Free and bound polyfunctional thiols and terpenols in dual-purpose hop varieties. First evidence of glutathione S-conjugates.

A review of two decades of hop research in Louvain-la-Neuve.

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This paper is the summary of a collaborative work in Louvain-la-Neuve, including 5 PhD theses (Dr. G. Lermusieau, C. Vermeulen, J. Gros, T.T.H. Tran, M.L. Kankolongo Cibaka) supervised by S. Collin.

First use of Gas chromatography-olfactometry (GC-O) to identify the hop aromatic compounds in beer

In 2001, aroma extract dilution analysis (GC-O-AEDA) was shown for the first time to be an efficient tool to identify the hop aromatic compounds in beer (Lermusieau, Bulens, Collin, 2001). The XAD-2 extraction procedure allowed for beer extracts to be obtained with odors representative of the original samples. Instead of only determining the chemical composition using gas chromatography/mass spectrometry. This alternative approach allowed the construction of aroma profiles and the identification of the most important compounds for hop aroma based on their odor quality and intensity.

The most potent compounds related to hop aroma in beer were shown to be 3-methyl-2-buten-1-thiol (MBT), dimethyltrisulfide, linalool, humuladienone, γ-nonalactone, β-damascenone and ethyl cinnamate. Yet several odorant sulfur compounds remained unidentified, especially in beers hopped with "cheesy" cultivars (such as Challenger).

Combinatorial synthesis and sensorial analysis of a library of polyfunctional thiols

In order to be able to identify the unknown sulfur compounds extracted from hops, we employed and optimized a combinatorial synthetic strategy to quickly obtain retention indices, mass spectra, and odors for a large series of commercially unavailable polyfunctional thiols. 10 Sulfanylalkyl ketones and secondary alcohols (Vermeulen et al., 2001), 13 sulfanylalkyl aldehydes (Vermeulen *et al*., 2002), 19 sulfanylalkyl primary alcohols and analogues (Vermeulen *et al*., 2003), and 21 sulfanylalkyl esters (Vermeulen and Collin, 2003) were characterized by combinatorial chemistry.

Polyfunctional thiols containing a linear chain of at least six carbon atoms usually imparted delicate odors of rhubarb/carrot/citrus, whereas most branched thiols of intermediate size had odor qualities that were described as onion-like, plastic like or pungent (Vermeulen *et al*., 2005).

MBT emerged as the most powerful compound of our thiol library (Vermeulen and Collin, 2006). Compared to other polyfunctional thiols, sulfanylalkyl esters showed relatively high chromatographic odor thresholds (BE-GC-LoADS). Among them, the lowest threshold value was obtained for 3-sulfanylhexyl acetate (3SHA).

Evidence of the key-role of polyfunctional thiols in beer

Vermeulen *et al.,* 2006 observed the presence of 10 polyfunctional thiols in commercial lager beers after a specific extraction with *p*-hydroxymercuribenzoic acid (*p*HMB) followed by analysis using GC coupled to mass spectrometry (MS), olfactometry (O), and pulsed-flame photometer detector (PFPD). Most of them had a 3-carbon distance between a chemical functional group (such as an alcohol, ester, carbonyl, etc.) and the SH group. The importance of these thiols on beer aroma was highlighted by spiking copper into the beers, which led to their complexation and removed their influence on the aroma profile. Also, all these polyfunctional thiols were absent from wort, suggesting that they were synthesized during fermentation

In the last decade, it has also been confirmed that polyfunctional thiols significantly contribute to the hop varietal aroma of late-hopped beers (Gros *et al*., 2011; Gros et al., 2012), bottle refermented beers (Nizet *et al*., 2013), and dry hopped beers (Kankolongo Cibaka *et al*., 2016).

Biosynthesis of thiols by the cysteine and homocysteine Ehrlich pathways

In all *p*HMB beer extracts, the empyreumatic 2-sulfanylethyl acetate (2SEA) emerged as the highest PFPD peak. Compared to other thiols, its sensorial threshold is relatively high (40 µg/L).

2-Sulfanylethanol (2SEol), 3-sulfanylpropanol (3SProl), and their corresponding acetates (2SEA and 3SPrA) were found to be derived from Ehrlich degradation of sulfur amino acids (Vermeulen, *et al*. 2006; Kankolongo Cibaka, Tran, et al., 2017). Yeast exerts a major impact on 2SEA concentration in fresh beer. This was recently shown by the production of two pilot beers hopped using the same Citra® hops but fermented with two different yeasts. The resulting beers had very different contents of 2SEA, 1.1 and 22.8 µg/L (Kankolongo Cibaka, Tran, et al., 2017). These result highlights the importance of yeast on 2SEA formation.

Hydrogen sulfide as precursor of thiols through fermentation

Different synthesis pathways of polyfunctional thiols were proposed from hop allylic alcohols such as hydrogen sulfide anti-Markovnikov radical addition (e.g. 2-sulfanyl-3-methylbutanol (2S3MBol)), electrophilic Markovnikov addition (e.g. 3-sulfanyl-3-methylbutanol (3S3MBol)), and/or nucleophilic substitution (e.g. MBT formation even in absence of light) (Vermeulen, *et al*. 2006).

Gros *et al.*, 2009 confirmed these mechanisms in pilot-scale beer productions by fermenting wort spiked with 10 mg/L of 3-methyl-2-buten-1-ol (MBOH) and subsequently detecting the onion-like 2S3MBol (1.8 µg/L) and less odorant 3S3MBol (7.0 µg/L) after fermentation. A strong freshly-cut onion off-flavor was also observed in the finished beer. An additional mechanism involving 2,3-epoxy-3-methyl-butanal was recently proposed by Shigekuni at the 2016 World Brewing Congress in Denver.

The 1,4 addition of hydrogen sulfide to α, β-unsaturated aldehydes or ketones in wort could also partially explain the synthesis of the grapefruit-like 3-sulfanylhexanol (3SHol from *trans*-2-hexenal) or the catty-like 4-sulfanyl-4-methyl-pentan-2-one (4S4M2Pone from 4-methyl-2-pentenal). Yeast reductases convert the resulting carbonyls to alcohols, while alcohol acetyltransferase can synthesize their corresponding esters (e.g. the production of passion fruit-like 3SHA from hop alcohols).

Occurrence of free thiols in hop

In 2011, forty-one free polyfunctional thiols were detected in hop pHMB extracts (specifically 13 β-sulfanylalkyl acetates, 14 β-sulfanylalkyl alcohols, 8 β-sulfanylalkyl carbonyls, and 6 others) (Gros *et al*., 2011). Each cultivar evaluated exhibited a unique thiol profile (Gros *et al*., 2011; Kankolongo Cibaka *et al*., 2015). Very low concentrations of polyfunctional thiols were detected in most of the low bitter varieties, such as Saaz.

The grapefruit/Muscat-like 3SHol (odor perception threshold of 55 ng/L in beer) was found in most dual-purpose and aromatic hop varieties. Cascade, Simcoe, Topaz, and Fuggle hop varieties were determined to contain the highest concentrations of 3SHol (80-120 µg/kg) (Gros *et al*., 2011).

3-Sulfanyl-4-methylpentan-1-ol (3S4MPol) (odor perception threshold of 70 ng/L in beer) has a grapefruit- / rhubarb- like flavor and was first proposed as one of the key compounds contributing to the specific “Sauvignon Blanc-like” note imparted to beer by Nelson Sauvin. Although it contains lower concentrations of other polyfunctional thiols, Hallertau Blanc was much richer in 3S4MPol compared to other varieties (e.g. concentrations in Hallertau Blanc were detected up to 109 µg/kg, while concentrations ranged from 25-46 µg/kg in Nelson Sauvin, Mosaic, and Amarillo hops) (Gros et al., 2011; Kankolongo Cibaka *et al*., 2015)

The floral-like 3-sulfanyl-2-ethylpropyl acetate (3S2EPrA) and the citrus/peach-like 3-sulfanyloctanal (3SOal) differentiated the super alpha Tomahawk from all the other thiol-rich varieties (Gros et al., 2011). High amounts of 2SEol (362 µg/kg), 2SEA (867 µg/kg), and 3SHA (27 µg/kg) were detected in Citra® (Kankolongo Cibaka *et al.,* 2015) while free 4S4M2Pone was found at higher levels in Sorachi Ace (Kankolongo Cibaka *et al.,* 2015).

First evidence of cysteine adducts in hop

As described by Gros *et al*., 2012, the thiol content of the final beer usually reaches higher values than might be expected on the basis of hopping rate and hop free thiol contents. Precursors were therefore suspected.

Yeast β-lyase activity was shown to release thiols after specific cleavage from hop S-cysteine conjugates (Kankolongo Cibaka *et al*., 2015; Gros *et al*. 2013). The rate of bioconversion was low during the initial stages of fermentation. Yet the nutrient-depleted conditions encountered after the initial fermentation were thought to promote β-lyase activity. Therefore, bottle refermentation and dry-hopping at the end of primary fermentation or during maturation emerge as brewing techniques which promote hoppy flavors (Nizet *et al*., 2013).

3-S-(1-hydroxyhexyl)-cysteine (Cys-3SHol) was first identified by HPLC-MS/MS in the Cascade cultivar (at 1.6 mg/kg free thiol equivalents) (Gros *et al.*, 2012). It has been further detected in many other varieties (e.g. 1.1 mg/kg free thiol equivalents in Sorachi Ace) (Kankolongo Cibaka *et al.,* 2015).

This thiol precursor and seven other S-conjugates have been evidenced in hops by means of enzymatic release assays using apotryptophanase (Kankolongo Cibaka *et al*., 2015; Gros *et al*., 2013). This commercial enzyme from *Escherichia coli* has been widely used to investigate cysteine and cysteinylglycine adducts in plants. It has been identified that a free amino function in the cysteinyl moiety is required for β-lyase activity.

Studies using these assays have revealed that the concentrations of this hidden potential in hops range 23 - 126 times more than the concentrations of the corresponding free thiols. Except for bound 3S4MPol, which was found only in Hallertau Blanc and was detected at a lower concentration (39 µg/kg) when compared to the corresponding free thiol content (109 µg/kg) (Kankolongo Cibaka *et al*., 2015).

While free 4S4M2Pone was discriminant for Sorachi Ace, the bound form was predominantly detected in Nelson Sauvin (Kankolongo Cibaka *et al*., 2015; Gros *et al.* 2013).

First evidence of glutathione adducts in hop

Three single varietal dry-hopped beers were produced with the Amarillo, Hallertau Blanc, and Mosaic dual-purpose hops (Kankolongo Cibaka *et al*., 2016). 3S4MPol was found in all three beers at much higher concentrations than expected based on the free thiol content in each of the hop varieties. Even concentrations of cysteinylated precursors were unable to explain the results observed. 3SHol also occurred in the dry-hopped Mosaic beer above the concentration calculated on the basis of the free and known bound thiol contents. Similarly to their occurrence and importance in grapes used to make wine, the occurrence of S-glutathione precursors in hops were therefore hypothesized to be the cause of these increases.

The analytical standards of S-3-(4-methyl-1-hydroxypentyl) glutathione (G-3S4MPol), never described before, and of S-3-(1-hydroxyhexyl) glutathione (G-3SHol), previously evidenced in grapes, were chemically synthesized. Unfortunately, G-3S4MPol displayed the same MS/MS fragmentation pattern as G-3SHol (major fragment m/z at 279, 262 and 162). Moreover, the very close structures of these compounds led to low chromatographic resolution on usual C18 HPLC columns. Acceptable separation and quantitation were finally achieved on a Cyclobond I 2000 RSP column.

An optimized extraction of glutathionylated precursors was then applied to Amarillo, Hallertau Blanc, and Mosaic hops. HPLC–ESI(+)MS/MS revealed, for the first time, the occurrence of G-3SHol and G-3S4MPol in hop, at levels well above those reported for their cysteinylated counterparts (up to 100 mg/kg) (Kankolongo Cibaka, *et al*., 2016). Similar results were further obtained in Citra® and Sorachi Ace hops (Decourrière *et al*., 2018).

G-3SHol emerged in all cases as the major adduct in hop. While, 3SHol seems to be relatively ubiquitous in free, cysteinylated, and glutathionylated form. The glutathione adduct of 3S4MPol (detected at 0.7 mg/kg in Hallertau Blanc) has never been evidenced in other plants up to now.

Very recently, the glutathione adduct of 3-sulfanylpentanol (3SPol) was also evidenced in hop (Chenot et al., 2019). Of all the samples investigated, Citra® emerged as the richest, with up to 18 mg/kg.

Potentialities of bottle refermentation

Bottle refermentation is used to promote carbonation, resistance against infection, and prevent oxidation in many Belgian beers. It has also long been known to accelerate ester hydrolysis. Overall, it is recognized that bottle refermentation improves flavor perception, by producing new pleasant odors and by reducing stale aldehydes (*trans*-2-nonenal, 3-methylthiopropionaldehyde, etc.) to low-odorant alcohols (Nizet, *et al*., 2013).

In order to understand which compounds improved beer quality, bottles that were and were not subjected to refermentation were investigated. The trained panel detected strong organoleptic differences between the samples. Chemically, *p*HMB thiol extraction was applied and the extracts were analyzed using GC-MS, GC-PFPD, and GC-olfactometry (AEDA). Many sulfanylalkyl alcohols, acetates, and carbonyls were shown to be produced during the refermentation process, especially after three weeks (Nizet, *et al*., 2013). Among them, MBT proved to be very important and was perceived at the sniffing port even after diluting the extract by a factor of 32768. The major thiol, 2SEA, also reached 10 µg/L.

Spiking 5 and 10 mg/L of Cys-3SHol to beer highlighted the ability of yeast to release free thiols during bottle refermentation. Further, spiking deuterated cysteine before bottle refermentation confirmed that the Ehrlich pathway was still active in the bottle (Nizet, *et al*., 2013). Therefore, the flavor profile and thiol concentrations in bottle-refermented beer is highly dependent on the control of the refermentation process in the package and requires an excellent management of yeast during conditioning

Fate of polyfunctional thiols through beer aging

During beer aging, based on their structures, thiols evolve in a number of different ways.

The term "ribes" refers to a characteristic taint encountered in some aged beers. It can develop within 4 weeks of bottling, but the note often fades and is no longer discernible after 6 months. Schieberle, 1991 identified 3-sulfanyl-3-methylbutyl formate (3S3MBF) as a possible driver of the “ribes” note. In our laboratory, both 2-sulfanyl-3-methylbutyl formate (2S3MBF) and acetate (2S3MBA) were synthesized (Tran *et al*, 2015), characterized and compared to the commercially available 3S3MBF and its corresponding acetate.

In contrast to the two acetates, which were much more piquant, both of the formates exhibited a typical “ribes” flavor. The sensorial threshold of 3S3MBF was much lower (57 ng/L in beer) than those measured for the other three esters. Also, when evaluating the beer extracts only 3S3MBF was perceived at the GC-O sniffing port. After one month of storage at 20°C, concentrations of 3S3MBF in pilot beers were measured up to 1230 ng/L. Fortunately, the compound was rarely detected in commercial beers that had greater dissolved oxygen control throughout the brewing process and implemented oxygen-scavenging packaging. Therefore, accelerated ageing in the presence of oxygen confirmed the key role of oxygen in the development of the “ribes” note (Tran *et al*, 2015).

For other thiols, synthesis was observed during the early period of storage, even in absence of yeast (Kankolongo Cibaka, Tran, *et al*., 2017*;* Tran *et al*, 2015*)*. Yet after one year of aging, most compounds were found to be strongly degraded regardless of the beer style evaluated.

Fresh filtered lager beer was spiked with non-volatile S-cysteine conjugates of 3SHol, 3S3MBol, MBT and 2SEA before aging (Kankolongo Cibaka, Tran, *et al*., 2017; Tran *et al*, 2015). Thiols were further extracted and analyzed by GC-PFPD while HPLC-ESI(+)-MS/MS allowed us to quantify the undegraded S-cysteine conjugates. Cysteine adducts were shown to be relatively stable in aqueous media. Yet in a complex medium like beer, many co-constituents could slightly facilitate the chemical degradation. It was suggested that the content of odorant thiols in aged beer could be increased by the presence of Maillard compounds formed during malt kilning (i.e. molar conversion of S-cysteine adducts into free thiol up to 0.3% at room temperature). Due to this result a modified “Strecker degradation” mechanism was proposed (Kankolongo Cibaka, Tran, *et al*., 2017).

Despite the release of 2SEA through beer aging, the determination and identification of Cys-2SEA in hops by direct HPLC-ESI(+)-MS/MS analyses or enzymatic assays has been inconclusive (Kankolongo Cibaka, Tran, *et al*., 2017). On the other hand, 2SEol was released in presence of apotryptophanase from some varieties (Tran *et al*, 2015). Chemical esterification into 2SEA could therefore occur in the bottle.

Additional contribution of terpenoids and terpenol glucosides to the flavor of dry-hopped beers

Terpenes and terpenoids have long been used to discriminate between aromatic and bitter hops. Farnesene and bergamotene have emerged as markers for noble aromatic hop varieties, while high amounts of esters, such as 3-methylbutyl isobutyrate, have only been found in bittering hop varieties (Perpète *et al*, 1998; Lermusieau *et al*., 2001). However, the arrival of dual-purpose hop varieties has completely modified these classifications. For example, despite its 12.3 % alpha acid level, Sorachi Ace contains more farnesene than the prestigious aromatic cultivar Saaz (Kankolongo Cibaka *et al*., 2015).

Kankolongo *et al.*, 2015 recently showed that the monoterpenic alcohols linalool (floral/lavender/coriander/citrus-like flavor), geraniol (floral/rose-like flavor), and β-citronellol (lemon/lime-like flavor) could be used to distinguish dual-purpose hop varieties from others. Linalool ranged from 100 – 312 mg/kg in Nelson Sauvin, Tomahawk, and Amarillo hops, while geraniol characterized Tomahawk, Amarillo, Citra®, and Mosaic hops (69 – 247 mg/kg). The highest β-citronellol content (33 mg/kg) was found in Sorachi Ace.

Terpenols were quantified by SBSE-GC-MS in five pilot dry-hopped beers produced with dual-purpose varieties (Kankolongo Cibaka*,* Silva Ferreira, *et al*., 2017). In all of them, concentrations of linalool and geraniol ranged from 72 – 178 µg/L and 7 – 57 µg/L, respectively, thus above their sensory thresholds (threshold of 8 µg/L for linalool and 4 µg/L for geraniol). Concentrations of β-citronellol were also measured above its threshold when Amarillo, Citra®, or Sorachi Ace were used for dry-hopping.

The terpenol glucoside fraction in hops and its potential contribution to beer flavor was also investigated (Kankolongo Cibaka, Silva Ferreira, *et al*., 2017). The hop glucoside potential was analysed by performing GC-MS after enzymatic degradation. A relative hydrolysis efficiency factor was applied to our data to take into account that the commercial β-glucosidase releases octan-1-ol, used as our internal standard, 2.8 times more efficiently than geraniol. β-Glucosidase treatment caused the release of linalool, α-terpineol, β-citronellol, and geraniol from all five dual-purpose cultivars, but in a much lower amount than the corresponding free terpenols (0.6 – 28.6 mg/kg aglycons vs. 7.8 – 109.2 mg/kg of free).

Conclusions and perspectives

Except the empyreumatic polyfunctional thiols issued from the Ehrlich pathway (e.g. 2SEA, 2SEol), most of the pleasant thiols found in beer (e.g. 3SHol, 3S4MPol) are extracted from hops. The involvement of cysteine and glutathione adducts has been proved and 41 free thiols in hops have been identified. Unfortunately, very little is known about the mechanisms able to release the huge hidden thiol potential during dry hopping. A modified Strecker degradation has been shown to promote the chemical degradation of S-cysteine conjugates in presence of Maillard dicarbonyls. Our research is now focused on finding the best enzymes, yeasts, or other chemical pathways allowing to improve the access to glutathione and cysteine adducts, with very challenging perspectives.

The sensorial impact of each individual thiol is usually assessed on the basis of individual odor thresholds. However, this might not be the best approach because strong synergistic effects have been evidenced between various thiols in wine (e.g. between the bacon-like 2-methylfuran-3-thiol and the fuel-like 3-sulfanyl-3-methylbutanal) (Bailly *et al*., 2006). To better understand dry-hop flavor, future studies should also investigate how mixtures of different analogues and concentrations of terpenoids and thiols synergize to yield higher perceptions.

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