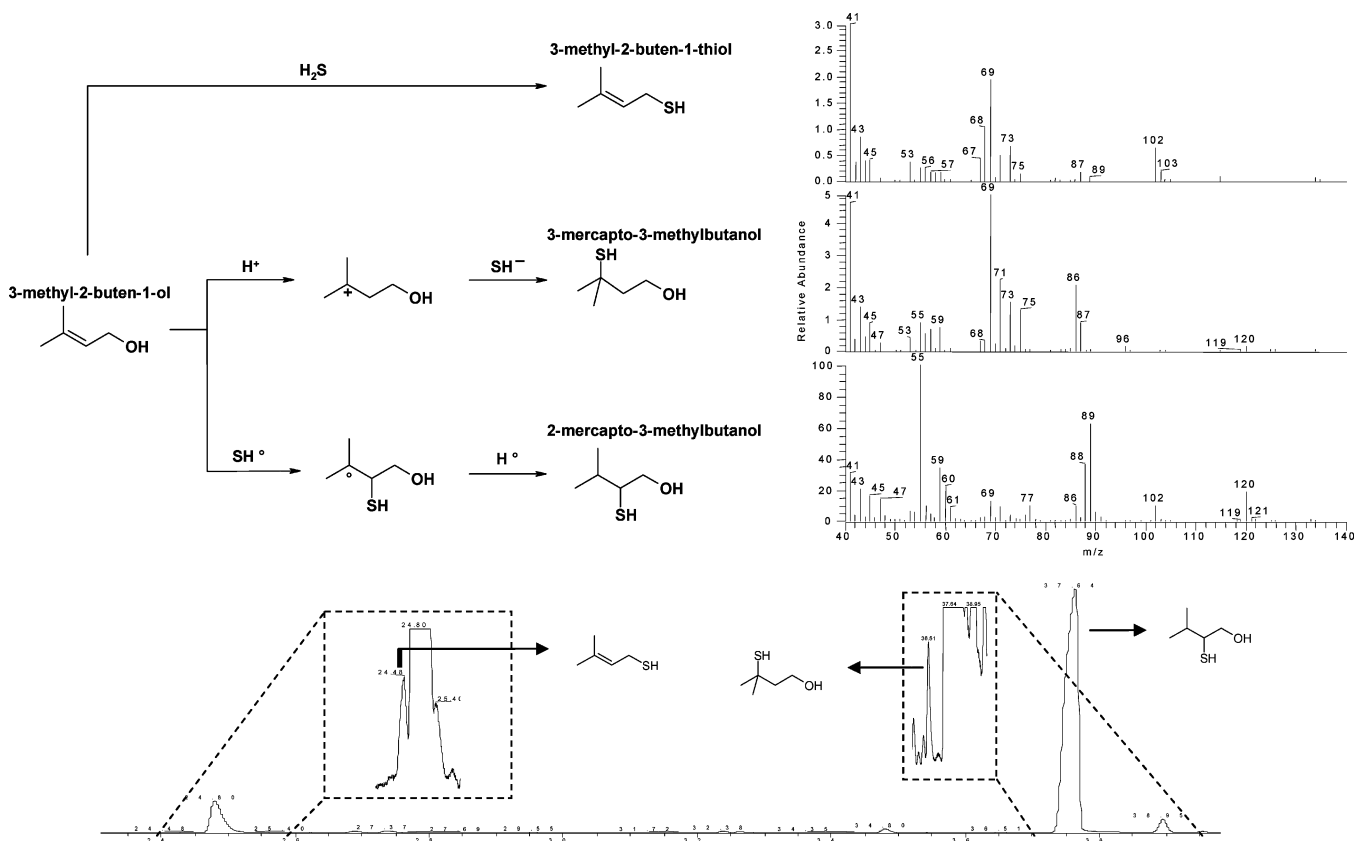


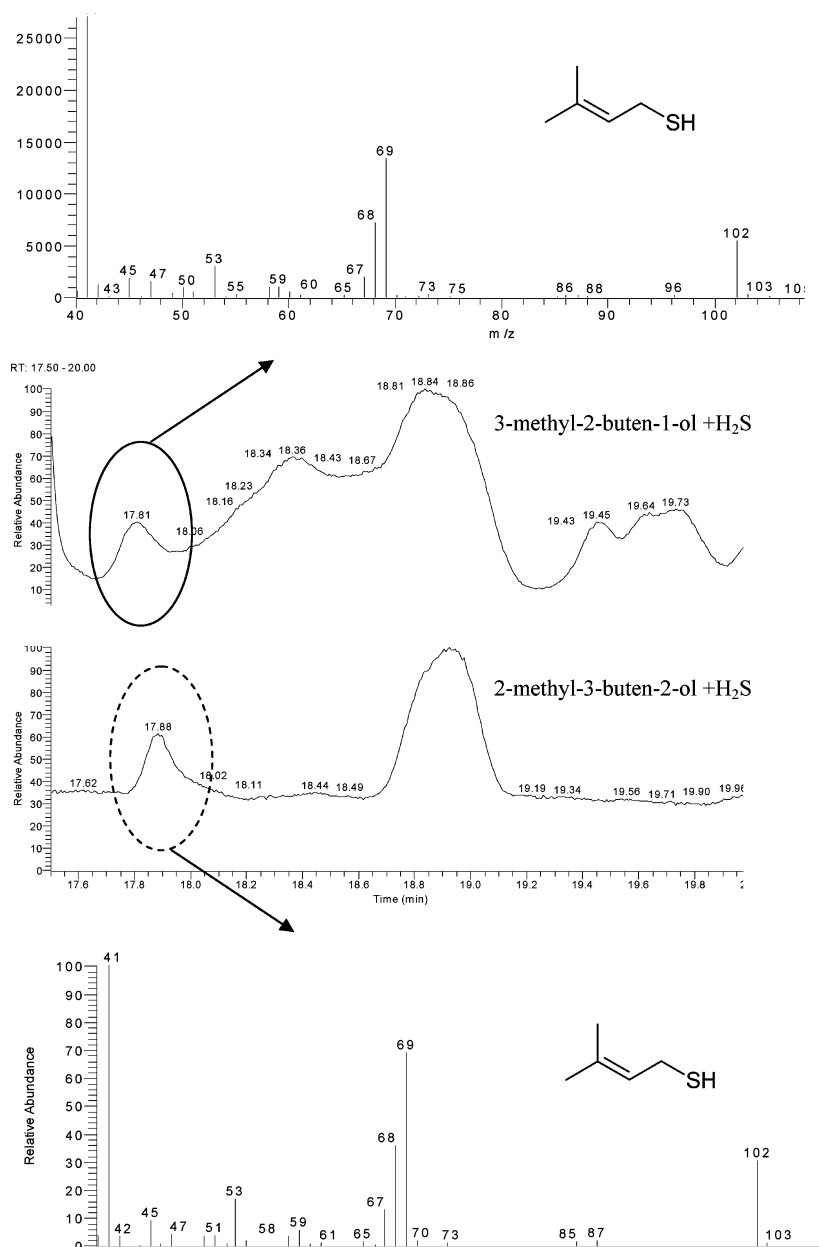
Figure 3. MS data from the Fe-catalyzed reaction between  $H_2S$  and 3-methyl-2-buten-1-ol and hypothetical formation pathway of MBT, 3-mercapto-3-methylbutanol, and 2-mercapto-3-methylbutanol in beer.

working independently. Retention indices were estimated by connecting the column to a FID maintained at 250 °C.

**Gas Chromatography Hyphenated to an Electronic Impact Mass Spectrometer (GC-MS).** Mass spectra ( $m/z$  40–380) 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 a 50 m × 0.32 mm i.d., WCOT apolar CP-Sil 5 CB MS capillary column (film thickness of 1.2 μm). The oven

temperature program was the same as that described for GC-FID. The spectral recording was automatic throughout elution; Xcalibur software was used.

**Gas Chromatography Hyphenated to a Pulsed-Flame Photometric Detector (GC-PFPD).** The injections were carried out in the splitless mode at 250 °C, with the split being turned on after 0.5 min. 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



**Figure 4.** MS data from the Fe-catalyzed reaction between H<sub>2</sub>S and 2-methyl-3-buten-2-ol compared with the H<sub>2</sub>S/3-methyl-2-buten-1-ol one.

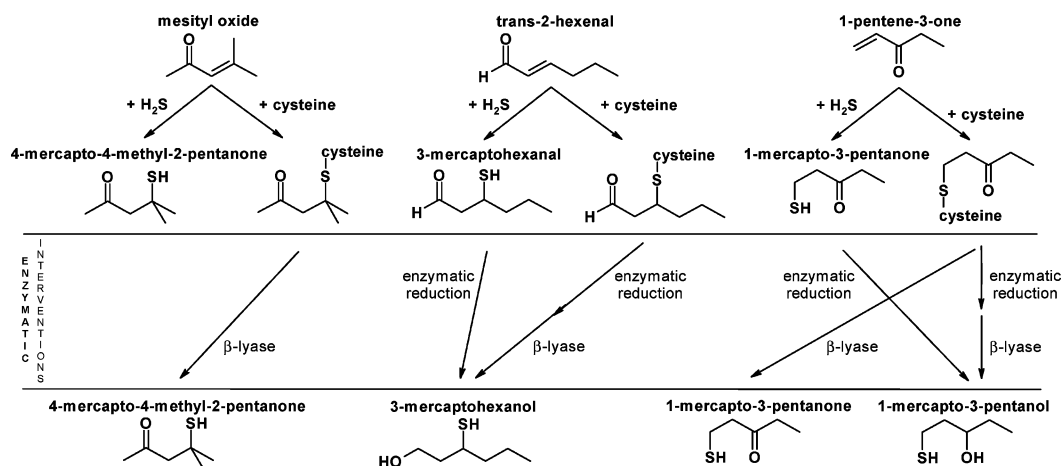
the gate delay, 600 mV as the trigger level, and 3.45 Hz as the pulse frequency. The chromatographic conditions were the same as for MS detection, but the last rate was set at 5 °C/min.

## RESULTS AND DISCUSSION

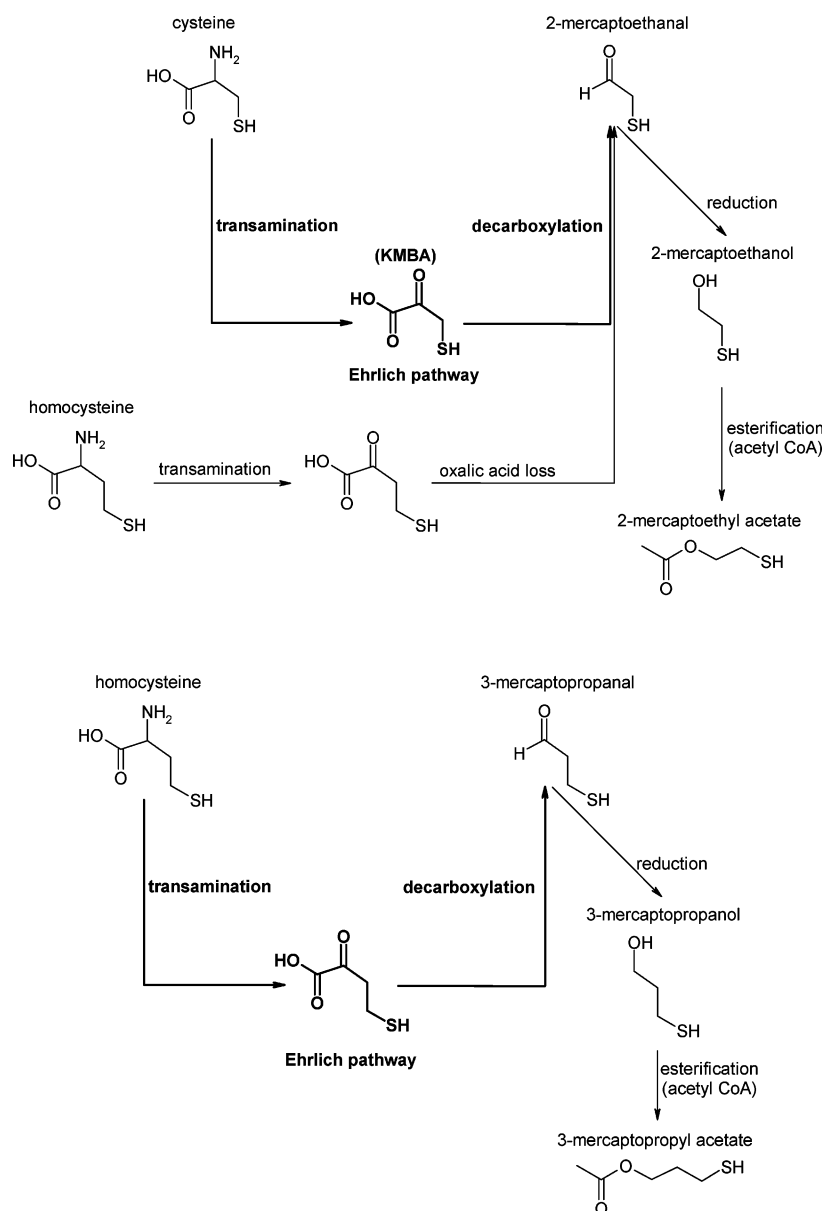
A total of 12 polyfunctional thiols were found in the 4 different fresh lager beers (Table 1). As depicted in Figure 1, GC-PFPD enabled us to detect 8 of them in the pHMB extracts [i.e., 2-mercaptoethanol (B), 3-mercaptopropanol (C), 2-methyl-3-furanthiol (D), 2-mercaptoethyl acetate (F), 3-mercapto-3-methylbutanol (H), 1-mercapto-3-pentanone (I), 2-mercapto-3-methylbutanol (J), 1-mercapto-3-pentanol (K), and 3-mercapto-propyl acetate (L)]. It was even possible to identify 6 of them by GC-MS (Figure 2). In addition, GC-O revealed the presence of 3 more compounds: 3-methyl-2-buten-1-thiol (odor: skunky), 4-mercapto-4-methyl-2-pentanone (odor: black-currant, catty), and 3-mercaptohexanol (odor: rhubarb, fruity). Among all of these compounds, only 2-methyl-3-furanthiol, 3-methyl-2-buten-1-thiol, 3-mercaptohexanol, 2-mercapto-3-methylbutanol, and 4-mercapto-4-methyl-2-pentanone have

previously been described in the literature as natural beer constituents (see the Introduction). None of them were found by GC-O or GC-PFPD in the pHMB wort extract, suggesting a key role of fermentation (Figure 1).

Because the analytical detectors (PFPD and MS) are not sensitive enough and because their perception threshold is very low, 3-methyl-2-buten-1-thiol (odor: skunky, beer, pungent, plastic) and another very powerful odorant (Ir<sub>CP-Sil 5 CB</sub> = 906) perfectly mimicking its skunky note were detected only by GC-O. Well-known to be photochemically synthesized from hop iso- $\alpha$ -acids under light (25), MBT seems also to be very important in light-protected beer extracts (4, 6). Because H<sub>2</sub>S is a very good nucleophile, we suspected 3-methyl-2-buten-1-ol [hop component (26)] to be its major precursor in the absence of light. To validate this hypothesis, we used a water/ethanol model medium set at pH 4.2 by a 0.2 M acetate buffer, saturated with a continuous flow of H<sub>2</sub>S, and spiked with catalytic traces of FeS and 3-methyl-2-buten-1-ol (100 ppm). As expected, GC-MS analysis of the mixture after 3 h revealed the presence of MBT. However, two other compounds [3-mercapto-3-meth-



**Figure 5.** Hypothetical formation pathways of 4-mercapto-4-methyl-2-pentanone, 3-mercaptohexanol, 1-mercapto-3-pentanone, and 1-mercapto-3-pentanol from  $\alpha,\beta$ -unsaturated ketones or aldehydes.














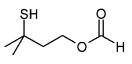
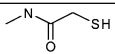
**Figure 6.** Hypothetical formation pathway of 2-mercaptoethanol, 3-mercaptoopropanol, and their corresponding acetates.

ylbutanol (H) and 2-mercapto-3-methylbutanol (J)] were also created (Figure 3). Hydrogen sulfide was thus able to induce three different kinds of reactions involving this allylic alcohol:

nucleophilic substitution, electrophilic addition (Markovnikov), and radical addition (anti-Markovnikov), leading respectively to MBT and to two mercapto alcohols (Figure 3). Note that



Table 2. Thiols Already Identified in Beer while Absent in Our pHMB Extracts

Name	Structure	Perception threshold in beer	Odor	Reference
<b>Linear thiols</b>				
Methanethiol	CH <sub>3</sub> -SH	2 ppb	Putrid, rotten egg, cabbage, drains, ester	(35)
Ethanethiol		1.7 ppb	Putrefaction, leek, onion, garlic, egg	(35)
Propanethiol		0.15 ppb	Putrefaction, onion, garlic, egg	(36)
Butanethiol		0.7 ppb	Putrefaction, onion, garlic, egg	(37)
<b>Branched thiols</b>				
2-Butanethiol		0.6 ppb	Putrefaction, onion, garlic, cheese, fish, egg	(37)
2-Methylpropanethiol		2.5 ppb	Putrefaction, onion, garlic, egg	(37)
2-Methyl-2-propanethiol		80 ppt	Unpleasant	(38)
2-Methylbutanethiol		2-3 ppb	-	(35)
2-Methyl-2-butanethiol		80 ppt	Putrefaction, onion, garlic, egg	(37)
s-Isoamylthiol		0.2 ppb	Putrefaction, onion, garlic, egg	(37)
t-Pentanethiol		70 ppt	Putrefaction, guava, peach, fish, catty	(37)
<b>Unsaturated thiols</b>				
Allylthiol		-	Garlic	(38)
<b>Mercaptoesters</b>				
3-Mercapto-3-methylbutylformate		-	Blackcurrant	(3)
<b>Mercaptoacetamide</b>				
N-Methylmercaptoacetamide		-	Sweat, sulfur	(5)

3-mercapto-3-methylbutanol (odor: broth, onion, sweat), already well-known in wine (16), has never been identified before in beer, in contrast to its corresponding formic ester, detected by Schieberle in an aged beer (3). The occurrence of 2-mercapto-3-methylbutanol [odor: onion, groundnut, sulfur; co-eluent of isomaltol on the CP-Sil 5 CB column (Figure 2)] may depend upon the antioxidant activity of the wort, explaining why this thiol seems to be more concentrated in beer 4 (Figure 1 and Table 1).

An additional MBT formation pathway involving another hop alcohol was considered. In this case, a combined H<sub>2</sub>S addition-H<sub>2</sub>O elimination reaction was expected on 2-methyl-3-buten-2-ol [another hop component (26)]. As depicted in Figure 4, this synthesis is quite efficient compared to the previous one involving 3-methyl-2-buten-1-ol and may also naturally occur in beer.

1,4 Addition of hydrogen sulfide to wort  $\alpha,\beta$ -unsaturated aldehydes or ketones is most likely another synthesis pathway of polyfunctional thiols, as indicated by the presence of 4-mercapto-4-methyl-2-pentanone, 1-mercapto-3-pentanone (I), 1-mercapto-3-pentanol (K), and 3-mercaptohexanol (Table 1). Via this reaction, these mercaptans should come respectively from mesityl oxide [hop (26) and beer (27) components], 1-penten-3-one [beer (27) component], and (*E*)-hexenal [barley (28), hop (26), and beer (29) components] (Figure 5). However,

biolysis of cysteine conjugates by yeast, as in Sauvignon wines, cannot be excluded (13–15).

2-Methyl-3-furanthiol (D), characterized by its typical odor of meat broth, was also present in beer samples. Its full-scan mass spectrum recorded in the pHMB extract confirmed our identification (Figure 2). The temperatures reached during mashing and boiling easily explain how such a Maillard product can be synthesized (30, 31).

Finally, Ehrlich degradation of sulfur amino acids (Figure 6 inspired from refs 32–34) is most likely involved in the synthesis of 2-mercaptoethanol [odor: roasted, sewage (B)] and 3-mercaptoopropanol [odor: roasted, broth, potato (C)], both identified by GC–O and GC–PFPD (Figure 1). Their corresponding acetates [odor: roasted (F and L)] were also detected by full-scan MS (Figure 2). Among all mercaptans, 2-mercaptoethyl acetate emerged as the most concentrated (Figure 1 and Table 1). An alternative pathway could be semisubstitution on diols such as 1,2-ethanediol or 1,3-propanediol by H<sub>2</sub>S. However, experiments conducted in model media indicated that this chemical reaction is not favored in beer.

A total of 14 thiols, expected to be found in beer according to the literature (Table 2), were absent in our pHMB extracts.

In the future, the hypothetical formation pathways described above will be checked by spiking the wort with labeled precursors. Sensorial analyses will also be performed to

determine the most relevant polyfunctional thiols in beer and its hedonic properties.

#### ABBREVIATIONS USED

EST, external standard; FID, flame ionization detector; GC, gas chromatography; IST, internal standard; MBT, 3-methyl-2-buten-1-thiol; MS, mass spectrometry; O, olfactometry; PFPD, pulse flame photometric detector; *p*HMB, *p*-hydroxymercuribenzoic acid.

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