

# Quantitation of Selected Terpenoids and Mercaptans in the Dual-Purpose Hop Varieties Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace

Marie-Lucie Kankolongo Cibaka, Jacques Gros, Sabrina Nizet, and Sonia Collin\*

Unité de Brasserie et des Industries Alimentaires, Earth and Life Institute (ELIM), Université Catholique de Louvain, Croix du Sud 2, Box L7.05.07, B-1348 Louvain-la-Neuve, Belgium

**ABSTRACT:** Free terpenoids and both free and bound polyfunctional thiols were investigated in five selected dual-purpose hop cultivars. Surprisingly, the dual-purpose Sorachi Ace variety was found to contain higher amounts of farnesene (2101 mg/kg) than aromatic hops such as Saaz but only traces of 3-methylbutylisobutyrate, a compound that usually distinguishes all bitter varieties. All five cultivars investigated here showed an exceptional citrus-like potential explained by either monoterpenic alcohols or polyfunctional thiols. Among the monoterpenic alcohols,  $\beta$ -citronellol at concentrations above 7 mg/kg distinguished Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace from Nelson Sauvin and Tomahawk, two previously investigated dual-purpose hops, while linalool (312 mg/kg) and geraniol (211 mg/kg) remained good discriminating compounds for Nelson Sauvin and Tomahawk, respectively. Regarding polyfunctional thiols, higher amounts of 3-sulfanylhexyl acetate (27  $\mu$ g/kg) characterized the Citra variety. Free 4-sulfanyl-4-methylpentan-2-one proved discriminant for Sorachi Ace, while the bound form is predominant in Nelson Sauvin. On the other hand, an S-conjugate of 3-sulfanylhexan-1-ol was found in Sorachi Ace at levels not far from those previously reported for Cascade, although the free form was undetected here. Both free and bound grapefruit-like 3-sulfanyl-4-methylpentan-1-ol (never evidenced before the present work) emerged as discriminating compounds for the Hallertau Blanc variety. The apotryptophanase assay also allowed us to evidence for the first time an S-conjugate of 2-sulfanylethan-1-ol.

**KEYWORDS:** hop (*Humulus lupulus* L.), flavor, terpenic alcohols, polyfunctional thiols, cysteine-S-conjugates, feline

## INTRODUCTION

In the past few decades, hop (*Humulus lupulus* L.) has entered a new age. Triploids obtained by crossing tetraploid and diploid parents appear very productive.<sup>1</sup> From such selections have emerged new dual-purpose varieties producing hop cones with both high bitter acid and high oil contents. The dual-purpose varieties increase the possibility of late hopping (addition of hop at the end of wort boiling) and dry hopping (addition of hop during beer maturation). These varieties notably include Amarillo, Citra, Centennial, Chinook, Cluster, El Dorado, Horizon, Mosaic, Simcoe, Sorachi Ace, and Tomahawk from U.S.A.; Dr Rudi, Green Bullet, Nelson Sauvin, and Rakau from New Zealand; Hallertau Blanc and Polaris from Germany; Boadicea, First Gold, and Wye Challenger from the United Kingdom; and Southern Promise from South Africa.<sup>2</sup>

As major constituents of hop oils, terpenoids have been extensively used to discriminate different varieties.<sup>3–7</sup> In a recent study comparing hop oils from the dual-purpose Tomahawk and Nelson Sauvin varieties on the one hand and traditional aromatic varieties on the other, bergamotene and  $\beta$ -farnesene were confirmed to be discriminating compounds for aromatic low bitter hops (undetected and <1.1 mg/kg in Tomahawk and Nelson Sauvin, respectively, versus >10 mg/kg for bergamotene and >150 mg/kg for  $\beta$ -farnesene in aromatic low-bitter hops).<sup>5,7,8</sup> On the other hand 3-methylbutylisobutyrate, because it derives from humulone degradation,<sup>5</sup> was logically found at higher concentrations in the Tomahawk and Nelson Sauvin varieties (>100 mg/kg).<sup>8</sup> A particular profile also emerged from the Tomahawk cultivar ( $\alpha$ - and  $\beta$ -selinenes >600 mg/kg IST

equivalents in total, methyl geranate >40 mg/kg IST equivalents, and geraniol >200 mg/kg), while Nelson Sauvin proved to be richer in linalool (>250 mg/kg).<sup>8</sup> On the other hand, the new dual-purpose Citra variety was claimed to be rich in geraniol (>40 mg/kg).<sup>9</sup>

Efforts have been made to understand the fate of each compound through the brewing and fermenting processes. The major hop oil compounds undergo heavy losses in the boiling kettle<sup>10,11</sup> and are therefore not detected in the final beer. Just a few, less apolar terpenoids such as linalool, geraniol,  $\beta$ -citronellol, and humuladienone can partially survive and impart hoppy notes to the final beer.<sup>3,11–15</sup>

Terpenoid glycosides have been evidenced in several hop varieties and conditionings (ethanolic extracts, spents, and tannin extracts). Linalool and  $\alpha$ -terpineol are released from hop extract subjected to enzymatic treatment.<sup>16,17</sup> Hop glycosides, more hydrosoluble in the wort than their corresponding aglycones, can be partially hydrolyzed or biochemically transformed into the corresponding aglycones through primary or secondary fermentations. These compounds can contribute to the kettle hop flavor of the beer.<sup>18</sup> This mechanism is particularly significant when procedures such as dry hopping or bottle refermentation are used.<sup>19,20</sup> The remaining glycosylated precursors in the beverage, still not converted to their

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corresponding volatiles, can be further transformed in the mouth. This leads to a long-lasting hoppy aroma.<sup>21</sup> This source of hoppy note must be viewed as part of the hop flavoring potential.

Besides free and bound terpenoids, polyfunctional thiols have recently been referred to as important hop-derived flavoring compounds in beer, especially when dual-purpose hop varieties are used. Forty-one volatile polyfunctional thiols have been reported in hop, most of them having a 3-carbon distance between the thiol and the oxygenated functions.<sup>8</sup> Rhubarb/grapefruit-like 3-sulfanyl-4-methylpentan-1-ol (3S4MPol) and blackcurrant-like 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone) constitute key compounds of the “Sauvignon-like” notes imparted to beer by Nelson Sauvin hop.<sup>14</sup> Floral-like 3-sulfanyl-2-ethylpropyl acetate (3S2EPra) and citrus/peach-like 3-sulfanyloctanal (3SOal) distinguish Tomahawk from all other thiol-rich varieties. Most low-bitter varieties, including Saaz, appear to be greatly lacking in polyfunctional thiols. Yet blackcurrant-like 4S4M2Pone and grapefruit-like 3-sulfanylhexan-1-ol (3SHol) have been described as key aroma compounds participating in the Muscat-like flavor perceived in Cascade (4.5–8%  $\alpha$ -acids) hopped beer.<sup>8,13,15,22</sup>

Whatever the cultivar, the thiol content of the final beer reaches much higher values than expected from the hop content. S-cysteine conjugates, recently evidenced for the first time in hop,<sup>23,24</sup> are partly responsible for the increases in thiol content from hop to wort and beer. The  $\beta$ -lyase activity of yeast, causing specific cleavage of the thiol from its conjugate, converts nonvolatile precursors to odorant thiols. The commercial apotryptophanase from *Escherichia coli* has been widely used to investigate cysteine- and cysteinylglycine adducts in plants<sup>24–27</sup> (free amine required for the enzyme activity-glutathione adducts not quantified by this assay<sup>27</sup>). Depending on the hop variety, the S-cysteine conjugate fraction represents a bound potential 23–126 times as high as the fraction of corresponding volatiles.<sup>24</sup> The rate of conversion by the action of yeast remains low during the main fermentation.<sup>28–30</sup> The late stage of bottle refermentation<sup>20</sup> and dry hopping during maturation constitute additional chances for yeast to convert these intact S-conjugates.<sup>19</sup> The nutrient-depleted conditions encountered after fermentation are thought to favor this yeast activity.<sup>19,30,31</sup>

Chemical information concerning the aromatic potential of other new dual-purpose varieties remains scarce. The aim of the present work was therefore to quantify free terpenoids and both free and bound polyfunctional thiols in five selected cultivars: Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace. The aromatic profiles of these cultivars were compared with those previously obtained for the dual-purpose varieties Tomahawk and Nelson Sauvin and for the low-bitter aromatic reference Saaz.<sup>8,24</sup>

## EXPERIMENTAL PROCEDURES

**Chemicals.** Ammonia (28%) was from VWR (Leuven, Belgium). *p*-Hydroxymercuribenzoic acid (pHMB), 37% HCl, S-benzyl-L-cysteine (IST for apotryptophanase assays), apotryptophanase, pyridoxal-5-phosphate, ethylenediaminetetraacetic acid, the Amberlite IR-120 resin, carvone (IST for Likens Nikerson extractions), myrcene, linalool, geraniol,  $\beta$ -caryophyllene,  $\alpha$ -humulene,  $\beta$ -farnesene, undecan-2-one, 3-sulfanylpropyl acetate (1)\*, 3-sulfanylpropan-1-ol (14)\*, 3-sulfanyl-3-methylbutan-1-ol (17)\*, 2-sulfanyloctanal (36)\*, 2-sulfanylethyl acetate (38)\*, and 2-acetylthiophene (EST for free thiol extractions) were purchased from Sigma-Aldrich (Bornem, Belgium). An asterisk means that numbering of compounds is conserved from refs 8 and 24. 4-Methoxy-2-methylbutan-2-thiol (IST for free thiol extractions), 3-sulfanylhexyl acetate (11)\*, 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.). Dichloromethane (99.9%) obtained from VWR (Leuven, Belgium) was distilled before use. Milli-Q water was used (Millipore, Bedford, MA, U.S.A.). NaOH and sodium sulfate (99%) were supplied by Janssen (Geel, Belgium). A strongly basic Dowex resin 1  $\times$  2, Cl<sup>-</sup> form (Sigma-Aldrich, Bornem, Belgium) was stored in hydrogen chloride (0.1 M). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany), and tris(hydroxymethyl)-aminomethane (Tris) was obtained from USB (Cleveland, Ohio, U.S.A.). Formic acid was provided by Fischer (Loughborough, U.K.). Polyvinylpyrrolidone was supplied by Spindal (Gretz-Armainvilliers, France).

**Reference Compounds Synthesized in Our Laboratory.** 3-Sulfanyl-2-ethylpropyl acetate (10)\* was synthesized prior to this work (reagents and complete procedure described in ref 32). 1-Sulfanylpentan-3-ol (19)\* and 3-sulfanylpentan-1-ol (21)\* were obtained with the reagents and procedure disclosed in ref 33. 3-Sulfanylheptanal (33) and 3-sulfanyloctanal (35)\* were obtained according to the procedure described in ref 34. 3-Sulfanyl-4-methylpentan-1-ol (26)\* was synthesized according to ref 35.

**Hop Samples.** Amarillo, Citra, Mosaic, and Sorachi Ace bred in the U.S.A. were provided by Yakima Chief (Louvain-la-Neuve, Belgium). Hallertau Blanc hop came from Hopsteiner (Germany). Their  $\alpha$ -acid contents were given by the suppliers.

**Extraction of Terpenoids and Other Non-Sulfur Hop Oils by the Likens Nickerson Method.** Steam distillation–solvent extraction was carried out in a microextractor (Alltech 8910) according to ref 36. Hop pellets (0.5 g) were milled and mixed with 50 mL of deoxygenated ultrapure water and 1.5 mL of a carvone solution at 20 mg/L (internal standard) in flask A. Dichloromethane and ultrapure, deoxygenated water (1.5 mL each) were introduced into the liquid/liquid extraction area. Dichloromethane (1.5 mL) was introduced into the organic-phase vessel (B). A few clean grains of carborundum were added into flasks A and B. Prior to the procedure, the entire system was purged with nitrogen (2–3 mL/min) for 5 min. Flask A was then heated in a 140 °C oil bath, and flask B was heated in a 70 °C water bath. The vapors were condensed by means of a coldfinger maintained at –10 °C by a cryostat. The entire steam distillation–solvent extraction procedure was carried out under a 2 mL/min nitrogen flow. The steam distillation was stopped after 45 min. The dichloromethane extract was then concentrated to 0.5 mL at 45 °C with a Kuderna–Danish concentrator. The method allows most of the terpenic compounds to be recovered with factors >92% and variation coefficients <6%.<sup>36</sup>

**Extraction of Free Polyfunctional Thiols by pHMB.** Polyfunctional thiols were extracted from hop according to ref 8, adapted from ref 37 (dichloromethane liquid/solid extraction of 15 g of hop, extraction of the resulting organic phase with a pHMB solution, loading of bound thiols onto a strong anion-exchange resin, release of free thiols by exchange with washed cysteine [4  $\times$  50 mL of dichloromethane for washing 640 mg of cysteine], final extraction with dichloromethane, and concentration to 250  $\mu$ L in a Kuderna–Danish apparatus and to 70  $\mu$ L in a Dufton column). 4-Methoxy-2-methylbutane-2-thiol was added as internal standard (IST, at 33.5  $\mu$ g/kg), and 2-acetylthiophene was added as external standard (EST, 1 mL at 200  $\mu$ g/L added before concentration).

**Extraction of Cysteine-S-conjugates from Hop and Release of the Corresponding Free Thiols by the Apotryptophanase Assay.** The extraction was conducted on the hop according to the procedure of ref 24 adapted from ref 27. Milled pellets (100 g) spiked with 1 mL of 1600 mg/L S-benzyl-L-cysteine were extracted with 1 L of hydroalcoholic solution (EtOH/H<sub>2</sub>O/HCOOH = 49.5:49.5:1, v/v/v) for 2 h. After centrifugation for 30 min at 1200g, the polyphenols present in the supernatant were partially removed by addition of 30 g of polyvinylpyrrolidone and stirring for 30 min. After a second centrifugation, the extract was purified on the IR-120 (H<sup>+</sup>) cation-exchange resin (100 g) conditioned with 100 mL of 6% HCl followed by 1 L of ultrapure water. After washing with 500 mL of water, sequential fractions were recovered by eluting with aqueous ammonia solutions (100 mL each) at the following concentrations: 0.3, 0.6, 0.9, 1.2, 1.5, 1.8,

**Table 1. Terpenoids (Top Part) and Other Non-Sulfur Hop Oil Constituents (Bottom Part) Determined by GC-MS (Concentrations in mg/kg of Hop, IST Equivalents for Compounds Carrying the IST Superscript), after Likens Nickerson Extraction (Mean of Duplicates, CV < 6%);<sup>36</sup> Values within a Row with Different Letters Are Significantly Different ( $p < 0.05$ ) According to the Student–Newman–Keuls Test**

retention index CP-Sil 5 CB	substance		$\alpha$ -acid content (% w/w)					previously reported cultivars <sup>8a</sup>	identification reliability (3 main $m/z$ ions given in parentheses)
	name	odor (GC-O)	Amarillo	Citra	Hallertau Blanc	Mosaic	Sorachi Ace		
			9.3	13.9	8.4	11.7	12.3		
982	myrcene	lime	2225.4 d	3945.2 b	3273.6 c	2370.0 d	4654 a	T c, NS e, S f	I* (41, 93, 69)
1086	linalool	floral	109.2 c	18.6 e	18.6 e	58.0 d	57.9 d	T b, NS a, S e	I* (71, 93, 41)
1212	$\beta$ -citronellol	lemon	10.6 c	7.8 d	13.0 b	10.0 c	33.3 a	T e, NS e, S e	I* (41, 69, 55)
1236	geraniol	floral	105.1 b	68.9 d	16.6 f	83.2 c	18.9 f	T a, NS e, S f	I* (69, 41, 68)
1313	methylgeranate <sup>b</sup> IST	greenery	1.7 e	0.3 e	1.6 e	0.9 e	1.0 e	T a, NS c, S d	I* (69, 41, 114)
1435	$\beta$ -caryophyllene	spicy	753.3 d	828.0 d	745.0 d	365.8 e	36.1 f	T a, NS b, S e	I* (41, 93, 69)
1469	$\alpha$ -humulene <sup>b</sup>	resin	1273.1 ab	1320.1 ab	1187.6 b	884.4 c	1328 a,b	T a, NS c, S ab	I* (93, 41, 121)
1475	bergamotene <sup>b</sup> IST	tea	1.7 c	3.1 c	1.9 c	1.4 c	1.2 c	T c, NS c, S a	I* (119, 93, 41)
1486	$\beta$ -farnesene <sup>b</sup>	woody	102.8 c	32.3 c	47.1 c	152.6 c	2101.0 a	T c, NS c, S b	I* (93, 41, 69)
1499	$\alpha$ -amorphenone <sup>b</sup> IST	woody	5.9 ef	8.4 e	14.6 d	3.9 ef	1.9 f	T a, NS b, S e	I* (105, 161, 94)
1548	$\alpha$ -selinene <sup>b</sup> IST	woody	35.8 cd	11.6 d	37.3 c,d	18.4 d	126.7 b	T a, NS b, S cd	I* (189, 93, 107)
1538	$\beta$ -selinene <sup>b</sup> IST	herbs	17.7 ef	6.4 f	36.9 de	7.7 f	77.0 c	T a, NS b, S e	I* (41, 105, 204)
1074	3-methylbutyl-isobutyrate <sup>b</sup>	sweet	69 d	111.9 c	180.7 a	125.7 bc	12.9 e	T a, NS b, S e	I* (43, 71, 70)
1279	undecan-2-one <sup>b</sup>	citrus	48.8 e	113.1 b	29.9 f	236.9 a	72 d	T f,g, NS cd, S c	I* (43, 58, 71)
	total		4760.1 cd	6475.7 b	5604.4 c	4318.9 d	8521.9 a	T a, NS c,d, S e	

<sup>a</sup>Data issued from ref 8; letters after T, NS, or S indicate the statistical group for the reported concentrations in Tomahawk, Nelson Sauvin, and Saaz. I\*, compound identified by mass spectrometry (full scan monitoring) and coincidence with the retention index. <sup>b</sup>Compounds previously selected for discriminating Cascade, Challenger, Hallertau Mittelfrueh, Lublin, Mount Hood, Northdown, Northern Brewer, Nugget, Saaz, Styrian Golding, and Target.<sup>5,7</sup>

and 2.1 M. The 1.2 and 1.5 M fractions were pooled, vacuum-evaporated, and lyophilized. The dried 1.2–1.5 M fraction (~100 mg) was suspended in 2 mL of potassium phosphate buffer (100 mM, pH 7.7) containing pyridoxal 5-phosphate (0.1 mM) and ethylenediamine-tetraacetic acid (1 mM). Five hundred microliters of a solution containing the commercial apotryptophanase from *Escherichia coli* (75–150 units/mg), freshly prepared (1 mg in 0.5 mL of buffer), were added. The incubation was kept at 28 °C for 30 min. A control without enzyme was conducted in parallel. The mixture was then stirred with 5 mL of bidistilled dichloromethane for 30 min. The organic phase was dried over sodium sulfate prior to concentration to 500  $\mu$ L in a Kuderna–Danish apparatus and to 70  $\mu$ L in a Dufton column.

**Gas Chromatography Hyphenated to Olfactometric and Flame Ionization Detectors (GC-O and GC-FID).** Two  $\mu$ L of extract (Likens Nikerson, pHMB free thiols or enzymatically released thiols) 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) column or 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 °C at 1 °C/min, and finally to 220 °C at 3 °C/min and held for 30 min. The column was connected to an FID detector (set at 250 °C with inlet of 21 mL/min H<sub>2</sub> and 210 mL/min air) and a GC-O port (Chrompack) maintained at 250 °C. The GC-O 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.

**Quantitation of Terpenoids and Other Non-Sulfur Hop Constituents.** For commercially available compounds, FID calibration curves (with areas relative to carvone (IST)) were used. For

commercially unavailable compounds, quantitations are given in IST equivalents (response coefficient relative to the IST set at 1; values carrying an IST superscript in Table 1).

**Gas Chromatography Hyphenated to a Pulsed-Flame Photometric Detector (GC-PFPD) and an Electronic Impact Mass Spectrometer (GC-MS).** Two  $\mu$ L of extract (Likens Nikerson, pHMB free thiols or enzymatically released thiols) were analyzed with an Agilent 6890N gas chromatograph. The injections were carried out in the splitless mode at 250 °C, the split being turned on after 0.5 min. The oven temperature program and the columns were the same as described for GC-FID and GC-O. The carrier gas was helium, and the pressure was set at 100 kPa (CP-Sil 5 CB) or 45 kPa (FFAP). The column was connected to a mass spectrometer ( $m/z = 40$ –380 recorded at 70 eV) and a PFPD port (O.I. Analytical PFPD, model 5380). The following parameters were selected for the PFPD detector: temperature, 220 °C; voltage, 590 V; gate width, 18 ms; gate delay, 6 ms; trigger level, 400 mV; pulse frequency, 3.33 Hz. PFPD and MS chromatograms were simultaneously recorded throughout elution; ChemStation software was used. Identifications were done as previously described in ref 8.

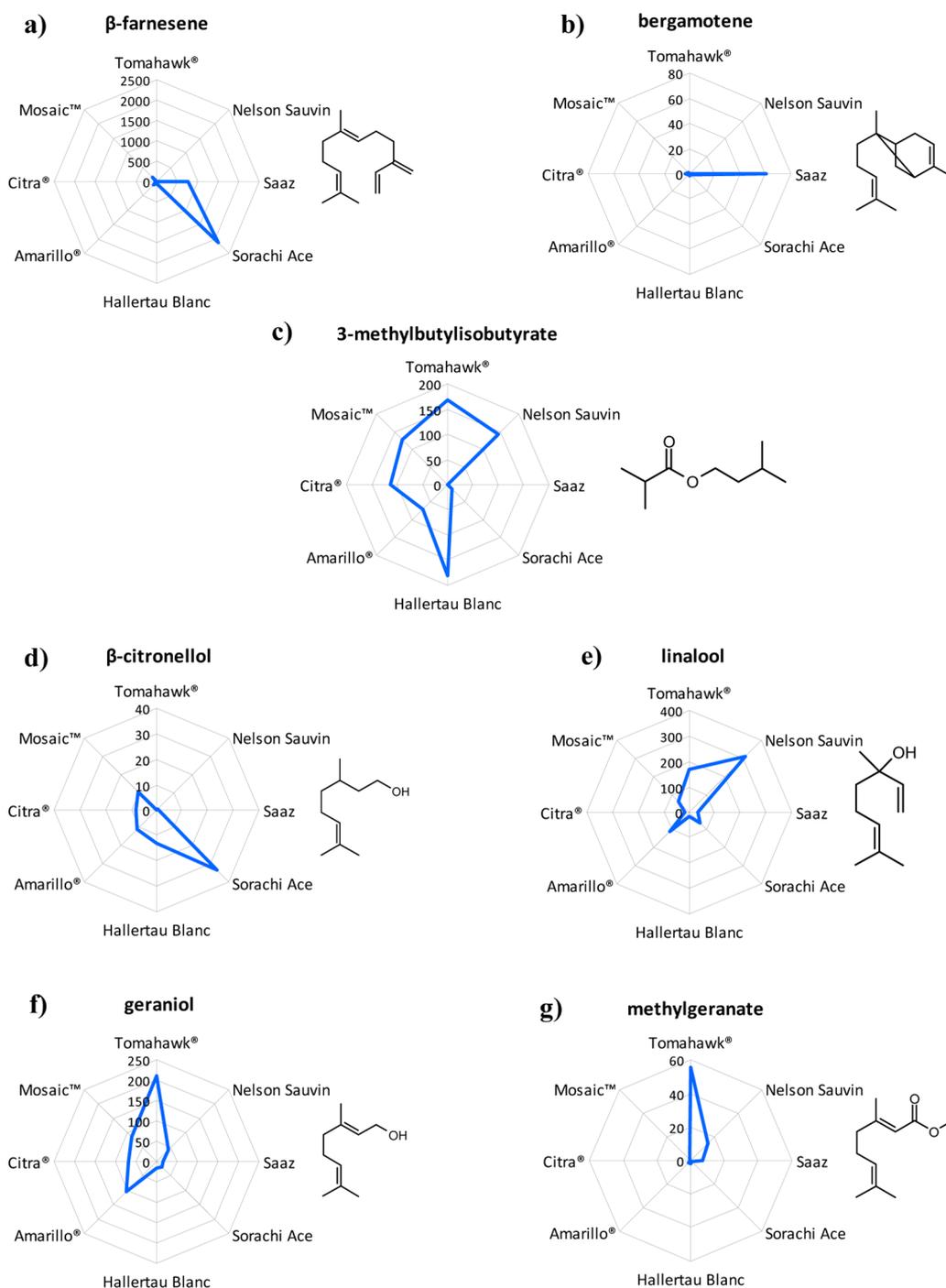
**Quantitation of Thiols (A) in pHMB Extracts.** The following general equation was used for compound A quantitation: concentration of A (in  $\mu$ g/kg) = IST concentration (in  $\mu$ g/kg)  $\times$  (A molecular weight/IST molecular weight)  $\times$  (A area/IST area)  $\times$  (IST molar response coefficient/A molar response coefficient)  $\times$  (IST recovery factor/A recovery factor).

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 2-sulfanylethan-1-ol (bad recovery at the second dichloromethane extraction; approximate concentrations given by applying a ratio of 0.1 assessed by standard addition). For commercially available thiols, complete calibration curves relative to the IST were used (use of the experimental response coefficient ratio in the above equation). For commercially unavailable compounds (values carrying an

**Table 2. Amounts of Polyfunctional Thiols Determined by GC-PPPD in Hop ( $\mu\text{g}/\text{kg}$ , IST Equivalents for Compounds Carrying the IST Superscript), Mean of Duplicates; Values within a Row with Different Letters Are Significantly Different ( $p < 0.05$ ) According to the Student–Newman–Keuls Test<sup>a</sup>**

no.	retention index	CP-Sil 5 CB	name	substance	odor (GC-O)	$\alpha$ -acid content (% w/w)				previously reported cultivars <sup>b</sup>	identification reliability (3 main $m/z$ ions given in parentheses)
						Amarillo	Citra	Hallertau Blanc	Mosaic		
37	1112	810	3-methyl-2-buten-1-thiol	MBT	coffee, skunky	9.3	13.9	8.4	11.7	12.3	I <sup>+</sup> (69, 53, 102)
29	1380	918	4-sulfanyl-4-methylpentan-2-one	4S4M2Pone	caty, black currant	nd c	d	nd c	nd c	30 b	I <sup>+</sup> (132, 75, 55)
38	1452	887	2-sulfanylethyl acetate	2SEA	toasted, grilled	d	867 a	d	8 b	nd b	I <sup>+</sup> (43, 60, 61)
36	1532	722	2-sulfanylethyl-1-ol	2SEol	grilled	d	362 a	d	d	d	I <sup>+</sup> (47, 60, 78)
1	1578	1007	3-sulfanylpropyl acetate	3SPrA	grilled	nd b	7 a	nd b	nd b	nd b	I <sup>+</sup> (74, 43, 47)
14	1623	849	3-sulfanylpropan-1-ol	3SProl	popcorn	nd a	d	d	d	nd a	I <sup>+</sup> (57, 58, 45)
17	1654	933	3-sulfanyl-3-methylbutan-1-ol	3S3MBol	sulfur, soup	d	d	d	d	d	I <sup>+</sup> (86, 69, 75)
33	1655	1113	3-sulfanylheptanal <sup>IST</sup>	3SHptal	lemon, candy	nd c	nd c	nd c	nd c	nd c	I <sup>+</sup> (102, 60, 69)
10	1657	1147	3-sulfanyl-2-ethylpropyl acetate <sup>IST</sup>	3S2EPPrA	floral, vinegar	nd c	nd c	nd c	nd c	nd c	I <sup>+</sup>
11	1717	998	3-sulfanylohexyl acetate	3SHA	passionfruit, grapefruit	5 bc	27 a	4 bc	8 b	nd c	I <sup>+</sup>
19	1722	970	1-sulfanylpentan-3-ol <sup>IST</sup>	1S3Pol	nettle	11 b	5 c	9 b	30 a	nd c	I
21	1760	1009	3-sulfanylpentan-1-ol	3SPol	citrus	10 a	6 b	d	d	nd c	I <sup>+</sup> (86, 69, 57)
35	1765	1214	3-sulfanyloctanal <sup>IST</sup>	3SOal	citrus, peach	nd c	nd c	nd c	nd c	nd c	I <sup>+</sup> (131, 83, 109)
26	1815	1186	3-sulfanyl-4-methylpentan-1-ol <sup>IST</sup>	3S4MPol	grapefruit	25 cd	10 de	109 a	46 b	nd e	I <sup>+</sup> (100, 55, 73)
23	1858	1095	3-sulfanylohexan-1-ol	3SHol	grapefruit/ rhubarb	d	12 a	d	d	nd c	I <sup>+</sup> (57, 41, 61)
total						54 b	1296 a	122 b	122 b	83 b	T b, NS b, S b

<sup>a</sup>d, detected under the quantitation limit (0.1  $\mu\text{g}/\text{kg}$ ) or unresolved. nd, not detected (<0.03  $\mu\text{g}/\text{kg}$ ). <sup>b</sup>Data issued from ref 8; letters after T, NS, or S indicate the statistical group for the reported concentrations in Tomahawk, Nelson Sauvin, and Saaz. I, compound 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>, additional confirmation by mass spectrometry (full scan monitoring); I<sup>-</sup>, compound 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).



**Figure 1.** Spider diagrams and corresponding chemical structures of discriminant nonsulfur constituents in seven dual-purpose hop varieties, as compared to Saaz. Concentrations in mg/kg of hop.

IST superscript in Table 2), the good equimolarity of the PFPD detector enabled us to set the IST-relative molar response coefficients at 1. The variation coefficients for thiols (extraction and analysis) were below 15%.

**Quantitation of Free Thiols (X) Released by Enzymatic Treatment.** For free thiol quantitation, the following equation was used:<sup>24</sup> concentration of X (in  $\mu\text{g}/\text{kg}$ ) = concentration of bound IST (in  $\mu\text{g}/\text{kg}$ )  $\times$  (molecular weight of free IST/molecular weight of bound IST)  $\times$  (peak area of X/peak area of free IST)  $\times$  (mass response coefficient of free IST/mass response coefficient of X).

The same bound-to-free thiol conversion rates were used for all compounds.<sup>24</sup> For commercially unavailable compounds, the mass

response coefficient of X was assessed as the molar response coefficient of free IST  $\times$  (molecular weight of X/molecular weight of free IST)

**Statistical Analyses.** All analyses were carried out in duplicate. Multiple comparisons of means were performed by means of Student–Newman–Keuls tests, with SAS software version 9.2 (SAS Institute, Inc., Cary, NC, U.S.A.). Values that do not share a letter indicating significance are significantly different ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

To extend the data provided by Gros et al.,<sup>8</sup> the terpenoid profiles of five new dual-purpose hops were determined (Figure 1

and Table 1). GC-MS analyses of hop Likens Nickerson extracts showed much diversity.

The most unexpected result was the concentration of  $\beta$ -farnesene (2101 mg/kg) in the Sorachi Ace variety, despite its high  $\alpha$ -acid content (12.3%) (Figure 1a). Amarillo (9.3%  $\alpha$ -acids) and Mosaic (11.7%  $\alpha$ -acids) also contained concentrations of  $\beta$ -farnesene (152.6 and 102.8 mg/kg, respectively) very close to those usually found in the finest aromatic varieties Lublin, Saaz, and Styrian Golding (discrimination level for these aromatic hop set at 150 mg/kg for fresh samples).<sup>5</sup> On the other hand, bergamotene, claimed to be the second discriminating sesquiterpene of aromatic varieties (>10 mg/kg),<sup>7</sup> was found only at trace levels in all dual-purpose varieties (1.4–3.1 mg/kg).

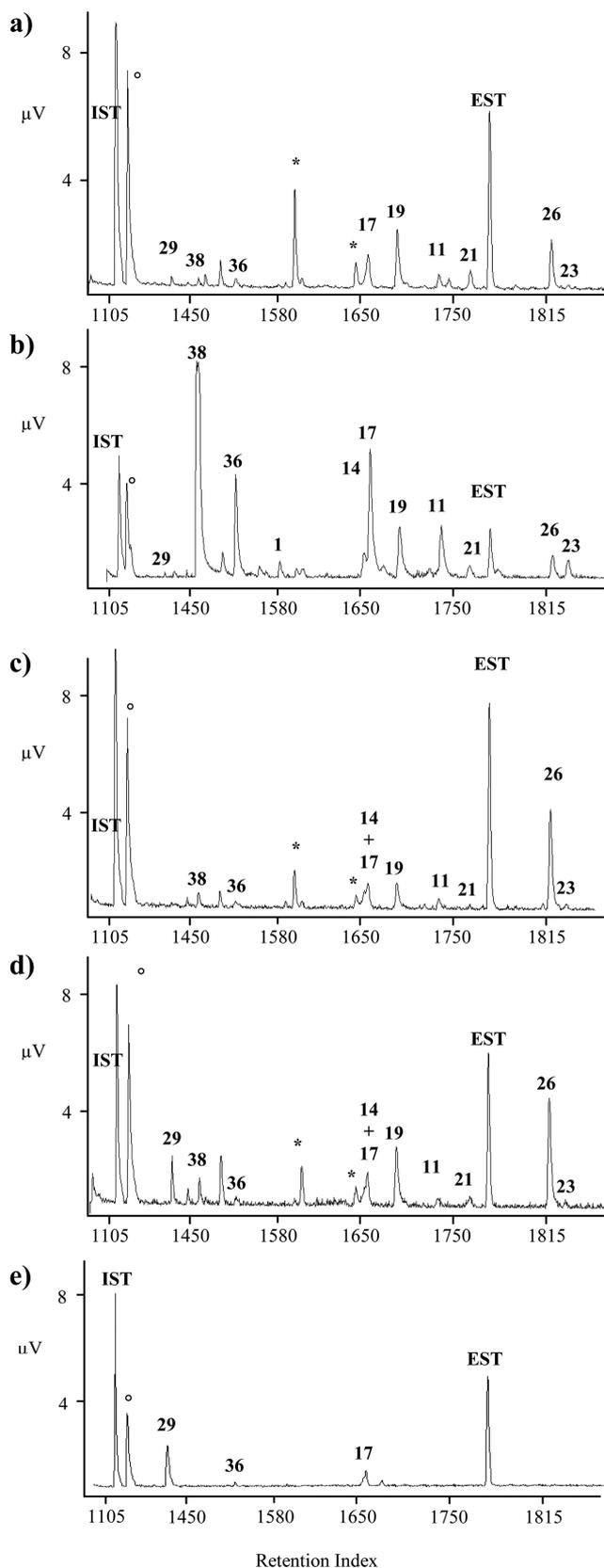
As expected for bitter varieties, 3-methylbutylisobutyrate derived from  $\alpha$ -acid lateral chain degradation was found at much higher levels (69–181 mg/kg) in Amarillo, Citra, Hallertau Blanc, and Mosaic than in Saaz (Figure 1c). Surprisingly, a low concentration was found in Sorachi Ace (13 mg/kg).

The concentration of  $\beta$ -citronellol was above 7 mg/kg in the five dual-purpose hops investigated here, while values under 1.2 mg/kg have been reported for Nelson Sauvin and Tomahawk (Figure 1d). Taking into account a dilution factor of  $\sim$ 500 between hop and beer (hopping rate generally  $\sim$ 200 g/hL) and a flavor threshold of 8  $\mu$ g/L,<sup>14</sup> we can assume an impact of this citrus-like terpenoid in the flavor of derived beers. On the other hand, the two other monoterpenic alcohols linalool (Figure 1e) and geraniol (Figure 1f) remain good discriminating compounds for Nelson Sauvin and Tomahawk, respectively (18–109 mg/kg linalool in the five hops investigated here versus 312 mg/kg in Nelson Sauvin; 16–105 mg/kg geraniol versus 211 mg/kg in Tomahawk, where more methylgeranate was also found, together with  $\alpha$ - and  $\beta$ -selinene and  $\alpha$ -amorphene). The fruity Mosaic variety was characterized by the presence of 237 mg/kg of undecan-2-one (<113 in all other varieties).

Polyfunctional thiols were selectively extracted from hops with an organomercuric reagent (pHMB). Identifications were achieved on two capillary columns (polar FFAP and apolar CP-Sil 5 CB) by the coincidence of retention times at the selective pulsed flame photometric detector (PFPD) and odors at the olfactometric port (GC-O). The selective PFPD enabled us to quantify 9 thiols (some peaks previously found in other hop extracts were here undetected or insufficiently resolved; see Table 2 and Figure 2). As previously evidenced for Nelson Sauvin and Tomahawk,<sup>8,13,35</sup> each variety investigated here exhibited a unique thiol profile.

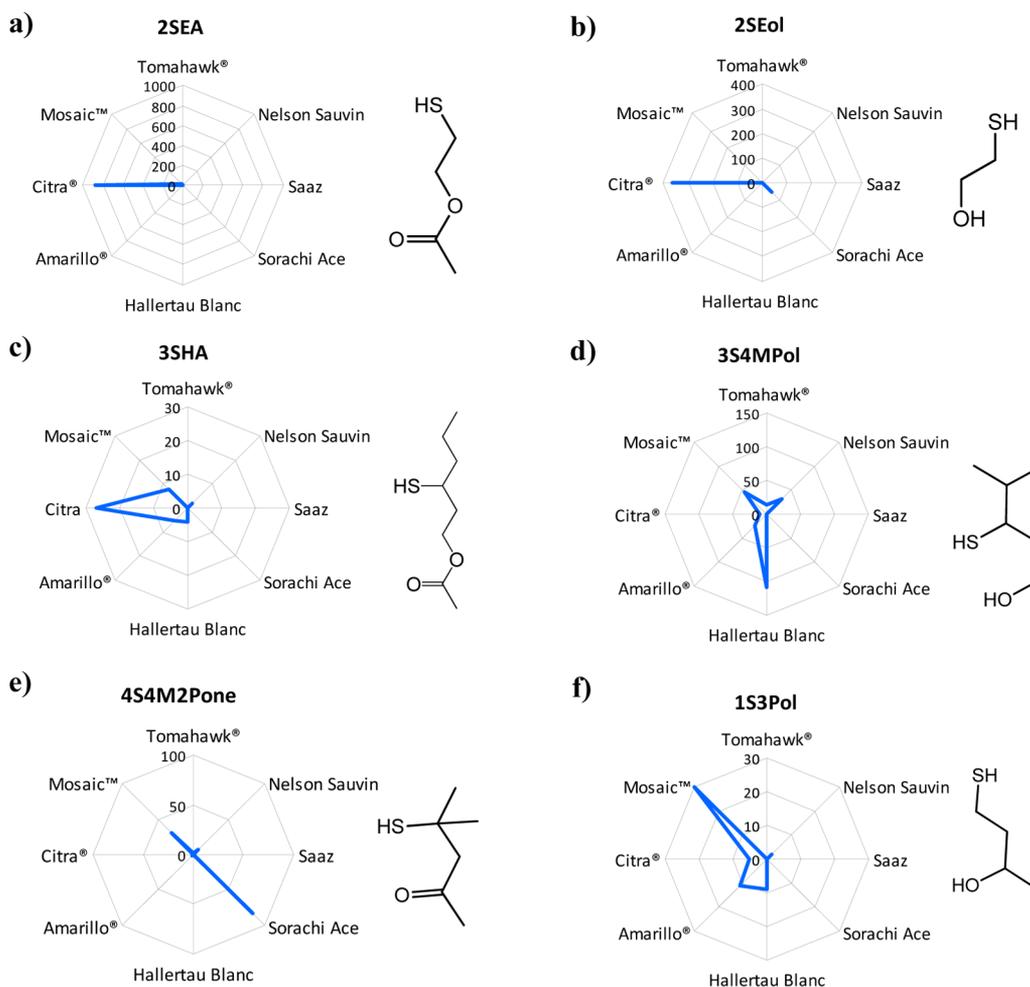
2-Sulfanylethyl acetate (no. 38, 2SEA, toasted, Figure 3a), 3-sulfanylpropyl acetate (no. 1, 3SPrA, grilled), and 2-sulfanylethyl-1-ol (no. 36, 2SEol, grilled, Figure 3b) were found in the Citra variety at levels never encountered in other hops (see also Figure 2b and Table 2). 2SEA and 2SEol explain 95% of the exceptionally high total amount of polyfunctional thiols found in Citra (1296  $\mu$ g/kg). Also worth stressing is the presence in the Citra variety of a very pleasant discriminating compound, 3-sulfanylhethyl acetate (no. 11, 3SHA, passionfruit, grapefruit, 27  $\mu$ g/kg, Figure 3c). By comparison with its threshold of 0.005  $\mu$ g/L in beer<sup>38</sup> and taking into account a dilution ratio of  $\sim$ 500 from hop to beer, 3SHA should have an impact in the flavor of Citra-hopped beers. This is probably not the case for the empyreumatic odorants discussed earlier (867  $\mu$ g/kg / 500 L/kg = 1.7  $\mu$ g/L of 2SEA for a threshold of 40  $\mu$ g/L).<sup>39</sup>

Although relatively poor in other polyfunctional thiols (total = 122  $\mu$ g/kg), the Hallertau Blanc cultivar proved much richer in 3-sulfanyl-4-methylpentan-1-ol (no. 26, 3S4MPol, grapefruit,



**Figure 2.** GC-PFPD chromatograms (FFAP column) of pHMB extracts obtained from Amarillo (a), Citra (b), Hallertau Blanc (c), Mosaic (d), and Sorachi Ace (e). °Impurity, \*unknown.

rhubarb, Figure 3d), although 3S4MPol was previously claimed to be specific to the Nelson Sauvin. Here we found it at



**Figure 3.** Spider diagrams and corresponding chemical structures of discriminant free polyfunctional thiols in seven dual-purpose hop varieties, as compared to Saaz. Concentrations in  $\mu\text{g}/\text{kg}$  of hop.

**Table 3.** Amounts of Thiols ( $\mu\text{g}/\text{kg}$ , IST Equivalents for Compounds Carrying the IST Superscript) Released from Purified Hop Extract by Apotryptophanase<sup>a,b</sup>

no.	retention index		substance		odor (GC-O)	Amarillo	Citra	Hallertau Blanc	Mosaic	Sorachi Ace	previously reported cultivars <sup>24c</sup>
	FFAP	CP-Sil 5 CB	name	acronym							
37	1112	810	3-methyl-2-buten-1-thiol	MBT	coffee, skunky	nd b	414 a	nd b	453 a	418 a	T a, NS a, S b
29	1380	918	4-sulfanyl-4-methylpentan-2-one	4S4M2Pone	catty, black currant	nd c	nd c	nd c	nd c	nd c	T b, NS a, S c
36	1532	722	2-sulfanylethan-1-ol	2SEol	grilled, gas	2499 a	2525 a	655 b	985 b	2087 a	T c, NS c, S c
14	1623	849	3-sulfanylpropan-1-ol	3SProl	popcorn	nd c	nd c	nd c	nd c	nd c	T a, NS b, S c
17	1654	933	3-sulfanyl-3-methylbutan-1-ol	3S3MBol	sulfur, soup	127 a	d	nd c	109 a	d	T c, NS c, S c
21	1760	1009	3-sulfanylpentan-1-ol	3SPol	catty, citrus	391 b	nd e	70 d	nd e	130 c	T d, NS a, S e
26	1815	1186	3-sulfanyl-4-methylpentan-1-ol <sup>IST</sup>	3S4MPol	grapefruit	nd b	nd b	39 a	nd b	nd b	T b, NS b, S b
23	1858	1095	3-sulfanylhexan-1-ol	3SHol	grapefruit	413 c	616 b	188 c,d	47 d	1158 a	T cd, NS cd, S cd
total						3430 a	3555 a	952 b,c	1594 b	3793 a	T bc, NS bc, S c

<sup>a</sup>Concentration determined by GC-PFPD. Mean of duplicates. Values within a row with different letters are significantly different ( $p < 0.05$ ) according to the Student–Newman–Keuls test. <sup>b</sup>nd, not detected ( $< 8 \mu\text{g}/\text{kg}$ ). d, detected but unresolved. <sup>c</sup>Data issued from ref 24; letters after T, NS, or S indicate statistical group for the reported concentrations in Tomahawk, Nelson Sauvin, and Saaz.

concentrations up to  $109 \mu\text{g}/\text{kg}$  (IST equivalents), compared to  $25\text{--}46 \mu\text{g}/\text{kg}$  in Mosaic, Nelson Sauvin, and Amarillo and  $10\text{--}13$

$\mu\text{g}/\text{kg}$  in Citra and Tomahawk. This compound was not detected at all in the Sorachi Ace variety.

4-Sulfanyl-4-methylpentan-2-one (no. 29, 4S4M2Pone, catty, Figure 3e), another proposed marker of the Nelson Sauvignon cultivar (7  $\mu\text{g}/\text{kg}$ ), was found at amounts 4-fold higher (30  $\mu\text{g}/\text{kg}$ ) in Mosaic and 11-fold higher (83  $\mu\text{g}/\text{kg}$ ) in Sorachi Ace. 1-Sulfanylpentan-3-ol (no. 19, 1S3Pol, nettle, Figure 3f) was found at the highest level in Mosaic (30  $\mu\text{g}/\text{kg}$ , IST equivalents).

3-Sulfanyl-2-ethylpropyl acetate (no. 10, 3S2EPrA, floral, vinegar) and 3-sulfanyloctanal (no. 35, 3SOal, citrus, peach) remained undetected in all five new dual-purpose varieties investigated here, whereas they were previously identified as markers of Tomahawk and Nelson Sauvignon.

S-cysteine conjugates of our five varieties were purified and subjected to apotryptophanase treatment (Table 3). Quantifications were done in IST equivalents, assuming that benzylthiol was released from its S-cysteine conjugate in the same way as all free amine hidden hop thiols.<sup>24</sup> Eight different adducts were identified, including two never before reported (2SEol and 3S4MPol).

The Sorachi Ace, Citra, and Amarillo varieties contained 1158, 616, and 413  $\mu\text{g}/\text{kg}$  of hidden 3SHol, respectively. These values should be compared to the 1641  $\mu\text{g}/\text{kg}$  previously found by Gros et al. in Cascade,<sup>24</sup> the hop variety where they identified the first S-cysteine conjugate by high-performance liquid chromatography (HPLC)/MS/MS. No obvious relation seems to exist between free and bound 3SHol. The following ratios of free to bound 3SHol were calculated: 1.9% for Citra, 4.3% for Tomahawk, and 7.1% for Cascade. For the Sorachi Ace variety, the free thiol was undetectable.

3-Sulfanyl-3-methylbutan-1-ol (3S3MBol) was also found to be released (127  $\mu\text{g}/\text{kg}$  from Amarillo and 109  $\mu\text{g}/\text{kg}$  from Mosaic). The thiol S-cysteine conjugate of 3S3MBol, known as felinine, has never been evidenced before in hop but was recently identified in cat urine.<sup>40</sup>

Never reported before, the 3-sulfanyl-4-methylpentan-1-ol S-conjugate was found in the Hallertau Blanc cultivar. Unlike the others, it occurred at lower concentration than the corresponding free thiol (39 and 109  $\mu\text{g}/\text{kg}$ , respectively).

Already discriminant in its free form for the Citra variety, 2-sulfanylethan-1-ol also proved to be enzymatically released in a high amount (2.5 mg/kg) from the Citra bound extract (free/bound ratio = 14%). A strong impact of the hop variety on the amount of 2SEol generated during beer fermentation has recently been reported.<sup>23</sup> From this first evidence of the presence of 2SEol S-conjugate, we can suspect that its cleavage might be an additional 2SEol synthesis pathway occurring during beer fermentation, together with Ehrlich degradation of cysteine.<sup>39,41</sup>

In conclusion, although much diversity was found among the investigated dual-purpose hops, all proved to contain an exceptional citrus-like potential. In Sorachi Ace,  $\beta$ -citronellol is probably the main compound responsible for such notes, while in others, polyfunctional thiols such as 3SHA (Citra) or 3S4MPol (Hallertau Blanc, Mosaic) could play a key role. Complementary investigations are now needed to assess which molecules involved in the citrus note must be preferred in beer, especially for increasing its flavor stability. The free/bound ratio of discriminating polyfunctional thiols also should be determined for successive harvest years.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: sonia.collin@uclouvain.be. Fax: +32-10-47 21 78.

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## ABBREVIATIONS USED

pHMB, *p*-hydroxymercuribenzoic acid; PFPD, pulse flame photometric detector; RI, retention index; IST, internal standard; EST, external standard

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