

# Occurrence of Odorant Polyfunctional Thiols in Beers Hopped with Different Cultivars. First Evidence of an S-Cysteine Conjugate in Hop (*Humulus lupulus* L.)

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**ABSTRACT:** Forty-one thiols, mainly  $\beta$ -sulfanylalkyl acetates,  $\beta$ -sulfanylalkyl alcohols, and  $\beta$ -sulfanylalkyl carbonyls, were recently evidenced in hop. In a beer hopped with the Tomahawk cultivar, most of them were found at higher levels than expected. The aim of the present work was to investigate the polyfunctional thiols in beers hopped with different varieties. A few thiols proved not to come only from hop (mainly 2-sulfanylethyl acetate,  $\mu\text{g/L}$  levels, and 1-sulfanylpentan-3-one and 1-sulfanylpentan-3-ol, ng/L levels, internal standard (IST) equivalents). The thiol profile of Saaz-hopped beer proved similar to that of the reference beer produced without hop. A high level of 3-sulfanyloctan-1-ol emerged as an indicator of the use of Tomahawk hop (140 ng/L, IST equivalents; FD (flavor dilution) = 65536). In both Cascade- and Tomahawk-hopped beers, 3-sulfanylhexan-1-ol and 3-sulfanylheptan-1-ol were smelled at high flavor dilutions, although only for the latter, significant amounts of the unreduced 3-sulfanylheptanal were found in hop. As already claimed for hop authentication, 3-sulfanyl-4-methylpentan-1-ol remains a good marker of Nelson Sauvin-hopped beers (548 ng/L, IST equivalents; FD = 65536), together with 4-sulfanyl-4-methylpentan-2-one (128 ng/L, FD = 4096). As illustrated by the huge production occurring during fermentation, accurate prediction of hop varietal impact requires quantitating thiol adducts in hop. S-3-(1-Hydroxyhexyl)cysteine was evidenced here for the first time in Cascade hop.

**KEYWORDS:** beer, polyfunctional thiols, hop (*Humulus lupulus*), varietal aroma, S-cysteine conjugate

## INTRODUCTION

Hop (*Humulus lupulus*) has been used as an ingredient of beer for over 800 years. It is appreciated for its flavoring (bitter taste<sup>1</sup> and hop aroma<sup>2</sup>), antispiling (bacteriostatic action against Gram-positive bacteria<sup>3</sup>), and foam stabilizer properties.<sup>4</sup> Through the ages, varieties have been improved and advanced breeding techniques have made it possible to obtain higher  $\alpha$ -acid contents. The low-bitter varieties (e.g., Saaz, Cascade) often used for their "aromatic" qualities are being increasingly replaced by "bitter" (e.g., Nelson Sauvin) and "Super Alpha" (e.g., Tomahawk) cultivars.

Among the numerous volatiles reported in essential oils,<sup>5</sup> discriminating sesquiterpenes such as  $\beta$ -farnesene,  $\beta$ -caryophyllene,  $\alpha$ -humulene,  $\beta$ -selinene, or bergamotene<sup>6–10</sup> are massively lost or degraded in the boiling kettle.<sup>11,12</sup> Just a few hop constituents can be found in beer above their threshold values (e.g., linalool, geraniol, humuladienone, geranyl acetate).<sup>12–18</sup> The "noble hop" or "kettle hop" flavor results mainly from oxidation and hydrolysis<sup>19–21</sup> followed by biotransformation during fermentation.<sup>22,23</sup>

Lermusieau et al. report that hopping with Challenger imparts more intense sulfur notes (mainly dimethyl disulfide and diethyl disulfide) than hopping with Saaz.<sup>17</sup> Ethyl esters derived from hop short-chain acids (e.g., ethyl 3-methylbutanoate, ethyl 4-methylpentanoate) appear associated with citrus characteristics imparted to Saaz-hopped beer.<sup>14</sup> 4-Sulfanyl-4-methylpentan-2-one (**29**; blackcurrant-like), geraniol (floral), (Z)-3-hexen-1-ol (green odorant), and 3-sulfanylhexan-1-ol (**23**; grapefruit-like) have been described as contributors to the Muscat-like flavor found in beers hopped with the Cascade

variety.<sup>14,18</sup> Takoi et al. identified 3-sulfanyl-4-methylpentan-1-ol (**26**; rhubarb, grapefruit) as a key contributor to the "Sauvignon-like" notes imparted to beer by the Nelson Sauvin hop variety.<sup>24</sup>

Very recently, Gros et al. investigated the distribution of 41 polyfunctional thiols among five hop cultivars: Tomahawk, Nelson Sauvin, Nugget, Cascade, and Saaz.<sup>25</sup> As compared to the other varieties, Tomahawk appeared particularly rich in 3-sulfanyl-2-ethylpropanal (**30**; skunky, plastic). Tomahawk and Nelson Sauvin proved to contain higher levels of 3-sulfanyl-2-ethylpropyl acetate (**10**; floral) and 3-sulfanyloctanal (**35**, citrus, peach), but only the latter was also characterized by higher amounts of **29** and total  $\beta$ -sulfanylalkyl alcohols.<sup>25</sup> Among the discriminating sulfanylalkyl alcohols found in Nelson Sauvin, **26** accounted for half of the total. **23** was found in all cultivars, but only Cascade reached high values, together with 3-sulfanylheptan-1-ol (**25**, lemon, hoppy).

In a wort boiled with the Tomahawk cultivar, in line with the results of Takoi et al. and Kishimoto et al., many polyfunctional thiols were found at higher levels than expected before boiling.<sup>24–26</sup> During fermentation, further strong increases were measured, leading to probably above-threshold concentrations of **10**, 3-sulfanylhexyl acetate (**11**, candy), and many sulfanylalkyl alcohols (**23**, **25**, **26**, and especially 3-sulfanyloctan-1-ol (**27**; catty, grapefruit)). A few of the thiols found lack

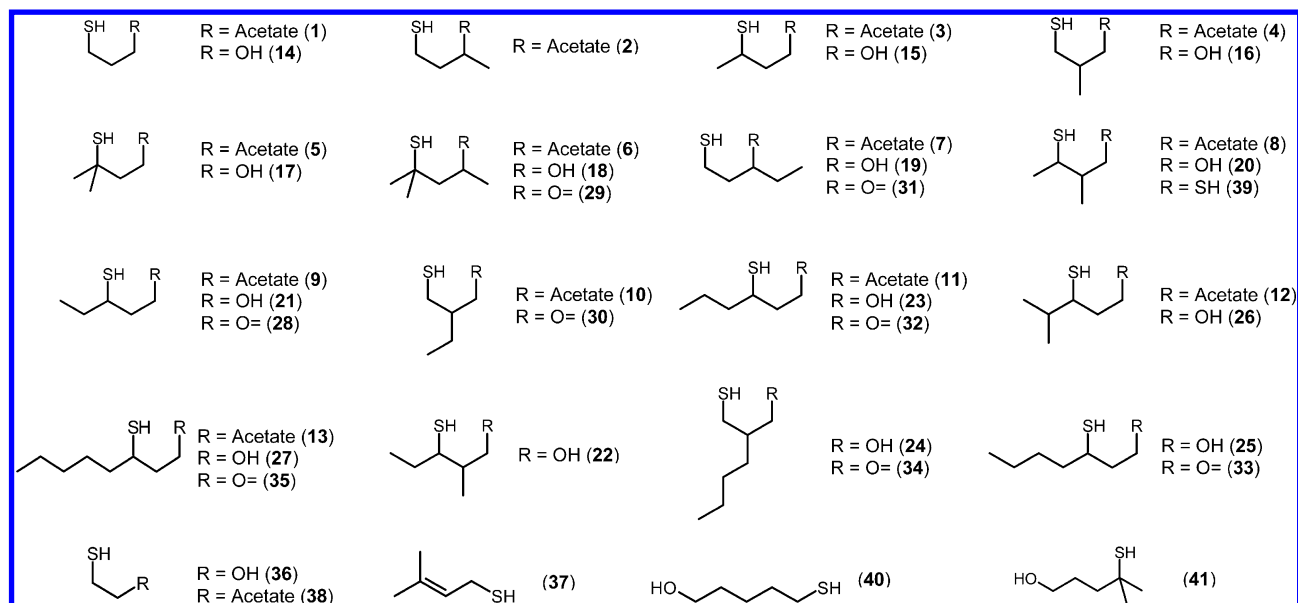
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**Figure 1.** Chemical structure and numbering of the thiols.

the  $\beta$ -sulfanylalkyl backbone. As for grapes, cysteine or glutathione adducts were therefore suspected.<sup>27</sup>

The aim of the present work was to determine how the free polyfunctional thiol content of hop could predict its total potential available for brewers. The odorant polyfunctional thiols found in beers hopped with Nelson Sauvín, Cascade, and Saaz hops (addition of pellets at the late stage of boiling) were compared to those quantified beforehand in the Tomahawk-derived beer. A beer produced without hop addition was also investigated. Just to roughly assess whether *S*-cysteine conjugates might constitute part of the hop thiol potential, 3-*S*-(1-hydroxyhexyl)cysteine was investigated in a Cascade hop extract.

## EXPERIMENTAL SECTION

**Chemicals.** 3-Sulfanylpropyl acetate (1), 3-sulfanylpropan-1-ol (14), 3-sulfanyl-3-methylbutan-1-ol (17), 2-sulfanylethan-1-ol (36), 2-sulfanylethyl acetate (38) (full chemical structures for the thiols are given in Figure 1), *p*-hydroxymercuribenzoic acid (*p*HMB), hydrochloric acid (37%), (*E*)-hexen-2-ol, *N*-Boc-*L*-cysteine (98.5%), citric acid, a strongly basic Dowex resin 1  $\times$  2, Cl<sup>-</sup> form, and the Amberlite IR-120 resin were purchased from Sigma-Aldrich (Bornem, Belgium). Ammonia (28%) was from VWR (Leuven, Belgium). 4-Methoxy-2-methylbutan-2-thiol, 3-sulfanylhexan-1-ol (23), and 3-methyl-2-buten-1-thiol (37) were obtained from Oxford Chemicals (Oxford, U.K.). 4-Sulfanyl-4-methylpentan-2-one (29) was from Frutarom (Hartlepool, U.K.). Cesium carbonate, sodium borohydride, and trifluoroacetic acid were sourced from Acros Organics (Geel, Belgium). Dichloromethane (99.9%) obtained from Romil (Cambridge, U.K.) was distilled before use. Milli-Q water was used (Millipore, Bedford, MA). NaOH and Na<sub>2</sub>SO<sub>4</sub> (99%) were supplied by Janssen (Geel, Belgium). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (Tris) from USB (Cleveland, OH). Formic acid was provided by Fischer (Loughborough, U.K.). Poly(vinylpyrrolidone) was supplied by Spindal (Gretz-Armainvilliers, France).

**Reference Compounds Synthesized in Our Laboratory.** 1-Sulfanyl-3-butyl acetate (2), 3-sulfanylbutyl acetate (3), 3-sulfanyl-2-methylpropyl acetate (4), 3-sulfanyl-3-methylbutyl acetate (5), 4-sulfanyl-4-methyl-2-pentyl acetate (6), 1-sulfanyl-3-pentyl acetate (7), 3-sulfanyl-2-methylbutyl acetate (8), 3-sulfanylpentyl acetate (9), 3-sulfanyl-2-ethylpropyl acetate (10), 3-sulfanylhexyl acetate (11), and 3-sulfanyloctyl acetate (13) were synthesized prior to this work

(reagents and complete procedure described by Vermeulen et al.<sup>28</sup>). 3-Sulfanylbutan-1-ol (15), 3-sulfanyl-2-methylpropan-1-ol (16), 1-sulfanyl-pentan-3-ol (19), 3-sulfanyl-2-methylbutan-1-ol (20), 3-sulfanylpentan-1-ol (21), 3-sulfanyl-2-methylpentan-1-ol (22), 3-sulfanyl-2-butylpropan-1-ol (24), 3-sulfanylheptan-1-ol (25), 3-sulfanyloctan-1-ol (27), 1-sulfanyl-pentan-3-one (31), and 3-sulfanyl-2-methylbutane-1-thiol (39) were obtained with the reagents and procedure disclosed in the publication of Vermeulen et al.<sup>29</sup> The materials and methods to obtain 4-sulfanyl-4-methylpentan-2-ol (18) are detailed in the work of Vermeulen et al.<sup>30</sup> 3-Sulfanylpentanal (28), 3-sulfanyl-2-ethylpropanal (30), 3-sulfanylhexanal (32), 3-sulfanylheptanal (33), 3-sulfanyl-2-butylpropanal (34), and 3-sulfanyloctanal (35) were previously produced according to Vermeulen et al.,<sup>31</sup> and 6-sulfanylhexan-1-ol (40) was produced as described in the paper of Vermeulen et al.<sup>32</sup> 3-Sulfanyl-4-methylpentyl acetate (12), 3-sulfanyl-4-methylpentan-1-ol (26), and 4-sulfanyl-4-methylpentan-1-ol (41) were synthesized according to Takoi et al.<sup>24</sup>

**Hop Samples.** As for Tomahawk pellets,<sup>25</sup> the varieties investigated here were from crop 2008. Saaz bred in the Czech Republic was provided by Hopsteiner (Mainburg, Hallertau, Germany). Cascade bred in the United States and Tomahawk CO<sub>2</sub> extract were provided by Yakima Chief (Louvain-la-Neuve, Belgium). Nelson Sauvín bred in New Zealand was provided by Hops Ltd. (Richmond, Nelson, New Zealand).

**Pilot Beer Production.** Beer was produced in a 60 L microbrewery (Coenco, Belgium) as described by Gros et al.<sup>25</sup> The following brewing process was used. A 12 kg portion of Pilsen Malt (2 rows, Malterie du Château, Belgium) was brewed in 60 L according to the following mashing program: 30 min at 50 °C, 30 min at 63 °C, and 30 min at 72 °C. The wort was then heated to 82 °C and filtered through the lauter tun. The 11°Plato wort thus obtained was boiled with 33 mg/L Tomahawk CO<sub>2</sub> extract for 75 min (8–11% evaporation). Ten minutes before the end, the selected hop variety was added at 1.78 g/L. The fermentation was conducted in cylindroconical fermenters with an ale-type yeast (INBR Bras268, propagated in a glucose/maltose/yeast extract/peptone medium). This strain was pitched at  $7.5 \times 10^6$  cells/mL. The fermentation was carried out at 22 °C for 4 days under an inner-tank pressure of 0.01 MPa. Maturation was held at 2 °C for 7 days with periodical purge of excess yeast from the bottom of the fermentor. After filtration on plates (0.5  $\mu$ m pores, BuonVinon CA), the beer was stored under carbon dioxide until extraction the next day.

**Extraction of Polyfunctional Thiols by *p*HMB.** Polyfunctional thiols were extracted from beer or wort according to Vermeulen et al.,<sup>33</sup>

adapted from Tominaga et al.<sup>34</sup> (dichloromethane liquid/liquid extraction of 500 mL of wort or beer, extraction of the resulting organic phase with a pHMB solution, loading of bound thiols onto a strong anion exchanger resin, release of free thiols by exchange with cysteine, final extraction with dichloromethane, and concentration to 250  $\mu$ L (for GC–O) in a Danish-Kuderna and to 70  $\mu$ L (for GC–MS and GC–PFPD) under nitrogen). 4-Methoxy-2-methylbutan-2-thiol was added as an internal standard (IST; at 0.67  $\mu$ g/L).

**Gas Chromatography–Olfactometric Detection (GC–O).** A 1  $\mu$ L volume of the pHMB extracts 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. Compounds were analyzed with a wall-coated open tubular (WCOT) apolar CP-Sil 5 CB (50 m  $\times$  0.32 mm i.d., 1.2  $\mu$ m film thickness) and a polar FFAP (25 m  $\times$  0.32 mm i.d., 0.3  $\mu$ m film thickness) capillary column. The carrier gas was nitrogen, and the pressure was set at 50 kPa (CP-Sil 5 CB) or 30 kPa (FFAP). The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 at 1 °C/min, and finally to 250 at 3 °C/min and held for 30 min. 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) prehumidified with an aqueous copper(II) sulfate solution. All extracts were analyzed immediately after extraction by two trained panelists. Complete aroma extract dilution analysis (AEDA<sup>35</sup>) was performed on pHMB extracts by one operator with the CP-Sil 5 CB column. The extracts were diluted stepwise with dichloromethane (1:1 by volume). The flavor dilution (FD) is defined as the highest dilution at which the compound could still be detected (FD = 2<sup>n</sup>, with n + 1 = number of dilutions applied on the extract until no odor was perceived). The precision of this AEDA analysis is n  $\pm$  1 (factor of 2 between FD values).

**Gas Chromatography–Electronic Impact Mass Spectrometry (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 an apolar CP-Sil 5 CB MS capillary column (50 m  $\times$  0.32 mm i.d., 1.2  $\mu$ m 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.

**Gas Chromatography–Pulsed-Flame Photometric Detection (GC–PFPD).** A 2  $\mu$ L volume of the pHMB extracts was analyzed on a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector. The injections were carried out in the splitless mode at 250 °C, the split being turned on after 0.5 min. Compounds were separated on the CP-Sil 5 CB column (50 m  $\times$  0.32 mm i.d., 1.2  $\mu$ m film thickness). The carrier gas was helium at a pressure of 90 kPa. The oven temperature program was the same as the one described for GC–O. At the OI Analytical PFPD detector (model 5380, combustor internal diameter 2 mm), 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.

**Identifications.** MS identifications were done by comparing the mass spectra obtained from each sample with those obtained with pure or synthesized compounds (from refs 28–32) injected under the same conditions and/or present in the National Institute of Standards and Technology (NIST) library. The retention indices (retention times normalized with respect to adjacently eluting *n*-alkanes) were determined by injection onto two capillary columns (CP-Sil 5 CB and FFAP-CB) connected to the olfactometric or the MS detector (identification checked by coinjection). In the case of PFPD (interesting for traces giving no GC–MS peak), injection of thioesters allowed translation into the alkane-related decimal numeral system.<sup>25</sup>

**PFPD Quantitations.** The following equation was used:

$$\begin{aligned} & \text{concentration(A, ng/L)} \\ &= \text{concentration(IST, ng/L)}(\text{molecular weight(A)} \\ & \quad / \text{molecular weight(IST)})(\text{area(A)/area(IST)}) \\ & \quad (\text{response coefficient(IST)/response coefficient(A)}) \\ & \quad (\text{recovery factor(IST)/recovery factor(A)}) \end{aligned}$$

For commercially available thiols, complete calibration curves relative to the IST were used. For commercially unavailable thiols, quantitations are given in IST equivalents (the good equimolarity of the PFPD detector allowed us to set the IST-relative 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 36 (bad recovery at the first dichloromethane extraction; approximate concentrations given in parentheses, obtained by applying a ratio of 0.1 assessed by standard addition).

**Synthesis of S-3-(1-Hydroxyhexyl)cysteine.** The synthesis was carried out according to the procedure described by Thibon et al.<sup>36</sup> (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 was done with trifluoroacetic acid according to Pardon et al.<sup>37</sup>

**First Evidence of a Cysteine Adduct in Cascade Hop.** A thiol precursor extraction procedure adapted from Starckenmann et al.<sup>38</sup> was applied to the Cascade hop variety. A 100 g portion of milled pellets was extracted with 1 L of a hydroalcoholic solution (H<sub>2</sub>O:EtOH:HCOOH = 49.5:49.5:1, v/v/v) for 2 h. After centrifugation for 30 min at 4000 rpm, the polyphenols present in the supernatant were partially removed by addition of 15 g of poly(vinylpyrrolidone) and stirring for 30 min. After a second centrifugation, the extract was purified on IR-120 (H<sup>+</sup>) cation exchange resin (100 g) conditioned with ultrapure water. After the extract was washed with 200 mL of water, sequential fractions were recovered by eluting with aqueous ammonia solutions at the following concentrations: 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 M. The 1.2 and 1.5 M fractions were pooled, vacuum evaporated, and lyophilized. The obtained extract (150 mg) was solubilized in 300  $\mu$ L of hydroalcoholic solution for analysis by RP-HPLC–HRMS/MS (ESI<sup>+</sup>; ESI = electrospray ionization). A 10  $\mu$ L volume of extract was injected onto a 150  $\times$  2.1 mm, 2  $\mu$ m C18 Prevail column (Grace, Deerfield, IL) and eluted with a linear gradient from 95% A (water/acetonitrile/formic acid, 98.9:1:0.1, v/v/v) to 100% B (acetonitrile) in 20 min, isocratic elution for 4.5 min, and a return to the initial conditions for 5 min. The flow rate was 200  $\mu$ L/min. An Accela system (Thermo Fisher, San Jose, CA) controlled with Xcalibur software version 2.2 was used. HRMS/MS analysis was carried out with the Q Exactive orbitrap (Thermo Fisher, San Jose, CA) equipped with an ESI source. The following ESI inlet conditions were applied in positive mode: spray voltage, 4 kV; heater temperature, 55 °C; capillary temperature, 320 °C; sheath gas, 45 psi; auxiliary gas, 15 psi. After the first monitoring at *m/z* 222.12, collision-induced dissociation spectra were recorded at 17% collision energy. Analysis of the Cascade hop extract confirmed an elemental formula of C<sub>9</sub>H<sub>19</sub>NO<sub>3</sub>S (experimental *m/z* of the pseudo molecular ion C<sub>9</sub>H<sub>20</sub>NO<sub>3</sub>S<sup>+</sup> = 222.11600, theoretical *m/z* = 222.11584,  $\delta$  = 0.72 ppm, well in the variation range of the apparatus) with a major fragmentation to *m/z* = 205.08954 (theoretical *m/z* of the deaminated C<sub>9</sub>H<sub>17</sub>O<sub>3</sub>S<sup>+</sup> = 205.08929,  $\delta$  = 0.82 ppm).

**Statistical Analyses.** All analyses were carried out in duplicate. Multiple comparisons of means were performed by means of Tukey's test, with SAS software version 9.2 (SAS Institute, Inc., Cary, NC). In the tables, values that do not share a common on-line Roman letter are significantly different (*p* < 0.05).





Table 2. Corresponding  $\beta$ -Sulfanylalkyl Alcohols<sup>a</sup>

RI	CP-Sil 5	FFAP	substance	odor (GC-O)	Tomahawk			Nelson Sauvin			Cascade			Saaz			nonhopped			identification reliability <sup>b</sup>
					wort	ale beer	FD	wort	ale beer	FD	wort	ale beer	FD	wort	ale beer	FD	wort	ale beer	FD	
14	849	1620	3-sulfanylpropan-1-ol	+	potatoes, pop corn	1024	184 a	16384 +	1024	165 a	16384 -	32 + b	4096 -	256 + b	4096 -	128	28 b	8192	I* (57-58-45)	
15	903	1628	3-sulfanylbutan-1-ol <sup>IST</sup>	+	perspiration, caty	1024	22	256 -	128 +	256 -	16 -	64 -	8 -	16 -	0	0	0	I* (106-72-61)		
16	922	1677	3-sulfanyl-2-methylpropan-1-ol <sup>IST</sup>	+	broth, leek	64	43	256 -	32 +	128 -	8 +	64 -	2 -	16 -	0	0	0	I		
17	938	1654	3-sulfanyl-3-methylbutan-1-ol	+	sulfur, soup	128	296 a	2048 -	64	275 a	2048 -	32	93 b	64 - c	1024 -	0 - c	0	0	I* (86-69-75)	
18	947	1547	4-sulfanyl-4-methylpentan-2-ol <sup>IST</sup>	+	fuel, caty	256 +	1024 +	1024 +	512 +	2048 +	32 +	128 -	0 -	64 -	0 -	0 -	0	0	I <sup>-</sup>	
19	977	1722	1-sulfanylpentan-3-ol <sup>IST</sup>	-	mushroom, nettle	8	317 a	4096 +	32	130 b	2048 +	32	34 b	512	17	128	86 b	2048	I* (102-87-59)	
20	989	1738	3-sulfanyl-2-methylbutan-1-ol <sup>IST</sup>	+	leek, hop	2048 +	1024 +	1024 +	2048	93	4096 -	128 +	128 -	16 -	128 -	8 -	256	2048	I* (120-86-60)	
21	1009	1760	3-sulfanylpentan-1-ol <sup>IST</sup>	-	caty, citrus	8	128 +	128 +	1024	39	4096 -	64 +	128 -	64 +	128 -	0 -	0	0	I	
22	1080	1822	3-sulfanyl-2-methylpentan-1-ol <sup>IST</sup>	19	gravy	1024 +	512 +	8192	30 c	512	24	512 +	128 +	4096 +	256 +	0	0	0	I* (74-100-83)	
23	1094	1858	3-sulfanylhexan-1-ol	68 b	grapefruit	1024	205 a	16384 +	1024	63 b	1024	92 a	2048	243 a	16384 + d	128	32 bc	1024	256	I* (57-41-61)
24	1191	1969	3-sulfanyl-2-butylpropan-1-ol <sup>IST</sup>	+	plastic	128 +	256 +	256 +	256 -	512 -	128 -	2048 -	256 +	512 -	0 -	0 -	0	0	0	I <sup>-</sup>
25	1199	1962	3-sulfanylheptan-1-ol <sup>IST</sup>	+	lemon, hoppy	256	96 b	16384 +	2048 +	2048 + b	2048 +	1024	160 a	16384 +	256 + b	512 -	0 - b	0	0	I* (114-81-55)
26	1208	1818	3-sulfanyl-4-methylpentan-1-ol <sup>IST</sup>	27 b	rhubarb, grapefruit	2048	86 b	4096	50 a	2048	548 a	65536 - c	64	36 b	2048 - c	4 - b	256 - c	0 - b	0	I* (100-55-73)
27	1308	2085	3-sulfanyloctan-1-ol <sup>IST</sup>	31 <sup>a</sup>	caty, grapefruit	2048	140 <sup>a</sup>	65536 +	1024 +	2048 +	2048 -	256 +	512 -	128 -	256 -	0 -	0	0	0	I
			sum of concns	247 a		1389 a	80 b	1337 a	92 b	566 b	17 c	118 c	22 c	83 c						

<sup>a</sup>Amounts determined by GC-PPPD in wort and beer (ng/L, IST equivalents for compounds carrying the IST superscript). GC-O AEDA analyses (FD) on the pHMB extracts. Assay in duplicate. All worts or beers that do not share a common on-line Roman letter are significantly different ( $p < 0.05$ ) according to Tukey's test. Statistics processed separately for worts and for beers. Key: +, detected under the quantitation limit; -, not detected ( $< 5-10$  ng/L). Quantitation limit = 25 ng/L for 14, 30 ng/L for 17, 28 ng/L for 23, and 15 ng/L for IST. Data for THW and THB are issued from Gros et al.<sup>22</sup> but given here in "beer equivalents". I indicates the compound was identified by coincidence with the GC-PPPD retention indexes and odor descriptor of the pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP), I<sup>+</sup> indicates additional confirmation by mass spectrometry (full scan monitoring), and I<sup>-</sup> indicates the compound was tentatively identified by coincidence with the GC-O retention indexes and odors of pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP). <sup>b</sup>Three main m/z ions given in parentheses.

Table 3. Sulfanylalkyls Carrying a Carbonyl Function at the  $\beta$  Position<sup>a</sup>

CP-Sil 5	RI	substance	odor (GC-O)	Tomahawk		Nelson Sauvin		Cascade		Saaz		Non Hopped		Identification reliability (3 main <i>m/z</i> ions given in parentheses)
				wort	ale beer	wort	ale beer	wort	ale beer	wort	ale beer	wort	ale beer	
28	907	3-sulfanylpentanal <sup>IST</sup>	hoppy, flower	16	1024	256	512	256	512	128	128	0	0	0 1
29	915	4-sulfanyl-4-methylpentan-2-one	catty, blackcurrant	32	40 b	256 23	4096	16	256	16	128	0	0	0 1* (132-75-55)
30	931	3-sulfanyl-2-ethylpropanal <sup>IST</sup>	skunky, plastic	8	256	16	128	32	128	32	64	0	0	0 1
31	947	1-sulfanylpentan-3-one <sup>IST</sup>	green, mineral	256	51 b	1024	40 b	256	4096	512	169 a	256	125 ab	8192 1* (61-85-118)
32	1013	3-sulfanylhexanal <sup>IST</sup>	flowery, lemon	128	33	2048	128	32	128	32	16	0	0	0 1* (99-114-132)
33	1113	3-sulfanylpentanal <sup>IST</sup>	lemon, candy	60 a	1024	512	64	16 b	128	64	64	0	0	0 1* (104-69-86)
34	1133	3-sulfanyl-2-butylpropanal <sup>IST</sup>	chicken soup, plastic	256	32	64	64	64	64	32	64	0	0	0 1*
35	1214	3-sulfanyloctanal <sup>IST</sup>	citrus, peach	27 a	512	256	256	128	64	128	64	0	0	0 1* (131-83-109)
			sum of concns	87 a	158 a	65 ab	169 a	16 c	44 b	169 a	169 a	125 ab		

<sup>a</sup>Amounts determined by GC-PPPD in wort and beer (ng/L, IST equivalents for compounds carrying the IST superscript). GC-O AEDA analyses (FD) on the *p*HMB extracts. Assay in duplicate. All worts or beers that do not share a common on-line Roman letter are significantly different ( $p < 0.05$ ) according to Tukey's test. Statistics processed separately for worts and for beers. Key: +, detected under the quantitation limit; -, not detected (<5-7 ng/L). Quantitation limit = 20 ng/L for 29 and 15 ng/L for IST. Data for THW and THB are issued from Gros et al.<sup>22</sup> but given here in "beer equivalents". I indicates the compound was identified by coincidence with the GC-PPPD retention indexes and odor descriptor of the pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP), I+ indicates additional confirmation by mass spectrometry (full scan monitoring), and I- indicates the compound was tentatively identified by coincidence with the GC-O retention indexes and odors of pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP).<sup>b</sup>Three main *m/z* ions given in parentheses.

## RESULTS AND DISCUSSION

To assess how a hop variety can affect the polyfunctional thiol profile of beer, pilot worts were hopped at the late stage of boiling (1.78 g/L) with four different cultivars, Tomahawk (TH), Nelson Sauvin (NS), Cascade (C), and Saaz (S), and compared with a nonhopped (NH) beer (these beers are henceforth referred to as THB, NSB, CB, SB, and NHB). As previously tested on hop pellets,<sup>25</sup> the pHMB-selective thiol extraction procedure was applied here to clarify worts (W) and beers (B). The extracts thus obtained were analyzed by GC-PFPD, GC-O, or GC-MS. By comparison with references issued from commercial standards or combinatorial syntheses,<sup>28–32</sup> 13  $\beta$ -sulfanylalkyl acetates (Table 1), 14  $\beta$ -sulfanylalkyl alcohols (Table 2), 8  $\beta$ -sulfanylalkyl carbonyls (Table 3), and 6 other polyfunctional thiols (Table 4) were identified in worts or beers (chemical structures shown in Figure 1).

**Polyfunctional Thiols Found in Nonhopped Beer.** As depicted in Figure 2, very few peaks were detectable at the PFPD detector in NHB. Among them, 2-sulfanylethyl acetate (**38**; burnt, grill) was found as the major polyfunctional thiol (2  $\mu\text{g/L}$ , Table 4). **38** arises from 2-sulfanylethan-1-ol (**36**; grilled, gas) esterification, itself issued from the Ehrlich degradation of cysteine.<sup>33</sup> When hop pellets were added into the boiling kettle, additional contributions from hop could explain higher amounts of **38**, especially in CB (30  $\mu\text{g/L}$ ). This value is very close to the threshold value, assessed at 40  $\mu\text{g/L}$  in wine model media.<sup>39</sup> Noteworthy, however, is the relatively low concentration measured in the corresponding wort (27  $\text{ng/L}$ ).

1-Sulfanylpentan-3-one (**31**; green, mineral, Table 3) and its derived alcohol, 1-sulfanylpentan-3-ol (**19**; mushroom, nettle, Table 2) were detected by GC-PFPD in all beer extracts (34–317  $\text{ng/L}^{\text{IST}}$ ), including the NHB extract (125 and 56  $\text{ng/L}^{\text{IST}}$ , respectively). The impact of **31** was much lower before fermentation (FD = 256–512 in three wort extracts; only the NSW reached FD = 1024, 20  $\text{ng/L}^{\text{IST}}$ ). With its  $\beta$ -sulfanylalkyl structure, **31** could be derived from addition of hydrogen sulfide onto 1-penten-3-one (wort lipid-oxidation-derived product).<sup>33,40</sup>

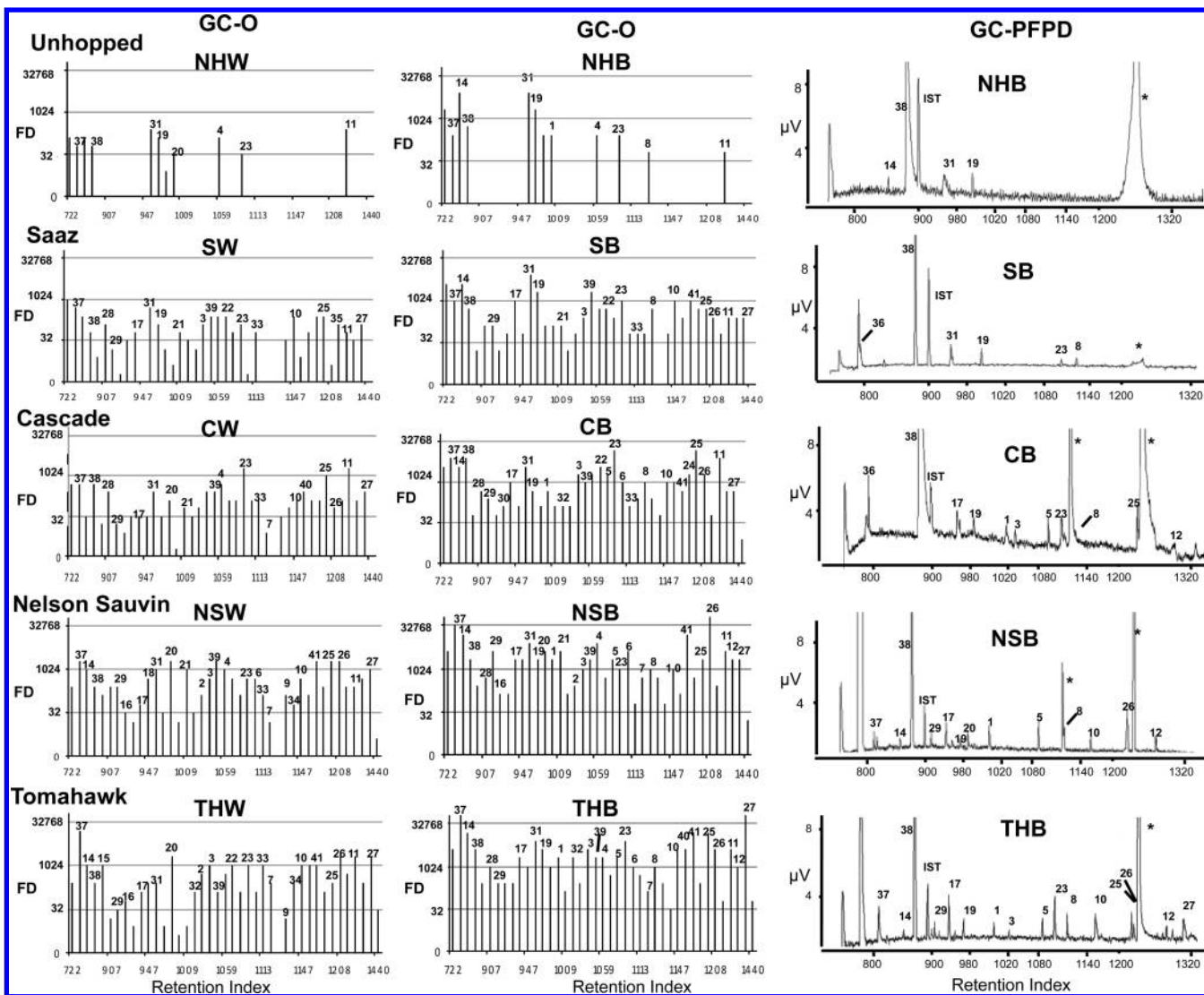
**Differences between Hopped Products.** The PFPD chromatograms for NHB and SB exhibited similar profiles (Figure 2). In SB only two additional compounds were quantifiable at the PFPD detector: 3-sulfanylhexan-1-ol (**23**; grapefruit, 32  $\text{ng/L}$ , close to its threshold assessed at 55  $\text{ng/L}$  in beer,<sup>14</sup> FD = 1024) and 3-sulfanyl-2-methylbutyl acetate (**8**; cooked meat, grill, 41  $\text{ng/L}^{\text{IST}}$ , FD = 512) (Tables 1 and 2). Their occurrence at trace levels was recently evidenced in Saaz hop pellets.<sup>25</sup> Our results indicate that Saaz contains both few thiols<sup>25</sup> and few thiol precursors.

The other three beers differed strongly from NHB and SB. Both the GC-O and the GC-PFPD profiles were completely different, with in all cases numerous odorant thiols with high FDs (Figure 2). In all three beers, despite protection from light throughout the extraction, 3-methyl-2-buten-1-thiol (MBT, **37**; coffee, skunky) emerged as one of the most odorant volatile thiols (FD = 8192–65536; concentrations 77–584  $\text{ng/L}$ , corresponding to a concentration to odor threshold<sup>41</sup> ratio (OAV<sup>35</sup>) of 17–292). MBT was previously detected in hop,<sup>25</sup> but the level found in hop could not explain the amounts found in beer (only 1/30 for TH, 1/13 for NS, and 1/21 for C). In the absence of light, some MBT is produced in the presence of fermentation-derived hydrogen sulfide by nucleophilic

Table 4. Other Polyfunctional Thiols Found in Hop Pellets<sup>a</sup>

RI	CP-Sil 5	FFAP	substance	odor (GC-O)	Tomahawk		Nelson Sauvin		Cascade		Saaz		nonhopped		identification reliability <sup>b</sup>	
					wort	ale beer	wort	ale beer	wort	ale beer	wort	ale beer	wort	ale beer		
36	722	1538	2-sulfanylethan-1-ol	grilled, gas	(170) b (32)	(5790) a (512)	(160) b (32)	(6610) a (512)	(330) b (64)	(5370) a (512)	(1170) a (128)	(4110) a (512)	(16)	(3090) a (256)	I <sup>+</sup> (47–60–78)	
37	810	1112	3-methyl-2-buten-1-thiol	coffee, skunky	+ 16384 584 a	65536 +	2048 194 b	32768 +	512 77 b	8192 +	512	28 b	1024	64	256	I <sup>+</sup> (69–53–102)
38	880	1454	2-sulfanylethyl acetate	burnt, grill	24 b	256 9998 b	4096 48 a	256 6889 b	2048 27 b	512 29918 a	8192 + c	64 2399 b	512	2055 b	512	I <sup>+</sup> (43–60–61)
39	1051	1780	3-sulfanyl-2-methylbutan-1-thiol <sup>IST</sup>	mushroom, white fruits	+ 128 15	2048 +	2048 +	2048 +	256 +	1024 +	256 +	256 +	2048	0	0	I
40	1170	2100	6-sulfanylhexan-1-ol <sup>IST</sup>	mushroom, flowers	+ 1024 27	4096	–	128	–	1024 +	256 +	8	–	256	–	I
41	1185	1838	4-sulfanyl-4-methylpentan-1-ol <sup>IST</sup>	celery	–	1024 +	16384 +	2048 +	16384 –	128	–	512	–	1024	–	I <sup>–</sup>

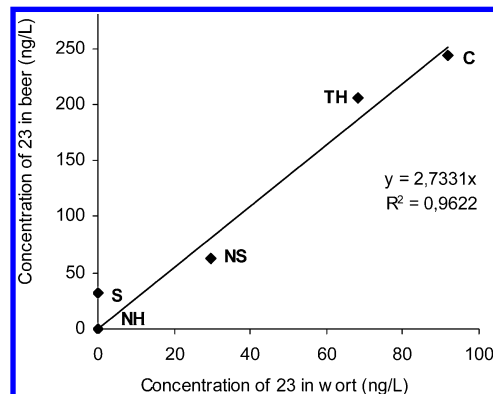
<sup>a</sup>Amounts determined by GC-PFPD in wort and beer (ng/L, IST equivalents for compounds carrying the IST superscript). GC-O AEDA analyses (FD) on the pHMB extracts. Assay in duplicate. All worts or beers that do not share a common on-line Roman letter are significantly different ( $p < 0.05$ ) according to Tukey's test. Statistics processed separately for worts and for beers. Key: +, detected under the quantitation limit; –, not detected (<5–7 ng/L). Quantitation limit = 20 ng/L for **37** and **38** and 15 ng/L for IST. The concentrations of **36** have been roughly assessed by applying an IST-relative recovery factor of 0.1 (concentrations given in parentheses, no correction applied on the FD values). Data for THW and THB are issued from Gros et al.<sup>22</sup> but given here in "beer equivalents". I indicates the compound was identified by coincidence with the GC-PFPD retention indexes and odor descriptor of the pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP). I<sup>+</sup> indicates additional confirmation by mass spectrometry (full scan monitoring), and I<sup>–</sup> indicates the compound was tentatively identified by coincidence with the GC-O retention indexes and odors of pure or synthesized compound on two capillary columns (CP-Sil 5 CB and FFAP). <sup>b</sup>Three main  $m/z$  ions given in parentheses.



**Figure 2.** Flavor dilution chromatograms (FD =  $2^n$ , with  $n + 1$  = number of dilutions applied on the extract until no odor was perceived, precision  $n \pm 1$  or a factor of 2 between FD values) and GC-PFPD chromatograms of *p*HMB extracts (IST 670 ng/L) of worts (W) and beers (B). Data for THW and THB are issued from Gros et al.<sup>22</sup> An asterisk indicates an unknown compound.

substitution on 3-methyl-2-buten-1-ol (MBOH; hop aglycon<sup>42</sup>).<sup>43</sup> The occurrence of MBOH being strongly linked to hop  $\alpha$ -acid content,<sup>44</sup> it was not surprising to find higher levels of MBT when the “Super Alpha” hop Tomahawk was used (MBT contents and FDs as follows: THB > NSB > CB > SB > NHB) (Table 4). 3-Sulfanyl-3-methylbutan-1-ol (17; sulfur, soup) previously described as the main product issued from the reaction of MBOH with hydrogen sulfide<sup>43</sup> also followed the same order (296 ng/L in THB, threshold 1500 ng/L in 12%, v/v, ethanol,<sup>45</sup> leading to OAV < 1) (Table 2). The acetate derived from 17 (5; pepper, plastic) was also found in all three beers (72–168 ng/L).

The Cascade variety has been distinguished from others by higher 23 (72–117  $\mu\text{g}/\text{kg}$ ) and 3-sulfanylheptan-1-ol (25; 25–52  $\mu\text{g}/\text{kg}$ ) contents.<sup>25</sup> As depicted in Figure 2, 23 and 25 also proved to be the most intense odorants in extracts of the derived worts (FD = 2048 and 1024, respectively) and beers (23 and 25 reached FD = 16384–243 and 160 ng/L, respectively). However, our data are not in line with the results of Kishimoto et al., who report close Charm values for 23 for beers hopped with Cascade and Saaz.<sup>14</sup> The concentration of

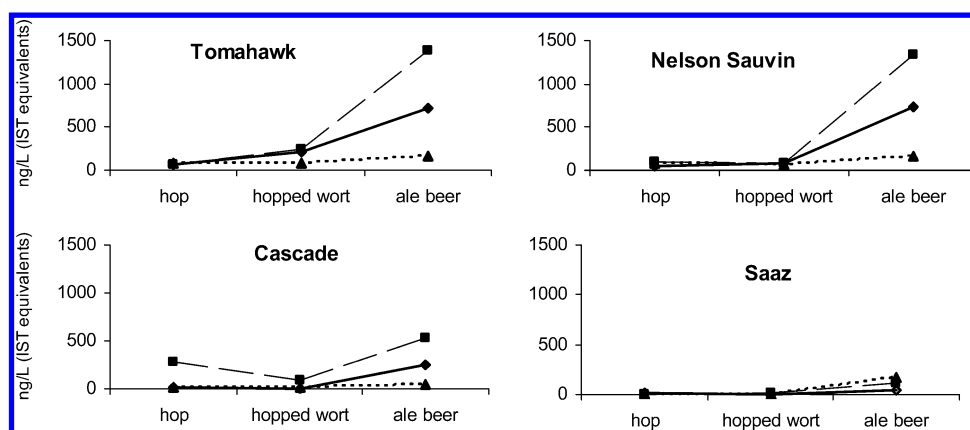


**Figure 3.** Relationship between wort and beer 3-sulfanylhexan-1-ol (23) contents.

23 in CB was clearly above its threshold value, assessed at 55 ng/L in beer<sup>14</sup> (OAV = 4).

23 and 25 also emerged as very important in THB (23, OAV = 4), although these free thiols were previously found





**Figure 4.** Evolution of the total amount of  $\beta$ -sulfanylalkyl alcohols (■),  $\beta$ -sulfanylalkyl acetates (◆), and  $\beta$ -sulfanylalkyl carbonyls (▲) from hop (concentrations converted into wort equivalents) to wort and beer (Tomahawk,<sup>22</sup> Nelson Sauvin, Cascade, and Saaz hopping).

at lower concentrations in TH hop (only 7–12  $\mu\text{g}/\text{kg}$  **23** and  $<1$   $\mu\text{g}/\text{kg}$  **25**).<sup>25</sup> However, as depicted in Figure 3, a linear correlation was found for **23** between the levels detected in worts and beers ( $R^2 = 0.962$ ). Yeast reductase activities are able to convert 3-sulfanylhexanal (**32**; flowery, lemon) into **23**,<sup>46</sup> but even in the TH wort, **32** was found under the quantitation limit. Most probably, TH balances its lower amount of **23** with a higher amount of precursors, partially released through boiling. The regression slope well above 1 confirms the key role of undetected precursors in wort.

On the other hand, **25** was detected only at trace levels in both worts, while found at 96 and 160  $\text{ng}/\text{L}^{\text{IST}}$  in THB and CB, respectively. The corresponding aldehyde **33** reached 60  $\text{ng}/\text{L}^{\text{IST}}$  in the TH-hopped wort but only 16  $\text{ng}/\text{L}^{\text{IST}}$  in the C-hopped wort.

Another very interesting  $\beta$ -sulfanylalkyl aldehyde is 3-sulfanyloctanal (**35**), previously highlighted as a marker of Nelson Sauvin (46–59  $\mu\text{g}/\text{kg}$ ) and Tomahawk (26–43  $\mu\text{g}/\text{kg}$ ) hops.<sup>25</sup> Only the Tomahawk-hopped beer contained high levels of the corresponding alcohol **27**, and even in that case, less than half of compound **27** (140  $\text{ng}/\text{L}^{\text{IST}}$ ) could have been issued from the free thiols **27** and **35** in the wort. This confirms the presence of precursors in hop. This strong catty/grapefruit odorant emerged as the most specific thiol in THB (FD = 65536).

In the NSB, total amounts of  $\beta$ -sulfanylalkyl alcohols were particularly high (1337  $\text{ng}/\text{L}$ ), but not significantly different from those found in THB (1389  $\text{ng}/\text{L}$ ) (Table 2). Both beers also showed similar  $\beta$ -sulfanylalkyl acetate concentrations (723/729  $\text{ng}/\text{L}$ ). Only a few odorant thiols differed strongly between these two beers. 3-Sulfanyl-4-methylpentan-1-ol (**26**) is probably the main contributor to the specific grapefruit-like flavor of the NSB (FD = 65536, 548  $\text{ng}/\text{L}^{\text{IST}}$ , OAV = 8 for a threshold of 70  $\text{ng}/\text{L}$  in beer<sup>24</sup>). The NS hop constituents 3-sulfanyl-2-methylbutan-1-ol (**20**; leek, hop), 3-sulfanylpentan-1-ol (**21**; citrus), and 4-sulfanyl-4-methylpentan-2-one (**29**) gave rise to only FD = 4096 in the NSB. Although previously considered responsible for the Muscat-like character of Cascade beers,<sup>14,18</sup> **29** was not quantifiable at the PFPD in CB pHMB extract (FD = 256) (Table 3). 4-Sulfanyl-4-methylpentan-2-ol (**18**) was also perceived at the sniffing port (FD = 2048, 1024, and 128 in NSB, THB, and CB extracts, respectively).

The isomer of **29** without the  $\beta$ -sulfanylalkyl backbone, 4-sulfanyl-4-methylpentan-1-ol (**41**; celery), also exhibited a high flavor dilution (FD = 16384) in NSB and THB extracts, as expected from the higher levels found in both hops (5–21  $\mu\text{g}/\text{kg}$ ).<sup>25</sup>

A few sulfanylalkyl esters gave rise to high FD values in beer. 3-Sulfanylhexyl acetate (**11**) reached FD = 8192 in CB (also characterized by high amounts of the corresponding alcohol (**23**)). 3-Sulfanyl-2-ethylpropyl acetate (**10**), previously claimed to be a marker of TH and NS hops (14–44  $\mu\text{g}/\text{kg}$ ), was found at 326 and 84  $\text{ng}/\text{L}^{\text{IST}}$  in THB and NSB while being nonquantifiable in the other beers. 4-Sulfanyl-4-methyl-2-pentyl acetate (**6**; grilled nut) was detected up to dilution 4096 in NSB.

As described above, volatile thiol profiles strongly differ from wort to beer. The observed beer thiol contents are much higher than might be expected on the basis of hop content<sup>25</sup> and hopping rate. As depicted in Figure 4, this was particularly true when the NS or TH hop was used. For  $\beta$ -sulfanylalkyl alcohols and acetates, the major increase took place during fermentation: 8–17-fold from NSW to NSB, 3–6-fold when C or TH was used. Accurate prediction of hop varietal impact thus requires quantitating thiol precursors.

In an attempt to evidence S-cysteine conjugates in hop, a Cascade extract was prepared according to Starckenmann et al.<sup>38</sup> and analyzed by RP-HPLC–HRMS/MS. For this experiment, Cascade hop was chosen for its high potential to release 3-sulfanylhexan-1-ol through the brewing process. S-3-(1-Hydroxyhexyl)cysteine was synthesized according to the procedure described by Thibon et al.<sup>36</sup> and Pardon et al.<sup>37</sup> As depicted in Figure 5, this RP-HPLC–HRMS/MS experiment enabled us to evidence, for the first time, an S-cysteine conjugate in hop: the compound eluting at 6.8 min was confirmed to be S-3-(1-hydroxyhexyl)cysteine ( $\text{C}_9\text{H}_{20}\text{NO}_3\text{S}^+$ , experimental  $m/z = 222.11600$ , theoretical  $m/z = 222.11584$ ,  $\delta = 0.72$  ppm).

In conclusion, both free compounds and bound precursors are available in hop to contribute to the amounts of polyfunctional thiols found in beer. The exact structures of the precursors present in each hop variety remain to be determined. Quantitative data are also required to accurately predict this hop varietal impact.

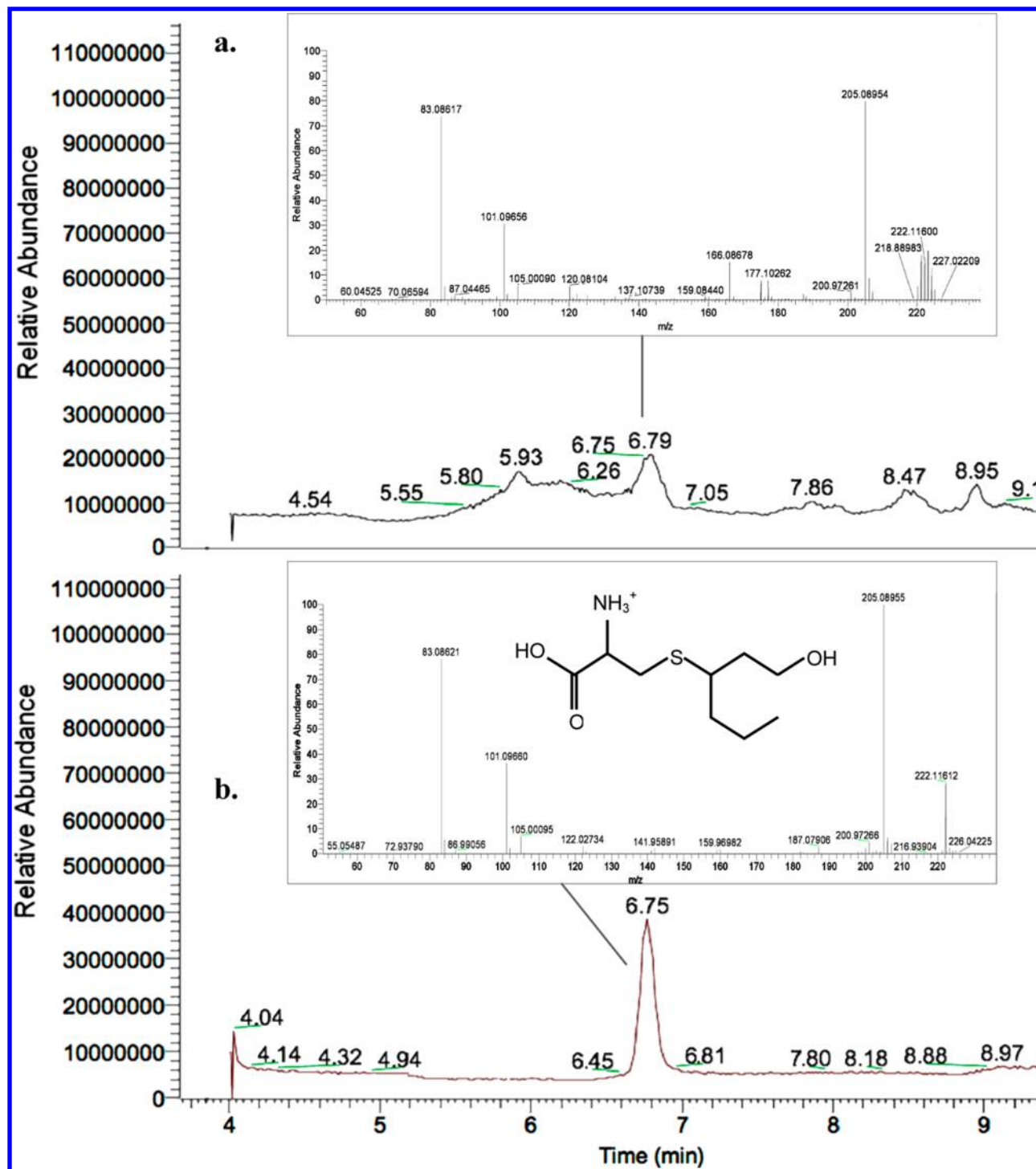


Figure 5. RP-HPLC–HRMS/MS ( $m/z$  222.12) chromatograms of (a) the Cascade hop extract and (b) the 3-S-(1-hydroxyhexyl)cysteine reference. HRMS/MS (ESI+) spectra at 6.8 min.

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### Notes

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## ABBREVIATIONS

FD, flavor dilution; OAV, odor activity value; *p*HMB, *p*-hydroxymercuribenzoic acid; PFPD, pulse flame photometric detection; RI, retention index; AEDA, aroma extract dilution analysis; IST, internal standard

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