JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# 3-Sulfanyl-4-methylpentan-1-ol in Dry-Hopped Beers: First Evidence of Glutathione *S*-Conjugates in Hop (*Humulus lupulus* L.)

Marie-Lucie Kankolongo Cibaka,[†] Laura Decourrière,[†] Celso-José Lorenzo-Alonso,[†] Etienne Bodart,[†] Raphaël Robiette,[§] 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

[§]Institute of Condensed Matter and Nanosciences (IMCN), Université catholique de Louvain, Place Louis Pasteur 1, Box L4.01.02, B-1348 Louvain-la-Neuve, Belgium

Supporting Information

**ABSTRACT:** Monovarietal dry-hopped beers were produced with the dual-purpose hop cultivars Amarillo, Hallertau Blanc, and Mosaic. The grapefruit-like 3-sulfanyl-4-methylpentan-1-ol was found in all three beers at concentrations much higher than expected on the basis of the free thiol content in hop. Even cysteinylated precursors proved unable to explain our results. As observed in wine, the occurrence of *S*-glutathione precursors was therefore suspected in hop. The analytical standards of *S*-3-(4-methyl-1-hydroxypentyl)glutathione, never described before, and of *S*-3-(1-hydroxyhexyl)glutathione, previously evidenced in grapes, were chemically synthesized. An optimized extraction of glutathionylated precursors was then applied to Amarillo, Hallertau Blanc, and Mosaic hop samples. HPLC-ESI(+)MS/MS revealed, for the first time, the occurrence of *S*-3-(1-hydroxyhexyl)glutathione and *S*-3-(4-methyl-1-hydroxypentyl)glutathione in hop, at levels well above those reported for their cysteinylated counterparts. *S*-3-(1-Hydroxyhexyl)glutathione emerged in all cases as the major adduct in hop. Yet, although 3-sulfanylhexan-1-ol seems relatively ubiquitous in free, cysteinylated, and glutathionylated forms, the glutathione adduct of 3-sulfanyl-4-methylpentan-1-ol, never evidenced in other plants up to now, was found only in the Hallertau Blanc variety.

**KEYWORDS:** *hop (Humulus lupulus L.), flavor, polyfunctional thiols, glutathione, dry-hopping, beer*

## INTRODUCTION

Dual-purpose hop cultivars are characterized by high contents of both bitter acids (>7% humulones) and essential oils.[1] Among the essential oils, polyfunctional thiols, present in much lower amounts (0.015−1.296 mg/kg) than terpenoids (4000−8500 mg/kg), are viewed as key contributors to hop flavor in beer.[1,2] Most of them have a 3-carbon distance between the SH group and the other chemical function (alcohol, ester, carbonyl, ...).[3] Forty-one volatile polyfunctional thiols have been found in hop, and each cultivar exhibits a unique thiol profile.[3−5] 3-Sulfanyl-4-methylpentan-1-ol (**26**) with grapefruit-/rhubarb-like flavors (odor perception threshold = 70 ng/L in beer)[5] was previously proposed as one of the key compounds contributing to the "Sauvignon Blanc-like" note imparted to beer by Nelson Sauvin hop.[5] Although relatively poor in other polyfunctional thiols, the Hallertau Blanc cultivar proved to be still much richer in **26**, with concentrations up to 109 μg/kg, compared to 25−46 μg/kg in Nelson Sauvin, Mosaic, and Amarillo hops.[1] 3-Sulfanylhexan-1-ol (**23**), also with a grapefruit-like flavor (odor perception threshold = 55 ng/L in beer),[4] has been described as one of the key aroma compounds participating in the "Muscat-like" flavor perceived in Cascade-hopped beers. This thiol has been found in most dual-purpose and aromatic hop varieties, but higher contents (80−120 μg/kg) are reported for Cascade, Simcoe, Topaz, and Fuggle hops.[3,6] To date, there are no available data about the enantiomeric distribution of **23** in hop and beer. In wine, its (*R*)- and (*S*)-enantiomers are described, respectively, to have grapefruit- and passionfruit-like flavors.[7] The corresponding ester, 3-sulfanylhexyl acetate (**11**), has been evidenced as one of the very pleasant discriminating odorant compounds (27 μg/kg) in Citra hop. This thiol with a passionfruit-/grapefruit-like flavor (odor perception threshold = 5 ng/L in beer)[6] has been found only as traces in the other cultivars.[1,3] As for **23**, the stereoisomeric distribution of **11** has not yet been reported for hop and beer.

Hoppy flavors can be enhanced in beer by applying either late hopping (addition of hop at the end of wort boiling or in the whirlpool) or dry-hopping (addition of hop during beer fermentation or maturation). In both cases, thermal degradation is minimized thanks to the short heat treatment or the low temperature applied.[8,9]

Surprisingly, as recently described by Gros et al.,[10] the thiol content of the final beer reaches higher values than might be expected on the basis of hopping rate and hop free thiol contents. Yeast is partly responsible for this increase, through bioconversion of heavy precursors to odorant thiols. Yeast β-lyase activity is known to release thiols after specific cleavage from *S*-cysteine conjugates.[10,11] The nutrient-depleted conditions encountered after the main fermentation are thought to favor this yeast activity, especially when dry-hopping is applied.[9,12,13] Moreover, yeast reductases convert carbonyls to alcohols,

Received: August 24, 2016
Revised: October 20, 2016
Accepted: October 23, 2016
Published: November 7, 2016





DOI: 10.1021/acs.jafc.6b03788
J. Agric. Food Chem. 2016, 64, 8572−8582

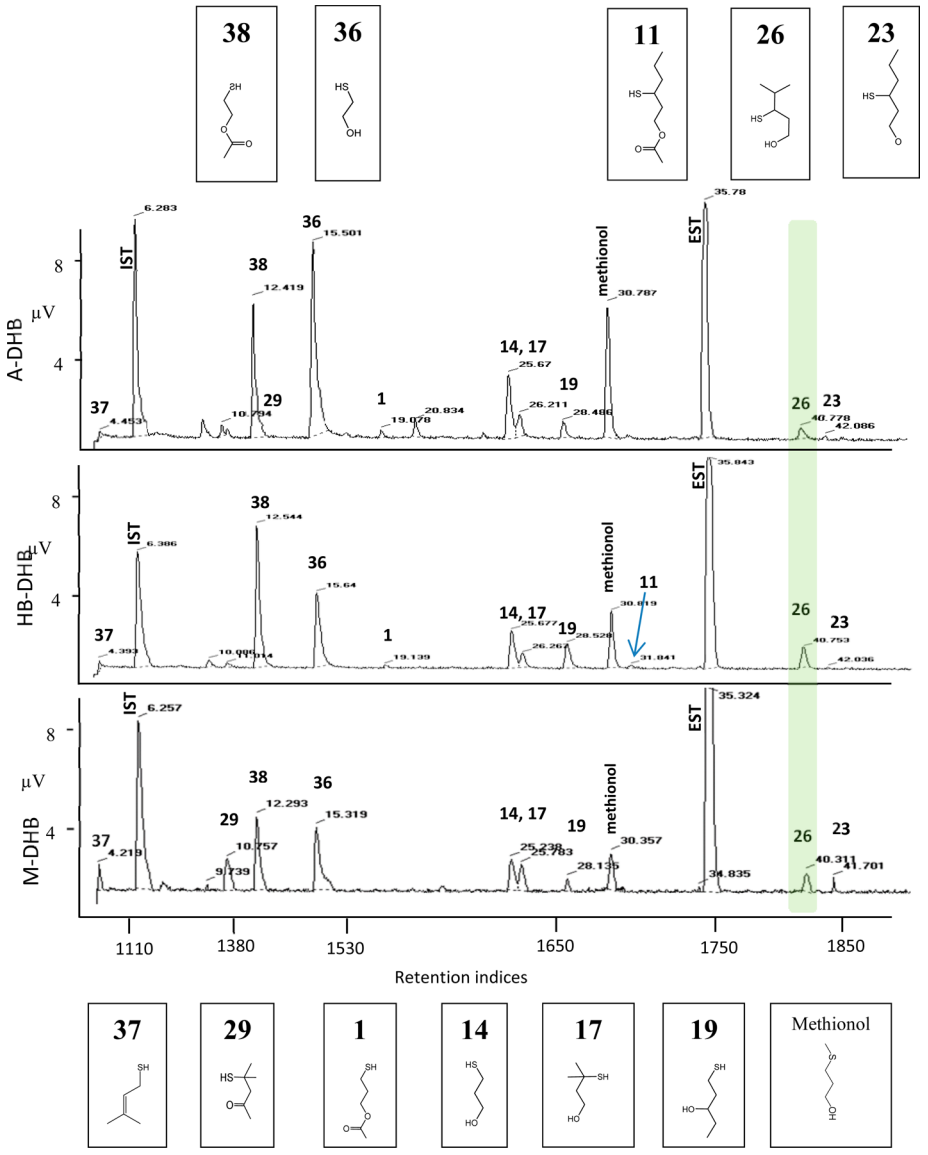


**Figure 1.** GC-PFPD chromatograms (FFAP column) of pHMB extracts obtained from pilot beers dry-hopped with Amarillo, Hallertau Blanc, and Mosaic hop cultivars. Numbering of compounds is conserved from refs 1 and 3. Only the chemical structures given at the top of the figure are discussed in the present paper.

**Table 1. Concentrations (ng/L) of Thiols in Pilot Dry-Hopped Beers**[a]

| no. | compound | odor[c] | A-DHB | HB-DHB | M-DHB | threshold[c] |
|---|---|---|---|---|---|---|
| **36** | 2-sulfanylethan-1-ol[b] | grilled | 19758 a | 21549 a | 6935 a | $2 \times 10^6$ |
| **38** | 2-sulfanylethyl acetate | toasted, grilled | 898 a | 1809 a | 869 a | $4 \times 10^4$ |
| **26** | 3-sulfanyl-4-methylpentan-1-ol$^{IST}$ | grapefruit | 107 b | 271 a | 118 b | 70 |
| **23** | 3-sulfanylhexan-1-ol | grapefruit/rhubarb | d b | d b | 43 a | 55 |
| **11** | 3-sulfanylhexyl acetate | passionfruit, grapefruit | nd b | 36 a | nd b | 5 |

[a]Numbering of compounds is conserved from refs 1 and 3. Measured concentrations were determined by GC-PFPD. Compound **26** carrying the IST superscript is measured in IST equivalents. d, detected below the quantitation limit (25 ng/L); nd, not detected (<10 ng/L). [b]Assessed by applying a relative recovery factor to the IST = 0.1. Standard deviations have been considered in the Student−Newman−Keuls test applied to the results to determine the statistical groups (represented by letters in superscript) in each row. Values in the same row that do not share a common letter are significantly different ($p < 0.05$). [c]Threshold (ng/L) and odor references: ref 26 for compounds **36** and **38**, ref 5 for compound **26**, ref 4 for compound **23**, and ref 6 for compound **11**.

whereas alcohol acetyltransferase synthesizes esters from hop alcohols.[6,14]

3-*S*-(1-Hydroxyhexyl)-cysteine (**Cys-23**) has been identified by HPLC-MS/MS in the Cascade variety.[10] This thiol precursor and seven other *S*-conjugates have been evidenced in other hop varieties by means of enzymatic release assays using apotryptophanase.[1,11] This commercial enzyme from *Escherichia coli* has been widely used to investigate cysteine and cysteinylglycine adducts in plants.[15−17] A free amino function in the cysteinyl moiety is required for $\beta$-lyase activity.[17] Such assays have revealed



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a hidden potential 23−126 times as high as the amount of the corresponding free volatiles, except for the bound fraction of **26**, found only in the Hallertau Blanc cultivar, and at lower concentration (39 $\mu$g/kg) than the corresponding free thiol content (109 $\mu$g/kg).[1]

In plants, cysteine-*S*-conjugates usually arise through the glutathione detoxification pathway, where the tripeptide is added to an $\alpha,\beta$-unsaturated carbonyl in the presence of glutathione-*S*-transferase. The resulting glutathione-*S*-conjugate is further converted to the corresponding *S*-cysteine conjugate after successive enzymatic cleavages of glycine and glutamate residues.[18−20] Although different *S*-cysteine conjugates have been evidenced in hop, the occurrence of glutathione *S*-conjugates has not yet been reported. In grape must, the content of *S*-3-(1-hydroxyhexyl)-glutathione (**G-23**) is 35 times as high as the amount of **Cys-23**.[21] Yet the yield of bioconversion to free **23** appears lower for **G-23** than for **Cys-23** (approximately 0.5 and 1%, respectively).[22,23]

The aim of the present work was to assess how the flavor potential of dual-purpose hops (Amarillo, Hallertau Blanc, and Mosaic cultivars) might help predict the contents of compounds **26** and **23** in dry-hopped beers. To determine if *S*-glutathione conjugates might also constitute part of the hop thiol potential, *S*-3-(1-hydroxyhexyl)glutathione (**G-23**) and *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**) were chemically synthesized to allow investigating their presence in different hop extracts by HPLC-ESI(+)MS/MS analyses.

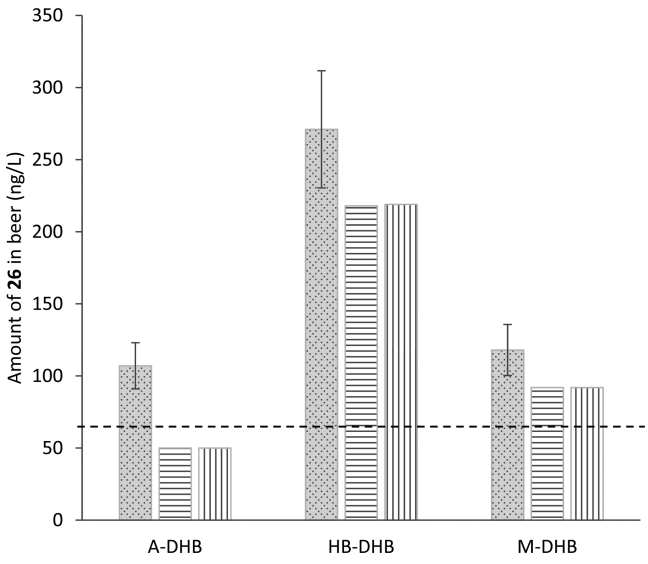


**Figure 2.** Amounts of 3-sulfanyl-4-methylpentan-1-ol in pilot beers dry-hopped with Amarillo, Hallertau Blanc, or Mosaic: bars from left to right in each grouping, measured concentration, expected concentration according to free thiol content in hop, and expected concentration according to free thiol content and bioconversion of cysteine-*S*-conjugate (1% bioconversion rate applied) in hop; the dashed horizontal line indicates the odor perception threshold in beer.

## EXPERIMENTAL PROCEDURES

**Chemicals.** Ammonia (28%), Amberlite IR-120 resin, formic acid, dichloromethane, acetone, ethanol, and diethyl ether (99.9%) were purchased from VWR (Leuven, Belgium). *p*-Hydroxymercuribenzoic acid (*p*HMB), 37% HCl, *S*-benzyl-L-cysteine, 2-sulfanylethan-1-ol (**36**), 2-sulfanylethyl acetate (**38**), 2-acetylthiophene, L-cysteine·HCl (97%), (*E*)-hexen-2-al, sodium borohydride (98%), and cesium carbonate were purchased from Sigma-Aldrich (Bornem, Belgium). 4-Methoxy-2-methylbutane-2-thiol, 3-sulfanylhexyl acetate (**11**), and 3-sulfanylhexan-1-ol (**23**) were obtained from Oxford Chemicals (Oxford, UK). Milli-Q water was used (Millipore, Bedford, MA, USA). NaOH, 4-methyl-2-pentenal, and sodium sulfate (99%) were purchased from Acros Organics (Geel, Belgium). A strongly basic Dowex resin 1X2, $Cl^-$ form (Sigma-Aldrich, Bornem, Belgium), was stored in hydrogen chloride (0.1 M). *N*-Boc-L-cysteine, $NaH_2PO_4$, and $Na_2HPO_4$ were obtained from Merck (Darmstadt, Germany), and tris(hydroxymethyl)-aminomethane (Tris) was obtained from USB (Cleveland, OH, USA). Ten gram C18 Sep-Pak cartridges were purchased from Waters Millipore. Polyvinylpyrrolidone was supplied by Spindal (Gretz-Armainvilliers, France).

**Reference Compounds Synthesized in Our Laboratory prior to This Study.** 3-Sulfanyl-4-methylpentan-1-ol (**26**) was synthesized according to the method of Takoi et al.[5] *S*-3-(1-Hydroxyhexyl)cysteine (**Cys-23**) was synthesized according to the method of Gros et al.[10]

**Hop Samples.** Amarillo and Mosaic hops bred in the United States were provided by Yakima Chief (Belgium). Hallertau Blanc hop bred in Germany was supplied by Hopsteiner (Germany).

**Pilot Beer Production.** Three beers were produced in a 60 L microbrewery (Coenco, Belgium). Pilsen malt (12.3 kg of 2-row spring malt, from Boortmalt, Belgium) was brewed in 33.9 L according to the following mashing program: 60 min at 60 °C and 25 min at 72 °C. The wort was then heated to 78 °C and filtered through the lauter tun. After sparging, 62 L of wort with a density of 12 °Plato was obtained. The wort was boiled with 126 mg/L Tomahawk $CO_2$ extract for 90 min (8−11% evaporation), and the final density was adjusted to 12 °Plato by the addition of water. The fermentation was conducted in cylindroconical fermenters with an ale-type yeast (INBR Bras212, propagated in a glucose/maltose/yeast extract/peptone medium). This strain was pitched at $7.5 \times 10^6$ cells/mL. Fermentation was carried out at 22 °C for 7 days. For each dry-hopping, a single hop variety was added to beer. For comparison purposes, the same hopping rate was applied (2 g/L, unmilled hop pellets in a cotton net). These hopping processes were conducted at 16 °C for 14 days in the presence of yeast. The beers were then kept at 4 °C for 7 days (maturation step). After filtration on plates (8 $\mu$m pores followed by 0.5 $\mu$m pores, BuonVinon CA) under a $CO_2$ atmosphere, the beers were carbonated at 5 g/L and stored at 4 °C until thiol extraction. The obtained beers contained 4−4.2% alcohol by volume.

**Extraction of Free Polyfunctional Thiols by *p*HMB.** Polyfunctional thiols were extracted from beer according to the procedure of Gros et al.[3] in the following steps: dichloromethane liquid/liquid extraction of 750 mL of beer, extraction of the resulting organic phase with a *p*HMB solution, loading of bound thiols onto a strong anion-exchanger resin, rinsing of the column with a pH 6 acetate buffer, release

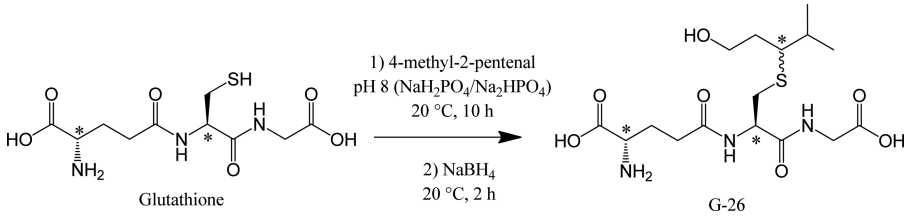


**Figure 3.** Chemical synthesis of *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**). Chiral centers are indicated by an asterisk (*).



DOI: 10.1021/acs.jafc.6b03788
J. Agric. Food Chem. 2016, 64, 8572−8582

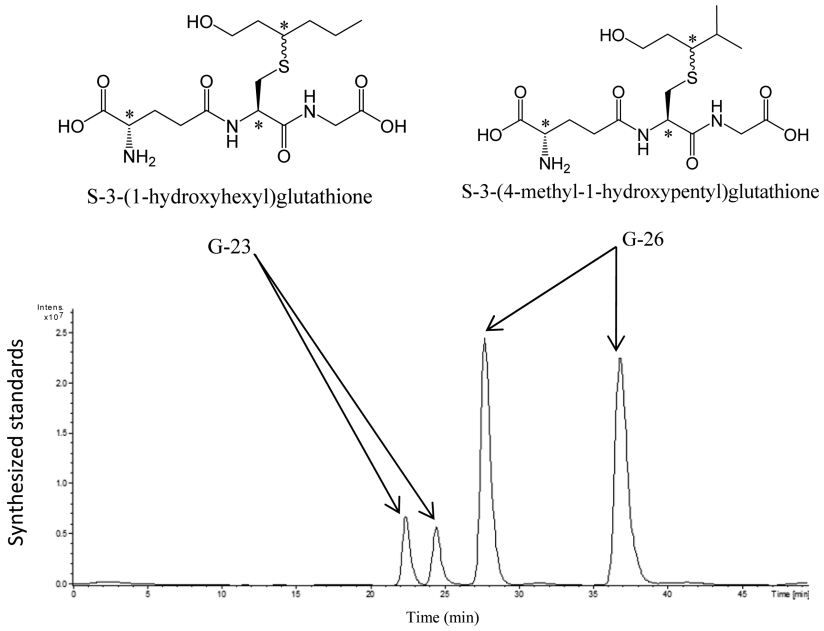


**Figure 4.** RP-HPLC-ESI(+)MS/MS ($m/z$ 408) chromatogram and chemical structures of synthesized *S*-3-(1-hydroxyhexyl)glutathione (**G-23**) and *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**). Chiral centers are indicated by an asterisk (∗).

of free thiols by exchange with washed cysteine (4 × 50 mL of dichloromethane for washing 640 mg of cysteine in 50 mL of water), final extraction with dichloromethane, and concentration to 250 $\mu$L in a Danish-Kuderna distillation apparatus and to 70 $\mu$L on a Dufton column. 4-Methoxy-2-methylbutane-2-thiol was added as internal standard (IST, at 670 ng/L in beer) and 2-acetylthiophene as external standard (EST, 1 mL at 200 $\mu$g/L added before concentration; final concentration at injection = 2857 $\mu$g/L).

**Gas Chromatography Hyphenated to a Pulsed-Flame Photometric Detector (GC-PFPD).** Two microliters of *p*HMB free thiol extract was analyzed with a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250 °C. Compounds were analyzed with a wall-coated open tubular (WCOT) polar FFAP (25 m × 0.32 mm i.d., 0.3 $\mu$m film thickness) capillary column. The carrier gas was helium, and the pressure was set at 45 kPa. 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 the OI Analytical PFPD detector (model 5380, combustor internal diameter = 2 mm). 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 chromatograms were recorded throughout elution; ChemStation software was used to process the resulting data. Identifications were done as previously described by Gros et al.[10]

**Quantitation of Free Thiols (A) in *p*HMB Extracts.** The following general equation was used for compound **A** quantitation: concentration of **A** (in $\mu$g/L) = IST concentration (in $\mu$g/L) × (**A** molecular weight/IST molecular weight) × (**A** area/IST area) × (IST molar response coefficient/**A** molar response coefficient) × (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 first dichloromethane extraction; approximate concentrations assessed by using a relative recovery factor to the IST of 0.1, previously assessed by standard addition).[3] 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 compound **26**, the good equimolarity of the PFPD detector enabled us to set the IST-relative molar response coefficients at 1.

**$^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) Spectra of Synthesized Thiol Precursors.** NMR spectra were recorded using the solvent $D_2O$ on a Bruker Avance spectrometer operating at 300 MHz. After simple pulse NMR experiments, resulting data were processed with Bruker TopSpin software (version 2.1). All chemical shifts ($\delta$) are reported in parts per million relative to the reference (TMS).

**High-Resolution Mass Spectrometry (HRMS/MS) of Synthesized Thiol Precursors.** Mass measurements and mass spectrum determinations were done with a Thermo Orbitrap Exactive mass spectrometer equipped with an electrospray ion source operating in positive mode (ESI+). Collision-induced dissociation spectra were recorded at a collision energy of 0.2 V for singly charged $[M + H^+]$ ions. The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 320 °C; sheath gas, nitrogen, 20 psi; auxiliary gas (nitrogen) flow rate, 5 mL/min. Synthesized compounds were solubilized in water and directly introduced into the mass spectrometer controlled with Xcalibur software version 2.0.7 (Thermo Fisher Scientific). The measured and calculated masses are given in daltons.

**Synthesis of *S*-3-(4-Methyl-1-hydroxypentyl)cysteine (Cys-26).** The procedure was adapted from the synthesis of *S*-3-(1-hydroxyhexyl)-cysteine (**Cys-23**) reported by Thibon et al.[24] and Gros et al.[10] A Michael addition of *N*-Boc-L-cysteine (500 mg, 2.26 mmol, 0.9 equiv) on 4-methyl-2-pentenal (0.292 mL, 2.51 mmol, 1 equiv) was performed overnight in anhydrous acetonitrile as solvent (7 mL) in the presence of cesium carbonate (350 mg, 1.13 mmol, 0.45 equiv). After evaporation of the solvent under reduced pressure, the obtained aldehyde was dissolved in 5 mL of methanol, and an aqueous solution of sodium borohydride (260 mg/4 mL, 6.87 mmol, 2.74 equiv) was added. The solution was stirred for 2 h. The pH was then adjusted to 2 with HCl (2 M), and 10 mL of water was added. The N-Boc-protected product was extracted three times with 25 mL of ethyl acetate. The combined organic phases were washed with 25 mL of water, dried with sodium sulfate, and concentrated under reduced pressure. Deprotection of the amine was achieved by reaction with trifluoroacetic acid (3.5 mL) in dichloromethane (10 mL) for 2 h. The solvent and the excess trifluoroacetic acid were evaporated under reduced pressure. The product was dissolved in 5 mL of ethanol, and 5 mL of 2 M HCl was added. After evaporation under reduced pressure, a white sticky solid was obtained. Yield of **Cys-26**, 74%; $^1$H NMR (300 MHz, $D_2O$), $\delta$ 0.93 (m, 6H, $-CH(CH_3)_2$), 1.61 (m, 1H, $-CH(CH_3)_2$), 1.91 (m, 2H, $-CH_2CH_2OH$), 2.77 (m, 1H, $CH$), 3.15 (m, 2H, $\beta CH_2$ in Cys), 3.73 (m, 2H, $-CH_2CH_2OH$), 4.22 (m, 1H, $\alpha CH$ in Cys); MS(ESI+) $m/z$ $[M + H]^+$ = 222;


DOI: 10.1021/acs.jafc.6b03788
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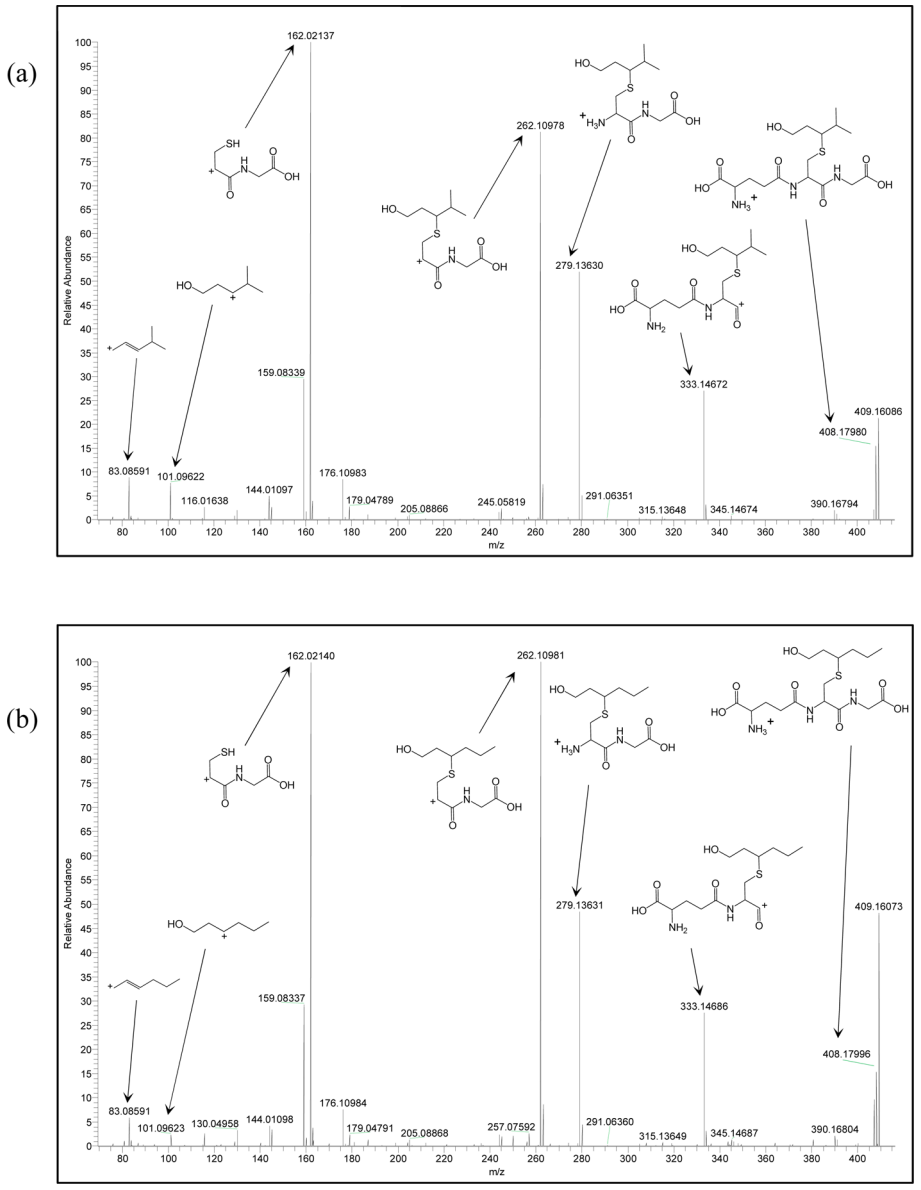


**Figure 5.** HR-MS/MS-ESI(+) ($m/z$ 408.179) mass spectra of (a) *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**) and (b) *S*-3-(1-hydroxyhexyl)glutathione (**G-23**).

HRMS(ESI+) calcd for $C_9H_{20}O_3N_1S_1$, 222.115841 Da; found, 222.115954 Da.

**Synthesis of *S*-3-(1-Hydroxyhexyl)glutathione (G-23).** This synthesis was adapted from the procedure described by Roland et al.[25] (*E*)-2-Hexenal was added in three steps (0.33 equiv every 3 h) to a solution of glutathione (500 mg, 1.63 mmol, 1 equiv) in phosphate buffer ($NaH_2PO_4/Na_2HPO_4$, 1 M, pH 8, 10 mL). The reaction mixture was stirred for 10 h (after the first addition) at room temperature. The obtained aldehyde derivative was reduced by adding dropwise to the reaction mixture an aqueous solution of sodium borohydride (177 mg/4 mL, 4.68 mmol, 2.87 equiv). After 2 h of stirring at room temperature, the pH was adjusted to 2 with 6 M HCl, and the solvent was evaporated under reduced pressure. To purify the product, a 10 g C18 Sep-Pak cartridge (Waters Millipore) was preconditioned with 200 mL of methanol and 300 mL of water. The product was dissolved in 5 mL of water and then loaded on the cartridge, washed with 100 mL of water, and eluted with 100 mL of acetonitrile/water/formic acid (89:10:1 v/v/v). The eluates were concentrated under reduced pressure. The resulting white solid residue was dissolved in 5 mL of aqueous 2 M HCl and washed three times with 15 mL of diethyl ether. The final product was obtained after concentration of the aqueous phase under reduced pressure. Yield of **G-23**, 16%; white solid; $^1$H NMR (300 MHz, $D_2O$), $\delta$ 0.87 (m, 3H, $-CHCH_2CH_2CH_3$), 1.39 (m, 1H, $-CHCH_2CH_2CH_3$), 1.54 (m, 1H, $-CHCH_2CH_2CH_3$), 1.72 and 1.83 (m, 2H, $-CH_2CH_2OH$), 2.20 (m, 2H, $\beta CH_2$ in Glu), 2.56 (m, 2H, $\gamma CH_2$ in Glu), 2.82 (m, 1H, $CH$), 2.89 and 3.04 (m, 2H, $\beta CH_2$ in Cys), 3.71 (m, 2H, $-CH_2CH_2OH$), 3.97 (m, 1H, $\alpha CH$ in Glu), 3.99 (s, 2H, $\alpha CH_2$ in Gly), 4.54 (m, 1H, $\alpha CH$ in Cys); MS(ESI+) $m/z$ $[M + H]^+$ = 408; HRMS(ESI+) calcd for $C_{16}H_{30}O_7N_3S_1$, 408.179897 Da; found, 408.179960 Da.

**Synthesis of *S*-3-(4-Methyl-1-hydroxypentyl)glutathione (G-26).** This method was similar to the above-described synthesis of **G-23**. 4-Methyl-2-pentenal was added in three steps (0.33 equiv every 3 h) to a solution of glutathione (500 mg, 1.63 mmol, 1 equiv) in a phosphate buffer ($NaH_2PO_4/Na_2HPO_4$, 1 M, pH 8, 10 mL). The reaction mixture was stirred for 10 h (after the first addition) at room temperature. The obtained aldehyde derivative was reduced with an aqueous solution of sodium borohydride (177 mg/4 mL, 4.68 mmol, 2.87 equiv) added dropwise to the reaction mixture. After 2 h of stirring at room temperature, the pH was adjusted to 2 with 6 M HCl, and the solvent was evaporated under reduced pressure. To purify the product, a 10 g C18 Sep-Pak cartridge (Waters, Millipore) was preconditioned


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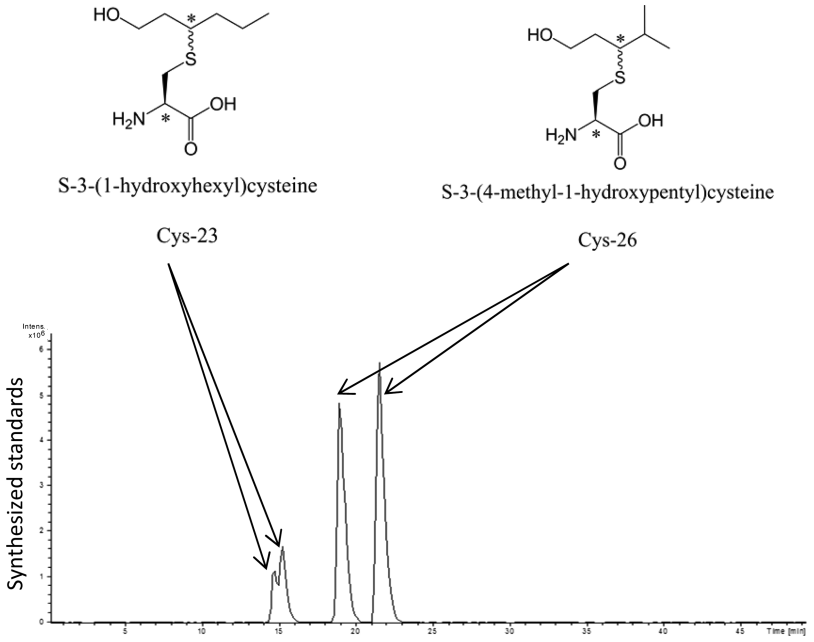


**Figure 6.** RP-HPLC-ESI(+)MS/MS ($m/z$ 222) chromatogram (Cyclobond I 2000 RSP column) of synthesized *S*-3-(1-hydroxyhexyl)cysteine (**Cys-23**) and *S*-3-(4-methyl-1-hydroxypentyl)cysteine (**Cys-26**). Chiral centers are indicated by an asterisk (∗).

with 200 mL of methanol and 300 mL of water. The product diluted in 5 mL of water was loaded on the cartridge, washed with 100 mL of water, and eluted with 100 mL of acetonitrile/water/formic acid (89:10:1 v/v/v). The eluates were concentrated under reduced pressure. The resulting white solid residue was dissolved in 5 mL of aqueous 2 M HCl and washed three times with 15 mL of diethyl ether. The final product was obtained after concentration of the aqueous phase under reduced pressure. Yield of **G-26**, 20%; white solid; melting point, 156−159 °C; $^1$H NMR (300 MHz, $D_2O$), $\delta$ 0.92 (m, 6H, $-CH(CH_3)_2$), 1.61 (m, 1H, $-CH(CH_3)_2$), 1.89 (m, 2H, $-CH_2CH_2OH$), 2.22 (m, 2H, $\beta CH_2$ in Glu), 2.58 (m, 2H, $\gamma CH_2$ in Glu), 2.68 (m, 1H, C*H*), 2.86 and 3.02 (m, 2H, $\beta CH_2$ in Cys), 3.71 (m, 2H, $-CH_2CH_2OH$), 3.98 (s, 2H, $\alpha CH_2$ in Gly), 4.09 (m, 1H, $\alpha CH$ in Glu), 4.51 (m, 1H, $\alpha CH$ in Cys); MS(ESI+) $m/z$ $[M + H]^+$ = 408; HRMS(ESI+) calcd for $C_{16}H_{30}O_7N_3S_1$, 408.179897 Da; found, 408.179800 Da.

**Extraction of Cysteine and Glutathione *S*-Conjugates: Optimization for a Better Recovery.** A thiol precursor extraction procedure adapted from refs 17 and 11 was applied to the Amarillo, Hallertau Blanc, and Mosaic hop varieties. *S*-Benzylcysteine (Cys-IST) was used as an internal standard at 8 mg/kg of hop. Milled pellets (100 g) were stirred with 1000 mL of a 1% (v/v) formic acid aqueous solution for 2 h at 45 °C. After centrifugation for 30 min at 8000 rpm, the supernatants were collected and loaded on a column of IR-120 cation exchange resin (100 g preconditioned with 100 mL of aqueous 2 M HCl followed by 1 L of water). The column was then washed with 500 mL of water, and sequential 100 mL fractions were recovered by elution with aqueous ammonia solutions at 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0, and 3.3 mol/L. As previously reported,[11] cysteine adducts are eluted in the 1.2−1.5 mol/L fractions. To determine in which fraction *S*-glutathione conjugates were eluted, a model solution of synthesized **G-23** (300 mL at 6.5 ppm) was loaded on an IR-120 cation exchange resin (100 g). **G-23** was found to elute mainly in the 1.2−2.4 mol/L fractions (also containing cysteine *S*-conjugates). These fractions were pooled and concentrated under reduced pressure. The obtained extract was dissolved in 2 mL of 0.1% formic acid aqueous solution for analysis by RP-HPLC-MS/MS.

**RP-HPLC-ESI(+)-MS/MS Analysis of Thiol *S*-Conjugates in Hop Extracts.** Analysis was first performed on a 150 mm × 2.1 mm, 2 $\mu$m, C18 Prevail column (Grace, Deerfield, IL, USA; previously used for cysteine adducts) and then on a 250 mm × 4.6 mm, 5 $\mu$m, Cyclobond I 2000 RSP (2-hydroxypropyl-$\beta$-cyclodextrin) chiral column (used here for its polarity and not for its chirality). The elution solvents were water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Gradient elution on the C18 column was as follows: for solvent A, from 95 to 80% in 4 min, from 80 to 50% in 2 min, from 50 to 42% in 3 min, from 42 to 0% in 3 min. Solvent B was finally kept at 100% for 4 min. An isocratic elution with 95% solvent A was applied to the Cyclobond I 2000 RSP column. The flow rate was 200 $\mu$L/min for the C18 column and 300 $\mu$L/min for the chiral column. Five microliters of sample was injected onto each column at room temperature. A system equipped with an autosampler and a quaternary pump (Agilent Technologies, 1200 series) was used. The system was controlled with Agilent Chem Station software. The mass spectra were acquired with a Bruker Daltonics Esquire 3000 ion trap mass spectrometer equipped with an electrospray ion source (Bruker) operated in positive mode (ESI+). The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 360 °C; nebulizer, nitrogen, 12 Psi. Nitrogen was also used as drying gas, at a flow rate of 8 mL/min. For identification, collision-induced dissociation MS/MS spectra were recorded at a relative collision energy of 0.2 V. For quantitation, the MRM mode was applied (relative collision energy of only 0.05 V to maximize the $[M + H^+]$ ions).

**Quantitation of Glutathione Adducts (G-T).** Calibration curves of G-T relative to Cys-IST were determined for all synthetic standards, and the following equation was used for each glutathione adduct (G-T) quantitation: concentration of G-T (in $\mu$g/kg) = concentration of Cys-IST (in $\mu$g/kg) × (peak area of G-T/peak area of Cys-IST) × (mass response coefficient of Cys-IST/mass response coefficient of G-T).

**Quantitation in Free Thiol Equivalents of Thiol (T) Bound to Glutathione (G-T).** Calibration curves of G-T relative to Cys-IST were determined for all synthetic standards, and the following equation was used for thiol T quantitation: concentration of T (in $\mu$g/kg) = (molecular weight of T/molecular weight of G-T) × concentration of Cys-IST (in $\mu$g/kg) × (peak area of G-T/peak area of Cys-IST) × (mass response coefficient of Cys-IST/mass response coefficient of G-T).

**Statistical Analyses.** All analyses were carried out in duplicate. Multiple comparisons of means were performed with Student−Newman−Keuls tests, with SAS software version 9.2 (SAS Institute, Inc., Cary, NC, USA). Values that do not share a common letter in the same row of result tables are significantly different ($p < 0.05$).

## RESULTS AND DISCUSSION

The Amarillo, Hallertau Blanc, and Mosaic dual-purpose hop varieties were selected to dry-hop a green beer just bittered in the


DOI: 10.1021/acs.jafc.6b03788
J. Agric. Food Chem. 2016, 64, 8572−8582

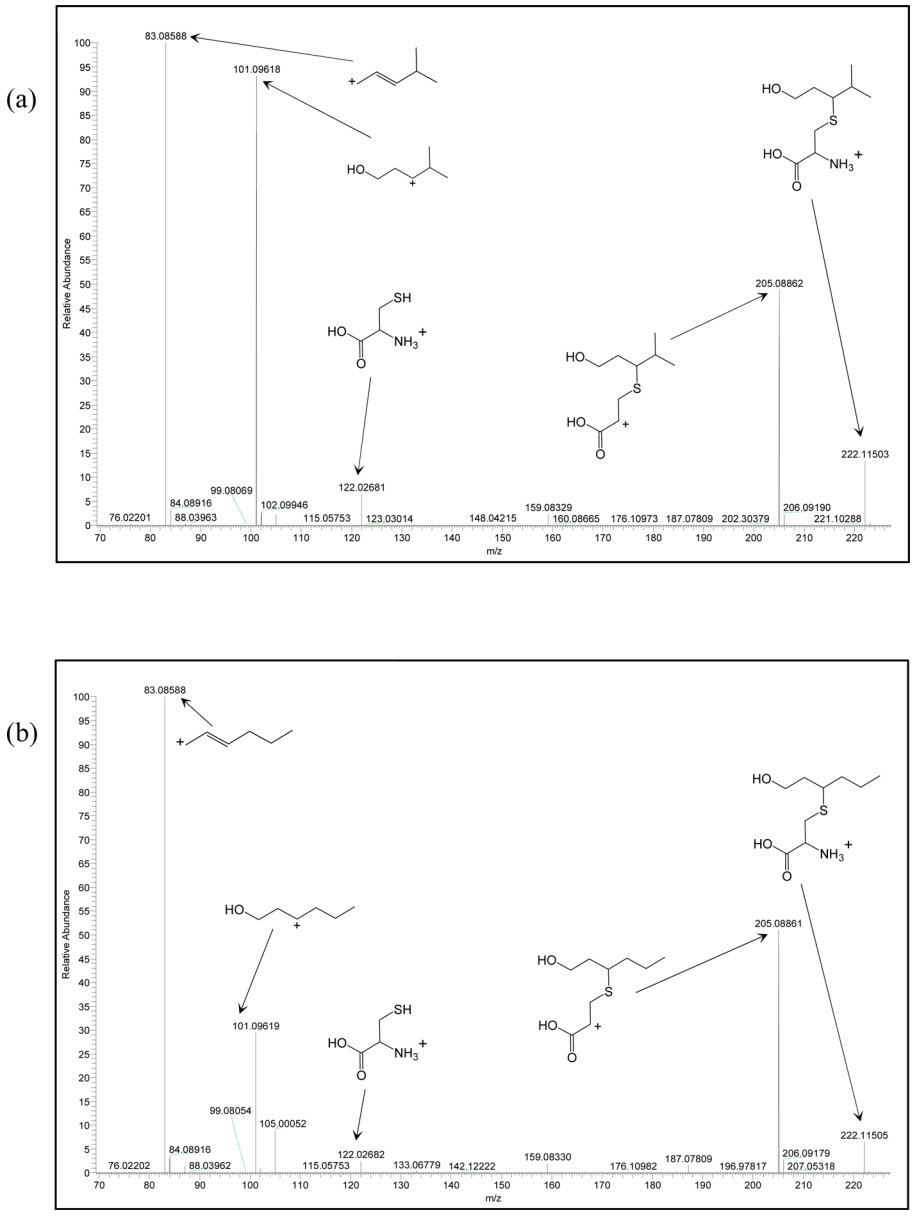


**Figure 7.** HR-MS/MS-ESI(+) ($m/z$ 222.115) mass spectra of (a) *S*-3-(4-methyl-1-hydroxypentyl)cysteine (**Cys-26**) and (b) *S*-3-(1-hydroxyhexyl)cysteine (**Cys-23**).

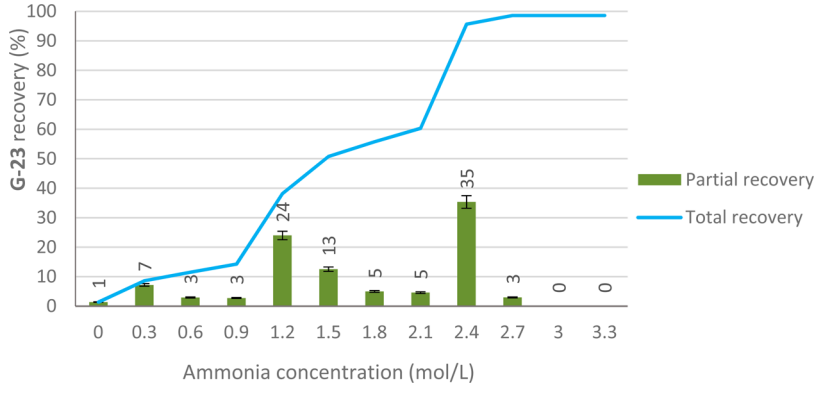


**Figure 8.** Recovery (%) of *S*-3-(1-hydroxyhexyl)glutathione (**G-23**) by elution from the cation exchange resin with increasing concentrations of ammonia (1.95 mg initially loaded).



DOI: 10.1021/acs.jafc.6b03788
*J. Agric. Food Chem.* 2016, 64, 8572−8582

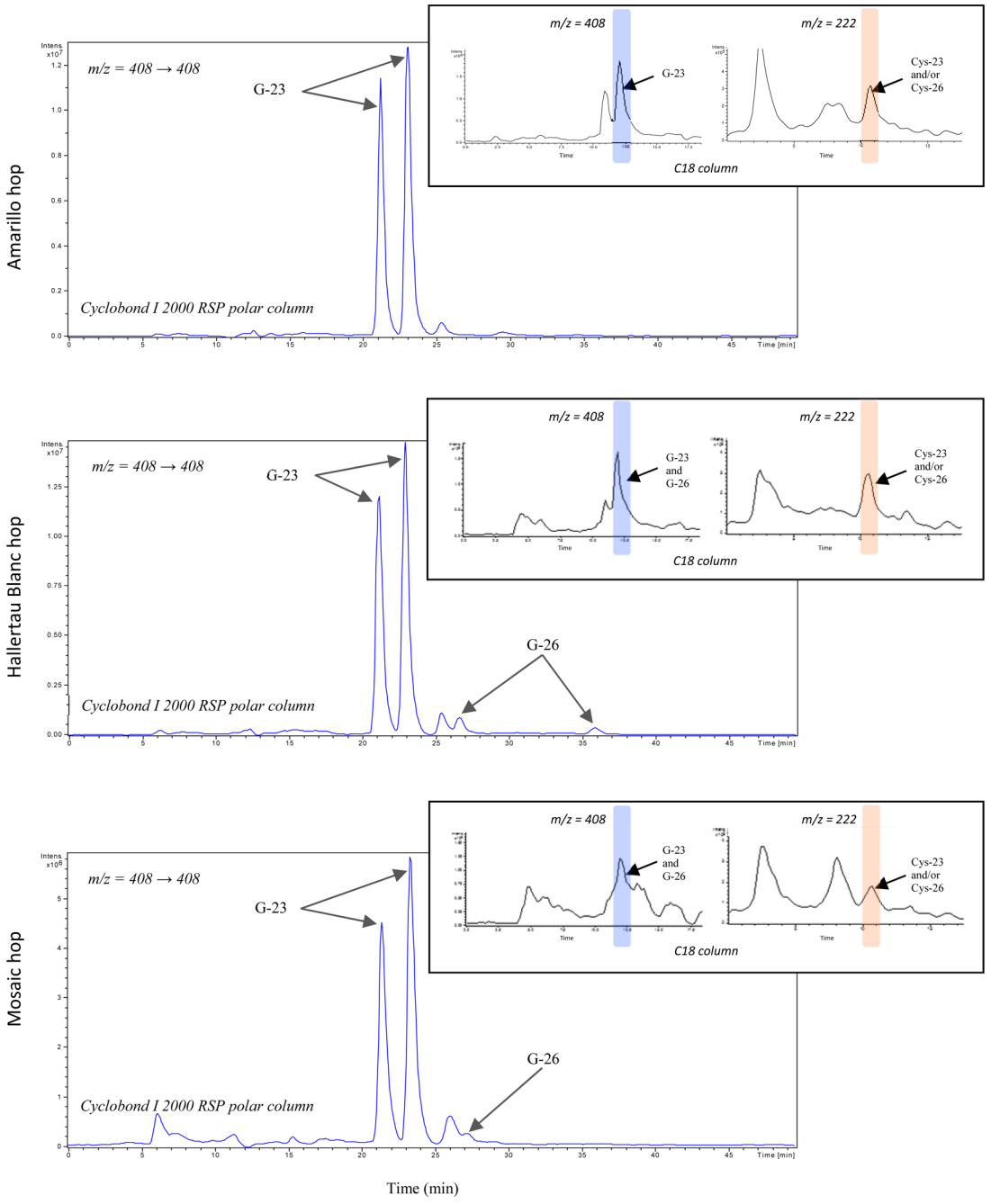


**Figure 9.** RP-HPLC-ESI(+)MRM ($m/z$ 408 → 408) analysis of *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**) and *S*-3-(1-hydroxyhexyl)-glutathione (**G-23**) diastereomers in Amarillo, Hallertau Blanc, and Mosaic hops on the Cyclobond I 2000 RSP column. Comparison for each hop variety with MS/MS analysis on the C18 column (coelutions of G-23 with G-26 ($m/z$ 408) and Cys-23 with Cys-26 ($m/z$ 222)).

boiling kettle with $CO_2$ hop extracts. The obtained beers were named A-DHB, HB-DHB, and M-DHB, respectively, for Amarillo, Hallertau Blanc, and Mosaic hops. Their polyfunctional thiol profiles were determined by GC-PFPD after selective pHMB extraction (Figure 1; Table 1).

As expected, the empyreumatic major thiols 2-sulfanylethan-1-ol (**36**) and 2-sulfanylethyl acetate (**38**) were found at levels well below their odor perception thresholds (Figure 1; Table 1).

On the other hand, all three beers contained the pleasant grapefruit-like 3-sulfanyl-4-methylpentan-1-ol (**26**) at concentrations above its odor perception threshold (70 ng/L;[5] Figure 2). HB-DHB displayed the highest level, with 271 versus 118 ng/L for M-DHB and 107 ng/L for A-DHB. In each beer, the measured concentration of **26** was higher than expected on the basis of the hop free thiol contents (109, 46, and 25 μg/kg, respectively; hopping rate = 2 g/L). This was also the case when the amount of the cysteinylated precursor previously evidenced in the Hallertau Blanc hop cultivar was taken into account (39 μg/kg,[1] yeast bioconversion of cysteine bound thiols estimated to be below 1%;[11] Figure 2). Another kind of thiol precursor thus must exist in hop. By analogy to grapes, a glutathione adduct was logically suspected.

The grapefruit-like 3-sulfanylhexan-1-ol (**23**) was detected at trace levels in HB-DHB and A-DHB, whereas its concentration reached 43 ng/L in M-DHB. Free **23** was found, however, at trace levels in all corresponding hop cultivars. Even the amounts


DOI: 10.1021/acs.jafc.6b03788
J. Agric. Food Chem. 2016, 64, 8572−8582

of cysteinylated precursors previously determined in hop[1] (413, 188, and 47 μg/kg in free thiol equivalents for Amarillo, Hallertau Blanc, and Mosaic, respectively) could not explain our observations. Although a bit below its published odor threshold (55 ng/L),[4] **23** might contribute to the pleasant hoppy flavors of the Mosaic beer by synergy with other thiols.

Lastly, the grapefruit/passionfruit-like 3-sulfanylhexyl acetate (**11**) was found above its perception threshold (5 ng/L,[6] much lower than the threshold of **23**) only in HB-DHB (36 ng/L). In addition to potential cysteine and glutathione adducts, **23** can also be considered as a precursor of **11** because yeast is able to catalyze ester synthesis.

To analyze glutathione adducts of **26** and **23** in hop, *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**) and *S*-3-(1-hydroxyhexyl)glutathione (**G-23**) were chemically synthesized. **G-26** has never been reported before. Its synthesis was adapted from that of **G-23** published by Roland et al.[25] After a Michael addition of L-glutathione to 4-methyl-2-pentenal, the obtained aldehyde was reduced to alcohol (Figure 3). **G-26** was obtained with a 20% yield. The purified product was a mixture of two diastereomers that are L-glutathione adducts of (*R*)- and (*S*)-**26**. The ratio of the two diastereomers was assessed at 50:50 by HPLC-MS/MS (Figure 4). **G-23**, which is not commercially available, was synthesized with a 16% yield (mixture of (*R*)- and (*S*)-**23** conjugated to L-glutathione with a diastereomeric ratio of 53:47; Figure 4). As depicted in Figure 5, **G-26** and **G-23** unfortunately displayed the same mass fragmentation pattern.

For further HPLC-MS/MS identifications, the synthesis of Cys-26 (Figure 6, not reported before) was adapted from that of Thibon et al.[24] for **Cys-23** (Figure 6). **Cys-26** was obtained with a 74% yield (mixture of (*R*)- and (*S*)-**26** conjugated to L-cysteine with a diastereomeric ratio of 45:55). As in the case of the glutathione adducts, **Cys-26** showed the same mass fragmentation pattern as **Cys-23** (Figure 7).

Chemically synthesized **G-23** was further used to optimize its extraction from hop on an Amberlite IR-120 cation-exchange resin column. An aqueous solution of **G-23** (300 mL at 6.5 mg/L) was loaded on the column, and the column was eluted with successive aqueous ammonia solutions (100 mL each) with concentrations increasing from 0 to 3.3 mol/L (in increments of 0.3 mol/L). As depicted in Figure 8, 99% elution of loaded **G-23** was reached after 2.4 mol/L ammonia, whereas for cysteine adducts, Gros et al.[10] previously showed that only fractions from 1.2 to 1.5 mol/L had to be kept. For our final protocol aiming at extracting both cysteine and glutathione adducts, fractions from 1.2 to 2.4 mol/L were collected (82% **G-23** recovered), as this proved to be the best compromise between high concentrations and cleanliness for HPLC injections.

This selected extraction method was then applied to the Amarillo, Hallertau Blanc, and Mosaic hop cultivars. The obtained hop extracts were analyzed by RP-HPLC-ESI(+)MS/MS, first with the C18 column previously used for cysteine *S*-conjugate investigations.[10] For the first time, glutathione adducts (*m*/*z* 408) were evidenced in hop together with the cysteine adducts (*m*/*z* 222), previously reported for Cascade[10] (Figure 9). Unfortunately, as for their cysteinylated counterparts, **G-23** and **G-26** proved not to be resolved with the C18 column, whatever the gradient. Moreover, as both molecules share the same mass spectrum (Figure 5), individual quantitations were not possible.

**G-23** and **G-26** were finally resolved by HPLC using the Cyclobond I 2000 RSP more polar column (depicted for synthesized standards, Figure 4). The MRM data given in Figure 9 confirm the evidence of **G-23** and **G-26** in hop. Both **G-23** diastereomers were found in all three cultivars (20−98 mg/kg; Table 2). The ratios between both diastereomers of **G-23** were in all cases close to 45:55. On the other hand, **G-26** occurred above the detection limit only in the Hallertau Blanc cultivar (0.7 mg/kg; Table 2). Traces were also detected in Mosaic hop. **G-23** was found in much greater amount than **G-26**.

**Table 2. Concentrations (mg/kg) of *S*-3-(4-Methyl-1-hydroxypentyl)glutathione (G-26) and *S*-3-(1-Hydroxyhexyl)glutathione (G-23) in Amarillo, Hallertau Blanc, and Mosaic Hop Cultivars[a]**

| compound | Amarillo hop | Hallertau Blanc hop | Mosaic hop |
|---|---|---|---|
| **G-23** | 98.6 a | 70.2 b | 20.1 c |
| **G-26** | nd b | 0.7 a | tr b |

[a]nd, undetected; tr, detected at trace level. The variation coefficients for the measurement of **G-26** and **G-23** were below 6%. Standard deviations have been considered in the Student−Newman−Keuls test applied to the results to determine the statistical groups (represented by letters) in each row. Values in the same row that do not share a common letter are significantly different ($p < 0.05$).

As summarized in Table 3, glutathionylated precursors emerged as the major fraction of polyfunctional thiols in hop.

**Table 3. Concentrations (μg/kg) of Free and Bound Potential (Given in Free Thiol Equivalents) of 3-Sulfanylhexan-1-ol (23) and 3-Sulfanyl-4-methylpentan-1-ol (26) in Amarillo, Hallertau Blanc, and Mosaic Hop Varieties**

| compound | Amarillo hop | Hallertau Blanc hop | Mosaic hop |
|---|---|---|---|
| **23** free thiol[a] | <1 a | <1 a | <1 a |
| **23** releasable from either cysteine or cysteinyl glycine adducts[a] | 413 a | 188 b | 47 c |
| **23 releasable from glutathione adducts** | **32469 a** | **23115 b** | **6634 c** |
| **26** free thiol[a] | 25 b | 109 a | 46 b |
| **26** releasable from either cysteine or cysteinyl glycine adducts[a] | nd b | 39 a | nd b |
| **26 releasable from glutathione adducts** | **nd b** | **230 a** | **tr b** |

[a]Data previously reported by Kankolongo et al.,[1] obtained using the indirect method with apotryptophanase enzymatic assays. The variation coefficients of these measurements were below 15%. nd, undetected; tr, detected at trace level (≤90 μg/kg). Standard deviations have been considered in the Student−Newman−Keuls test applied to the results to determine the statistical groups (represented by letters) in each row. Values in the same row that do not share a common letter are significantly different ($p < 0.05$).

This is particularly true for **23**, the glutathione adduct of which could bring 6634−32469 μg/kg of free thiol (versus <1 μg/kg for the free form and 47−413 μg/kg for the cysteinylated adduct). As previously reported in other plants, including grapes,[15−17,21−23] 3-sulfanylhexan-1-ol and its bound fractions **Cys-23** and **G-23** seem relatively ubiquitous in dual-purpose hops. **G-26**, on the other hand, never evidenced before, proved to be relatively specific to the Hallertau Blanc variety (as was the case for the cysteinylated adduct).[1]

In conclusion, assessing the sensorial profile of a dry-hopped beer on the basis of hop analyses alone remains delicate. Complementary research is needed to determine the conversion efficiency of yeast for each adduct, depending on the medium composition. Moreover, the contribution of malt cannot be excluded.[27]



DOI: 10.1021/acs.jafc.6b03788
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## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03788.

$^1$H NMR spectra of the synthesized *S*-3-(4-methyl-1-hydroxypentyl)glutathione (**G-26**), *S*-3-(1-hydroxyhexyl)glutathione (**G-23**), and *S*-3-(4-methyl-1-hydroxypentyl)cysteine (**Cys-26**); concentrations (ng/L) of here-undiscussed thiols in the three pilot dry-hopped beers (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

*(S.C.) E-mail: sonia.collin@uclouvain.be. Fax: +32-10-47 21 78.



### Funding

We are grateful to the Barth-Haas Group for the 2016 grant. R.R. is a *Chercheur Qualifié* of the Fonds de la Recherche Scientifique-FNRS (F.R.S-FNRS).


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank the hop providers Yakima Chief and Hopsteiner.

## ■ ABBREVIATIONS USED

*p*HMB, *p*-hydroxymercuribenzoic acid; PFPD, pulse flame photometric detector; IST, internal standard; EST, external standard; ESI, electrospray ionization

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DOI: 10.1021/acs.jafc.6b03788
*J. Agric. Food Chem.* 2016, 64, 8572−8582