

# Malt and Hop as Sources of Thiol S-Conjugates: Thiol-Releasing Property of Lager Yeast during Fermentation

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**ABSTRACT:** The contribution of polyfunctional thiols (PFTs) to the overall flavor of a lager beer has been well documented, but their origin remains unclear. In comparison with *Saccharomyces cerevisiae* strains previously investigated (same conditions: 7 days at 24 °C, 3 days at 4 °C), we examined how *Saccharomyces pastorianus* yeasts are able to produce these PFTs from cysteinylated (Cys-) and glutathionylated (G-) conjugates. Up to 0.35% release was observed from G-conjugates against less than 0.08% for all yeasts studied so far. Lowering the wort nitrogen level and the fermentation temperature (12 °C) strongly increased the release efficiency and the ester/alcohol ratio from Cys-conjugates. However, it did not improve the release from G-conjugates and even prevented the yeast from producing any acetate. Although poor in free PFTs and their Cys-precursors, both Saaz hop and pale malt (classical ingredients for premium lager beers) confirmed to be significant sources of G-conjugates (especially those of 3-sulfanylhexanol and 3-sulfanyl-4-methylpentanol).

**KEYWORDS:** polyfunctional thiols, fermentation, glutathione conjugates, *Saccharomyces pastorianus*

## INTRODUCTION

Despite the big leap of craft beers, premium lager beers remain topmost in worldwide production. Their drinkability results from a delicate balance between malt-derived smoothness, some fruity flavors issued from yeast esters, dimethylsulfide, and noble hop aromas obtained by late hopping in the wort kettle. Many have tried to identify the key contributors to that complex kettle aroma issued from low-bitter hop varieties.<sup>1–3</sup> The typical spicy/herbal descriptor pointed out in beers brewed with Saaz could be brought by oxygenated sesquiterpenoids,<sup>4</sup> while linalool,  $\beta$ -ionone,  $\beta$ -damascenone, and some esters and aldehydes have been identified as responsible of their citrus, floral, fruity, and green notes.<sup>3,5</sup> Along with Lublin and Styrie, the noble hop Saaz also distinguishes itself from many other cultivars by higher levels of bergamotene (50 mg·kg<sup>-1</sup> in fresh hops, stable through aging) and farnesene (800 mg·kg<sup>-1</sup> in fresh hops, unstable through aging).<sup>6</sup> As compared to bitter varieties, Saaz also appears poor in sulfur aroma compounds, especially thioesters and polysulfides responsible for the aggressive cheesy and sulfury notes displayed by some beers (e.g., Challenger-derived beers).

In 2006, Vermeulen et al. and Kishimoto et al. pointed out the key role of polyfunctional thiols (PFTs) in commercial lager beers made with Saaz.<sup>5,8</sup> Even at trace levels, these compounds, already identified as key contributors to flavors of various foods and beverages,<sup>9,10</sup> proved to influence lager beer aroma. This was confirmed by sensory analysis of lager beers spiked or not with copper ions.<sup>11</sup> No PFTs were present in the wort, suggesting that yeast produced them. At that time, no thiol precursors had been evidenced in hop or malt.

In the past decade, the organoleptic role played by PFTs has been well documented in late- and dry-hopped beers,

especially when dual hops are used (Figure 1).<sup>12–14</sup> Among these compounds, 3-sulfanylhexanol (3SHol; grapefruit, rhubarb), 3-sulfanyl-4-methylpentanol (3S4MPol; passion fruit), and their corresponding acetates [3-sulfanylhexyl acetate (3SHA) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA); passion fruit] are usually found above their sensory thresholds (55,<sup>5</sup> 70,<sup>15</sup> 5,<sup>16</sup> and 160<sup>15</sup> ng·L<sup>-1</sup>, respectively). 3-Sulfanylpentanol (3SPol; citrus), found at lower levels and in fewer hop samples, exhibits a much higher threshold (620 ng·L<sup>-1</sup>).<sup>17</sup> The Saaz-free PFT profile can be described as poor, as compared to those of dual-purpose varieties.<sup>15,18</sup>

Today, we know that the major part of the hop thiol potential consists of cysteinylated and glutathionylated conjugates (Cys- and G-conjugates). G-3SHol and G-3SPol are ubiquitous and represent the majority of these thiol pools in hops, with surprisingly just as much in Saaz as in dual-purpose cultivars (95.7 and 2.5 mg·kg<sup>-1</sup> G-3SHol and G-3SPol, respectively, in Saaz vs 20.1–118.2 and 1.4–9.8 mg·kg<sup>-1</sup> in dual hops). G-3S4MPol, on the other hand, appears peculiar to some varieties (e.g., Hallertau Blanc and Polaris).<sup>19</sup> As for Cys-conjugates, up to 1.9 mg·kg<sup>-1</sup> Cys-3SHol, 0.2 mg·kg<sup>-1</sup> Cys-3SPol, and 65  $\mu$ g·kg<sup>-1</sup> Cys-3S4MPol have been evidenced in hops (only 0.5 mg·kg<sup>-1</sup> Cys-3SHol in Saaz).<sup>19–21</sup>

Several *Saccharomyces cerevisiae* brewing yeasts are able to release thiols from S-conjugates. 3SHol was found at 5.5  $\mu$ g·L<sup>-1</sup> after bottle refermentation of a beer spiked with 10 mg·L<sup>-1</sup>

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		Free	Cys-	G-
3SPol (R = CH <sub>2</sub> CH <sub>3</sub> )	In malt (μg.kg <sup>-1</sup> )	/	*	*
	In hop (μg.kg <sup>-1</sup> )	10	162	18100
	In late-hopped beer (ng.L <sup>-1</sup> )	39	/	/
	In dry-hopped beer (ng.L <sup>-1</sup> )	/	/	/
3S4MPol (R = CH(CH <sub>3</sub> ) <sub>2</sub> )	In malt (μg.kg <sup>-1</sup> )	/	*	*
	In hop (μg.kg <sup>-1</sup> )	305	65	3600
	In late-hopped beer (ng.L <sup>-1</sup> )	548	/	/
3SHol (R = CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )	In malt (μg.kg <sup>-1</sup> )	/	6	700
	In hop (μg.kg <sup>-1</sup> )	125	1930	118200
	In late-hopped beer (ng.L <sup>-1</sup> )	243	/	/
	In dry-hopped beer (ng.L <sup>-1</sup> )	210	/	/

**Figure 1.** Chemical structures and overview of the maximal amount of S-conjugates and free thiols found in malt, hop, and beers.<sup>13,14,19,25</sup> \*Data exposed for the first time here.

synthesized Cys-3SHol (release efficiency: 0.09%).<sup>22</sup> More recently, investigators have studied the effects of primary fermentation parameters and ale yeast strains on thiol release from both cysteinylated and glutathionylated precursors. Maturation appeared as the crucial step for free thiol excretion from yeast cells (no thiol was recovered in fermented worts without maturation). After 7 days at 24 °C and additional 3 days at 4 °C, SafAle K-97 yeast reached the highest release efficiencies (up to 0.45 and 0.08% release from Cys- and G-conjugates, respectively). Significant differences were observed between yeast strains, especially in terms of the acetate ratio [released acetate/released (alcohol + acetate), varying from 0 to 80%]. This ratio was also greatly affected by wort density (higher esterification at 17 °Plato than at 15 and 12 °Plato).<sup>23</sup> As for the nitrogen level of wort, known to greatly affect the ability of yeasts to release thiols in the oenological process (nitrogen catabolite repression of the gene for  $\beta$ -lyase *Irc7p*), the influence of this parameter has not been assessed under brewing conditions.<sup>24</sup>

The aim of this study was to determine how delicate PFT flavors can be produced by lager yeasts in premium lager beers made only with hops containing very few free PFTs. First, the same methodology as used recently on ale yeasts<sup>23</sup> was applied to assess the efficiency with which seven lager yeasts (*Saccharomyces pastorianus*) release sulfanylalkyl alcohols (3SPol, 3SHol, and 3S4MPol) from cysteine and glutathione S-conjugates and esterify them. The influences of fermentation temperature and wort nitrogen level were also studied. Then, because good releasing activity is useful only in the presence of a substantial amount of substrates, the S-conjugate profiles of pale malt and Saaz hop, the classical ingredients of premium lagers, were investigated. Roland et al.<sup>25</sup> already evidenced in malt up to 6 μg.kg<sup>-1</sup> Cys-3SHol and 700 μg.kg<sup>-1</sup> G-3SHol. Yet as these compounds co-elute from most HPLC columns, an adapted procedure for distinguishing 3S4MPol from 3SHol conjugates was applied here.<sup>19,26</sup>

## MATERIALS AND METHODS

**Chemicals.** Absolute ethanol, acetonitrile, Amberlite IR-120 resin, 28% ammonia, dichloromethane, formic acid, 37% hydrochloric acid, and sodium chloride were purchased from VWR (Leuven, Belgium). 2-Acetylthiophene, apotryptophanase, 6 mL Discovery Ag-ion SPE tubes, >98% L-cysteine hydrochloride monohydrate, 4-methoxy-2-methylbutane-2-thiol, S-benzyl-L-cysteine, and S-hexylglutathione were purchased from Sigma-Aldrich (Bornem, Belgium). Anhydrous sodium sulfate and sodium hydroxide were purchased from Acros Organics (Geel, Belgium). Pyridoxal 5-phosphate (PLP) was purchased from Alfa Aesar (Haverhill, Massachusetts, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker Chemicals (Radnor Township, Pennsylvania, USA). Di-potassium hydrogen phosphate was purchased from Merck (Darmstadt, Germany). Milli-Q water was used (Millipore, Bedford, MA, USA).

**Synthesis of Previously Investigated Reference Conjugates.** As described in Chenot et al.,<sup>23</sup> all the S-conjugates (Cys-3SHol, G-3SHol, Cys-3S4MPol, G-3S4MPol, Cys-3SPol, and G-3SPol) were synthesized prior to this work according to the methods of Gros et al.,<sup>12</sup> Kankolongo Cibaka et al.,<sup>27</sup> and Chenot et al.<sup>19</sup> Mixtures of S-conjugate diastereomers were obtained. Accurate weights of the pure solids (>99%) were diluted into 1 mL water just before spiking them into the wort.

**Yeasts.** Four lager yeast strains (BRAS37, BRAS25, BRAS51a, and BRAS45; propagation in YPS liquid media at 28 °C) from the INBR UCLouvain collection (Louvain-la-Neuve, Belgium) and three active dry yeasts (Saflager S-23, Saflager W34-70, and Saflager S-189; hereinafter referred to as S23, W3470, and S189) from Fermentis Lesaffre (Marcq-en-Barœul, France) were used for fermentation trials.

**Fermentation of Wort Spiked with Cys-3SPol, Cys-3SHol, and Cys-3S4MPol or G-3SPol, G-3SHol, and G-3S4MPol.** Wort samples were produced from pale malt (malt I, Boortmalt) in a 60-L-scale pilot plant (Coenco, Oostkamp, Belgium). The 17 °Plato unhopped wort was obtained after 90 min of boiling and freezing after clarification until the fermentation trials. Lager yeasts were pitched at 0.5 g.L<sup>-1</sup> if dry or 5 million cells/mL if liquid into 250 mL wort at 15 °Plato (obtained by diluting the original 17 °Plato wort with water). For the trial focusing on the impact of the nitrogen level, the 15 °Plato wort was diluted (50:50) with a 15 °Plato glucose syrup solution. The worts were spiked beforehand with Cys-3SPol, Cys-3SHol, and Cys-3S4MPol (10 mg.kg<sup>-1</sup> each) or G-3SPol, G-3SHol, and G-3S4MPol (15 mg.kg<sup>-1</sup> each). The first screening trials were conducted for 7 days at 24 °C with the seven above-mentioned yeasts (or 12 °C for

two additional fermentation trials, with yeast BRAS45 only) under shaking at 80 rpm (Labwit ZWY-240 incubator shaker). The fermented worts were kept at 4 °C for 3 days before extraction of free thiols (see the next section). Experiments were done in duplicate.

**Free Thiol Extraction from Fermented Media with an Ag Cartridge.** Thiol-specific extraction, initially reported by Takazumi et al.,<sup>14</sup> was performed with a Discovery Ag-ion SPE cartridge as described by Chenot et al.<sup>23</sup> After subjecting the fermented wort to liquid/liquid extraction with dichloromethane, the organic extract was loaded onto the preconditioned cartridge. After washing, thiols were eluted from the cartridge with a cysteine solution and re-extracted in bidistilled dichloromethane. The final extract was concentrated to 70  $\mu\text{L}$  and stored at  $-80\text{ }^\circ\text{C}$  until injection into the GC-PFPD system.

**Gas Chromatography-Pulsed-Flame Photometric Detection.** One microliter of free thiol extract (obtained by Ag extraction or released upon apotryptophanase treatment) was analyzed with an Agilent 6890N gas chromatograph equipped with a splitless injector maintained at 250 °C. Compounds were separated with a wall-coated open tubular (WCOT) apolar CP-Sil5-CB capillary column (50 m, 0.32 mm i.d., 1.2  $\mu\text{m}$  film thickness). The carrier gas was helium and the pressure was set at 90 kPa. The oven temperature was programmed to rise from 36 to 85 °C at 20 °C per min, then to 145 °C at 1 °C per min, and finally to 220 °C at 3 °C per min and was held for 30 min. The column was connected to an OI Analytical PFPD detector (model 5380, combustor, internal diameter: 2 mm). The following parameters were selected for the PFPD detector: temperature, 250 °C; voltage, 600 V; gate width, 18 ms; gate delay, 6 ms; trigger level, 400 mV; and pulse frequency, 3.33 Hz. PFPD chromatograms were recorded throughout elution; the ChemStation software was used to process the resulting data. Identifications were done as previously described by Gros et al.<sup>18</sup> The following equation was used for commercially available 3SHA and 3SHol (X) quantitation [calibration curves and internal standard (IST)-relative molar response coefficients detailed in Section S1]

$$\begin{aligned} \mu\text{g L}^{-1} \text{ of X} &= \mu\text{g L}^{-1} \text{ of IST} \times \frac{\text{X area}}{\text{IST area}} \\ &\times \frac{\text{IST molar response coefficient}}{\text{X molar response coefficient}} \\ &\times \frac{\text{X molar weight}}{\text{IST molar weight}} \times \frac{\text{IST recovery factor}}{\text{X recovery factor}} \end{aligned}$$

For commercially unavailable 3SPA, 3SPol, 3S4MPA, and 3S4MPol (X), the good equimolarity of the PFPD detector enabled us to set the IST-relative molar response coefficients at 1. The corrective molar weight ratio was applied. For all thiols, the IST-relative recovery factor was set at 1 (experimental values from 0.8 to 1.2, determined beforehand by standard addition).

**Release Efficiency Determination.** The efficiency of release of free  $\text{X}_{\text{OH}}$  (sulfanylalkyl alcohol) from bound  $\text{X}_{\text{OH}}$  was calculated with the following equation

$$\begin{aligned} \text{X}_{\text{OH}} \text{ release efficiency (\%)} &= \frac{\mu\text{g L}^{-1} \text{ X}_{\text{OH}}}{\mu\text{g L}^{-1} \text{ added bound X}_{\text{OH}}} \times \frac{\text{bound X}_{\text{OH}} \text{ molar weight}}{\text{free X}_{\text{OH}} \text{ molar weight}} \\ &\times 100 \end{aligned}$$

For the corresponding esters, the efficiency of release of free  $\text{X}_{\text{A}}$  (sulfanylalkyl acetate) from bound  $\text{X}_{\text{OH}}$  was calculated in alcohol equivalents

$$\begin{aligned} \text{X}_{\text{A}} \text{ release efficiency (\%)} &= \frac{\mu\text{g L}^{-1} \text{ X}_{\text{A}}}{\mu\text{g L}^{-1} \text{ added bound X}_{\text{OH}}} \\ &\times \frac{\text{bound X}_{\text{OH}} \text{ alcohol molar weight}}{\text{free X}_{\text{A}} \text{ acetate molar weight}} \times 100 \end{aligned}$$

The results (detailed in Section S2) are given as mean values of duplicates.

**Acetate Ratio Determination.** The ratio of acetate is calculated using the following equation

$$\text{acetate ratio (\%)} = \frac{\mu\text{g L}^{-1} \text{ X}_{\text{A}}}{(\mu\text{g L}^{-1} \text{ X}_{\text{A}} + \mu\text{g L}^{-1} \text{ X}_{\text{OH}})} \times 100$$

**Hop and Malt Samples.** Saaz pellets (Czech Republic) from two different harvest years, purchased from Brouwland (Belgium), were analyzed. Three pale malts (I, II, and III) were purchased from Boortmalt (Belgium). Malt I was a six-row winter variety (Etincel, harvest 2019), while malts II and III were two-row spring varieties (Pilsen style, harvests 2018 and 2020, respectively).

**S-Conjugate Extraction from Malt and Hop.** Extraction of Cys- and G-conjugates was performed as described by Chenot et al.<sup>19,26</sup> Milled malt (150 g) or milled hop pellets (100 g) were stirred with 1000 mL acidified hydroalcoholic solution ( $\text{H}_2\text{O}/\text{EtOH}/\text{HCOOH}$  79:20:1, v/v/v) for 2 h at 45 °C. After centrifugation, the supernatant was collected and loaded on 100 g of IR-120 cation exchange resin. The resin was then washed and sequential 100 mL fractions were recovered by elution with aqueous ammonia solutions at increasing concentrations, from 0.3 to 2.4 mol·L<sup>-1</sup>. The 1.2–2.4 mol·L<sup>-1</sup> fractions were pooled and concentrated under reduced pressure. The extract was dissolved either in 2 mL of 0.1% aqueous formic acid solution for analysis by liquid chromatography or in 2 mL potassium-phosphate-buffered solution for enzymatic assays and further gas chromatography analysis. S-benzyl-L-cysteine was added as the IST to the milled solid sample before liquid extraction at a concentration chosen according to the quantitation method to be used (2–8 mg·kg<sup>-1</sup> in samples for liquid chromatography quantitation, 200  $\mu\text{g}\cdot\text{kg}^{-1}$  in samples for gas chromatography quantitation).

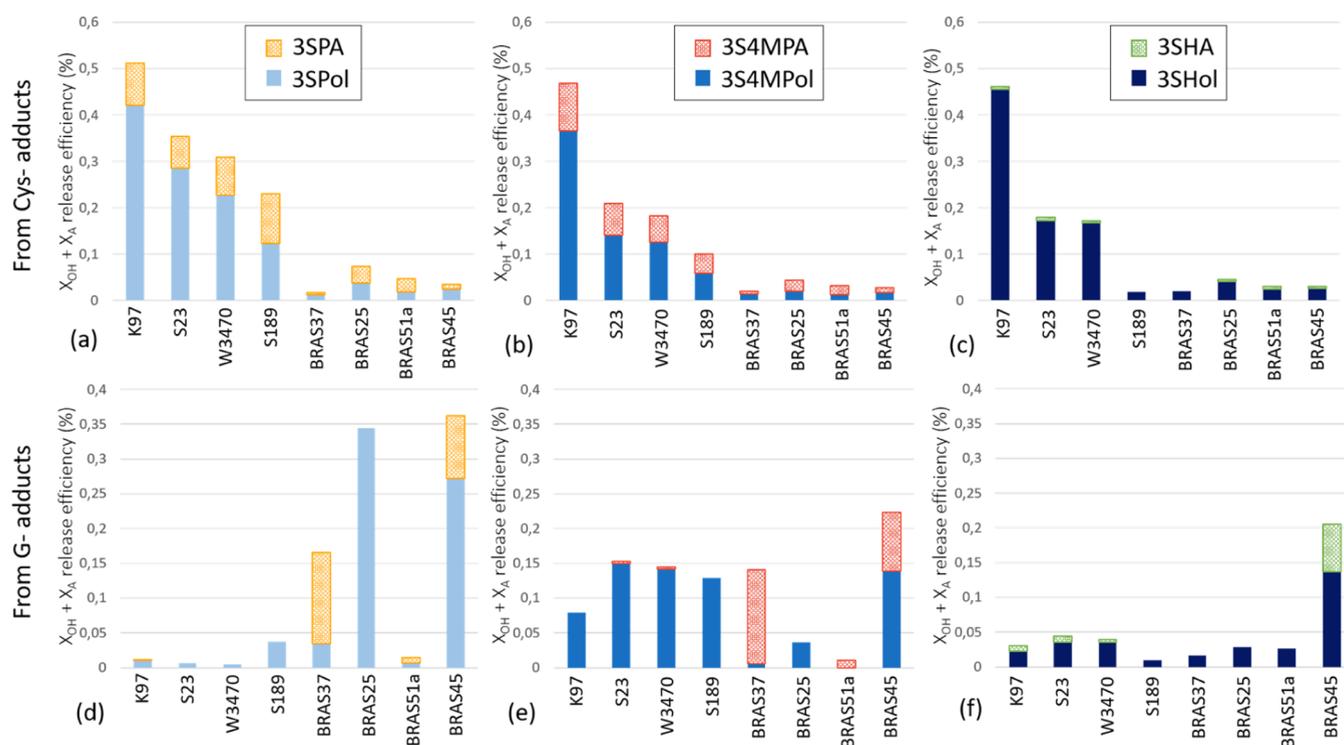
**Direct Quantitation of S-Conjugates by High-Performance Liquid Chromatography–Multiple Reaction Monitoring.** The above-described synthesized cysteinylated and glutathionylated precursors of 3SPol, 3SHol, and 3S4MPol were used for identification (MS/MS mode) and quantitation (calibration curves, MRM mode).

A 100 mm  $\times$  2.1 mm, 3  $\mu\text{m}$  Hypersil GOLD aQ column (a polar endcapped C18 phase offering superior retention of polar compounds, ThermoFisher) was used to quantify Cys-3SPol, Cys-3SHol + Cys-3S4MPol, and G-3SPol. A 250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  Astec Cyclobond I 2000 RSP (chiral column used here for its polarity and not for its chirality, Sigma-Aldrich) was used to quantify G-3SHol and G-3S4MPol separately.

The chromatographic and spectroscopic parameters of this two-complementary-columns strategy have been thoroughly described by Chenot et al.<sup>19,26</sup>

Calibration curves of conjugates relative to IST were determined for all synthetic standards (detailed in Section S3). The following equation was used for each conjugate quantitation: concentration of conjugate (in  $\mu\text{g}\cdot\text{kg}^{-1}$ ) = concentration of IST (in  $\mu\text{g}\cdot\text{kg}^{-1}$ )  $\times$  (peak area of conjugate/peak area of IST)  $\times$  (response coefficient of IST/response coefficient of conjugate).

**Indirect Quantitation of Cysteine Conjugates by Apotryptophanase Treatment.** To confirm the amount of Cys-3SPol and to deduce the Cys-3SHol/Cys-3S4MPol ratio from the amount of Cys-3SHol + Cys-3S4MPol obtained by HPLC-MRM, an enzymatic treatment described by Gros et al.<sup>20</sup> was applied to hop precursor extracts. Commercial *Escherichia coli* apotryptophanase (75–150 units·mg<sup>-1</sup>) was added to the S-conjugate extract dissolved in a potassium-phosphate-buffered solution (containing 0.1 mM PLP as a cofactor and 1 mM EDTA). The mixture was kept at 28 °C for 30 min and then stirred with 5 mL bidistilled dichloromethane for 30 min. The organic phase was recovered and concentrated to 500  $\mu\text{L}$  in a Danish–Kuderna apparatus and to 70  $\mu\text{L}$  in a Dufton column. Then, 1  $\mu\text{L}$  of the obtained extract was analyzed by GC-PFPD (described above).



**Figure 2.** Efficiencies (%) of sulfanylalkyl alcohol (solid fill) and acetate (striped pattern fill) release from Cys-conjugates (a–c) or G-conjugates (d–f) after 7 days of fermentation at 24 °C and 3 days of maturation at 4 °C. S-Conjugates were added to a 15 °Plato wort pitched with the lager yeasts. (Previously obtained results for SafAle yeast K-97 are added as a benchmark.)<sup>23</sup> The data are given as mean values of duplicates (variation coefficients under 15%).

**Table 1. Acetate Ratios (Acetate/Alcohol + Acetate, %) Measured for Different Yeast Strains after Spiking with Cys- or G-Conjugates**

	spiking with Cys-Conjugates								BRAS45 12 °C	BRAS45 12 °C, FAN135
	S23	W3470	S189	BRAS37	BRAS25	BRAS51a	BRAS45	BRAS45		
3SPA	20	27	46	29	49	59	28	33	59	
3S4MPA	33	31	42	31	55	63	37	26	64	
3SHA	4	3	0	0	10	20	16	8	20	
	Spiking with G-Conjugates								BRAS45 12 °C	BRAS45 12 °C, FAN135
	S23	W3470	S189	BRAS37	BRAS25	BRAS51a	BRAS45	BRAS45		
3SPA	0	0	0	79	0	56	25	0	0	
3S4MPA	2	2	0	96	0	100	38	0	0	
3SHA	19	10	0	0	0	0	34	0	0	

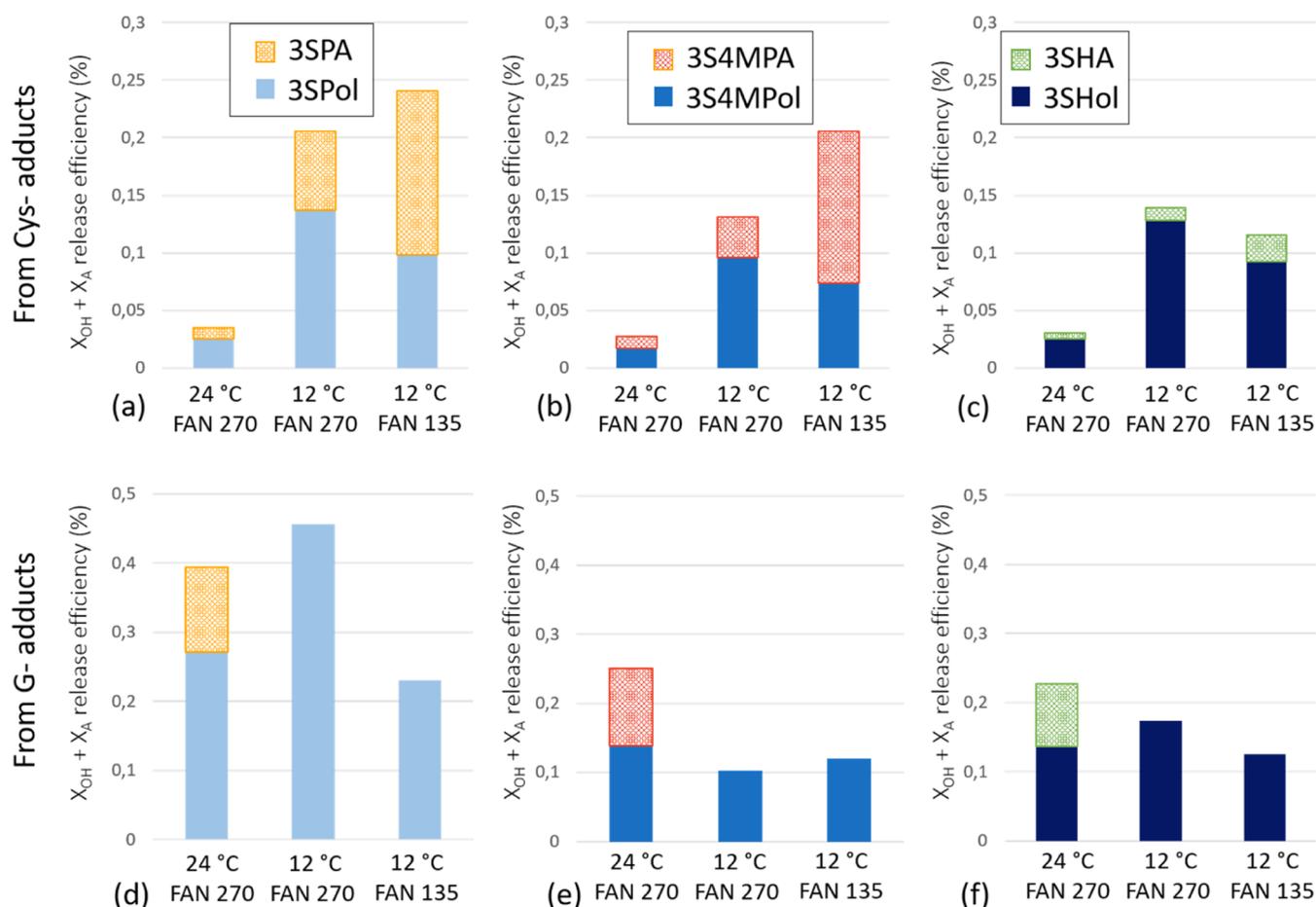
## RESULTS AND DISCUSSION

Despite the fact that lager yeasts are used at 10–15 °C in the brewing industry, the efficiency with which seven *S. pastorianus* strains released thiols from S-conjugates was first assessed in the exact same fermentation conditions as those previously applied with *S. cerevisiae* top fermentation yeasts (15 °Plato initial wort density, 7 days of fermentation at 24 °C, and 3 days of maturation at 4 °C). The obtained results are therefore compared to the performance of SafAle K-97, exhibiting the best release efficiency among the tested ale yeasts.<sup>23</sup> The spiking levels of the PFT precursors have been optimized here to ensure an accurate quantitation of the derived free PFTs (10–15 mg·L<sup>-1</sup>; much higher than the realm of what one might expect from a hopped wort). Yet the release efficiencies revealed not to be significantly affected when threefold less Cys- and G-conjugates were added.

### Ability of Lager Yeasts to Release Thiols from S-Cysteinyllated Conjugates.

As depicted in Figure 2a–c, the yeasts were tested for the efficiency of release of sulfanylalkyl alcohols and acetates from Cys-conjugates (from 0.02 to 0.35% total release). Yeast S23 emerged as the best at releasing sulfanylalkyl alcohols generally, followed closely by yeast W3470 (and S189, except for 3SHol/3SHA results). These three dry yeasts appeared more active than the four liquid yeasts. However, none of the investigated lager yeasts proved outstanding, especially as compared to SafAle K-97 (release efficiency up to 0.51%).<sup>23</sup>

Six of the seven lager yeasts showed a preference for Cys-3SPol hydrolysis (3SPol + 3SPA release) (yeast BRAS37 being the exception with no preferential hydrolysis). None of them displayed for 3SPA an acetate/(alcohol + acetate) ratio above 59% (this ratio can reach 79% with some ale yeasts)<sup>23</sup> (Table 1).



**Figure 3.** Efficiencies (%) of sulfanylalkyl alcohol (solid fill) and acetate (striped pattern fill) release from Cys-conjugates (a–c) or G-conjugates (d–f) after 7 days of fermentation at 24 or 12 °C and 3 days of maturation at 4 °C. S-Conjugates were added to a 15 °Plato wort (FAN: 270 mg·L<sup>-1</sup>) or a 50:50 wort/glucose solution (FAN: 135 mg·L<sup>-1</sup>) pitched with lager yeast BRAS45. The data are given as mean values of duplicates (variation coefficients under 15%).

**Table 2.** S-Conjugate Contents (in  $\mu\text{g}\cdot\text{kg}^{-1}$ ) of Three Pale Malt Samples and Two Saaz Hop Samples<sup>a</sup>

Malt	3SPol		3S4MPol		3SHol	
	Cys-	G-	Cys-	G-	Cys-	G-
sample I	15	d	10	nd	nd	d
sample II	3	d	8	35200	6	nd
sample III	nd	200	nd	nd	nd	1400
Saaz Hop						
harvest 2019	630	3300	1434	nd	5350	18600
harvest 2020	d	3800	nd	nd	3914	48400

<sup>a</sup>nd, undetected; d, detected at trace level ( $<0.5 \mu\text{g}\cdot\text{kg}^{-1}$  for Cys-conjugate quantitation by the indirect GC method;  $<50 \mu\text{g}\cdot\text{kg}^{-1}$  for G-conjugate quantitation by the direct HPLC method).

**Ability of Lager Yeasts to Release Thiols from S-Glutathionylated Conjugates.** Depending on the thiol nature, up to five of the lager yeasts (S23, W3470, BRAS25, BRAS51a, and BRAS45) were found to release free thiols from glutathionylated conjugates even more efficiently than SafAle K-97 (up to 0.36%)<sup>23</sup> (Figure 2d–f). Surprisingly, no correlation emerged between the release from Cys-conjugates and the release from G-conjugates (e.g., S23 acted the most efficiently on Cys-conjugates and BRAS45 on G-conjugates). Yeasts BRAS37 and BRAS45 even exhibited a higher release

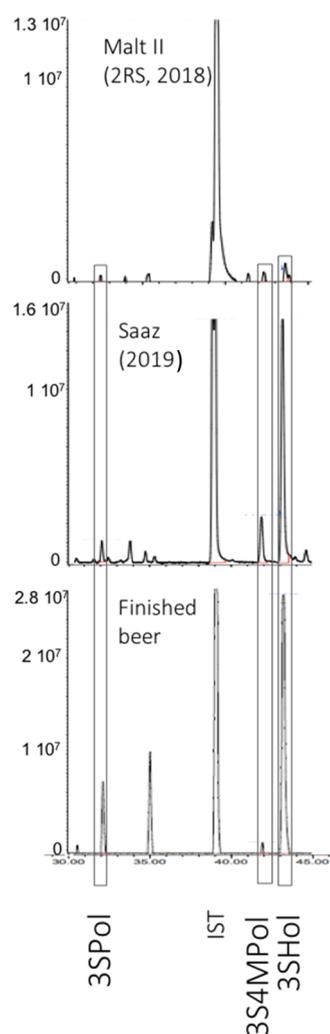
efficiency with G-conjugates than with Cys-conjugates (also the case of BRAS25 for 3SPol conjugates). Yeast BRAS45 definitely stood out from the others, with release efficiencies reaching 0.36% for 3SPol + 3SPA, 0.23% for 3S4MPol + 3S4MPA, and 0.20% for 3SHol + 3SHA.

BRAS37, BRAS25, and BRAS45 showed a preference for 3SPol release; S23, W3470, and S189 for 3S4MPol release; and BRAS51a for 3SHol release. While some yeasts released these three thiols with similar efficiency, others exhibited a strong preference for one structure (e.g., BRAS25 released 0.34% 3SPol but less than 0.05% 3S4MPol and 3SHol).

Only three yeasts (BRAS37, BRAS51a, and BRAS45) displayed efficient esterification of sulfanylalkyl alcohols (especially 3S4MPol to 3S4MPA), with an acetate/(alcohol + acetate) release ratio reaching 100% for BRAS51a (the absolute level of acetates was higher for BRAS37) (Table 1).

**Impact of the Fermentation Temperature.** Considering the outstanding results of release efficiency from G-conjugates, *S. pastorianus* BRAS45 was further used to assess the fermentation conditions most favorable to the release of free thiols from G- and Cys-conjugates.

Because the fermentation temperature for lager beers is usually around 10–15 °C, trials at 24 °C (see above) were compared with the ones at 12 °C. As illustrated in Figure 3a–c, lowering the fermentation temperature significantly improved (up to sixfold) the release from Cys-conjugates. Although



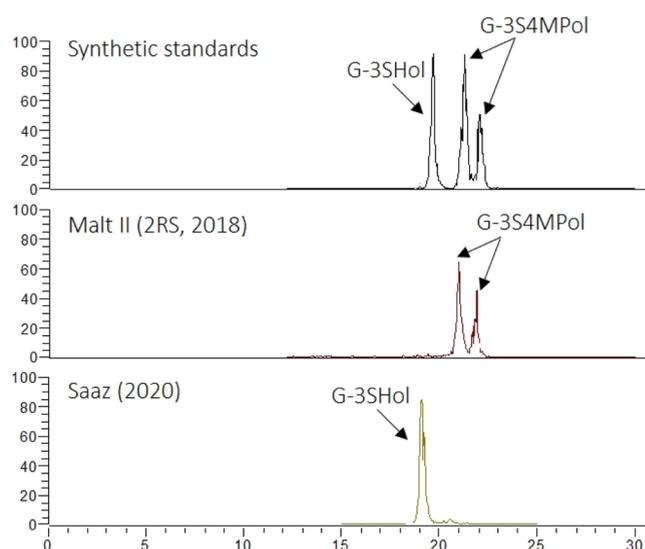
**Figure 4.** GC-PFPD chromatograms of malt II (2RS, harvest 2018), harvest 2019 Saaz hop, and finished beer *S*-conjugate extracts after incubation with apotryptophanase (IST = benzylthiol released from *S*-benzyl-L-cysteine).

acetate ratios were lower for 3S4MPA and 3SHA at 12 °C (Table 1), absolute acetate levels were much higher.

The results for G-conjugates were quite different (Figure 3d–f): lowering the fermentation temperature to 12 °C caused only a slight (1.5-fold) increase in 3SPol and 3SHol concentrations, and no trace of the corresponding sulfanylalkyl acetates was detected. At 24 °C, release from G-conjugates was up to eightfold higher than from Cys-conjugates, whereas at 12 °C, release efficiencies for Cys- and G-conjugates were similar.

**Impact of the Wort Nitrogen Level.** To assess whether nitrogen starvation might further improve free thiol production with BRAS45, a 15 °Plato low-nitrogen wort [free amino nitrogen (FAN): 135 mg·L<sup>-1</sup>] was made by mixing (50:50 v/v) the original 15 °Plato wort (FAN: 270 mg·L<sup>-1</sup>) with a 15 °Plato glucose solution.

In the trials with Cys-conjugates, as depicted in Figure 3a–c, a strong increase in the acetate ratio was measured, often together with a greater total release of free thiols (except for 3SHol + 3SHA). Our results confirm previous data obtained under oenological conditions<sup>12</sup> (higher β-lyase activity) and also evidence a positive impact of low nitrogen level on sulfanylalkyl alcohol esterification. Unfortunately, no similar



**Figure 5.** RP-HPLC-ESI(+)MRM ( $m/z$  408 → 262 for G-3SHol and G-3S4MPol) performed with the Cyclobond I 2000 RSP column and applied to a model medium containing synthetic G-3SHol and G-3S4MPol standards as compared to malt II (2RS, harvest 2018) and harvest 2020 Saaz hop *S*-conjugate extracts.

**Table 3. Theoretical Concentrations (in ng·L<sup>-1</sup>) of Free Thiols Reached in a 5% v/v Ethanol Beer Produced with Pale Malt (Sample II in Table 1), 200 g·L<sup>-1</sup> Saaz Hop (Harvest 2020 in Table 2), and BRAS45 Lager Yeast at 12 °C (15 °Plato Wort)**

	3SPol	3S4MPol	3SHol
sensorial threshold (in beer)	620 <sub>(in water)</sub>	70	55
from malt (II)	0.4	1733.6	0.7
from Saaz (harvest 2020)	10.4		58.7
total	10.8	1733.6	59.4

improvement was measured at low FAN with G-conjugates (Figure 3d–f).

Because lager yeasts appear to act quite readily on G-conjugates but premium lager beers are usually made only with pure Pilsen malt and traditional aromatic hops such as Saaz (having a low free thiol content), it appeared interesting to investigate the *S*-conjugate profiles of malt and Saaz hop to determine from which G-conjugates *S. pastorianus* might release odorant sulfanylalkyl alcohols and acetates in these beers.

#### Potential Use of Malt *S*-Conjugates by Lager Yeast.

Using both the direct method with two different HPLC columns<sup>19,26</sup> and the indirect GC-PFPD procedure (after apotryptophanase incubation),<sup>20</sup> we identified, for the first time in malt samples, the Cys- and G-conjugates of 3SPol and 3S4MPol (Table 2). As already reported by Roland et al.<sup>25</sup> for 3SHol, the contents measured for 3SPol and 3S4MPol *S*-conjugates differed considerably from one malt sample to another, with up to 8 μg·kg<sup>-1</sup> Cys-3S4MPol and 35 mg·kg<sup>-1</sup> G-3S4MPol in malt II (2RS, harvest 2018) (Figures 4 and 5) and none at all in malts I and III. A beer with a 5% v/v ethanol content could end up with up to 1.7 μg·L<sup>-1</sup> 3S4MPol (far above its threshold) if brewed with malt II (15 °Plato wort) and fermented at 12 °C with BRAS45 yeast (Table 3). However, it is sure that a big part of the malt thiol potential is lost through wort mashing and filtration, explaining why a non-

hopped beer always contains very few PFTs.<sup>12,15</sup> Yet these results do explain why beers sometimes exhibit free 3S4MPol slightly above the amount expected for the contribution of hop (e.g., 53–275 ng·L<sup>-1</sup> in beers late-hopped with the Mandarina Bavaria cultivar, on average 4.5 times more than expected for 100% transfer of the free form, the sole detected form in that hop).<sup>15</sup>

**Potential Use of S-Conjugates from the Aromatic Hop Saaz by Lager Yeast.** Despite their very low levels of free PFTs, Saaz hop pellets (both harvest years) proved richer in S-conjugates than some dual varieties (up to 4 and 48 mg·kg<sup>-1</sup> G-3SPol and G-3SHol, respectively) (Table 2). As for 3S4MPol conjugates, the cysteinylated form was found only in the 2019 sample (Figure 4), while no trace of G-3S4MPol was observed in either sample (Figure 5). All these S-conjugate levels are within the same range as observed in a previously investigated Saaz sample (harvest 2017<sup>19</sup>).

Without taking into account malt contribution, the production of a late-hopped beer with Saaz (200 g·h L<sup>-1</sup> Saaz 2020 in a 15 °Plato wort) and with fermentation at 12 °C by BRAS45 yeast could yield up to 10 ng·L<sup>-1</sup> 3SPol and 59 ng·L<sup>-1</sup> 3SHol (just above its threshold) in the resulting product (Table 3).

In conclusion, our results highlight some lager yeasts as great producers of free thiols from G-conjugates (up to 0.35%). Such strains seem essential to getting the most out of malt and Saaz glutathione S-conjugates in premium lagers. Studies should now investigate the impact of initial wort density (a high-density wort being commonly used in this industrial process) and maturation duration in an attempt to still improve the highly appreciated organoleptic profiles of these premium lagers.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c07272>.

Calibration curves of IST (4-methoxy-2-methylbutane-2-thiol), 3SHol, and 3SHA and corresponding to IST-relative response coefficients obtained from GC-PFPD analyses; detailed data of release efficiencies available in Figures 2 and 3; calibration curves of Cys-3SHol and G-3SHol relative to IST (S-benzyl-L-cysteine); and relative response coefficients for all the quantitated compounds obtained from HPLC-MRM analyses (PDF)

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## Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

3SHA, 3-sulfanylhexyl acetate; 3SHol, 3-sulfanylhexan-1-ol; 3SPA, 3-sulfanylpentyl acetate; 3SPol, 3-sulfanylpentan-1-ol; 3S4MPA, 3-sulfanyl-4-methylpentyl acetate; 3S4MPol, 3-sulfanyl-4-methylpentan-1-ol; Cys-, cysteinylated; EST, external standard; G-, glutathionylated; GC, gas chromatography; HPLC, high-pressure liquid chromatography; IST, internal standard; MRM, multiple reaction monitoring; MS, mass spectroscopy; PFPD, pulsed flame photometric detector; PFTs, polyfunctional thiols

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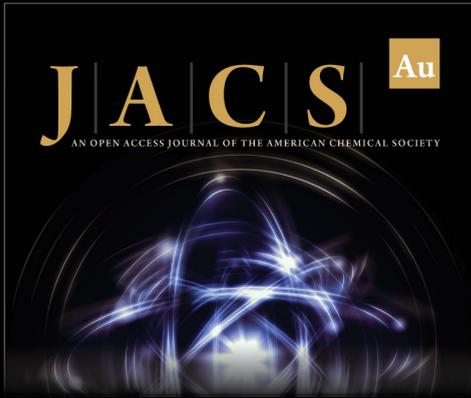
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