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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*)

Aurélie Roland,^{a*} Clément Viel,^a Florence Reillon,^a Stéphane Delpech,^a Patrick Boivin,^b Rémi Schneider^a and Laurent Dagan^a



Abstract: Since ten years, many studies conducted on beers showed an important impact of polyfunctional thiols of the aroma profiles. Among them, three thiols responsible for blackcurrant bud, passion fruit, citrus and rhubarb notes have been intensively studied: the 4-methyl-4-mercaptopentan-2-one (4MMP), the 3-mercaptohexan-1-ol (3MH) and its corresponding acetate. Their origin was very complex in beers since they probably came from odourless precursors present either in hops or in malts. Our work focused on the formal identification of thiol precursors in hops and on their quantification. By using pure synthetic standards and mass spectrometry characterization, we formally identified for the first time the occurrence of glutathionylated conjugates of 4MMP and 3MH and the cysteinylated conjugate of 4MMP in hops. First quantification results obtained on 10 hop varieties, showed that 3MH conjugates were more ubiquitous than 4MMP ones. Conjugates of 3MH occurred at very high level until 20 mg kg⁻¹ in Cascade hop, which was considerably higher than concentrations found in grapes. Then, we compared the proportion of bound and free thiol fractions and we demonstrated that more than 99 % of 3MH occurred as precursors in hops. On the contrary, free 4MMP fraction represented the most important source of 4MMP in hops. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: hops; thiol; thiol precursor; Aroma potential

Introduction

Hops are a key ingredient for beer production since it brings bitter taste, contributes to the protein precipitation in wort and exhibits antimicrobial properties to stabilize beers. Hops and associate derivatives are mainly composed of bitter taste compounds, crude proteins, crude fibres and N-free extractable matter and in a less extend essential oils.^[1] A great number of plants, flowers and fruits are rich in aroma precursors that are called the 'aroma potential', as, in a technological point of view, they may be converted into volatile compounds during different steps of their processing. In theory, it is assumed that an aroma precursor is an odourless molecule that lead to a volatile aroma compound after one or two chemical or enzymatic rearrangements.^[2] Thus, parts of the original skeleton of the precursor compounds are still recognizable in formed volatile compounds.

As already described in literature,^[3] many similarities exist between wine and beer aroma compounds from biogenesis point of view. In the enological field, the grape aroma potential is composed of three main families that could be defined as (i) dimethylsulfide potential, (ii) glycoconjugate precursors and (iii) thiol precursors. The present work is focused on the third category of aroma potential: the thiol precursors.

Polyfunctional thiols occurred in beers^[4] and some of them are responsible for pleasant notes like rhubarb, citrus, passion fruit or blackcurrant bud: the 3-mercaptohexan-1-ol (3MH), the

3-mercaptohexylacetate (3MHA) and the 4-methyl-4-mercaptopentan-2-one (4MMP), respectively. These three thiols were described as key odorant compounds in beers^[5,6] but their biogenesis during brewing is not well understood.

Intensively studied in enology for 20 years, the thiol biogenesis in wine is complex and not yet totally understood. Indeed, they originated from 3 different categories of odourless precursors present in grapes called the (i) S-cysteinylated,^[7] (ii) S-glutathionylated^[8–12] and (iii) S-(Cys-Gly)^[13,14] conjugates. Another biogenesis route involved the C6 unsaturated compounds through hexenal^[15] and hexenol^[16] which can release 3MH in wine under enological conditions. These S-conjugates precursors were cleaved during the alcoholic fermentation through the C-S lyase activity of yeast. Conversion yields of precursors into thiols were always very low (less than 10 %) and depend on the type of yeast, the fermentation temperature, the grape juice turbidity, the amount of available nitrogen in grape must and on the winemaking conditions (oxygen management). Up to now,

* Correspondence to: Aurélie Roland, Nyseos, 53 Rue Claude François, Parc 2000, 34080, Montpellier, France. E-mail: aurelie@nyseos.fr

^a Nyseos, 53 Rue Claude François, Parc 2000, 34080, Montpellier, France

^b Institut Français des Boissons de la Brasserie Malterie, 7 Rue du Bois de la Champelle BP 267, 54512 Vandoeuvre-lès-Nancy Cedex, France

all the studies allowed to explain the presence between 35% and 80% of 3MH^[10–12,17–19] in wine, suggesting that the thiol precursor panorama remains incomplete.

Concerning the brewing industry, the occurrence of 4-methyl-4-mercaptopentan-2-one, 3-mercaptohexan-1-ol and its corresponding acetate in hops were firstly identified as their free forms. From the sensorial point of view, several hop cultivars as Nelson Sauvin and Cascade analysed by GC-olfactometry exhibited rhubarb, blackcurrant bud and grape fruit notes and these odours have been linked with the occurrence of both 4MMP and 3MH.^[20] These thiols occurred in hops at ppb levels and might be affected by several field treatments like the copper treatments on the plants.^[21] Actually, the presence of copper in this type of treatment might oxidize a part of thiol into the corresponding disulfides or metal-complexes. In the same time, it has been hypothesized the occurrence of thiol precursors in both malts and hops^[22] for the first time. Few years later, the S-cysteinylated precursor of 3MH (Cys3MH) was formally identified by High Resolution Mass Spectrometry (HRMS) in Cascade hop variety.^[6] Later, it was evidenced that Cys3MH could be either enzymatically degraded into 3MH by yeast during bottle re-fermentation^[23] or during Belgian beer ageing^[24] via chemical mechanisms. Very low molar conversion yields ranging from 0.03 to 0.19 % were observed during beer ageing and they hypothesized that Cys3MH could be chemically degraded through a modified Strecker pathway involving dicarbonyl compounds.

From the analysis point of view, first quantification results based on an indirect method (enzymatic cleavage of precursors and analysis of the released thiols) revealed that Cys3MH might be present until 1641 ppb as 3MH equivalent in Cascade.^[25] In addition, the same authors hypothesized the occurrence of 4MMP precursors in hops since they observed the release of 4MMP (up to 600 ppb equivalent of 4MMP in Nelson Sauvin variety) under their analytical conditions.

Thus, the aim of this work was to (i) identify and (ii) quantify the hop aroma potential in order to (iii) give some possible considerations about its management and release during beer production.

Experimental

Chemicals

All chemical products have been purchased from Sigma-Aldrich (Saint Quentin en Fallavier, France). Solvents used for analyses were from Biosolve (Dieuze, France).

Samples

Eight hop varieties either as cones (Aramis Alsace, Saaz, Mistral, Barbe Rouge, Mandarina) or pellets (Cascade, Chinook, Strisselspat) conditionings were studied. They were provided by the local distributor (Rolling beers, Vendargues, France) from 2014 and 2013 crop years.

Five different categories of beers (two Pale Ale, five Belgium, three Pils, five IPA, eight experimental beers) representing a total of 23 samples, were analysed in terms of thiol contents. They were supplied or kindly offered as experimental beers by the Institut Français des Boissons, de la Brasserie et de la Malterie.

Extraction procedure

Ten hop varieties were collected (Rolling beers distributor) either as cones or as pellet conditioning for characterization. Hop cones were preliminary crushed at room temperature to obtain a homogeneous sample. The

general procedure consisted in an extraction step with a mixture of water/ethanol composed as follows: 11 % ethanol; 4 g.L⁻¹ of tartaric acid; pH = 3,5 for 45 min at room temperature. For thiol precursor analysis, extraction mixture was slightly acidified with 0.1 % formic acid. After the extraction step, samples were centrifuged (10 min, 4 °C 10000 rpm) and aliquoted for further analysis (free thiols and thiol precursors). Samples assigned for thiol analysis were directly analysed to avoid any oxidation process whereas aliquots for precursors were stored at -20 °C prior to analysis.

Thiol precursors analysis

Hop samples (200 mg) were extracted with a mixture of water/ethanol (90/10; 3 mL) acidified with 0.1 % formic acid. After centrifugation (12000 rpm; 15 min), deuterated precursors, synthesized as already described,^[10] were added to supernatants (1 mL) as follows: Cys3MH-d₂: 111 µg.L⁻¹, G3MH-d₂: 197 µg.L⁻¹, Cys4MMP-d₁₀: 22 µg.L⁻¹ and G4MMP-d₁₀: 20 µg.L⁻¹. Extracts were then purified and analysed by nanoLC-MS/MS (HPLC Infinity 1260 Agilent and triple quadrupole 6460 Agilent) by adapting a published method.^[26] Samples (1 mL) were firstly purified using a cationic exchange resin (DOWEX, 50 mg), previously conditioned with water (600 µL), HCl 2 M (600 µL) and water (4 mL) to reach a neutral pH. Samples were then loaded into the resin, rinsed with water (1 mL), and elution was carried out using ammonium phosphate buffer (NH₄⁺ H₂PO₄⁻, 1 M, 1 mL). Extracts were then desalted on C18 cartridge (Sep-Pak) previously conditioned with methanol (1 mL), then with water (2 × 1 mL). Extracts were loaded into the cartridges, washed with water (600 µL) and the final elution was performed using methanol (600 µL). The final extracts were concentrated to dryness, and then dissolved into an accurate volume of water (50 µL) before analysis by nanoLC-MS/MS (HPLC Agilent series 1260 Infinity hyphenated with Chip Cube and connected to an Agilent 6460 triple quadrupole mass spectrometer). Chromatographic separation was performed using an HPLC-polymeric Chip that integrates a 360 nL-enrichment column and a reverse phase LC separation column (150 mm × 75 µm), both being packed with 3 µm Polaris C18A particles (180 Å pore size), together with a nanospray emitter. Samples were firstly loaded into the enrichment column in a mobile phase containing 7 % of B using the capillary pump at a flow rate of 4 µL.min⁻¹. Following the enrichment, analytes were backflushed into the separation column using the nano pump at 0.4 µL.min⁻¹. The mobile phases consisted in (A) water and (B) acetonitrile, both acidified with 0.1 % formic acid. The gradient started at 7 % of B and increased up to 30 % of B in 3 min, then increased up to 50 % of B in 5 min, and finally increased up to 80 % of B in 7 min before reconditioning stationary phase at 7 % of B for 5 min. The total run time was equal to 20 min. Source parameters were as follows: gas temperature equal to 325 °C, gas flow equal to 2,5 L.min⁻¹ and capillary voltage equal to 1900 V under the positive mode. Ionization was carried out using positive electrospray (ESI+) and, under multiple reaction monitoring mode (MRM). Quantification and qualification transitions are described in Table 1.

Thiol analysis

3-mercaptohexanol (3MH)

Hop samples (100 mg) were extracted by a mixture of water/ethanol (90/10; 1.5 mL) then centrifuged (12000 rpm; 15 min) and the resulting supernatants were derivatized and subsequently analysed by nanoLC-MS/MS. For beer, CO₂ gas was removed using filtration on glasswool, and the corresponding liquid (1 mL) was derivatized and subsequently analysed by nanoLC-MS/MS.

Briefly, deuterated thiols (3MH-d₂ and 3MHA-d₅) were introduced into the hop extract (1 mL) at 1000 and 250 ng.L⁻¹, respectively. Samples were then subsequently treated with ammonium bicarbonate buffer (1 M; 300 µL) and N-phenylmaleimide solution (25 mM; 120 µL). The derivatization reaction lasted 1 h at room temperature and was then quenched using iced acetic acid (200 µL). The samples were then purified by SPE using Bond Elut Plexa cartridges (200 mg of sorbent). After a conditioning step using methanol (2 mL), water (2 mL) and then acetic acid in water at 20 %

Table 1. MS/MS conditions for thiol precursors

Compounds	Quantification transition	Precursor ion (m/z)	Product ion (m/z)	Fragmentor	Collision energy (V)
Cys3MH	✓	222	205	40	4
		222	102	40	4
		222	83	40	12
Cys3MH-d ₂	✓	224	207	45	4
		224	104	45	16
		224	85	45	8
G3MH	✓	408	333	65	12
		408	279	65	8
		408	262	65	12
G3MH-d ₂	✓	408	162	65	20
		410	335	45	12
		410	281	45	8
G3MH-d ₂	✓	410	264	45	12
		410	162	45	20
		410	162	45	20
Cys4MMP	✓	220	202	55	0
		220	122	55	4
		220	99	55	8
Cys4MMP-d ₁₀	✓	226	208	45	4
		226	122	45	4
		226	105	45	8
G4MMP	✓	406	331	55	12
		406	259	55	12
		406	179	55	16
G4MMP-d ₁₀	✓	416	341	40	12
		416	269	40	12
		416	179	40	16

(2 mL), the samples were loaded into the cartridge, washed with water (2 mL) and water/acetonitrile (60/40; V/V; 2 mL) and eluted with acetonitrile/2,2,2-trifluoroethanol (90/10, V/V; 1.5 mL). After concentration to dryness, the extracts were dissolved in water/acetonitrile (98/2; V/V; 75 μ L), acidified with formic acid at 0.1 % and analysed by nanoLC-MS/MS (HPLC Agilent series 1260 Infinity hyphenated with Chip Cube and connected to an Agilent 6460 triple quadrupole mass spectrometer). Chromatographic separation was performed using an HPLC-polymeric Chip that integrates a 40 nL enrichment column (4 mm) and a reverse phase LC separation column (43 mm \times 75 μ m), both of them packed with 5 μ m ZORBAX 300 SB-C18 particles, as well as a nanospray emitter. The Agilent Mass Hunter ChemStation software (version B 04.01) was used for data acquisition and processing. The mobile phase components were (A) water and (B) a mixture of acetonitrile/TFE (90/10), both containing 0.1 % (v/v) formic acid. Samples were firstly loaded into the enrichment column in mobile phase containing 2 % of B using the capillary pump at a flow rate of 4 μ L.min⁻¹. Following the enrichment step, analytes were backflushed into the separation column using the nano pump at 0.6 μ L.min⁻¹. The injection volume was 6 μ L, and thiols were separated by the following gradient: from 0 to 10 min 2–80 % of B then 80 % of in isocratic mode from 10.1 to 14 min. The column was equilibrated with 98 % of A for 6 min and the total run time was 20 min per analysis. Between each sample injection, the chromatographic system was flushed with a mixture of ACN/TFE (50/50). Concerning the detection conditions, analyses were made under positive electrospray ionization (ESI+) and multiple reaction monitoring mode. Quantification was performed using the following transitions: 308.1 m/z \rightarrow 208.0 m/z for 3MH, 310.2 m/z \rightarrow 208.1 m/z for 3MH-d₂, 350 m/z \rightarrow 290 m/z for 3MHA and 355.2 m/z \rightarrow 292.1 m/z for 3MHA-d₅.

4-methyl-4-mercaptopentan-2-one (4MMP)

Hop samples (50 mg) were extracted by a mixture of water/Ethanol (90/10; 3 mL) then filtered on glasswool. The resulting filtrate (1.5 mL) was spiked with 4MMP-d₁₀ at 45 ng.L⁻¹ and then diluted in water (7 mL) with NaCl

(4 g). For beer, CO₂ gas was firstly removed using filtration on glasswool and the corresponding liquid was aliquoted and prepared using the similar procedure as for hop extracts. Derivatization, standardization and analyses by GC-MS/MS (Varian; GC3800; MS4000) were adapted from a published procedure.^[27] Derivatization reaction was carried out using an automatic CombiPal system (CTC Analytics, Zwingen, Switzerland) as follows: each vial was spiked with 500 μ L of three different solutions of EDTA (50 g.L⁻¹), L-cystein hydrochloride monohydrate (32 g.L⁻¹) and o-methylhydroxylamine hydrochloride (20 g.L⁻¹). Then they were stirred for 45 min at 55 °C and extracted by SPME fiber (DVB/CAR/PDMS) for 30 min at 55 °C. Finally, compounds were desorbed into the GC inlet at 250 °C for 3 min. Chromatographic separation was performed on DB-WAX capillary column (60 m \times 0.25 mm \times 0.25 μ m) from Supelco. The oven temperature program was as follows: 35 °C for 3 min, raised to 80 °C at 15 °C.min⁻¹, raised to 130 °C at 3 °C.min⁻¹ then raised to 245 °C at 20 °C.min⁻¹ for 10 min. The carrier gas was helium with a constant flow rate of 1 mL.min⁻¹ and the injector temperature was set at 250 °C. Injection was performed by splitless mode for 3 min then a split of 1/20 was operated. The trap and transfer line temperatures were set at 150 °C and 170 °C, respectively. Ionization was performed by positive chemical ionization using isobutane (pressure 1.5 bars). Detection was performed in MS/MS conditions in resonant mode. The m/z 163 and m/z 173 ions were chosen for 4MMP and 4MMP-d₁₀ as parent ions, respectively, with an isolation window of 3 amu. The excitation amplitude was set at 0.6 V and the excitation storage level was adjusted to m/z 53.5 (q = 0.3) and m/z 56.8 (q = 0.3) for m/z 163 and m/z 173, respectively. The scan ranged from m/z 57 to m/z 182. The quantification was performed using m/z 128 and 138 as daughter ions for the natural and labeled compounds, respectively.

Results and discussion

As already mentioned, for 20 years, thiol precursors were intensively studied in the enology field.^[28] Indeed, these odourless

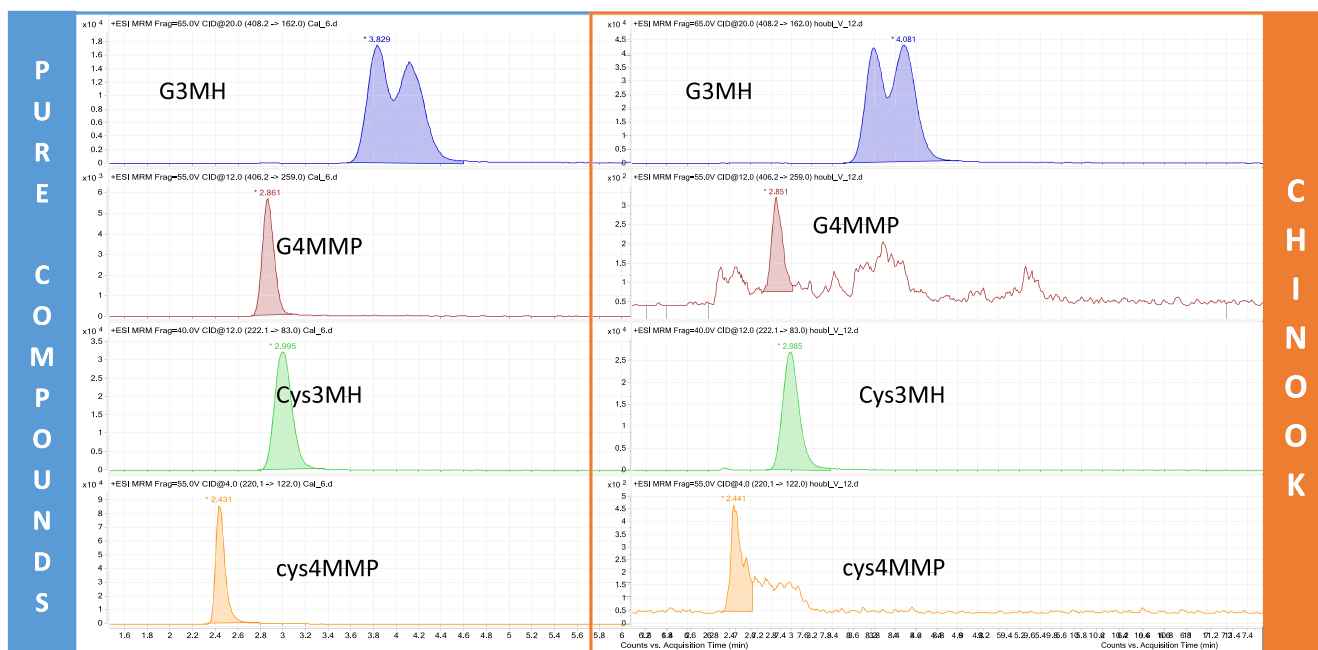


Figure 1. chromatograms of pure synthetic compounds and a chinook hop extract

compounds are responsible for the biogenesis of varietal thiols such as 3-mercaptohexanol^[29] and 4-methyl-4-mercaptopentan-2-one.^[30,31] Firstly identified in wine, these aroma compounds conferred olfactive notes that are particularly appreciated by the consumers. These two compounds were later identified in fresh lager beers,^[4] and in Cascade,^[32,33] Nelson Sauvvin^[34] and Super Alpha Tomahawk^[20] hop cultivars. In addition, a S-cysteinylated precursor of 3MH in hops^[6] able to release the 3MH under enzymatic mechanism^[25] was recently identified. Considering these aspects and by analogy with the enology field, we analysed ten hop varieties to demonstrate (i) the occurrence of both S-cysteinylated and S-glutathionylated conjugates of 3MH and

4MMP and (ii) to quantify them to have an approximation of their concentration ranges.

The sampling we performed was not sufficient to consider this study as a formal characterization of thiol and thiol precursors in the respective varieties. It gives only a general idea of the occurrence of those compounds and the variability of their amounts.

Thiol precursor identification

The 4-S-glutathionyl-4-methylpentan-2-one (G4MMP), the 4-S-cysteiny-4-methylpentan-2-one (Cys4MMP), the 3-S-glutathionylhexan-1-ol (G3MH) and the 3-S-cysteinyhexan-1-ol

Table 2. Identification criteria of thiol precursors in pure synthetic standard mixture and in hops

Compounds	Transitions (m/z)	Pure synthetic standard (abundance %)	Hop samples (abundance %)							
			Chinook	Saaz	Mistral	Barbe Rouge	Cascade	Aramis Alsace	Strisselspat	Mandarina
Cys3MH	222 → 205	42	43	42	41	36	43	37	40	43
	222 → 83	58	57	58	59	64	57	63	60	57
	Retention time (min)	3.04	2.98	2.96	2.93	3.01	2.85	2.97	2.95	3.10
G3MH	408 → 333	6	6	6	6	6	6	6	6	6
	408 → 279	23	23	23	22	23	23	23	23	22
	408 → 262	22	21	21	22	22	22	22	22	22
	408 → 162	49	50	50	50	49	50	50	49	51
Retention time (min)	3.95	4.08	4.07	4.04	4.05	4.06	4.16	4.05	3.74	
Cys4MMP	220 → 122	65	58	63	/	55	73	68	73	83
	220 → 99	35	42	37	/	45	27	32	27	17
	Retention time (min)	2.48	2.45	2.47	/	2.46	2.46	2.52	2.55	2.48
G4MMP	406 → 331	6	6	/	/	/	/	/	/	/
	406 → 259	50	51	/	/	/	/	/	/	/
	406 → 179	44	44	/	/	/	/	/	/	/
	Retention time (min)	2.83	2.85	/	/	/	/	/	/	/

Table 3. LOD and LOQ of thiol precursors and thiols in hops and beers

Thiol Precursors	LOD	LOQ	Matrix	Units
Cys3MH	1.0	3.3	Hop	$\mu\text{g.kg}^{-1}$
G3MH	19	64	Hop	$\mu\text{g.kg}^{-1}$
Cys4MMP	0.9	3.0	Hop	$\mu\text{g.kg}^{-1}$
G4MMP	0.5	1.7	Hop	$\mu\text{g.kg}^{-1}$
Thiols	LOD	LOQ	Matrix	Units
3MH (Perception threshold = 55 ng.L^{-1})	0.3	1.0	Hop	$\mu\text{g.kg}^{-1}$
	2.6	8.8	Beer	ng.L^{-1}
3MHA (Perception threshold = 4 ng.L^{-1})	0.5	1.6	Hop	$\mu\text{g.kg}^{-1}$
	2.8	9.3	Beer	ng.L^{-1}
4MMP (Perception threshold = 1.5 ng.L^{-1})	0.1	0.3	Hop	$\mu\text{g.kg}^{-1}$
	0.9	3.1	Beer	ng.L^{-1}

(Cys3MH) were synthesized according to published methods (cysteinyated precursors from Dagan *et al.*,^[35] G3MH from Roland *et al.*^[11] and G4MMP from Fedrizzi *et al.*^[9]) and fully characterized ^1H NMR using trimethylsilylpropionic acid as internal standard. NMR characterization of each compound is available as supporting information.

Cysteinyated and glutathionylated conjugates were considered as identified in the samples when meeting the following criteria specified in Commission decision 2002/657/EC^[36]:

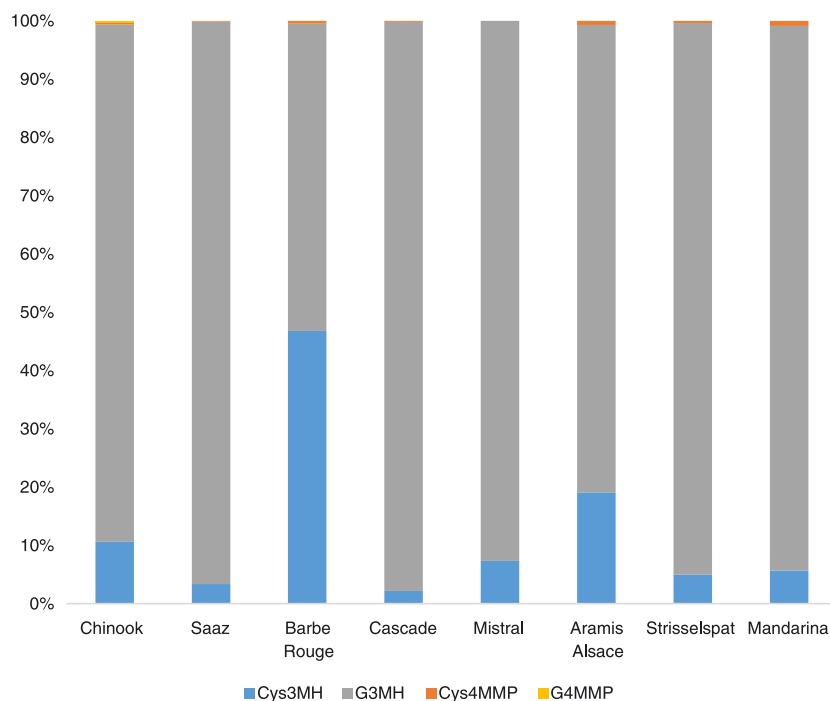
- The relative retention time of an analyte in a sample and a calibration solution has to be within the $\pm 5\%$.
- The presence of four identification points (parent ion and three daughter ions).
- The relative ion intensities have to comply with the $\pm 30\%$ permitted tolerances.

In practice, a mixture of pure synthetic standards in water was analysed by nanoLC-MS/MS and the retention times and MRM

spectra were compared with those obtained from natural extract of hops. Both Cys3MH and G3MH occurred in all hop varieties whereas 4MMP precursors were only identified in Chinook for G4MMP (Figure 11) and in all varieties except for Mistral hop for Cys4MMP (Table 2). Retention times, transition specificity and relative abundance of transitions were in the authorized ranges to warrant the best possible identification without using high resolution mass spectrometry or NMR technologies. As for grapes,^[26,37] 3MH precursors were more ubiquitous than 4MMP precursors. For G3MH, it is important to note that both diastereomers are present in hops with an equal distribution of the two compounds.

Thiol precursors quantification

The analysis of thiol precursors has been carried out by nanoLC-MS/MS using a validated method^[26] based on Stable Isotope Dilution Assay (SIDA) to guarantee reliable results. This


Figure 2. Distribution of thiol precursors in several hop varieties

method was optimized for hop samples to reach the best possible sensitivity. Limits of detection (LOD) and quantification (LOQ) were evaluated for 10 hop samples using the 'peak-to-peak' algorithm on Mass Hunter software (Agilent Technologies) to measure the signal to noise ratio (S/N) of targeted peaks. LOD and LOQ were averaged for the whole collection of samples considering S/N = 3 and S/N = 10, respectively (Table 3). Our method was highly sensitive since LOD and LOQ were below ppb level for all the precursors except for G3MH for which the values were slightly higher.

More than 99 % of the thiol potential was composed by 3MH precursors that is to say both G3MH and Cys3MH (Figure 2). By comparison, 3MH conjugates present in grapes of Sauvignon Blanc, Gewürztraminer, Muscat, Albariño, Verdejo, Grenache and Tempranillo varieties were also described as the most known abundant precursors.^[37,38]

Among 3MH precursors, G3MH represented more than 80 % of the total amount except for Barbe Rouge hops where the proportion of Cys3MH and G3MH was 50/50 (Figure 2). Concentration of G3MH ranged from 1.2 to 19 mg.kg⁻¹ whereas Cys3MH levels were from 0.1 to 1.1 mg.kg⁻¹ (Table 4). These levels were in accordance with those previously reported for Cys3MH amounts in Sorachi Ace^[5] (1.158 mg.kg⁻¹), Citra^[5] (0.616 mg.kg⁻¹), Amarillo^[5] (0.413 mg.kg⁻¹) and Cascade^[6] (1.641 mg.kg⁻¹) hops even if the used analytical strategies were very different. Indeed, these authors used an indirect analytical method to quantify Cys3MH in hops since they quantified the 3MH enzymatically released from the precursor. On the other hand, our method was based on a direct quantification of each thiol precursors by Stable Isotope Dilution Assay (SIDA), providing reliable results.

Hops were particularly rich in thiol precursors compared to grapes. Indeed, G3MH concentrations could reach 19 mg.kg⁻¹ in Cascade hop, which is at least 10 times higher than fresh grapes.^[37] This important difference may be explained by the moisture degree of samples. Indeed, hop samples contained less than 10 % of moisture whether it be cones or pellets in comparison with grapes and may result in higher concentrations.

4MMP precursors consisted mainly in Cys4MMP with concentrations ranging from five to 40 µg.kg⁻¹. G4MMP was only identified in one hops (Chinook) and the level did not exceed 12 µg.kg⁻¹ (Table 4). Cys4MMP levels in hops were systematically higher than G4MMP ones as already reported for grapes of Melon B, Sauvignon Blanc, Riesling, Gewürztraminer and Merlot.^[26,38]

Thiol analysis

Analysis of 3-mercaptohexan-1-ol, its acetate and the 4-methyl-4-mercaptopentane-2-one in wine or other alcoholic beverages has always been challenging due to their low concentrations and their high reactivity.^[18] For hop samples, analysis of thiols was carried out by nanolC-MS/MS for 3MH and 3MHA and by GC-MS/MS for 4MMP, both methodologies being based on SIDA quantification. The signal to noise ratio (S/N) of targeted peaks was measured on five hop samples using the 'peak-to-peak' algorithm on either Mass Hunter (Agilent Technologies) or Workstation (Varian) softwares. LOD and LOQ were averaged for the whole collection of samples considering S/N = 3 and S/N = 10, respectively (Table 3). For both methods, LOD and LOQ were inferior or close to the perception threshold of these molecules, underlining the high sensitivity of our analyses dedicated for hops and beers.

Free thiols were also quantified and occurred at ppb levels for 3MH and 4MMP that is to say inferior or close to their perception

Table 4. Chemical characterization of several hop varieties (nd: not detected)

Names	Years	Conditioning	Thiol precursors				Thiols	
			Cysteinylated conjugate of 3MH	cysteinylated conjugate of 4MMP	Glutathionylated precursor of 3MH	Glutathionylated precursor of 4MMP	3-mercaptohexan-1ol	4-mercapto-4-methylpentan-2-one
			Cys3MH µg.kg ⁻¹	Cys4MMP µg.kg ⁻¹	G3MH µg.kg ⁻¹	G4MMP µg.kg ⁻¹	3MH µg.kg ⁻¹	4MMP µg.kg ⁻¹
Chinook	2013	pellets	535	19	4479	11	nd	3.1
Saaz	2013	cones	179	5	5112	nd	nd	nd
Barbe Rouge	2014	cones	1140	12	1283	nd	nd	nd
Cascade	2014	pellets	429	35	19056	nd	12.1	3.5
Mistral	2014	cones	225	nd	2838	nd	nd	0.2
Aramis Alsace	2014	cones	562	21	2363	nd	1.3	nd
Strisselspat	2014	pellets	479	39	9141	nd	nd	0.1
Mandarina	2014	cones	129	20	2119	nd	nd	0.1

thresholds (Table 4). The concentrations were in the vicinity of those reported in literature with 3MH and 4MMP amounts up to 12 and 83 ppb in Citra and Sorachi Ace hop varieties, respectively.^[5] In our samples, 3MH was less ubiquitous than 4MMP since free 3MH only was present in Chinook and Barbe Rouge varieties. In contrast, 4MMP occurred at trace levels in all varieties except for Barbe Rouge, Cascade and Strisselspat. When both free thiols occurred in the same variety, 3MH amount was systematically higher than 4MMP.

The 3MHA was not detected in our samples whereas other data reported its occurrence in hops,^[5] suggesting that this compound depends on enzymatic activities likely to be present in hops.

Comparison of free and bound fraction of thiols in hops

The next step of our work consisted in the comparison of free and bounded fractions of thiols in hops. For this purpose, the corresponding bounded fractions of 3MH and 4MMP were expressed as follows:

- 'equivalent 3MH' (ppb) = $\frac{[\text{Cys3MH}]}{\text{MW}_{\text{Cys3MH}}} + \frac{[\text{G3MH}]}{\text{MW}_{\text{G3MH}}} \times \text{MW}_{\text{3MH}}$
- 'equivalent 4MMP' (ppb) = $\frac{[\text{Cys4MMP}]}{\text{MW}_{\text{Cys4MMP}}} + \frac{[\text{G4MMP}]}{\text{MW}_{\text{G4MMP}}} \times \text{MW}_{\text{4MMP}}$

with MW referring to the molecular weight of each molecule.

The results are summarized in Table 5 and showed important variability according to the hop varieties. Indeed, 3MH mainly occurred as precursors since the free 3MH fraction represented less than 1 % in all hop varieties. In literature, similar experiments revealed that free to bounded fraction ratios equal to 1.9 % for Citra, 4.3 % for Tomahawk and 7.1 % for Cascade.^[5]

On the contrary, free 4MMP represented the most important fraction (up to 95–100 %) for Saaz and Mistral varieties where Cys4MMP and G4MMP were either present at trace levels or not detected. For other varieties, free 4MMP proportions ranged from 1 % to 23 % except for Barbe Rouge, Cascade and Strisselspat samples where free 4MMP was not detected. Free and bounded fractions of 4MMP seemed to be more imparted by the hop varieties than those of 3MH, and the amount of free 3MH was negligible whatever the considered sample.

Even if no obvious link allowed explaining such variability, we hypothesize that the hop transformation process or the storage

conditions might influence the free and bounded fractions since free thiols are highly reactive and oxidizable.

How to drive thiol release in beers?

The last step of our work consisted in characterizing some beers from five different categories (Pale ale, Belgium origin, IPA, Pils and experimental beers) in order to build some hypotheses about thiol origins. Our benchmark (Figure 3) revealed that there was no obvious link between beer style and thiol profiles. Even if our sampling was limited and not perfect (sample freshness, type of cap liner), we observed important variability of thiol concentrations in the same category of beers. Several beers contained up to 1200 ng.L⁻¹ of 3MH and 300 ng.L⁻¹ of 4MMP which is particularly high, especially for 4MMP. Surprisingly, 3MHA occurred only in experimental beers except for IPA1 and the levels were higher than those of 3MH. 3MHA came from the acetylation of 3MH by yeast during fermentation and the reported concentrations were often close to 10 % of free 3MH in wine. Specific yeasts or fermentation conditions used during the elaboration of experimental beers might be responsible for high 3MHA contents. Another surprising point concerned the IPA thiol profile. Indeed, we expected that these beers exhibited important concentrations of thiols due to an intensive hopping but 3MH levels did not exceed 180 ng.L⁻¹ except for IPA4.

In regard of this high thiol level variability, the benchmark pointed a major question: how to take advantage of thiol precursors contained in hops to better drive beer aroma? Many hypotheses may be formulated considering our previous results to explain thiol origins in beers. First, we just considered only the free thiol fraction contained in hops. Free 3MH occurred up to 12 µg.kg⁻¹ in hops and by assuming a hopping of 400 g.hL⁻¹ and a theoretic extraction yield equal to 100 %, we may expect a maximum concentration of 3MH ranging from 5 to 48 ng.L⁻¹ in final beers, according to the considered hop variety (Table 6). An alternative process more dedicated to IPA beers with both kettle and dry hopping at 400 and 500 g.hL⁻¹ respectively, might result in higher 3MH amounts that we estimated to be up to 109 ng.L⁻¹ for the Chinook variety. Obviously, hopping conditions may impact on 3MH content in beers but the simulated amounts remains far from those found in commercial products. For 4MMP, the same hypothesis with a kettle hopping at 400 g.hL⁻¹ might result in concentrations ranging from 1 and 14 ng.L⁻¹ in beers. Even if 4MMP that could be transferred from

Table 5. Comparison of free and bounded fractions of thiols in several hop varieties (Eq3MH = $\frac{[\text{Cys3MH}]}{\text{MW}_{\text{Cys3MH}}} + \frac{[\text{G3MH}]}{\text{MW}_{\text{G3MH}}} \times \text{MW}_{\text{3MH}}$) (Eq4MMP = $\frac{[\text{Cys4MMP}]}{\text{MW}_{\text{Cys4MMP}}} + \frac{[\text{G4MMP}]}{\text{MW}_{\text{G4MMP}}} \times \text{MW}_{\text{4MMP}}$)

Hop varieties	Eq3MH (µg.kg ⁻¹)	Free 3MH (µg.kg ⁻¹)	Free/bounded fractions	Eq4MMP (µg.kg ⁻¹)	Free 4MMP (µg.kg ⁻¹)	Free/bounded fractions
Chinook	1799	12,1	0,7 %	15	3,4	23 %
Saaz	1792	nd	0,0 %	3	3,1	95 %
Barbe Rouge	1114	1,3	0,1 %	7	0,0	0 %
Cascade	6534	nd	0,0 %	21	0,0	0 %
Mistral	1071	nd	0,0 %	0	0,1	100 %
Aramis Alsace	1119	nd	0,0 %	12	0,2	2 %
SStrisselspat	3300	nd	0,0 %	23	0,0	0 %
Mandarina	776	nd	0,0 %	12	0,1	1 %

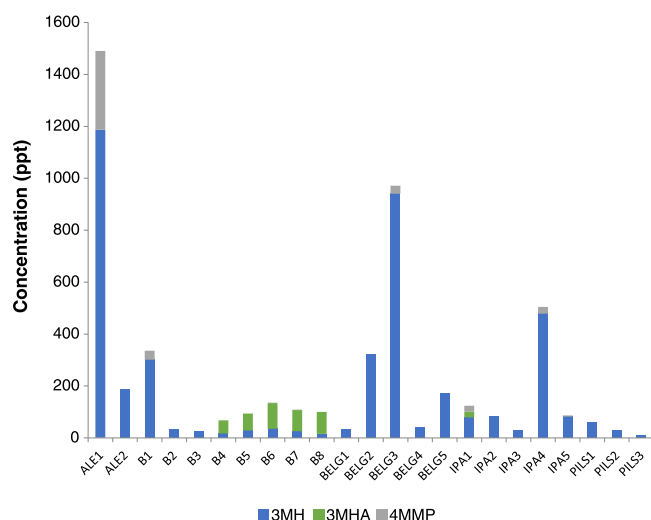


Figure 3. Quantification of 3MH, 3MHA and 4MMP in 23 different beers (Ale : Pale Ale ; B1 to B8 : experimental beers, Belg : Belgium beers)

hops into beers seemed limited, the sensory impact has to be taken into account since its perception threshold is equal to 1.5 ng.L^{-1} . In all the previous scenarios we hypothesized, we considered a theoretic extraction yield of 100 % but thiols are highly reactive from the chemical point of view. They could undergo some oxidation reactions leading to disulfides by-products or react with electrophiles to form thioether compounds conducting

to a concentration decrease in alcoholic beverages. Consequently, we consider the bounded thiol fraction in a second time to explain higher thiol release in beers.

For each free thiol, we considered the sum of the corresponding cysteinylated and glutathionylated precursors. By taking into account the similar hopping conditions as previously described and by assuming a conversion yield of precursors close to 3 % (as average value reported for wines^[11,17]), we could explain between 93 and 784 ng.L^{-1} of 3MH that could be released throughout the action of the yeast. For 4MMP, the same calculations conducted in hypothetical amounts ranging from 0.4 to 2.8 ng.L^{-1} .

In conclusion, 3MH and 4MMP origins seemed to be clearly distinct in beers. Indeed, 3MH might probably be most originating from precursors whereas 4MMP origins seemed to be from both bound and free fractions.

In addition, both bound and free fractions of 3MH and 4MMP, are not sufficient to explain the entire thiol amount that could occur in beers. These observations highlighted four important points:

- Thiol precursor amounts in hops may reach higher values for other varieties.
- Other precursors have to be investigated such as the Cys-Gly and γ -Glu-Cys conjugates, already hypothesized in grapes.^[13]
- Conversion yields have to be measured during brewing because the yeast strains usually used for enology and brewing do have great differences.

Table 6. Hypothetical hopping and brewing conditions to investigate the thiol release in beers

Hop varieties	3MH precursors				Free 3MH		
	<i>Eq3MH</i> ($\mu\text{g.kg}^{-1}$)	<i>Kettle hopping</i> (g.L^{-1})	<i>Conversion Yied</i> (%)	<i>3MH hypothetically released</i> (ng.L^{-1})	<i>Free 3MH</i> ($\mu\text{g.kg}^{-1}$)	<i>Kettle hopping</i> (g.L^{-1})	<i>3MH hypothetically released</i> (ng.L^{-1})
Chinook	1799	400	3	216	12,1	400	48
Saaz	1792	400	3	215	nd	400	0
Barbe Rouge	1114	400	3	134	1,3	400	5
Cascade	6534	400	3	784	nd	400	0
Mistral	1071	400	3	129	nd	400	0
Aramis Alsace	1119	400	3	134	nd	400	0
Strisselspat	3300	400	3	396	nd	400	0
Mandarina	776	400	3	93	nd	400	0
Hop varieties	4MMP precursors				Free 4MMP		
	<i>Eq4MMP</i> ($\mu\text{g.kg}^{-1}$)	<i>Kettle hopping</i> (g.L^{-1})	<i>Conversion Yied</i> (%)	<i>4MMP hypothetically released</i> (ng.L^{-1})	<i>Free 4MMP</i> ($\mu\text{g.kg}^{-1}$)	<i>Kettle hopping</i> (g.L^{-1})	<i>4MMP hypothetically released</i> (ng.L^{-1})
Chinook	15	400	3	1,8	3,4	400	13,6
Saaz	3	400	3	0,4	3,1	400	12,4
Barbe Rouge	7	400	3	0,8	0	400	0
Cascade	21	400	3	2,5	0	400	0
Mistral	0	400	3	0,0	0,1	400	0,4
Aramis Alsace	12	400	3	1,4	0,2	400	0,8
Strisselspat	23	400	3	2,8	0	400	0
Mandarina	12	400	3	1,4	0,1	400	0,4

- Hops might not be the only source of thiol precursors for brewers and malts have also to be considered as already hypothesized by previous work.^[33]

Conclusion

Using published and validated analytical methods dedicated to grapes and wines, this work allowed completing the thiol precursor panorama in hops. The aromatic potential concept, hugely used in enology to characterize and drive the wine aroma quality, appeared also of interest for hop manufacturers and brewers.

The possibilities to pilot the beer aroma profile provided by this aroma reservoir are important compared to the grape and wine sector. As for enology, the hop aroma potential may be considered as a key parameter during the brewing process in order to adapt hopping conditions to the raw material composition and to the beer aroma profile. Obviously, further studies will have to be performed in order to (i) better characterize the hop aroma potential associated with the influence of culture conditions and the post-harvest processes, to (ii) determine the best hopping conditions able to exploit the different components of this aroma potential, and finally to (iii) examine the sensory effect of the thiols released in the various types of beer.

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Abbreviations and Nomenclature: 3MH, 3-mercaptohexanol; 4MMP, 4-methyl-4-mercaptopentan-2-one; GC-MS/MS, Gas-Chromatography hyphenated with tandem Mass Spectrometry; LC-MS/MS, Liquid Chromatography hyphenated with tandem Mass Spectrometry; Cys3MH, Cysteinylated precursor of 3-mercaptohexanol; Cys4MMP, Cysteinylated precursor of 4-methyl-4-mercaptopentan-2-one; G3MH, Glutathionylated precursor of 3-mercaptohexanol; G4MMP, Glutathionylated precursors of 4-methyl-4-mercaptopentan-2-one; NMR, Nuclear Magnetic Resonance; LOD, Limit Of Detection; LOQ, Limit of Quantification; SIDA, Stable Isotope Dilution Assay; S/N, Signal to Noise ratio.

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