Research article

Received: 22 September 2020 Revised: 8 December 2020 Accepted: 11 December 2020 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jib.636

Ability of the Mandarina Bavaria hop variety to release free odorant polyfunctional thiols in late-hopped beers

Cécile Chenot and Sonia Collin*

The cysteinylated and glutathionylated precursors of 3-sulfanylpentanol (Cys-3SPol up to 197 μg/kg, G-3SPol up to 14 mg/kg), recently reported in hops, were quantitated for the first time in the Mandarina Bavaria variety, along with the ubiquitous cysteinylated and glutathionylated forms of 3-sulfanylhexanol (Cys-3SHol up to 897 μg/kg, G-3SHol up to 46 mg/kg). In contrast to findings with another new German cultivar, Polaris, no trace of 3-sulfanyl-4-methylpentanol adducts (Cys- and G-3S4MPol) was found. To assess the transfer rate of thiols from hops to finished beer, the same pilot Mandarina Bavaria hopped wort was fermented with two different dry yeasts, bottle refermented or not, and analysed. The data were compared with results obtained for a similarly produced commercial beer. In conclusion, despite significant variation between harvest years, Mandarina Bavaria appears not to contain outstanding amounts of free thiols or thiol S-conjugates. Its S-conjugate pool is sufficient, however, to bring 3SHol above its odour threshold. This work also suggests that since 3S4MPol was found near or above its threshold in most late-hopped beers it does not originate from hops and that malt may be its main contributor. © 2021 The Institute of Brewing & Distilling

Keywords: Mandarina Bavaria; hops; polyfunctional thiols; late-hopped beers

Introduction

Hop *(Humulus lupulus* L.*)* made its first appearance in the brewing process at the end of the 8th century AD *(1)*, its main purpose being to prevent beer spoilage through the bacteriostatic effect against Gram+ bacteria *(2)*. It quickly became one of the main ingredients imparting bitterness and flavour. For a long time, hops were classified as either being high bitterness or aromatic, on the basis of their α-acid content ($>$ or $<$7 %, respectively). Yet over the past few decades, new dual purpose varieties have been produced, characterised by hop cones rich in both α-acids and essential oils. These dual purpose hops are a direct response to the growing demand of brewers, especially craft brewers, for varieties that can increase the possibility of late hopping and new dry hopping processes *(3)*.

US hop growers were the first to respond to this increasing demand for dual hops. Since the early 2000's, they have been providing hop varieties with strong differentiating aroma notes, such as Centennial, Chinook, Citra, and Amarillo. In 2018, 59% of the worldwide hop acreage was used to produce these flavour hops.

Noble and high alpha varieties with traditional hoppy characteristics have monopolised German hop breeding for decades *(4)*. In 2006, to pave the way of German hops for use by craft brewers, a new breeding programme was launched at the Hop Research Centre Huell. Its objective was to develop hops combining distinctive fruity, citrusy, floral, and exotic aroma impressions, more characteristic of US flavour hops, with the progeny disease resistance, agronomic performances, and traditional herbal, wood, and spicy notes typical of European cultivars. For this, crosses were made between the US Cascade and male Huell. From all the hop breeding lines and brewing trials, a few varieties have been selected and registered. Among them, Mandarina Bavaria (7-10% w/w α-acids; 1.5-2.2 ml/100g total oils/dried cones) is described as fruity with pronounced mandarin and citrus notes combined with traditional hoppy nuances. This aromatic profile, with its similarities to those of Cascade and Centennial US hops, is unique in the Huell hop portfolio *(4, 5)*. Since its commercialisation, Mandarina Bavaria has already been widely used for late and dry hopping applications. The resulting beers appear to be appreciated, with excellent drinkability, a pronounced mandarin orange aroma, and high fruity citrusy potential *(4, 6)*.

To determine the varietal contributors of this unique aroma and flavour impression to beers, there have been a number of studies of the essential oil composition of Mandarina Bavaria hop. The later the harvest, the higher the total oil content *(7)*. With 140 mg/kg, geraniol is quite prominent in this variety. A comparison of all the new dual German varieties has revealed linalool, known as a hop derived key aroma compound in hoppy beers *(8, 9)*, as playing the least variety dependent role *(6)*. In late-hopped beers, however, linalool can achieve concentrations (42.1 μg/L) above its odour threshold (5-44 μg/L) *(10)*, while geraniol usually remains below one flavour unit *(11)*. Beer terpenols can arise not only through direct transfer of the free form from hop, but also through release from precursors in which they are glycosidically bound or through yeast biotransformation *(12)*. For example, geraniol can be metabolised to linalool or β-citronellol, especially after late hopping *(13)*. On the other hand and whatever the cultivar, delaying

* Correspondence: Sonia Collin, Unité de Brasserie et des Industries Alimentaires, LIBST Institute, Faculté des Bioingénieurs, Université Catholique de Louvain. Croix du Sud, 2 box L7.05.07, B-1348 Louvain-la-Neuve, Belgium. Email: sonia.collin@uclouvain.be

Unité de Brasserie et des Industries Alimentaires, LIBST Institute, Faculté des Bioingénieurs, Université Catholique de Louvain, Croix du Sud, 2 box L7.05.07, Louvain-la-Neuve B-1348, Belgium

hop addition makes it possible to avoid geraniol consumption during the yeast growth phase *(11)*.

Regarding polyfunctional thiols, levels of 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone) and 3-sulfanylhexan-1-ol (3SHol) are very low in Mandarina Bavaria (1.1 and 5.7 μg/kg, respectively) compared to American, New Zealand and Australian dual hops (up to 36.6 in Citra and 23.2 μg/kg in Ekuanot, respectively) *(14)*. Yet in the late-hopped beers, 3SHol can be found at concentrations well above its threshold (10 times as high as expected, considering the hopping rate and free thiol content), suggesting the occurrence of glutathionylated (G-) or cysteinylated (Cys-) precursors. Roland et al *(15)* have confirmed this by identifying and quantifying for the first time S-conjugates forms of 4S4M2Pone and 3SHol in Mandarina Bavaria (20 μg/kg of Cys-4S4M2Pone, G-4S4M2Pone not detected, 129 and 2119 μg/kg of Cys- and G-3SHol, respectively). This is not the case of 3-sulfanyl-4-methyl-pentan-1-ol (3S4MPol), whose level in beer was proposed to be strictly determined by the hop free content *(14)*. In malt, only the S-conjugates of 3SHol have been found so far *(16)*.

The aim of this paper was to complete the reported thiol adduct profile of Mandarina Bavaria hop using two complementary HPLC-MS methods recently applied to Polaris *(17)*, another German dual hop. These analyses focused especially on the cysteinylated and glutathionylated adducts of three sulfanylalkyl alcohols, recently reported in other dual hop varieties (structures and abbreviations detailed in Figure 1). Pilot beers late hopped with Mandarina Bavaria were further analysed by GC-PFPD after selective pHMB extraction, in order to assess the release of free thiols from cysteinylated and glutathionylated adducts through boiling and fermentation. One commercial Mandarina Bavaria late-hopped beer was also investigated.

Materials and methods

Hop samples

Two Mandarina Bavaria hop samples (2017 and 2019 harvest, 7.1 and 8.1% alpha acids, respectively) were kindly provided by Hopsteiner (Germany) for S-conjugates analyses. The former was used to produce pilot beer samples, the later for the commercial beer.

Pilot-scale production of Mandarina Bavaria late-hopped beers

Beers were produced in a 60 L microbrewery (Coenco, Belgium) as described previously *(18)*. In the brewing process, 13.65 kg of malt, 12.75 kg of Heineken type pale malt (Boortmalt, Belgium) and 0.9 kg of Cara 50 malt (Goldswean, Belgium) were brewed with 37.6 L of brewing water according to the following mashing program: 60 min at 63°C and 20 min at 73°C. The wort was then heated to 78°C and filtered through the lauter tun at a 0.8 L/min flow. The 13° Plato (60L) wort obtained was boiled with 0.58 g/L of Mandarina Bavaria pellets for 90 min (10% evaporation). Just before the whirlpool, Mandarina Bavaria pellets were added at 2 g/L. The remaining cold clarified wort was divided into two and fermented in cylindroconical fermentation tanks pitched with dry top fermented yeast at 0.5 g/L, one with BE-134 Fermentis yeast strain (BE-134 and BE-134* samples) and one with BE-256 Fermentis yeast strain (BE-256* sample). The fermentation was at 20°C for 7 days, followed by maturation for 3 days at 4°C. After filtration on plates (0.5 μM pores, Buon Vino, Cambridge, Canada), BE-134 sample was stored under carbon dioxide until extraction, while for samples BE-134* and BE-256*, bottle refermentation

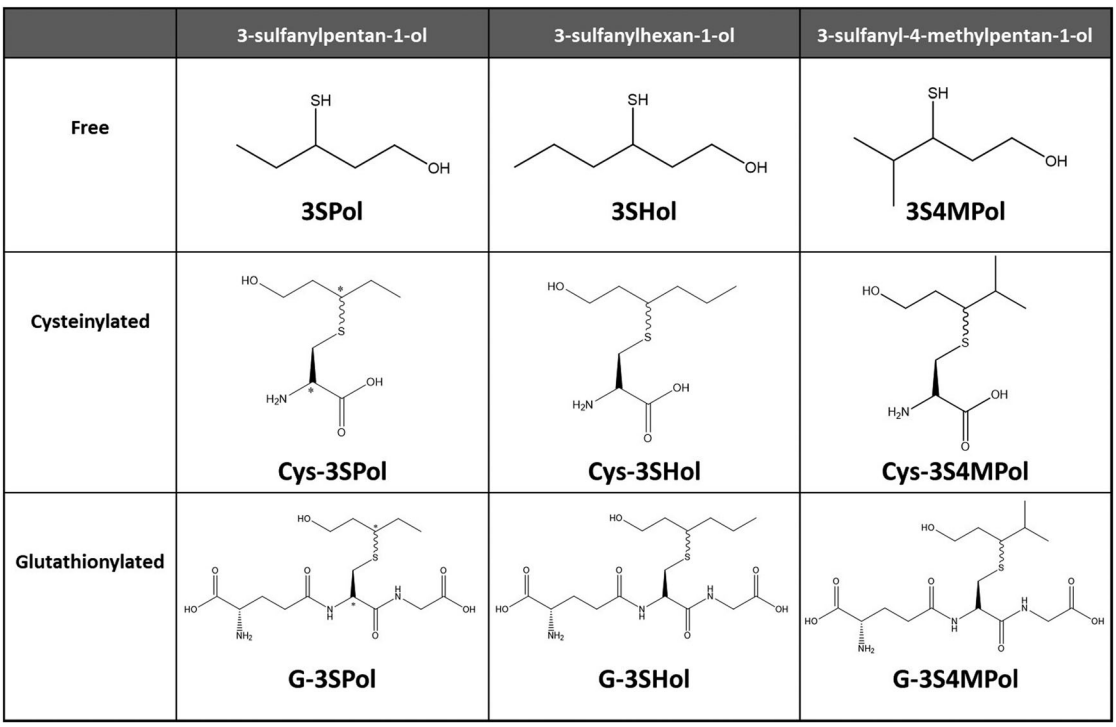

Figure 1. Chemical structures of three odorant sulfanylalkyl alcohols and their corresponding precursors.

was applied (with BE-256 Fermentis yeast strain pitched at 100,000 cells/mL/ °alcohol and the addition of 12g/L of sucrose). The * symbol is used throughout the text to indicate which samples were bottle refermented.

Commercial Mandarina Bavaria late-hopped beers

The refermented commercial bottled beer was selected for its recipe which was very similar to the pilot scale production. The beer contained a small percentage of 50 °EBC special malt, was late-hopped with 2 g/L Mandarina Bavaria, and BE-256 Fermentis yeast strain was used for pitching.

Chemicals

Absolute ethanol, Amberlite IR-120 resin, 28% ammonia, dichloromethane, formic acid, and 37% hydrochloric acid were purchased from VWR (Leuven, Belgium). 2-Acetylthiophene, apotryptophanase, Dowex resin 1x2 chloride form, 4-(hydroxymercuri) benzoic acid sodium salt (*p*HMB), >98% L-cysteine hydrochloride monohydrate, 4-methoxy-2-methylbutane-2-thiol, *S*-benzyl-L-cysteine and *S*-hexylglutathione were purchased from Sigma-Aldrich (Bornem, Belgium). Sodium hydroxide was purchased from Acros Organics (Geel, Belgium). Pyridoxal 5-phosphate was purchased from Alfa Aesar (Haverhill, Massachusetts, USA). Ethylenediaminetetraacetic acid was purchased from JT Baker Chemicals (Radnor Township, Pennsylvania, USA). di-Potassium hydrogen phosphate was purchased from Merck (Darmstadt, Germany). Milli-Q water was used (Millipore, Bedford, MA, USA).

Basic analyses of beer samples

The Analytica EBC *(19)* method 9.35 was used for beer pH. Original, real, and apparent extract, density, and alcohol content (% v/v) were determined with an Anton Paar DMA 4500M (approved by Analytica EBC).

Free thiol extraction from hops and beer samples

Polyfunctional thiols were extracted from hop pellets/beers according to the procedure *(18)*. In the following steps: solid-liquid or liquid-liquid extraction from 15 g milled pellets or 750 mL beer with extraction of the resulting organic phase with 2 x 30 mL *p*HMB solution. Loading of the combined aqueous phase onto a strong anion-exchanger resin (preconditioned with 50 mL sodium hydroxide (2 M), 100 mL water, 50 mL hydrochloric acid (2 M), 100 mL water), rinsing impurities from the column with acetate buffer pH 6. Release of free thiols from *p*HMB by percolating with washed cysteine solution (4 × 50 mL dichloromethane for washing 640 mg cysteine in 50 mL water), final extraction with 1 x 10 and 1 x 15 mL bidistilled dichloromethane. Concentration to 250 µL in a Danish-Kuderna distillation apparatus and to 70 μL on a Dufton column. 4-Methoxy-2-methylbutane-2-thiol was added as internal standard (IST, at 67 µg/kg in hops and at 1.34 µg/kg in beer samples) and 2-acetylthiophene as external standard (EST, 1 mL at 200 µg/L added before concentration). Extraction from a beer sample is illustrated in Figure 2.

Free thiol quantitation by GC-PFPD

*p*HMB free thiol extract (1 μL) was analysed with a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250°C. Compounds were

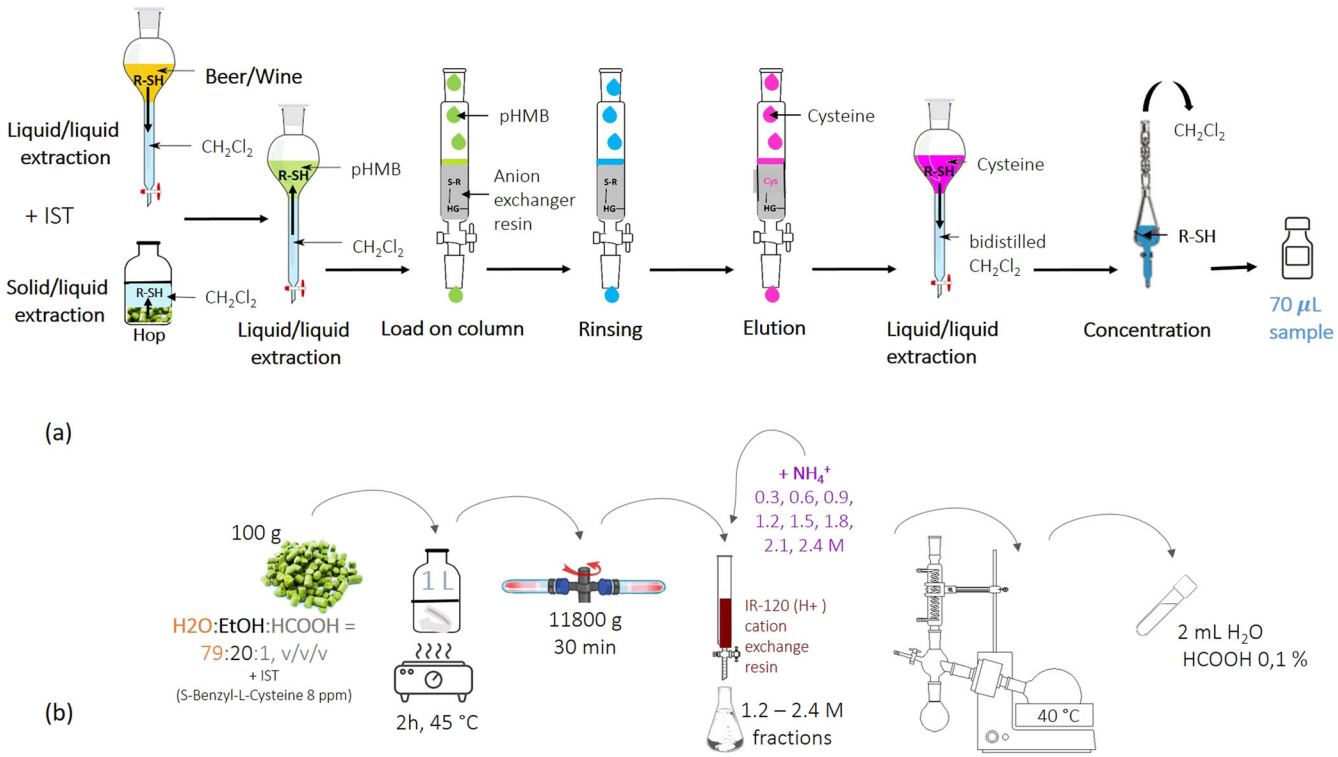

Figure 2. Sequential specific extraction of (a) free thiols (from hop or beer), (b) bound thiols (from hops).

analysed with a wall coated open tubular (WCOT) apolar CP-Sil5-CB (50 m X 0.32 mm i.d., 1.2 μm 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, 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. Identification was as previously described *(18)*. 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). The good equimolarity of the PFPD detector enabled the setting the IST-relative molar response coefficients at 1.

Bound thiol extraction from hop samples

Extraction of cysteinylated and glutathionylated thiol precursors from hop pellets was performed according to *(17)* (Figure 2b). Milled pellets (100 g) were stirred with 1000 mL H_2O:EtOH: HCOOH (79:20:1, v/v/v) for 2 h at 45°C. After centrifugation, the supernatants were collected and loaded on a column of IR-120 cation exchange resin (100 g preconditioned with 100 mL aqueous 2 M HCl followed by 1 L water). The column was then washed with 800 mL 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. The 1.2 to 2.4 mol/L fractions were pooled and concentrated under reduced pressure. The extract was either dissolved in 2 mL of 0.1 % aqueous formic acid solution for analysis by HPLC-MS or dissolved in 2 mL of potassium phosphate buffered solution for enzymatic assay.

Bound thiol quantitation by High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MRM)

The previously synthesised *(17, 20, 21)* cysteinylated and glutathionylated precursors of 3SPol, 3SHol, and 3S4MPol were used for identification and quantitation. The two complementary columns - strategy described by *(17)* - was applied to identify and quantify these molecules in hop samples.

A 100 mm x 2.1 mm, 3 μm Hypersil GOLD™ aQ column (a polar endcapped C18 phase offering superior retention of polar compounds, ThermoFisher) was first used to quantify Cys-3SPol, Cys-3SHol+Cys-3S4MPol, and G-3SPol. A 250 mm × 4.6 mm, 5 μm Astec® Cyclobond® I 2000 RSP (chiral column used here for its polarity and not for its chirality, Sigma Aldrich) was then used to quantify G-3SHol and G-3S4MPol distinctively.

For both columns, the elution solvents were acidified (0.1% formic acid) water (solvent A) and acetonitrile (solvent B) (0.005% formic acid in water with the Cyclobond to remain above pH 4, within the pH stability range of the column). When using the Hypersil GOLD™ aQ column, the gradient elution was as follows: 100% of 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 μL/min. Ten microliters of sample were injected onto the column at 50 °C. As for Astec® Cyclobond® I 2000 RSP column, 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, then back to the original conditions in 3 min for 12 min. The flow rate was set at 800 μL/min. Sample (10 μL) was injected onto the 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. 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; nebulizer pressure and flow rate of the drying gas (nitrogen) 40 Psi and 8 mL/min. To provide optimised detection and quantitation of each kind of precursors, the MS was tuned with two commercially available cysteine and glutathione conjugates (*S*-benzyl-cysteine, also used as internal standard 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. The following *m/z* were screened: *m/z* 208 for Cys-3SPol, *m/z* 222 for Cys-3SHol and Cys-3S4MPol, *m/z* 394 for G-3SPol, *m/z* 408 for G-3SHol and G-3S4MPol. For quantitation, the Multiple Reaction Monitoring (MRM) mode, consisting in quantifying only a selected ion issued from the fragmentation reaction of the molecular ion in the second mass spectrometer stage *(22)*, was applied. A relative collision energy of 0.8 V was used to maximise the fragmentation of the molecular ion and the major fragment for each compound was selected: *m/z* 208 → 191 for Cys-3SPol, *m/z* 394 → 248 for G-3SPol, *m/z* 222 →205 for Cys-3SHol and Cys-3S4MPol, *m/z* 408 → 262 for G-3SHol and G-3S4MPol.

Calibration curves of adducts relative to IST were determined for all synthetic standards and the following equation was used for each adduct quantitation: concentration of adduct (in μg/kg) = concentration of IST (in μg/kg) × (peak area of adduct/peak area of IST) × (response coefficient of IST/response coefficient of adduct).

The following equation was used to express adduct concentrations in free thiol equivalents: concentration of free thiol equivalent (in μg/kg) = concentration of adduct (in μg/kg) × (molecular weight of thiol/molecular weight of the corresponding adduct).

Distinctive cysteine adducts quantitation by enzymatic assay and GC-PFPD

To establish the Cys-3SHol/Cys-3S4MPol ratio from the Cys-3SHol +Cys-3S4MPol amount obtained by HPLC-MRM, an enzymatic treatment *(20)* was applied to hop precursors extracts. After dissolution in 2 mL of potassium phosphate buffered solution (100 mM, pH 7.7) containing pyridoxal 5-phosphate (0.1 mM) and ethylenediaminetetraacetic acid (1 mM), the extract was mixed with 0.5 mL of a solution containing the commercial apotryptophanase from *Escherichia coli* (75–150 units/mg) freshly prepared (1 mg in 0.5 mL of buffer). The mixture was kept at 28°C for 30 min and then stirred with 5 mL of bi-distilled dichloromethane for 30 min. A control without enzyme was conducted in parallel. The organic phase was recovered, dried over sodium sulphate and concentrated to 500 μL in a Danish-Kuderna and to 70 μL in a Dufton column. 1 μL of the obtained extract was analysed by GC-PFPD.

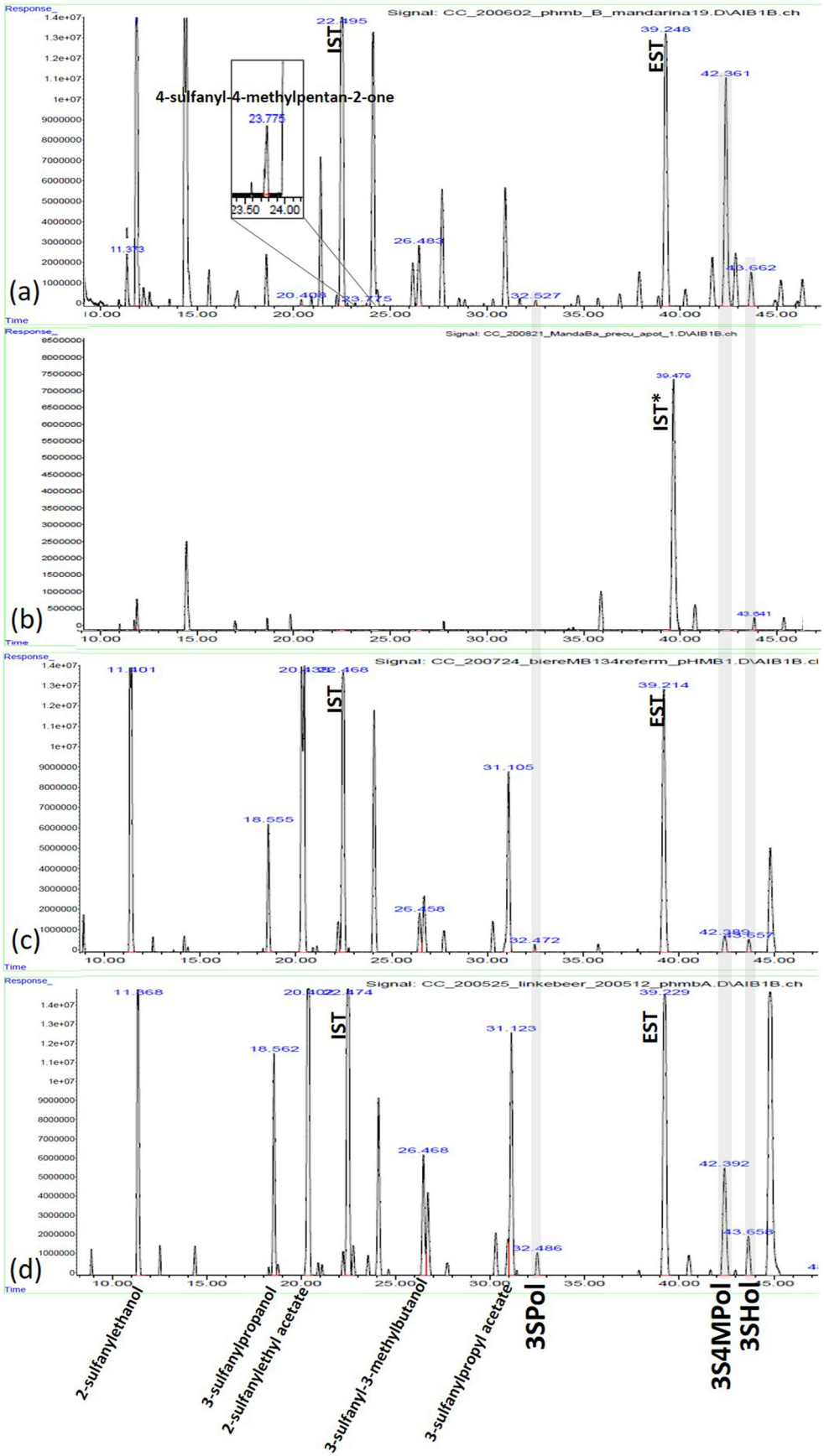

Figure 3. GC-PFPD chromatograms of pHMB extracts issued from (a) Mandarina Bavaria hop (2019 crop year), (b) Mandarina Bavaria hop (2019 crop year) after apotryptophanase incubation (IST*= benzylthiol released from S-benzyl-L-cysteine), (c) BE-134* pilot beer sample and (d) commercial Beer*.

Results and discussion

Free sulfanylalkyl alcohols in Mandarina Bavaria hop

The GC-PFPD chromatogram obtained after selective pHMB extraction of free polyfunctional thiols is presented for the 2019 harvest in Figure 3a. Table 1 details the concentrations of 3SPol, 3SHol, and 3S4MPol in both harvests (2017 and 2019). Compared to other dual varieties such as Tomahawk, Nelson Sauvin, Cascade, Citra, Ekuanot, and Hallertau Blanc *(14, 18)*, Mandarina Bavaria was not rich in free thiols (0.4-6.1 μg/kg 3SHol vs 117 μg/kg in Cascade, nd. - 0.5 μg/kg 3SPol vs 10 μg/kg in Amarillo, nd. - 0.3 μg/kg 4S4M2Pone vs 37 μg/kg in Citra *(14)*, 1.8-6.6 μg/kg 3-sulfanyl-3-methylbutan-1-ol vs 36 μg/kg in Nelson Sauvin *(14)*).

Significant differences were found, however, between the two harvests. As for other plants, climate during growth or date of harvesting probably affect the abiotic or biotic stresses linked to the occurrence of thiols. In 2019, the rhubarb/grapefruit-like 3S4MPol reached 29.1 μg/kg, a level above those usually found in Cascade (6 μg/kg) and Galaxy (26 μg/kg) hops. Only Ekuanot, Hallertau Blanc, and Nelson Sauvin appear even richer (282, 295, and 305 μg/kg, respectively) *(14)*. The sample from harvest 2019 was more consistent with published values (5.7 and 61 μg/kg for 3SHol and 3S4MPol, as reported previously *(14)*). If only free forms were available and considering a 100% recovery rate from hop to beer, the richer harvest (2019) would have to be added at 0.24 kg/hL to reach the sensory threshold of 3S4MPol (70 ng/L in beer).

Bound thiols in Mandarina Bavaria hop

For quantification of precursors, a strategy using two HPLC columns was applied as previously described for analysis of Polaris *(17)*. With the first column (Hypersil GOLD™ aQ column), both cysteine and glutathione adduct can be quantitated (Figure 4a, two peaks not always completely resolved for each adduct, because of diastereoisomers). Unfortunately, as this column does not distinguish 3SHol precursors from 3S4MPol precursors (same retention time and m/z, positional isomers), the more polar Astec® Cyclobond® I 2000 RSP column is preferred to quantitate G-3SHol and G-3S4MPol distinctively (Figure 4b). For quantitation of Cys-3SHol and Cys-3S4MPol, the enzymatic assay using apotryptophanase was applied (Figure 3b) *(20)*.

As reported for all previously studied varieties, glutathione adducts emerged with the biggest aroma potential (for 3SHol, 10^6 times as much adduct as free form). Also noteworthy is that the G-3SHol content reached 3 to 6 times the G-3SPol content *(17)*. As recently reported for Polaris, *trans*-2-hexenal (precursor of G-3SHol) is from the major oxidation pathway of linolenic acid, while G-3SPol requires the involvement of minor routes *(17)*. Both the Astec® Cyclobond® I 2000 RSP HPLC column and the apotryptophanase enzymatic assay revealed the total absence of 3S4MPol adducts (Cys- and G-3S4MPol) in Mandarina Bavaria, despite the presence of 8.7 μg/kg free form.

Free polyfunctional thiols transferred to beer by late hopping

To highlight the organoleptic impact of late hopping with Mandarina Bavaria, polyfunctional thiols were quantified in three pilot beers (BE-134, BE-134*, BE-256*) after pHMB extraction. One commercial refermented Belgian bottled beer (Beer*) produced in a similar way was also investigated. As expected, both the yeast strain and the bottle refermentation process strongly influenced the real extract and alcohol content (Table 2). *S. diastaticus* strain BE-134 was more attenuating, resulting in 3.8°P and 6.2% ABV. Bottle refermentation (BE-134*) pushed further the attenuation to 3.2°P real extract and 7.1% ABV.

Free polyfunctional thiols were extracted from pilot and commercial beer samples with the pHMB procedure and analysed with the PFPD specific detector (Figures 3c, 3d and Table 3).

Among the major polyfunctional thiols found in beer samples (Table 3), 2-sulfanylethan-1-ol (2SEol), 2-sulfanylethyl acetate (2SEA), 3-sulfanylpronan-1-ol (3SProl), and 3-sulfanylpropyl acetate (3SPrA) are known to be associated with the yeast Ehrlich pathway

Table 1. Free and bound forms of three sulfanylalkyl alcohols in Mandarina Bavaria hop, compared to the maximum values reported in literature for other varieties. All contents are given in free form equivalents (true values in parentheses)

	Free form (μg/kg)			Cysteinylated form (μg/kg)			Glutathionylated form (mg/kg)		
	Mandarina Bavaria[a]		Maximum found in another variety *(3, 14, 18)*	Mandarina Bavaria[b]		Maximum found in another variety *(15, 17)*	Mandarina Bavaria[b]		Maximum found in another variety *(15, 17)*
	2017	2019		2017	2019		2017	2019	
3SPol	nd.	0.5	10.0 (Amarillo)	d.	114 (197)	93 (161) (Polaris)	1.8 (5.9)	4.3 (14.1)	5.4 (18.1) (Citra)
3SHol	0.4	6.1	117.1 (Cascade)	93[c] (155)	542[c] (897)	2708 (4484) (Polaris)	10.6 (33.3)	15.0 (45.6)	37.8 (118.2) (Polaris)
3S4MPol	8.7	29.1	305.0 (Nelson Sauvin)	n.d.[c]	n.d.[c]		n.d.	n.d.	1.1 (3.6) (Polaris)

[a] Data obtained by specific sodium 4-(hydroxymercuri)benzoate extraction of free thiols and determined by GC-PFPD (μg/kg hop, IST equivalents). nd, undetected.

[b] Data obtained by S-conjugate-specific extraction and determined by HPLC-MS/MS (mg/kg hops, calibration curves relative to IST). d, detected at trace level (< 50 μg/Kg). nd, undetected. i. identified but not quantified. /, no data. [c]Distinctive data obtained using the indirect method with apotryptophanase enzymatic assay.

(25, 26). On the basis of their sensory thresholds, these Ehrlich derived thiols should not significantly affect the perceived flavour in any sample. Surprisingly, in contrast to other beers, all samples contained lower amounts of 3SProl than of its ester 3SPrA (3SProl/3SPrA ratios of 0.58, 0.36, 0.34, and 0.44 for BE-134, BE-134*, BE-256*, and Beer*, respectively).

Among the three pilot beers, BE-134* exhibited notably higher amounts of 2SEol and its corresponding ester 2SEA (31.4 and 7.2 μg/L, respectively vs about 20 and 2.5 μg/L). Compared to BE-134, the additional bottle refermentation significantly increased the amount of both Ehrlich compounds issued from cysteine. BE-256*, on the other hand, exhibited the highest amount of 3SProl and its corresponding ester 3SPrA (1.1 and 3.4 μg/L respectively vs about 0.4 and 0.9 μg/L), both issued from homocysteine. These results suggest that BE-256 yeast could be better at metabolising homocysteine than BE-134.

All the varietal sulfanylalkyl alcohols found in Mandarina Bavaria hop were detected in beer samples (except 3SPol in BE-134). Because of the presence of bound forms in hops, all showed levels 3 to 67 times higher than expected on the basis of hopping rate and a 100% transfer rate of free thiols from hop to beer.

The 33-50 ng/L 3SHol found in the pilot samples (close to the 55 ng/L threshold) was 33-50 times the concentration expected for a 100% transfer rate of the free form. Yet reaching such levels should have required breakdown of only 0.02% of the 33 mg/kg G-3SHol found in Mandarina Bavaria (harvest 2017). The 12 ng/L 3SPol found in BE-134* could be explained by enzymatic or chemical release of 0.3% of its glutathione adduct. Likewise, the 113 ng/L 3SHol (well above its threshold) and 19 ng/L 3SPol found in the commercial sample could easily be derived from their S-conjugates as quantitated in hop harvest 2019.

Although felinine (Cys-3S3MBol) was not investigated in this work, it can be assumed that bound forms can also be degraded to produce up to 267 ng/L 3S3MBol in beer (still far below the threshold). Otherwise, hydrogen sulphide electrophilic addition on 3-methylbutenal has been shown to be a major pathway leading to the onion-like 3S3MBol in beer *(27)*.

On the other hand, for the three pilot beers as well as the commercial sample, 3S4MPol cannot originate solely from hops (53-275 ng/L in beer, on average 4.5 times higher than the concentration expected for a 100% transfer rate of the free form). In this case neither the cysteinylated nor the glutathionylated S-conjugate have been found in hops. This constitutes the first

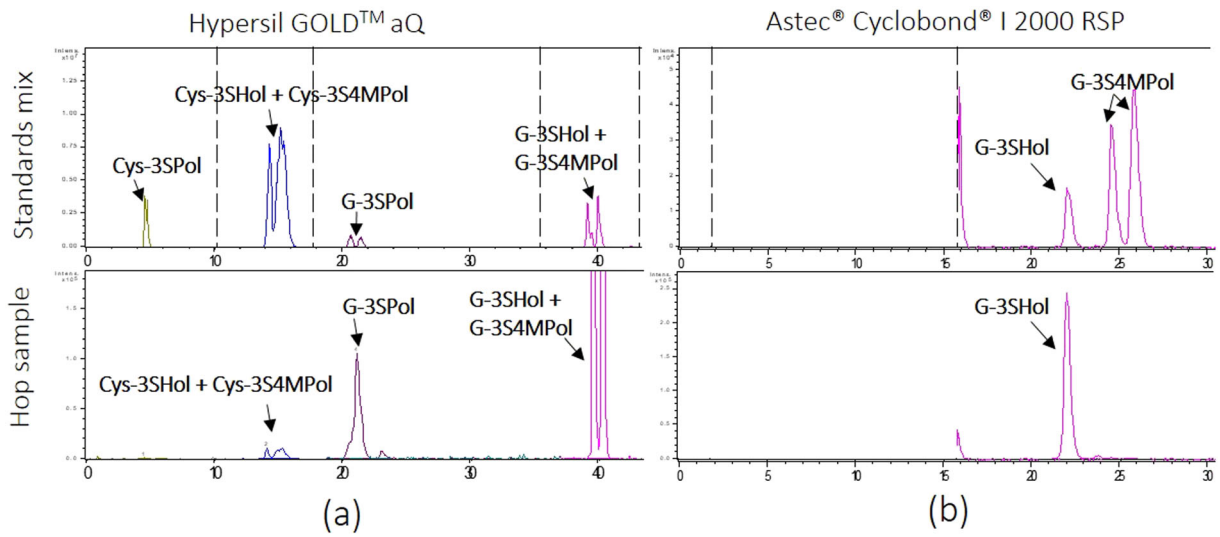

Figure 4. RP-HPLC-ESI(+)MRM (m/z 208 → 191 for Cys-3SPol, m/z 222 → 205 for Cys-3SHol and Cys-3S4MPol, m/z 394 → 248 for G-3SPol, m/z 408 → 262 for G-3SHol and G-3S4MPol) performed with (a) the Hypersil Gold aQ column and (b) the Cyclobond I 2000 RSP column, applied to a standard medium of adducts compared to the Mandarina Bavaria hop extract (2017 crop year).

Table 2. Description of the investigated pilot (BE-134, BE-134* and BE-256*) and commercial (Beer*) beers

Samples	pH	Extract (°P)			Alcohol (% v/v)
		original	real	apparent	
BE-134	4.2	13.3	3.8	1.5	6.2
BE-134*	4.2	14.1	3.2	0.6	7.1
BE-256*	4.0	13.9	5.1	3.0	5.8
Beer*	4.2	13.7	5.3	3.3	5.5

* Bottle refermented beers

Table 3. Polyfunctional thiol contents (ng/L) in Mandarina Bavaria late-hopped pilot (BE-134, BE-134* and BE-256*) and commercial (Beer*) beers. Max = Maximal transfer of free thiols and potential release of bound forms from hop (harvest 2017 for pilot samples, 2019 for commercial sample) to beer based on hopping rate (200 g/hL)

				Harvest 2017						Harvest 2019				Sensory threshold (ng/L)
		Substance (odour)	**RI** CP-Sil5	**BE-134 (ng/L)**	**BE-134* (ng/L)**	**BE-256* (ng/L)**	Max (ng/L) from *free*	Max (ng/L) from *Cys-*	Max (ng/L) from *G-*	**Beer* (ng/L)**	Max (ng/L) from *free*	Max (ng/L) from *Cys-*	Max (ng/L) from *G-*	
Ehrlich derived	**2SEol**	**2-sulfanylethan-1-ol** (soup, grilled, gas)	722	19333^{b}	31432^{a}	20847^{b}				8881^{c}				2.10^{6}
	2SEA	**2-sulfanylethyl acetate** (brunt, grill)	880	2820^{b}	7230^{a}	2133^{b}				2487^{b}				4.10^{4}
	3SProl	**3-sulfanylpronan-1-ol** (potatoes, popcorn)	849	470^{b}	409^{b,c}	1161^{a}				319^{c}				4.10^{5}
	3SPrA	**3-sulfanylpropyl acetate** (grilled)	1000	814^{b}	1113^{b}	3412^{a}				730^{b}				4.10^{4}
Hop derived	**3S3MBol**	**3-sulfanyl-3-methylbutan-1-ol** (cooked leek, soup)	938	**62^{c}**	**65^{c}**	**267^{a}**	4			**203^{b}**	13			1500
	3SPol	**3-sulfanylpentan-1-ol** (citrus)	1009	**nd.^{a}**	**12^{a}**	**6^{a}**	-	-	3600	**19^{a}**	1	228	8600	620 (in water)
	3S4MPol	**3-sulfanyl-4-methylpentan-1-ol** (rhubarb, grapefruit)	1084	**53^{b}**	**73^{b}**	**70^{b}**	17	-	-	**275^{a}**	58	-	-	70
	3SHol	**3-sulfanylhexan-1-ol** (grapefruit, passion fruit)	1094	**33^{b}**	**50^{b}**	**44^{b}**	1	186	21200	**113^{a}**	12	1794	30000	55

Data obtained by specific sodium 4-(hydroxymercuri)benzoate extraction of free thiols followed by GC-PFPD (ng per L of beer, IST equivalents). nd, undetected (< 5-10 ng/L). 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 ($p > 0.05$). Sensory threshold values from literature *(21, 23, 24)*. *Bottle refermented beers

evidence that malt could be a significant contributor of 3S4MPol. Of course, in the case of beers dry-hopped with 3S4MPol-rich varieties, the hop contribution should remain the major contributor (up to 3.2 μg/L in beer dry-hopped with Mosaïc) *(28)*.

Conclusions

This work provides, for the first time, evidence of 3SPol S-conjugates in Mandarina Bavaria, in addition to the Cys- and G-3SHol adducts previously investigated. Even though this variety does not contain outstanding amounts of precursors as compared to other dual or aromatic varieties, it contributes enough potential to release some appreciated volatiles, especially 3SHol, to levels above or near their odour thresholds. The amount of 3S4MPol, on the other hand, raises questions. Other S-conjugate origins should now be investigated.

Conflict of Interest Statement

The authors declare there are no conflicts of interest.

References

1. Edwardson JR. 1952. Hops: their botany, history, production and utilization, *Econ Bot 6*:160–175.
2. Simpson WJ, and Smith AR. 1992. Factors affecting antibacterial activity of hop compounds and their derivatives. *J Appl Bacteriol 72*:327–334. https://doi.org/10.1111/j.1365-2672.1992.tb01843.x
3. Kankolongo M-L, Gros J, Nizet S, and Collin S. 2015. Quantitation of selected terpenoids and mercaptans in the dual-purpose hop varieties Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace. *J Agric Food Chem 63*:3022–3030. https://doi.org/10.1021/jf5058556
4. Lutz A, Kneidl J, Kammhuber K, and Seigner E. 2013. Breeding of special flavor hops to pave the way to the craft brewers. International Hop Growers Convention, Proceedings of the scientific commission, Kiev, Ukraine, p 21–24. https://www.lfl.bayern.de/mam/cms07/ipz/dateien/issn_1814-2206__proceedings__kiew-2013.pdf
5. Lutz A, Kammhuber K, and Seigner E. 2012. New trend in hop breeding at the Hop Research Center Huell. *Brew Sci 65*:24–32.
6. Forster A, and Gahr A. 2013. On the fate of certain hop substances during dry hopping. *Brew Sci 66*:93–103.
7. Schnaitter M, Wimmer A, Kollmannsberger H, Gastl M, and Becker T. 2016. Influence of hop harvest date of the 'Mandarina Bavaria' hop variety on the sensory evaluation of dry-hopped top-fermented beer. *J Inst Brew 122*:661–669. https://doi.org/10.1002/jib.382
8. Kaltner D, Steinhaus M, Mitter W, Biendl M, and Schieberle P. 2003. (R)-Linalool as key flavour for hop aroma in beer and its behaviour. *Monatsschrift fü Brauwissenschaft 56*:192–196.
9. Fritsch HT, and Schieberle P. 2005. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian Pilsner-type beer. *J Agric Food Chem 53*:7544–7551. https://doi.org/10.1021/jf051167k
10. Peacock VE, and Deinzer ML. 1981. Chemistry of hop aroma in beer. *J Am Soc Brew Chem 39*:136–141.
11. Takoi K, Tokita K, Sanekata A, Usami Y, Itoga Y, Koie K, Matsumoto I, and Nakayama Y. 2016. Varietal difference of hop-derived flavour compounds in late-hopped/dry-hopped beers. *Brew Sci 69*:1–7.
12. Kankolongo Cibaka M-L, Silva Guimarães Ferreira C, Decourrière L, Lorenzo-Alonso C J, Bodart E, and Collin S. 2017. Dry hopping with the dual-purpose varieties Amarillo, Citra, Hallertau Blanc, Mosaic, and Sorachi Ace: minor contribution of hop terpenol glucosides to beer flavors. *J Am Soc Brew Chem 2017*:122.
13. Takoi K, Koie K, Itoga Y, Katayama Y, Shimase M, Nakayama Y, and Watari J. 2010. Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer. *J Agric Food Chem 58*:5050–5058. https://doi.org/10.1021/jf1000524
14. Takazumi K, Takoi K, Koie K, and Tuchiya Y. 2017. Quantitation method for polyfunctional thiols in hops (*Humulus lupulus* L.) and beer using specific extraction of thiols and gas chromatography-tandem mass spectrometry. *Anal Chem 89*:11598–11604. https://doi.org/10.1021/acs.analchem.7b02996
15. Roland A, Viel C, Reillon F, Delpech S, Boivin P, Schneider R, and Dagan L. 2016. First identification and quantification of glutathionylated and cysteinylated precursors of 3-mercaptohexan-1-ol and 4-methyl-4-mercaptopentan-2-one in hops (*Humulus lupulus*). *Flavour Fragr J 31*:455–463. https://doi.org/10.1002/ffj.3337
16. Roland A, Delpech S, and Dagan L. 2020. Chapter 4: How to monitor positive aromatic thiols during winemaking and brewing, Hop Flavor and Aroma: Proc 2nd Int Brewers Symp, p 49-70. Shellhammer TH, Lafontaine SR (Ed), American Society of Brewing Chemists and Master Brewers Association of Americas, USA.
17. Chenot C, Robiette R, and Collin S. 2019. First evidence of the cysteine and glutathione conjugates of 3-sulfanylpentan-1-ol in hop (*Humulus lupulus* L.). *J Agric Food Chem 67*:4002–4010. https://doi.org/10.1021/acs.jafc.9b00225
18. Gros J, Nizet S, and Collin S. 2011. Occurrence of odorant polyfunctional thiols in the super alpha Tomahawk hop cultivar. Comparison with the thiol-rich Nelson Sauvin bitter variety. *J Agric Food Chem 59*:8853–8865. https://doi.org/10.1021/jf201294e
19. EBC Analysis Committee. 2008. *Analytica-EBC*. Verlag Hans Carl, Nurnberg.
20. Gros J, Tran TTH, and Collin S. 2013. Enzymatic release of odourant polyfunctional thiols from cysteine conjugates in hop. *J Inst Brew 119*:221–227. https://doi.org/10.1002/jib.80
21. Kankolongo M-L, Decourrière L, Lorenzo-Alonso C-J, Bodart E, Robiette R, and Collin S. 2016. 3-Sulfanyl-4-methylpentan-1-ol in dry-hopped beers: first evidence of glutathione S-conjugates in hop (*Humulus lupulus* L.). *J Agric Food Chem 64*:8572–8582. https://doi.org/10.1021/acs.jafc.6b03788
22. de Hoffmann E. 1996. Tandem mass spectrometry: A primer. *J Mass Spectrom 31*:129–137. https://doi.org/10.1002/(SICI)1096-9888(199602)31:2<129::AID-JMS305>3.0.CO;2-T
23. Roubelakis-Angelakis KA. 2009. *Grapevine Molecular Physiology & Biotechnology*. Springer Science & Business Media.
24. Tran TTH, Gros J, Bailly S, Nizet S, and Collin S. 2012. Fate of 2-sulphanylethyl acetate and 3-sulphanylpropyl acetate through beer aging. *J Inst Brew 118*:198–204. https://doi.org/10.1002/jib.24
25. Vermeulen C, Lejeune I, Tran TTH, and Collin S. 2006. Occurrence of polyfunctional thiols in fresh lager beer. *J Agric Food Chem 54*:5061–5068. https://doi.org/10.1021/jf060669a
26. Gros J, Peeters F, and Collin S. 2012. Occurrence of odorant polyfunctional thiols in beers hopped with different cultivars. First evidence of an S-cysteine conjugate in hop (*Humulus lupulus* L.). *J Agric Food Chem 60*:7805–7816. https://doi.org/10.1021/jf301478m
27. Gros J, Nizet S, and Collin S. 2009. Hop allylic alcohols are precursors of sulfur-containing odorants in fresh beer. *Acta Hortic 848*:273–278.
28. Silva Guimarães Ferreira CA, Bodart E, Cibaka M-LK, and Collin S. 2016. What is unique in Belgian dry-hopped beers? Poster at World Brewing Congress 2016, Denver, USA.