

First Evidence of the Cysteine and Glutathione Conjugates of 3-Sulfanylpentan-1-ol in Hop (*Humulus lupulus* L.)

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Supporting Information

ABSTRACT: After evidence of the cysteinylated precursors of 3-sulfanyl-4-methylpentan-1-ol (**Cys-26**) and 3-sulfanylhexan-1-ol (**Cys-23**) in hop, *S*-glutathione precursors (**G-23** and **G-26**) were recently discovered in different dual-purpose hop varieties. Because free 3-sulfanylpentan-1-ol (**21**) has also been detected in hop, the present work aimed to identify its potential precursors. The compounds *S*-3-(1-hydroxypentyl)cysteine (**Cys-21**) and *S*-3-(1-hydroxypentyl)glutathione (**G-21**) were first synthesized and characterized by nuclear magnetic resonance and high-resolution mass spectrometry. High-performance liquid chromatography–positive electrospray ionization–tandem mass spectrometry evidenced both for the first time in hop. Both *S* conjugates were further quantitated in six hop samples: the well-known Saaz, Amarillo, Citra, Hallertau Blanc, Nelson Sauvin, and Polaris. Similar to **G-23**, **G-21** appeared ubiquitous to all varieties. Of all of the samples investigated here, Citra (harvest 2017) emerged as the richest in **G-21**, with 18 mg/kg of dry matter. **Cys-21** was found in all samples at a much lower concentration (up to 0.2 mg/kg of dry matter in Polaris, harvest 2017). Model media spiked with **Cys-21** or **G-21** allowed for the confirmation that brewing yeast is able to release free compound **21** from them.

KEYWORDS: hop (*Humulus lupulus* L.), polyfunctional thiols, cysteine conjugates, glutathione conjugates

INTRODUCTION

Besides its bacteriostatic effect against Gram-positive bacteria¹ and its contribution to beer foam stability,² hop (*Humulus lupulus* L.) is mainly used by brewers to increase bitterness (when added at the beginning of wort boiling) and flavor (when added at the end of boiling/late hopping or during beer fermentation and maturation/dry hopping).^{3–5} Among the numerous studies on key hop components, extensive work has focused on terpenes and terpenoids, the major constituents of hop essential oils.^{7–10} More recently, polyfunctional thiols were also shown to be key contributors to hop flavor in beer. According to the length of the main chain, these molecules, which often have a 3-carbon distance between the SH group and the other chemical function (alcohol, ester, carbonyl, etc.), can impart rather unpleasant flavors (e.g., 3-methyl-2-butene-1-thiol) or very delicate exotic/fruity/citrusy aromas.¹¹

A total of 41 polyfunctional thiols have now been found in hop, with each variety having a unique profile.^{12,13} The blackcurrant-like 4-sulfanyl-4-methylpentan-2-one (**29**, in Figure 1), first detected from Cascade hops by Steinhaus et al.,¹⁴ was evidenced in numerous hop cultivars (up to 114 $\mu\text{g kg}^{-1}$ in Citra).^{15,16} While 3-sulfanylhexan-1-ol (**23**, in Figure 1) (grapefruit-like flavor, with an odor perception threshold of 55 ng L^{-1} in beer) proved to be relatively ubiquitous in hop (17 varieties investigated by Kishimoto et al., up to 125 $\mu\text{g kg}^{-1}$ found in Simcoe),¹⁵ its isomer 3-sulfanyl-4-methylpentan-1-ol (**26**, in Figure 1) (grapefruit-like flavor, with an odor perception threshold of 70 ng L^{-1} in beer) emerged as more specific to some hop varieties (first proposed as the key component of Nelson Sauvin¹³ but later found up to

109 $\mu\text{g kg}^{-1}$ in Hallertau Blanc).^{12,13,17} 3-Sulfanylpentan-1-ol (**21**, in Figure 1, citrus, catty olfactory descriptors), also mentioned as a wine constituent,¹⁸ has been evidenced in a few hop varieties, including Nelson Sauvin, Citra, and Amarillo (up to 10 $\mu\text{g kg}^{-1}$).^{4,12,13} This compound could also be derived from malts.¹³ Despite its high threshold (620 ng L^{-1} in water), it is proposed to contribute to wine flavor by an additive effect, together with 3-sulfanylhexan-1-ol and 3-sulfanylheptan-1-ol.¹⁸

Unexpectedly, the thiol contents of the final beers usually reached higher values than might be expected on the basis of the hopping rate and hop free thiol contents.¹⁹ Levels of compounds **21**, **23**, **26**, and **29** were, for instance, multiplied by 10–40 after fermentation.²⁰ These findings led investigators to suspect the presence of precursors in hop. The first cysteine *S* conjugates had been discovered just before in Sauvignon Blanc grapes.^{21,22} In 2012, the first evidence of *S*-3-(1-hydroxyhexyl)-cysteine (**Cys-23**) in Cascade hop confirmed the suspicions.²⁰ Subsequently, these compounds were quantitated directly [high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) analysis]^{20,23} and indirectly [after release by apotryptophanase]²⁴ in several hop varieties. Up to 1 mg kg^{-1} of **Cys-23** was found in Cascade hop,²⁰ while *S*-3-(4-methyl-1-hydroxypentyl)cysteine (**Cys-26**) again proved to be more specific to the Hallertau Blanc variety (39 $\mu\text{g kg}^{-1}$).²⁵ *S*-4-(4-Methylpentan-2-one)cysteine (**Cys-29**)

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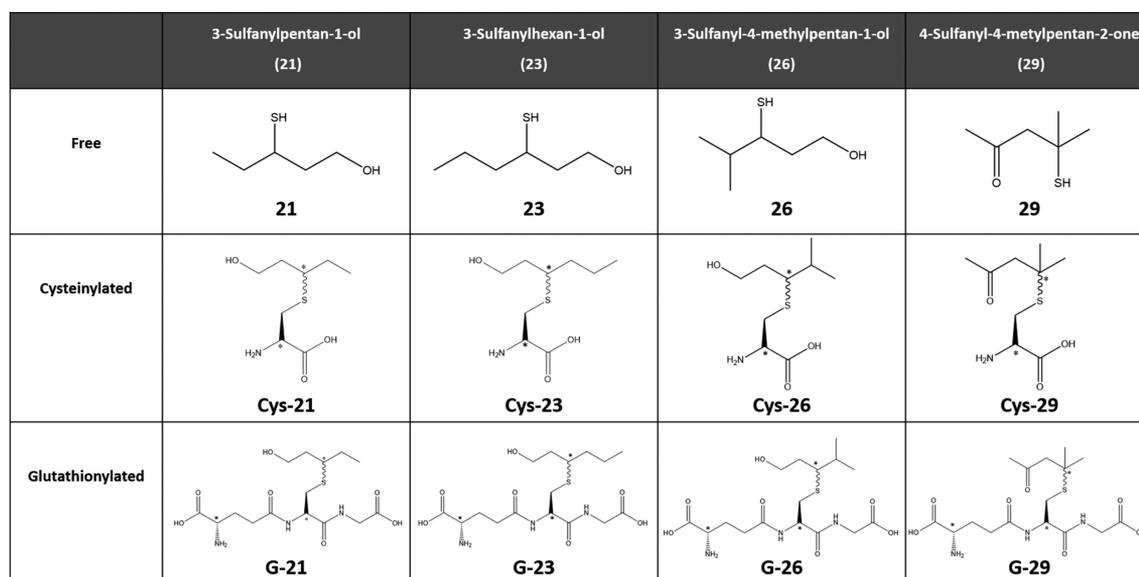


Figure 1. Chemical structures of free, cysteinylated, and glutathionylated 3-sulfanylpentan-1-ol (21), 3-sulfanylhexan-1-ol (23), 3-sulfanyl-4-methylpentan-1-ol (26), and 4-sulfanyl-4-methylpentan-2-one (29). Asterisks indicate chiral centers.

was also found by the direct method in the Citra cultivar, at concentrations up to $35 \mu\text{g kg}^{-1}$.²³ On the other hand, cysteinylated 3-sulfanylpentan-1-ol (Cys-21) was detected only by the indirect apotryptophanase assay ($391 \mu\text{g kg}^{-1}$ in Amarillo and $724 \mu\text{g kg}^{-1}$ in Nelson Sauvin).^{4,24} According to Lafontaine et al.,²⁶ early harvest hops may contain higher cysteine conjugate concentrations and may therefore be better suited for kettle addition, while later harvested hops may be richer in free thiols, as expected for dry hopping.

First identified in Sauvignon Blanc as an intermediate in the biosynthesis of cysteinylated conjugates, S-3-(1-hydroxyhexyl)-glutathione (G-23) was recently found in hop, together with S-3-(4-methyl-1-hydroxypentyl)glutathione (G-26) and S-4-(4-methylpentan-2-one)glutathione (G-29).^{27–29} While G-23 appeared to be ubiquitous, even in malt,³⁰ G-26 and G-29 were found to be peculiar to the Hallertau Blanc and Chinook hop varieties, respectively.^{23,25} With concentrations reaching up to 100 mg kg^{-1} , glutathione conjugates could constitute the main thiol potential of hop compared to free thiols and cysteinylated conjugates, provided that the yeast is able to break it down through fermentation.^{31,32}

S-3-(Hexan-1-ol)cysteinylglycine (CysGly-23) has been evidenced in Sauvignon Blanc grape must, with levels relative to Cys-23 and G-23 greatly dependent upon the must origin.^{33,34} The alternative dipeptide S conjugate, S-3-(hexan-1-ol)- γ -glutamylcysteine (GlyCys-23), has also been found in some fermentation media.³⁴ Cysteinylglycine S conjugates can be easily obtained from the glutathionylated analogue by exogenic enzymatic treatment with γ -glutamyl transpeptidase, also called γ GT.^{35–37} Dipeptide S conjugates have not been reported in hop thus far.

Although Cys-21 and G-21 have never been found in other plants, the aim of this work was to evidence them in hop by direct HPLC–MS/MS. To this end, standards of both compounds were first chemically synthesized with traditional nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) characterizations. To compare the levels of Cys-21 and G-21 to those of other β -sulfanylalkyl alcohol conjugates previously evidenced in hop, high-performance liquid chromatography–positive electrospray ionization–multiple

reaction monitoring (HPLC–ESI(+)-MRM) was further applied to six hop samples arbitrarily chosen among different varieties, countries, and harvest years. Lastly, the potential of brewing yeast to release free compound 21 from G-21 and Cys-21 was assessed in spiked model media made of unhopped wort.

MATERIALS AND METHODS

Chemicals. Absolute ethanol, acetonitrile, Amberlite IR-120 resin, 28% ammonia, dichloromethane, diethyl ether, ethyl acetate, formic acid, 37% hydrochloric acid, and methanol were purchased from VWR (Leuven, Belgium). 2-Acetylthiophene, anhydrous acetonitrile, cesium carbonate, deuterium oxide, Dowex resin 1 \times 2 chloride form, 4-(hydroxymercuri)benzoic acid sodium salt (pHMB), >98% L-cysteine hydrochloride monohydrate, >98% L-glutathione reduced, (E)-penten-2-ol, 4-methoxy-2-methylbutane-2-thiol, S-benzyl-L-cysteine, S-hexylglutathione, and sodium borohydride were purchased from Sigma-Aldrich (Bornem, Belgium). Anhydrous sodium sulfate, sodium hydroxide, and trifluoroacetic acid were purchased from Acros Organics (Geel, Belgium). N-Boc-L-cysteine, monosodium phosphate, and disodium phosphate were obtained from Merck (Darmstadt, Germany). Milli-Q water was used (Millipore, Bedford, MA, U.S.A.). The 10 g C18 Sep-Pak cartridges were purchased from Waters Millipore (Milford, MA, U.S.A.). γ GT type IV from bovine kidney was purchased from MyBioSource (San Diego, CA, U.S.A.).

Synthesis of Reference Conjugates Previously Investigated. Cys-23, Cys-26, G-23, and G-26 were synthesized prior to this work according to the method of Gros et al.²⁰ and Kankolongo et al.²⁵

Hop Samples. Vacuum-packed T90 hop pellets (7–9% moisture) from different varieties, harvest years, and countries were arbitrary selected and stored at $-20 \text{ }^\circ\text{C}$ until used. Saaz (Czech Republic, harvest 2017; α acid, 2.7%; oil content, 0.5%) was provided by Brouwland (Belgium); Hallertau Blanc (Germany, harvest 2015; α acid, 11.3%; oil content, 1.2%) and Polaris (Germany, harvest 2017; α acid, 19.1%; oil content, 3.6%) were provided by Hopsteiner (Germany); Nelson Sauvin (New Zealand, harvest 2018; α acid, 12.4%; oil content, 1.1%) was provided by Brouwerij Anders!; and Citra (U.S.A., harvest 2017; α acid, 12.3%; oil content, 2.3%) and Amarillo (U.S.A., harvest 2015; α acid, 9.2%; oil content, 1.7%) were provided by Yakima Chief (Belgium).

¹H NMR Spectra of Synthesized S Conjugates. To confirm the chemical structure of the synthesized products, Cys-21 and G-21 were analyzed by NMR. After dissolution of the product in deuterium oxide, NMR spectra were recorded on a Bruker Avance II 300

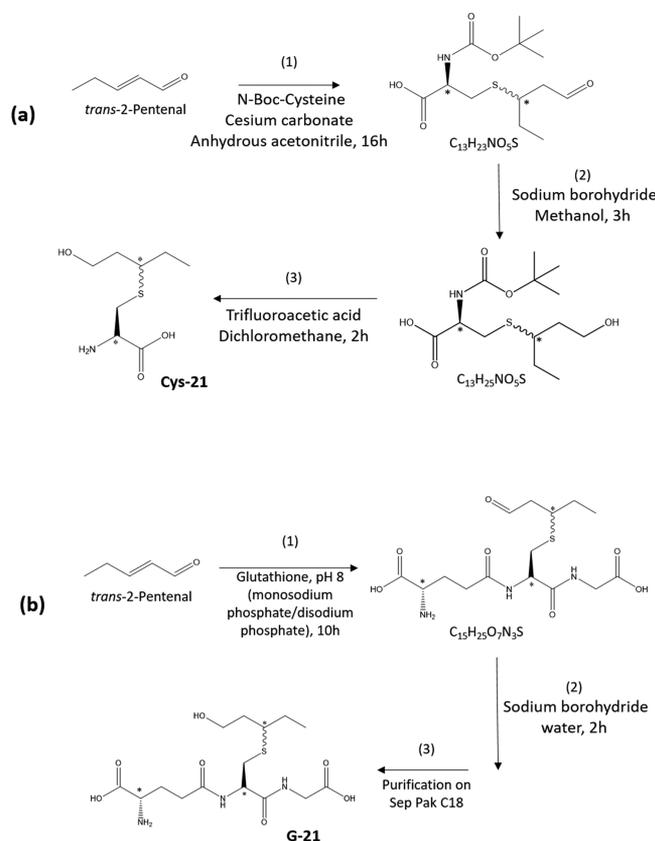


Figure 2. Chemical synthesis of (a) *S*-3-(1-hydroxypentyl)cysteine (Cys-21) and (b) *S*-3-(1-hydroxypentyl)glutathione (G-21). Asterisks indicate chiral centers.

spectrometer operating at 300 MHz. After simple-pulse NMR experiments, the resulting data were processed with Bruker TopSpin software (version 2.1). All chemical shifts (δ) are reported in parts per million relative to the reference [tetramethylsilane (TMS)]. The ^1H NMR spectra are detailed at the end of each synthesis section.

HRMS of Synthesized S Conjugates. To confirm the molecular formula of the synthesized products, Cys-21 and G-21 were analyzed by HRMS. Mass spectrum determinations were performed with a Q-Exactive Orbitrap Exact mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ion source operating in positive mode (ESI+). The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 320 °C; sheath gas (nitrogen), 20 psi; and auxiliary gas (nitrogen) flow rate, 5 mL min⁻¹. 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 at the end of each synthesis section.

Synthesis of *S*-3-(1-Hydroxypentyl)cysteine (Cys-21). The procedure was adapted from the synthesis of Cys-23 reported by Thibon et al.,³⁸ Gros et al.,²⁰ and Kankolongo et al.²⁵ A Michael

addition of *N*-Boc-*L*-cysteine (500 mg, 2.26 mmol, 0.9 equiv) on (*E*)-2-pentenal (0.245 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) (Figure 2). After evaporation of the solvent under reduced pressure, 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 hydrochloric acid (2 M), and 10 mL of water was added. The *N*-Boc-protected product was extracted 3 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 amine was achieved by reaction with trifluoroacetic acid (3.5 mL) in dichloromethane (10 mL) for 2 h. The solvent and excess trifluoroacetic acid were evaporated under reduced pressure. The product was dissolved in 5 mL of ethanol, and 5 mL of 2 M hydrochloric acid was added. After evaporation under reduced pressure, a white sticky solid was obtained. Yield of Cys-21, 35%; ^1H NMR (300 MHz, deuterium oxide), δ 0.94 (m, 3H, $-\text{CHCH}_2\text{CH}_3$), 1.50–1.95 (m, 4H, $-\text{CHCH}_2\text{CH}_3$ and $-\text{CH}_2\text{CH}_2\text{OH}$), 2.80 (m, 1H, *CH*), 3.11 (m, 2H, $-\text{CH}_2\text{S}-$), 3.68 (m, 2H, $-\text{CH}_2\text{CH}_2\text{OH}$), 4.28 (m, 1H, αCH in Cys); MS(ESI+) m/z $[\text{M} + \text{H}]^+$ 208; HRMS(ESI+) calcd for $\text{C}_8\text{H}_{18}\text{O}_3\text{NS}$, 208.10019 Da; found, 208.10023 Da.

Synthesis of *S*-3-(1-Hydroxypentyl)glutathione (G-21). This synthesis was adapted from the procedure described by Kankolongo et al.²⁵ for the synthesis of G-23. (*E*)-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 phosphate buffer (monosodium phosphate/dipotassium phosphate, 1 M, pH 8, 10 mL) (Figure 2). 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 hydrochloric acid and the solvent was evaporated under reduced pressure. To purify the product, a 10 g C18 Sep-Pak cartridge (Waters Millipore) was pre-conditioned 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 hydrochloric acid and washed 3 times with 15 mL of diethyl ether. The final product was obtained after concentration of the aqueous phase under reduced pressure. Yield of G-21, 56%; pale yellow solid; ^1H NMR (300 MHz, deuterium oxide), δ 0.95 (m, 3H, $-\text{CHCH}_2\text{CH}_3$), 1.45–1.91 (m, 4H, $-\text{CHCH}_2\text{CH}_3$ and $-\text{CH}_2\text{CH}_2\text{OH}$), 2.22 (m, 2H, βCH_2 in Glu), 2.60 (m, 2H, γCH_2 in Glu), 2.71–2.95 (m, 2H, $-\text{CH}_2\text{S}-$), 3.01 (m, 1H, $-\text{CHS}-$), 3.71 and 4.10 (m, 2H, $-\text{CH}_2\text{CH}_2\text{OH}$), 4.01 (s, 2H, αCH_2 in Gly), 4.29 (m, 1H, αCH in Glu), 4.54 (m, 1H, αCH in Cys); MS(ESI+) m/z $[\text{M} + \text{H}]^+$ 394; HRMS(ESI+) calcd for $\text{C}_{15}\text{H}_{28}\text{O}_7\text{N}_3\text{S}$, 394.16425 Da; found, 394.16426 Da.

Enzymatic Production of *S*-3-(1-Hydroxypentyl)-cysteinylglycine (CysGly-21). Unpurified CysGly-21 (just for identification purposes, with no quantitation in the present work) was obtained from the previously synthesized G-21 by enzymatic release of the

Table 1. Concentrations (mg kg_{dm}⁻¹) of Bound 3-Sulfanylpentan-1-ol (21), 3-Sulfanylhexasan-1-ol (23), and 3-Sulfanyl-4-methylpentan-1-ol (26) in Hop, Determined by RP-HPLC–ESI(+)-MRM^a

	Amarillo (U.S.A., 2015)	Citra (U.S.A., 2017)	Hallertau Blanc (Germany, 2015)	Nelson Sauvin (New Zealand, 2018)	Polaris (Germany, 2017)	Saaz (Czech Republic, 2017)
Cys-21	d	d	d	d	0.162 a	d
G-21	7.5 bc	18.1 a	3.0 cd	1.4 d	9.8 b	2.5 cd
Cys-23 + Cys-26	2.1 b	0.3 d	1.3 c	0.2 d	4.9 a	0.4 d
G-23	101.0 a	91.0 a	77.1 ab	20.1 c	118.2 a	95.7 a
G-26	nd	nd	0.3 b	d	3.6 a	d

^and, undetected; d, detected at a trace level (<50 μg kg⁻¹). Variation coefficients are under 15%. Standard deviations have been considered in the Student–Newman–Keuls test. Values in the same row that do not share a common letter are significantly different ($p > 0.05$).

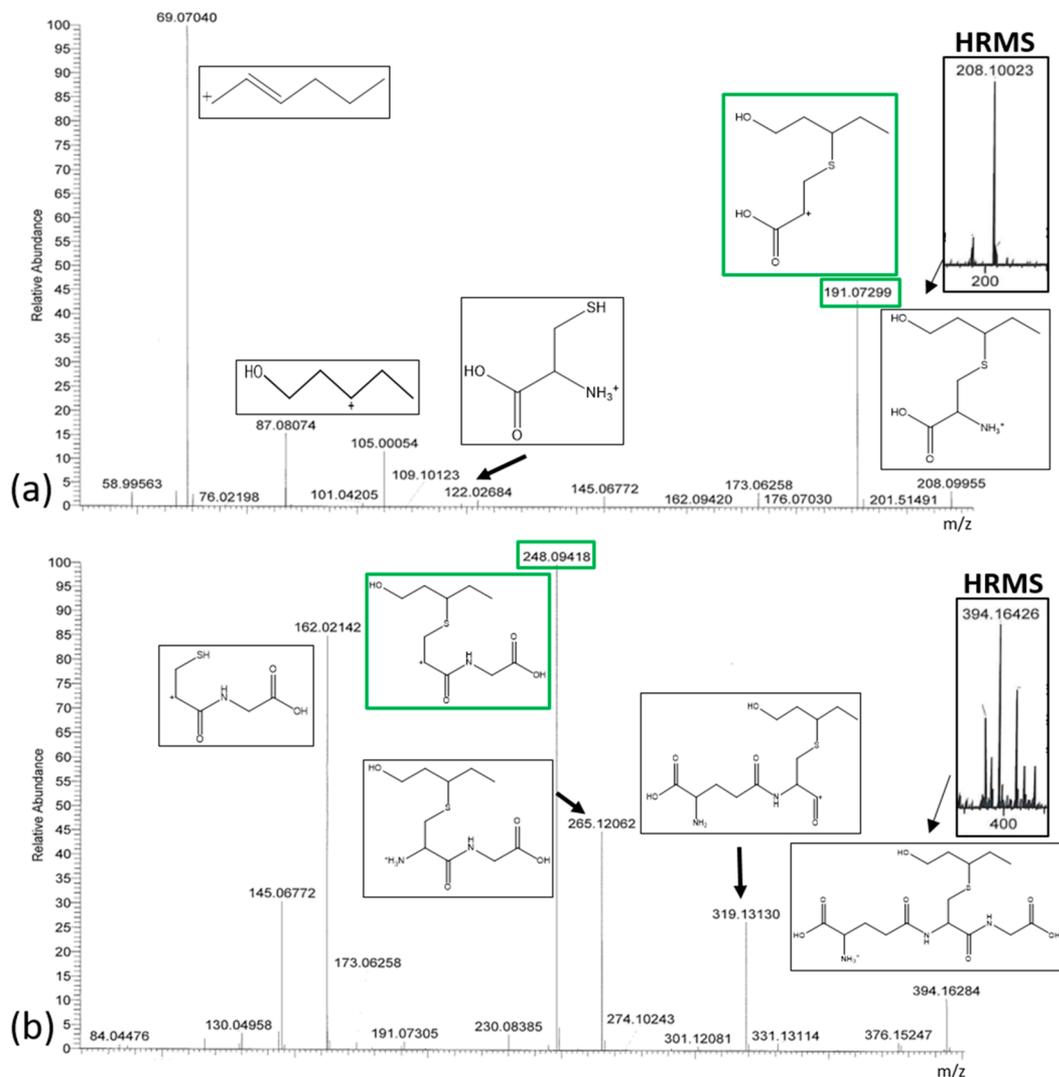


Figure 3. HRMS and MS/MS–ESI(+) mass spectra of (a) S-3-(1-hydroxypentyl)cysteine (Cys-21, m/z 208) and (b) S-3-(1-hydroxypentyl)-glutathione (G-21, m/z 394).

γ -glutamyl moiety in the presence of γ GT. A 200 mg kg^{-1} aqueous G-21 solution adjusted to pH 7 with sodium hydroxide was treated with 1 mg of γ GT (around 24 units). The medium was lightly stirred in a 37 °C bath for 30 min. This medium was compared to a reference, where 200 mg kg^{-1} aqueous G-21 solution followed the same treatment but without enzyme. The structure of the expected dipeptide conjugate was confirmed by RP-HPLC–ESI(+)-MS/MS (m/z 264).

Extraction of Cysteine and Glutathione S Conjugates from Hop Pellet Samples. S-Benzyl-L-cysteine was used as the internal standard (IST) at 8 mg kg^{-1} of hop. Milled pellets (100 g) were stirred with 1000 mL of water/ethanol/formic acid (79:20:1, v/v/v) for 2 h at 45 °C. After centrifugation for 30 min at 11800g, 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 hydrochloric acid, followed by 1 L of water). The column was then washed with 800 mL of water, and sequential 100 mL fractions were recovered by elution with aqueous ammonia solutions at 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, and 2.4 mol L^{-1} . According to previous optimizations,^{20,25} the 1.2–2.4 mol L^{-1} fractions were pooled and concentrated under reduced pressure. The obtained extract was dissolved in 4 mL of 0.1% formic acid aqueous solution

Reversed-Phase High-Performance Liquid Chromatography–Positive Electrospray Ionization [RP-HPLC–ESI(+)] Analyses of Cys-21, G-21, and Cys-23 + Cys-26 on a Hypersil GOLD aQ Column. Analyses were performed on a 100 \times 2.1 mm, 3 μm

Hypersil GOLD aQ column, a polar end-capped C18 phase offering superior retention of polar compounds (Thermo Fisher Scientific). The elution solvents were water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. Gradient elution was as follows: 100% solvent A for 10 min, from 100 to 98.6% in 15 min, maintained for 5 min, from 98.6 to 85% in 20 min, decrease to 10% in 1 min, 10 min of washing, and back to the original conditions in 5 min for 15 min. The flow rate was set at 350 $\mu\text{L min}^{-1}$. A total of 10 μL of sample was injected into the column at 50 °C. A system equipped with an autosampler and a quaternary pump (Agilent Technologies, 1200 Series) was used. The system was controlled with Agilent ChemStation software. 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, 365 °C; and nebulizer pressure and flow rate of the drying gas (nitrogen), 40 psi and 8 mL min^{-1} . To provide optimized detection and quantitation of each kind of precursor, MS was tuned with two commercially available cysteine and glutathione conjugates (S-benzyl-cysteine, also used as IST, and S-hexyl-glutathione). For identification by tandem mass spectroscopy (MS/MS), collision-induced dissociation spectra were recorded at a relative collision energy of 0.5 V. For quantitation, the multiple reaction monitoring (MRM) mode, consisting of quantifying only a selected ion issued

from the fragmentation reaction of the molecular ion in the second mass spectrometer stage,³⁹ was applied. A relative collision energy of 0.8 V was used to maximize the fragmentation of the molecular ion, and the major fragment for each compound was selected: m/z 208 \rightarrow 191 for Cys-21, m/z 394 \rightarrow 248 for G-21, and m/z 222 \rightarrow 205 for Cys-23 and Cys-26.

Selective Analysis of G-23 and G-26 by RP-HPLC–ESI(+)-MS/MS or MRM on an Astec Cyclobond I 2000 2-Hydroxypropyl- β -cyclodextrin (RSP) Column. Analyses were performed on a 250 \times 4.6 mm, 5 μ m, Astec Cyclobond I 2000 RSP chiral column (used here for its polarity and not for its chirality, as advised by Kankolongo et al.,²⁵ for resolving G-23 from G-26). Water with 0.005% formic acid (to remain above pH 4, within the pH stability range of the column) was used as solvent A, and acetonitrile with 0.1% formic acid was used as solvent B. The gradient elution was as follows: for solvent A, 95% for 5 min, from 95 to 50% in 5 min, 50% maintained for 25 min, from 50 to 10% in 1 min, 10% maintained for 9 min, and then back to the original conditions in 3 min for 12 min. The flow rate was set at 800 μ L min^{-1} . A total of 10 μ L of sample was injected into the column at room temperature. The equipment and software were the same as those used with the Hypersil GOLD aQ column. The ESI inlet conditions also remained identical, except for the nebulizer pressure and flow rate of the drying gas, which were raised to 65 psi and 11 mL min^{-1} . For the MRM mode, m/z 408 \rightarrow 262 was selected for G-23 and G-26.

Quantitation of Cysteine and Glutathione Conjugates in Hop. The hop water content of each hop sample (7–9%) was determined according to Method 7.2 of *Analytica EBC*.⁴⁰ The concentration of S conjugates was expressed in milligrams per kilogram of dry matter ($\text{mg kg}_{\text{dm}}^{-1}$). Calibration curves of conjugates relative to IST were determined for all synthetic standards. Water with 0.1% formic acid, 10–25–50–75–100 mg kg^{-1} of the synthesized conjugates, and 100 mg kg^{-1} of IST were used to plot the linear curves (area ratio against concentration ratio). The obtained slopes gave the response coefficient ratio to the IST ($R^2 > 0.97$). The following equation was used for each conjugate quantitation: concentration of the conjugate in hop ($\text{mg kg}_{\text{dm}}^{-1}$) = concentration of IST ($\text{mg kg}_{\text{dm}}^{-1}$) \times (conjugate peak area/IST peak area) \times (response coefficient of IST/response coefficient of conjugate).

Statistical Analyses. All hop conjugate analyses were carried out in duplicate. Multiple comparisons of means were performed with Student–Newman–Keuls tests. Values that do not share a common letter in the same row of Table 1 are significantly different ($p < 0.05$).

Fermentation of Wort Spiked with Cys-21/Cys-23/Cys-26 and G-21/G-23/G-26. Wort was produced from pale malt (Boortmalt) in a 50 L scale pilot plant (Coenco, Oostkamp, Belgium). The 15 °P wort was diluted to 11 °P before boiling. A 12 °P wort was obtained after 75 min of boiling. A dry top-fermentation yeast (K97, Fermentis, Marcq-en-Baroeul, France) was pitched at 50 g hL^{-1} into 250 mL of wort spiked beforehand with Cys-21, Cys-23, and Cys-26 or G-21, G-23, and G-26 (10 mg kg^{-1} each). The fermentation temperature was maintained at 24 °C for 10 days. The fermented wort was directly subjected to free thiol extraction (see the next section). Experiments were performed in duplicate.

Extraction of Polyfunctional Thiols from Fermented Spiked Media by pHMB. Polyfunctional thiols were extracted from fermented spiked media according to the procedure of Gros et al.¹² in the following steps: 300 mL of dichloromethane liquid/liquid extraction of 750 mL of fermented wort, extraction of the resulting organic phase with a pHMB solution, loading of the pooled aqueous phase onto a strong anion-exchanger resin, rinsing of impurities from the column with pH 6 acetate buffer, release of free thiols from pHMB by percolating a washed cysteine solution (4 \times 50 mL of dichloromethane for washing 640 mg of cysteine in 50 mL of water), final extraction with bidistilled dichloromethane, and concentration to 250 μ L in a Kuderna–Danish distillation apparatus and to 70 μ L on a Dufton column. 4-Methoxy-2-methylbutane-2-thiol was added as the IST (at 1.3 $\mu\text{g L}^{-1}$ in fermented wort), and

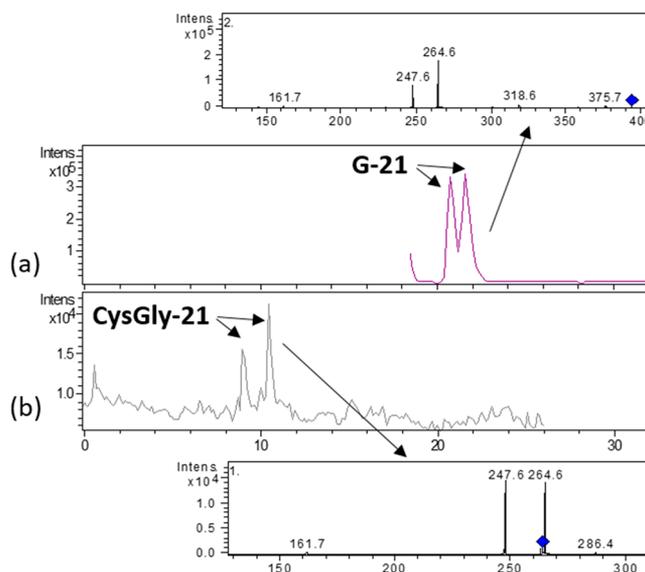


Figure 4. RP-HPLC–ESI(+)-MS/MS performed with a Hypersil Gold aQ column and applied to (a) unmodified G-21 (m/z 394) and (b) the same molecule subjected to γ GT enzymatic treatment (CysGly-21, m/z 264).

2-acetylthiophene was added as the external standard (EST, 1 mL at 200 $\mu\text{g L}^{-1}$ added before concentration).

Quantitation of Free Thiols in the Fermented Spiked Media by Gas Chromatography–Pulsed Flame Photometric Detection (GC–PFPD). A total of 1 μ L of pHMB free thiol extract was analyzed with a Thermo Finnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250 °C. Compounds were analyzed with a wall-coated open tubular (WCOT) apolar CP-Sil 5 CB (50 m, 0.32 mm inner diameter, and 1.2 μ m film thickness) capillary column. The carrier gas was helium, and the pressure was set at 50 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, 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, with a combustor internal diameter of 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; and pulse frequency, 3.33 Hz. PFPD chromatograms were recorded throughout elution. ChemStation software was used to process the resulting data. Identifications were performed as previously described by Gros et al.¹² The following equation was used for compounds 21, 23, and 26 (X) quantitation: concentration of X ($\mu\text{g L}^{-1}$) = concentration of IST ($\mu\text{g L}^{-1}$) \times (X peak area/IST peak area) \times (X molecular weight/IST molecular weight) \times (IST molar response coefficient/X molar response coefficient) \times (X recovery factor/IST recovery factor). The IST relative recovery factor was set at 1 (experimental values from 0.8 to 1.2, previously determined by standard addition).¹² The good equimolarity of the PFPD detector enabled us to set the IST relative molar response coefficients at 1.

RESULTS AND DISCUSSION

Synthesis of S-3-(1-Hydroxypentyl)cysteine (Cys-21).

Because Cys-21 synthesis has never been reported before, it was adapted from that of Cys-23 and Cys-26 reported by Thibon et al., Gros et al., and Kankolongo et al.^{20,25,38} After a three-step synthesis (1, Michael addition; 2, aldehyde reduction; and 3, cysteine deprotection; Figure 2a), Cys-21 was obtained in 35% yield (weight ratio of final product to reagents in mole equivalents – racemic mixture of (R)- and (S)-21 conjugated to L-cysteine). Mass fragmentation is detailed in Figure 3a. From the right to the left are observed

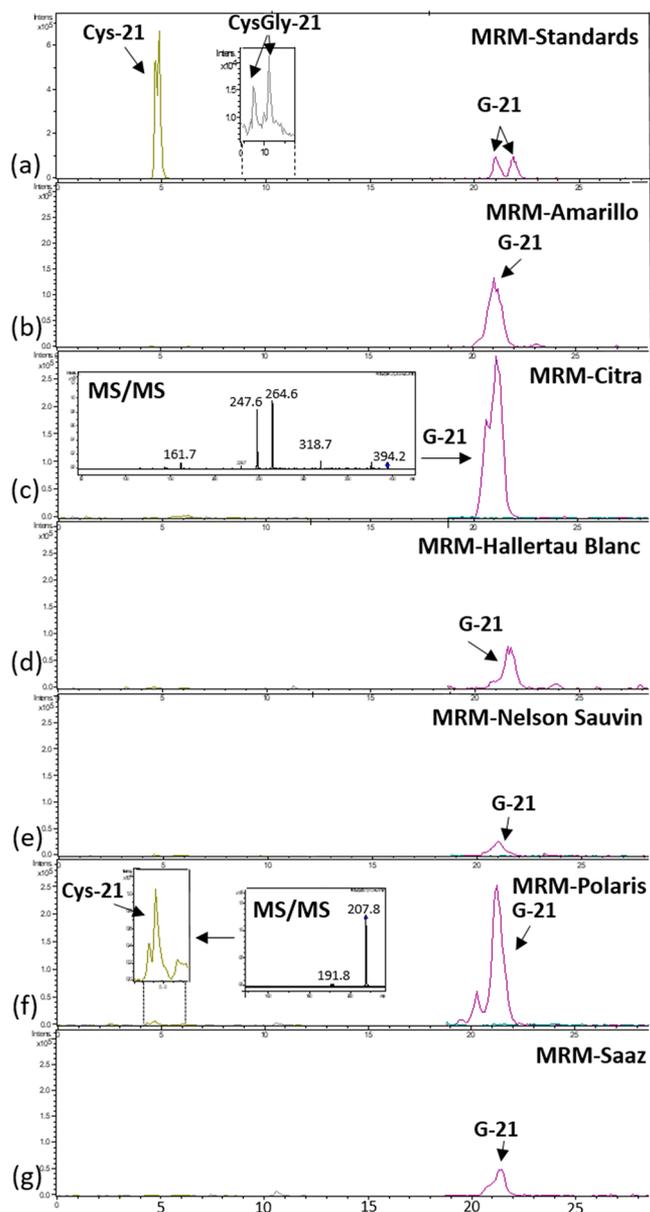


Figure 5. RP-HPLC-ESI(+)-MRM (m/z 208 \rightarrow 191 for Cys-21 and m/z 394 \rightarrow 248 for G-21) performed with a Hypersil Gold aQ column and applied to (a) a standard medium compared to hop extracts: (b) Amarillo, (c) Citra, (d) Hallertau Blanc, (e) Nelson Sauvvin, (f) Polaris, and (g) Saaz. Experimental ESI(+)-MS/MS spectra in hop extracts (G-21 in Citra and Cys-21 in Polaris).

the pseudo-molecular ion (m/z 208), the ion corresponding to loss of NH_3 (m/z 191), the pseudo-molecular ion of cysteine (m/z 122), the carbocation of the volatile thiol (m/z 87), and its dehydrated form (m/z 69). The major fragment (m/z 191) was used for RP-HPLC-ESI(+)-MRM quantitation. A double peak (a mixture of two diastereomers, slightly separated) is observed at 4.5–5.3 min when injected onto the Hypersil GOLD aQ column.

Synthesis of S-3-(1-Hydroxypentyl)glutathione (G-21). Similar to its cysteinylated counterpart, G-21 was synthesized for the first time on the basis of the previous synthesis of G-23 and G-26 reported by Kankolongo et al.²⁵ Because no protected glutathione was commercially available, the final deprotection step of the synthesis of the cysteine conjugate was replaced with purification on Sep Pak C18 (Figure 2b).

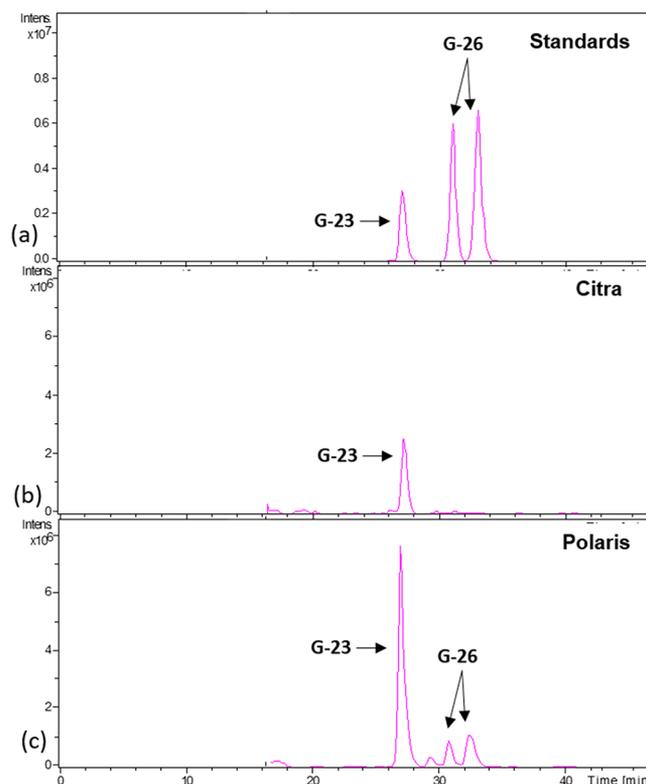


Figure 6. RP-HPLC-ESI(+)-MRM (m/z 408 \rightarrow 262 for G-23 and G-26) performed with a Cyclobond I 2000 RSP column and applied to (a) a standard medium compared to hop extracts: (b) Citra and (c) Polaris.

A pale yellow powder was obtained in 56% yield (weight ratio of final product to reagents in mole equivalents – racemic mixture of (*R*)- and (*S*)-21 conjugated to L-glutathione). The mass fragmentation pattern is depicted in Figure 3b. From the right to the left are found the pseudo-molecular ion (m/z 394), the conjugate resulting from glycine loss (m/z 319), that resulting from glutamyl loss (m/z 265), which can, in turn, lose NH_3 (m/z 248), and the dipeptide cysteinylglycine ion (m/z 162). The major fragment (m/z 262) was chosen for further quantitation by RP-HPLC-ESI(+)-MRM. Two peaks (the two diastereomers, hardly resolved) are eluted at 20.4–22.5 min when injected onto the Hypersil GOLD aQ column.

Enzymatic Production of S-3-(1-Hydroxypentyl)-cysteinylglycine (CysGly-21). Two diastereomers of CysGly-21 (t_R of 9.3 and 10.6 min on the Hypersil GOLD aQ column) were obtained after 30 min of γ GT treatment applied to G-21, as confirmed by RP-HPLC-ESI(+)-MS/MS analysis (Figure 4). The dipeptide conjugate appeared unstable over time, showing its maximum area after a 30 min γ GT treatment. Because its recovery was unknown through our *S* conjugate extraction procedure, only qualitative analysis of CysGly-21 was undertaken in hop.

First Identification of Cys-21 and G-21 in Hop by RP-HPLC-ESI(+)-MS/MS. A Hypersil Gold aQ column was used to investigate Cys-21, CysGly-21, and G-21 in our six hop samples: Amarillo, Citra, Hallertau Blanc, Nelson Sauvvin, Polaris, and the aromatic reference Saaz.

For the first time, RP-HPLC-ESI(+)-MS/MS revealed in hop the compounds Cys-21 and G-21 (both diastereomers in each case) (Figure 5). G-21 appeared, similar to G-23, ubiquitous to all of the studied varieties. No trace of CysGly-21

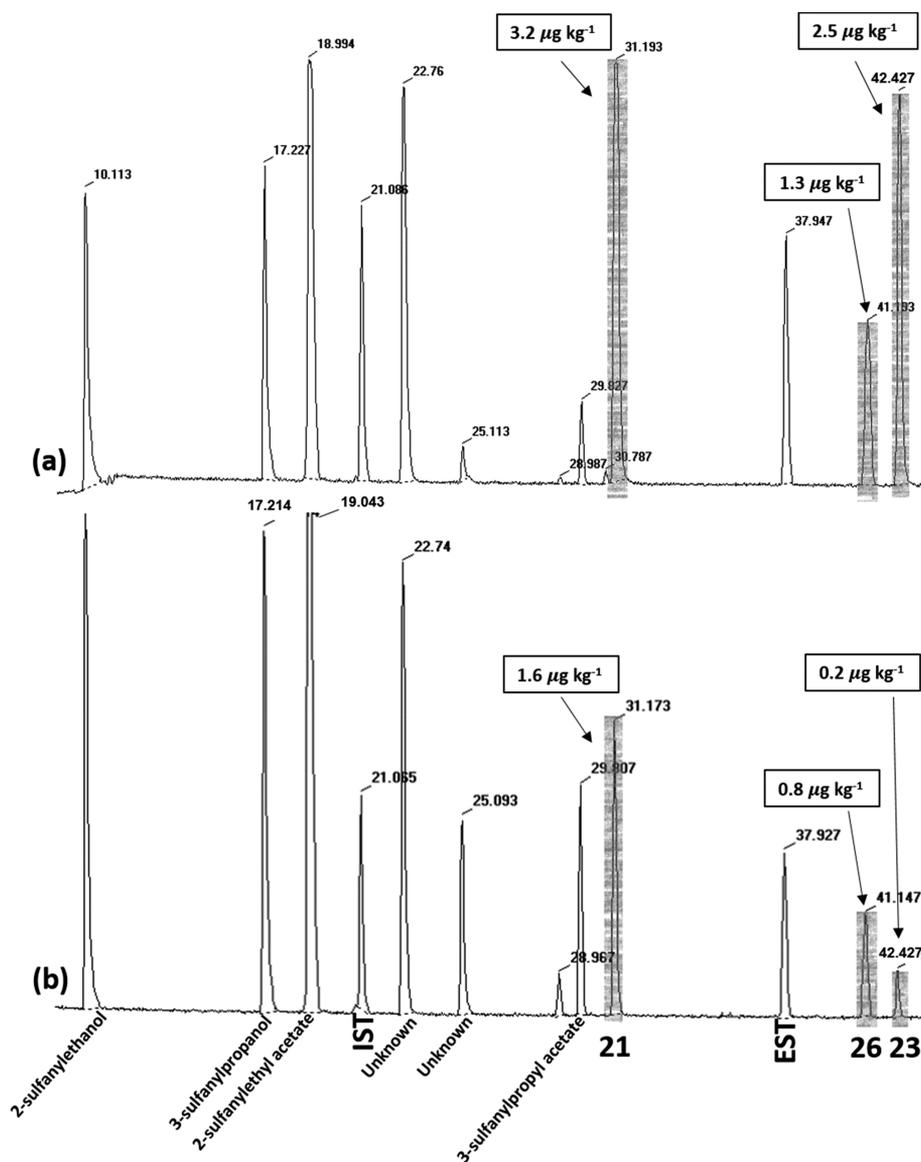


Figure 7. GC–PFPD chromatogram (CP-Sil 5 CB column) of pHMB extracts obtained from fermented unhopped worts spiked with 10 mg kg^{-1} of (a) Cys-21, Cys-23, and Cys-26 and (b) G-21, G-23, and G-26 (variation coefficient of duplicates under 15%).

was evidenced in hop. However, additional investigations should be undertaken to confirm that this dipeptide was not degraded through our hop extraction procedure. Cys-21 was found in all samples but at a trace level in most of them.

Quantitation of Cys-21 and G-21 in Hop by RP-HPLC–ESI(+)-MRM and Comparison to Other Conjugates. A strategy using two complementary columns was used to analyze thiol conjugates in hop (Figures 5 and 6). Besides the hypersil Gold aQ column ensuring Cys-21 and G-21 analyses (together with Cys-23 + Cys-26, unfortunately not separated on this column), the Cyclobond I 2000 RSP column was used to distinguish the chemical isomers G-23 and G-26 (but not Cys-23 and Cys-26, which co-elute from this column with major co-constituents, such as amino acids).

Amounts of all conjugates are depicted in Table 1. Up to $18.1 \text{ mg kg}_{\text{dm}}^{-1}$ of G-21 ($5518 \mu\text{g kg}_{\text{dm}}^{-1}$ free thiol equivalents) was found in Citra. Polaris, a relatively recent dual hop variety from Germany, emerged here as another interesting source of G-21 ($9.8 \text{ mg kg}_{\text{dm}}^{-1}$), along with Amarillo ($7.5 \text{ mg kg}_{\text{dm}}^{-1}$). Surprisingly, even the aromatic Saaz variety, known to be very

poor in free polyfunctional thiols,¹² turned out to contain $2.5 \text{ mg kg}_{\text{dm}}^{-1}$ of G-21.

In all hop samples, G-23 is present at a much higher concentration than G-21 (often above $70 \text{ mg kg}_{\text{dm}}^{-1}$, the concentration ratio is about 5 for Citra and over 10 for the other varieties). The lowest levels of both compounds are found in the same variety (20.1 and $1.4 \text{ mg kg}_{\text{dm}}^{-1}$ of G-23 and G-21 in Nelson Sauvin). While the former is known to come from the addition of glutathione on *trans*-2-hexenal (the major aldehyde from linolenic acid 13-hydroperoxide), G-21 is possibly produced by the addition of glutathione on 2-penten-1-ol (issued from the same hydroperoxide by a minor pathway) with further hydration or from 14-hydroperoxide (only chemically synthesized).

Analyzed for the first time in this paper, Polaris also revealed to be an interesting source of G-26 ($3.6 \text{ mg kg}_{\text{dm}}^{-1}$), even surpassing the previously investigated Hallertau Blanc. It also contains significant amounts of Cys-21 ($0.2 \text{ mg kg}_{\text{dm}}^{-1}$) and Cys-23 + Cys-26 ($4.9 \text{ mg kg}_{\text{dm}}^{-1}$), making it very interesting for late hopping or dry hopping combined with bottle refermentation.

For more sensitive quantitation of Cys-21, the indirect method (analysis of the free thiols after enzymatic treatment with apotryptophanase enzyme)²⁴ might be advisable, although the relative efficiency of the enzyme for different conjugates is not yet well understood. At least 50 $\mu\text{g kg}^{-1}$ of conjugate in hop samples is required for detection by our direct HPLC–MRM method, whereas GC–PFPD can easily detect just a few micrograms per kilogram of the corresponding released thiol.

Potentiality of Yeast To Release Free Compound 21 from G-21 or Cys-21. To assess if hop Cys-21 and G-21 could really release free compound 21 through beer fermentation, spiked unhopped worts were fermented by *Saccharomyces cerevisiae* K97 for 10 days at 24 °C. The first model medium contained cysteine conjugates of compounds 21, 23, and 26 (10 mg kg^{-1} each), while the corresponding glutathione conjugates were added to the second medium.

Amounts of free polyfunctional thiols were determined by GC–PFPD after a selective pHMB extraction. Besides two unknowns, 2-sulfanylethanol, 2-sulfanylethyl acetate, 3-sulfanylpropanol, and 3-sulfanylpropyl acetate, all issued from the yeast Ehrlich pathway,²⁰ were found in all fermented media, even in the absence of spiking. As depicted in Figure 7a, up to 3.2 $\mu\text{g kg}^{-1}$ of compound 21 was released by the brewing yeast from spiked Cys-21 (molar conversion of 0.05%). A similar degradation was found for Cys-23 (2.5 $\mu\text{g kg}^{-1}$ = 0.04%), while only half of this amount was released from Cys-26 (1.3 $\mu\text{g kg}^{-1}$). The ability of *S. cerevisiae* to release compounds 23 and 29 from S-cysteine conjugates had already been highlighted in grape must.^{41–44} More interesting was a notable release of the free thiols 21, 23, and 26 from glutathione S conjugates (Figure 7b). Half of the amount released from Cys-21 was detected here from G-21 (1.6 $\mu\text{g kg}^{-1}$) yet corresponding to an equivalent molar conversion (0.05%). A slightly less efficient release was observed from G-23 (0.2 $\mu\text{g kg}^{-1}$), but other yeast strains and fermentation parameters should be investigated for more extensive comparisons.

In conclusion, the present evidence and quantitation of 3-sulfanylpentan-1-ol conjugates in hops confirm the tremendous potential of glutathione conjugates. Fermentation of spiked unhopped worts also confirmed the status of Cys-21 and G-21 as potential precursors of free compound 21 in beer, provided that a sufficient contact occurs between hop constituents and yeast. Complementary research should focus on finding the best fermentation conditions to improve the access to this aromatic potential, which has been often wasted thus far.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00225.

¹H NMR spectra of synthesized S-3-(1-hydroxypentyl)-cysteine (Cys-21) and S-3-(1-hydroxypentyl)glutathione (G-21) (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

EST, external standard; IST, internal standard; γ GT, γ -glutamyl transpeptidase; kg_{dm} , kilogram of dry matter; MS/MS, tandem mass spectroscopy; MRM, multiple reaction monitoring; pHMB, 4-(hydroxymercuri)benzoic acid sodium salt; RP-HPLC–ESI(+), reversed-phase high-performance liquid chromatography–positive electrospray ionization

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