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Unusual Profile of Thiol Precursors in Special Malts: First Evidence of Chemical Glutathione-/γGluCys- and CysGly-/Cys- Conversions

Cécile Chenot[a], Sonia Collin[a], Lucas Suc[b] and Aurélie Roland[b]

[a]Unité de Brasserie et des Industries Alimentaires, Louvain Institute of Biomolecular Science and Technology (LIBST), Faculté des Bioingénieurs, Université catholique de Louvain, Louvain-la-Neuve, Belgium; [b]Sciences pour l'œnologie (SPO), Institut Agro Montpellier, Montpellier, France

ABSTRACT

Polyfunctional thiols (PFTs) present in beers or in wines are known to be released by yeast, during fermentation, from bound forms originally found in raw materials. In the brewing field, huge amounts of *S*-conjugates have been evidenced in several dual-purpose hop varieties. Malt, however, being the major raw material of beer, could also be a significant contributor of PFTs to beer (cysteinylated, Cys- and glutathionylated, G- precursors of 3SHol already identified in a few samples of barley and malt). Forty-two barley malts from 2 to 1500 EBC and five other malted cereals were screened to characterize their thiol precursor profile (G- and Cys- as well as dipeptidic bound CysGly- and γGluCys- forms of 3SHol). First, it was confirmed that G-3SHol was ubiquitous reaching up to 320 µg/kg in some samples, whereas Cys-3SHol remained at a trace level of up to 13 µg/kg. Moreover, for the first time, dipeptidic bound forms of 3SHol were evidenced in malt (up to 10 and 11 µg/kg for CysGly-3SHol and γGluCys-3SHol, respectively). In pale malts, the level of the CysGly- form was shown to be proportional to that of G-3SHol (in situ γGT during the malting process). This appeared to be no longer true for special malts (ranging from 5 to 45°EBC), whose CysGly-3SHol level correlated instead with Cys-3SHol (suspected chemical conversion from the dipeptide conjugate). As for γGluCys-3SHol, it was only found in the special barley malts (indicating another chemical break of the Cys-Gly bond, here on G-3SHol) and in malted rye, spelt, and wheat. No precursors were found in roasted malt.

Abbreviations: Cys-: Cysteinylated; CysGly-: Cysteinylglycinylated; γGluCys-: γ-Glutamylcysteinylated; G-: Glutathionylated; 3SHol: 3-sulfanylhexanol; 3SPol: 3-sulfanylpentanol; 3S4MPol: 3-sulfanyl-4-methylpentanol; 4S4M2Pone: 4-sulfanyl-4-methylpentan-2-one

KEYWORDS

barley malt; cysteinylated conjugates; glutathionylated conjugates; polyfunctional thiols

Introduction

Famous for their very low thresholds (ng/L level) and pleasant flavors (fruity or floral odors for those with five or more carbon atoms), polyfunctional thiols (PFTs)[1–3] play a key role in the organoleptic profiles of hopped-forward beers[4,5] as well as wines.[6] A slight proportion of them can arise simply through solid-liquid extraction during late or dry hopping. Up to 41 different free PFTs have been identified in dual-purpose hop varieties.[3, 7,8]

Most PFTs, however, as in the case of grapes and the resulting wines,[9] are released by yeast (*Saccharomyces cerevisiae* or *S. pastorianus*) during fermentation from bound forms originally found in raw materials.[10–12] In plants, these non-volatile precursors arise through the detoxification pathway of *alpha, beta*-unsaturated carbonyls, involving glutathione (GSH). The resulting glutathionylated (G-) conjugates can be further metabolized enzymatically (through γ-glutamyltransferase and carboxypeptidase activity) to two intermediate dipeptide conjugates, cysteinylglycine (CysGly-) and γ-glutamylcysteine, (γGluCys-) conjugates and ultimately to cysteinylated (Cys-) conjugates (structures and interconversions given in Figure 1).[13] At this stage, yeast β-lyase activity will be able to cleave the sulfur-carbon bond, releasing the odorant thiol moiety.[14,15]

Huge amounts of glutathionylated conjugates, especially G-3SHol (bound 3-sulfanylhexanol), but also G-3SPol (bound 3-sulfanylpentanol), G-3SPal (bound 3-sulfanylpentanal), G-3S4MPol (bound 3-sulfanyl-4-methylpentanol), and G-4S4M2Pone (bound 4-sulfanyl-4-methylpentan-2-one) (thiol moieties in Figure 1), have been evidenced in several hop varieties, mostly dual-purpose ones (up to several mg/kg in cultivars such as Citra, Mosaic, and Polaris).[16–19] The hop S-conjugate profile appears highly variety-dependent,[16–19] and the date of ripening and harvest year may also have an impact.[20] In contrast to the situation with grapes, the terroir impact is still largely unknown.

Unlike wine, made solely from grapes, beer is made from several raw materials liable to contribute PFTs. Malt is the major one: its final proportion is 1/9-1/5, versus 1/500-1/100

CONTACT Cécile Chenot cecile.chenot@uclouvain.be
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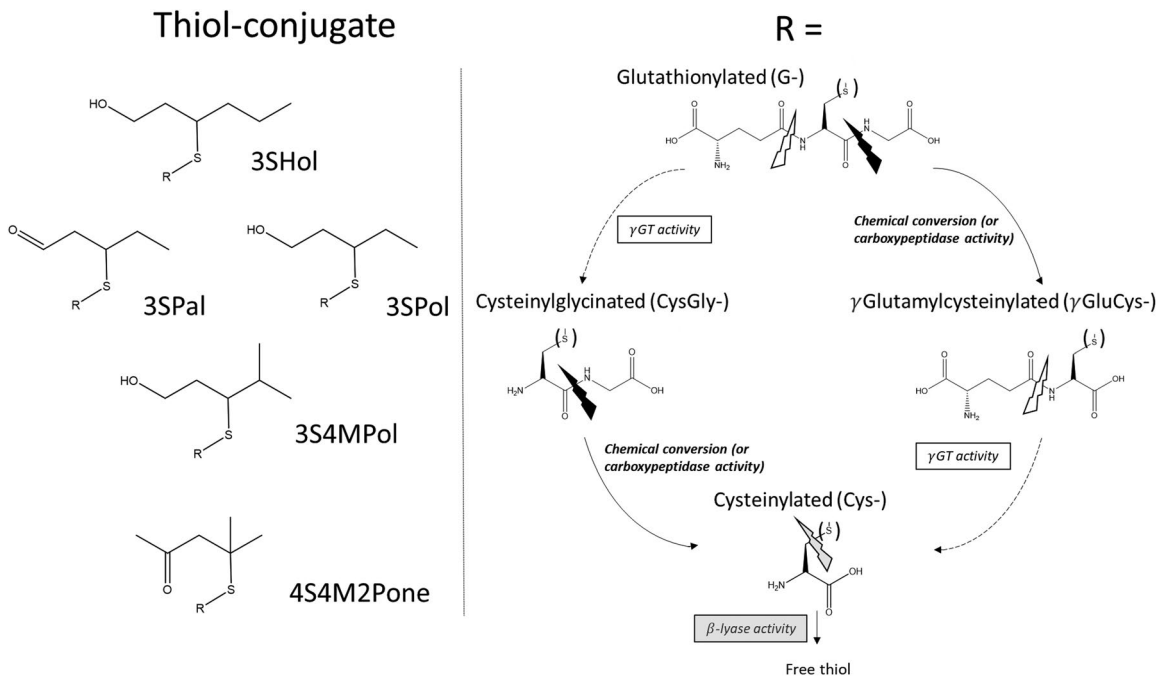

Figure 1. Chemical structures and interconversions between PFT conjugates found in malt and hop.

for hops.[21] Recent investigations have evidenced traces of cysteinylated and glutathionylated precursors of thiols in barley and malt.[22] In un-malted barley grains, about 2 µg/kg Cys-3SHol and up to 60 µg/kg G-3SHol have been found. In four out of six samples, the level of G-3SHol was shown to be slightly increased through the malting process (*in situ* formation from (E)-2-hexenal and glutathione suspected).[22,23] While steeping and germination could induce the synthesis of PFT precursors (trapping of aldehydes arising through malt lipoxygenase activity, which is enhanced as various other barley enzymes including amylases), the kilning step (temperatures from 80 to 84 °C for pale malts and up to 120 °C or more for special malts) most probably degrades some of these neo-formed *S*-conjugates together with *S*-methylmethionine (precursor of dimethylsulfide) and various Maillard reaction intermediates.[21] As shown by Roland et al., the G-3SHol content appears to be strongly malt-sample-dependent (e.g., 700 µg/kg in one pale malt but this compound was undetected in a 1150°EBC special malt). Yet because only a limited number of malt samples (12) were analyzed, no clear relationship could be established between °EBC or barley variety and the *S*-conjugate profile.[22]

More recently, additional *S*-conjugates have been identified in malt (up to 15 µg/kg Cys-3Pol, 200 µg/kg G-3SPol, 8 µg/kg Cys-3S4MPol, and 35 mg/kg G-3S4MPol).[24] Moreover, the G-conjugate of 3-sulfanylheptanol (G-3SHptol) was found at 10 µg/kg in a green (unkilned) malt sample but was no longer detected in the corresponding finished kilned malt.[25] Although these results may explain why beers sometimes exhibit free 3S4MPol slightly above the amount expected from the hop contribution,[16, 26] it is sure that a big part of the malt thiol potential is lost through wort mashing and filtration (thiols are able to strongly interact with quinones kept in the spent grain, together with undegraded polyphenols still offering functional properties for health and wellness benefits[27,28]). This explains why PFT levels in a non-hopped beer are always very low.[1,2]

The aim of the present work was to investigate the occurrence of G-3SHol, both derived dipeptides, and Cys-3SHol in a panel of experimental and commercial pale barley malts (30 samples) and to compare the results with *S*-conjugate profiles of special barley malts (12 samples up to 1500°EBC), and other malted grains.

Experimental

Chemicals

Formic acid (99%), methanol (99.98%) and water (99.98%) (UPLC/MS - CC/SFC grade), used for UPLC analyses, were purchased from Biosolve Chemicals (Dieuze, France). Methanol (> 99.99%) (Optima LC/MS grade), used for malt extract preparation, was purchased from Fisher Chemical (U.S.A.).

Malt samples

Pale barley malts were either experimental (numbered from 1 to 24 in Table 1) or commercial samples (purchased from Rolling Beers, France; listed from 25 to 30 in Table 1 with their commercial name, industrial producer, and color). Special barley malts, also purchased from Rolling Beers (France) are listed in Table 1 from 31 to 42, with their commercial name, producer, and color. All other malted cereals (listed in Table 2), produced by Weyermann, came also from Rolling Beers.

Extraction of PFT precursors from malt

PFT precursors were extracted from malt as follows: 10 g malt was ground in a mixer. One gram, accurately weighed, was

Table 1. Pale and special barley malt samples.

Number	Commercial name	Producer	Color (°EBC)
1	/	Experimental	3
2	/	Experimental	3
3	/	Experimental	3
4	/	Experimental	3
5	/	Experimental	3
6	/	Experimental	3
7	/	Experimental	3
8	/	Experimental	3
9	/	Experimental	3
10	/	Experimental	3
11	/	Experimental	3
12	/	Experimental	3
13	/	Experimental	3
14	/	Experimental	3
15	/	Experimental	3
16	/	Experimental	3
17	/	Experimental	3
18	/	Experimental	3
19	/	Experimental	3
20	/	Experimental	3
21	/	Experimental	3
22	/	Experimental	3
23	/	Experimental	3
24	/	Experimental	3
25	Pilsen	Soufflet*	3
26	Pilsen	Soufflet*	3
27	Pilsen	Weyermann*	3
28	Extra Pale Premium Pilsner	Weyermann*	2
29	Diastatic	Weyermann*	3
30	Extra Pale Maris Otter	Crisp*	3
31	Carapils	Weyermann*	5
32	Vienna	Weyermann*	6
33	Pale Ale	Weyermann*	8
34	Acid	Weyermann*	9
35	Munich	Weyermann*	15
36	Munich	Soufflet*	15
37	Carahell	Weyermann*	20
38	Munich	Weyermann*	25
39	Carabelge	Weyermann*	35
40	Abbaye	Weyermann*	45
41	Biscuit	Malterie du Chateau*	50
42	Carafa	Weyermann*	1500

*Purchased from Rolling Beers (France).

Table 2. Other malted cereals.

Code	Cereal	Producer	Color (°EBC)
A	Rye	Weyermann	7
B	Spelt	Weyermann	/
C1	Wheat	Weyermann	4
C2	Wheat	Weyermann	16
C3	Wheat	Weyermann	1000

further added to a 10 mL demineralized water-methanol 50:50 solution. The mixture was stirred for 15 min, sampled, spiked with deuterated internal standards, and filtered on a CHROMAFIL syringe filter (0.45 µm) before LC-MS/MS analysis.

Previously synthesized natural and deuterated thiol precursors

Natural and deuterated G-3SHol/G-3SHol-d_2,[29] CysGly-3SHol/CysGly-3SHol-d_2,[30,31] γGluCys-3SHol/γGluCys-3SHol-d_2,[30,31] Cys-3SHol/Cys-3SHol-d_2,[32] G-4S4M2Pone/G-4S4M2Pone-d_{10},[33] and Cys-4S4M2Pone/Cys-4S4M2Pone-d_6[34] were synthesized prior to this work according to published methods. The purity of the synthetic natural and deuterated compounds was assessed by ^{1}H NMR[35] (above 87% except for Cys-conjugates at around 50% because of trifluoro acetic acid salt formation).

Analysis of S-conjugates in malt by ultra-high-performance liquid chromatography – mass spectroscopy (UPLC – MS/MS)

Analyses were performed on a Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 µm), a polar end-capped C18 phase offering superior retention of polar compounds (Thermo Fisher, Waltham, MA, U.S.A.). The elution solvents were water (solvent A) and methanol (solvent B), both containing 0.1% v/v formic acid. The flow rate was set at 0.6 mL/min. The elution gradient was as follows: 95% of A for 1 min, from 95 to 65% in 9 min, from 65 to 2% in 3 min, held for 2 min, then back to the original conditions in 10 sec and held for 2 min.

The analytical system consisted of a 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, U.S.A.) hyphenated to a 6470B Agilent Triple Quadrupole. Source parameters were as follows: gas temp: 230 °C; gas flow: 4 L/min; nebulizer at 3.79 bar; sheath gas temp: 400 °C; sheath gas flow: 11 L/min; capillary voltage: 3000 V in positive mode; nozzle voltage: 0 V; positive Delta EMV set at 400 V. Ionization was carried out by positive electrospray (ESI+) and detection was performed by Multiple Reaction Monitoring (MRM; parameters detailed in Table A in online Supplemental material).

As depicted in Figure 2 (synthetized standards in a; samples of pale and darker malts in b, and c), each 3SHol S-conjugate counterpart appears as a double peak due to the presence of two diastereomers (R and S configurations of the 3SHol moiety and L configuration of the amino acid/peptide moiety).

Quantitation of S-conjugates in malt with a stable isotope dilution assay (SIDA)

A calibration curve of each natural precursor relative to its deuterated counterpart was determined. Solutions at various concentrations (adapted for each precursor to the range usually found in samples, detailed in Table B in online Supplemental material) were used to plot linear curves (concentration ratio versus area ratio). The slope gave the natural conjugate-to-deuterated conjugate response coefficient ratio ($R^2 > 0.99$). The following equation was used for conjugate quantitation: concentration of the natural conjugate in the extract (µg/kg) = ((concentration of deuterated conjugate in the extract (µg/kg) × (natural conjugate peak area/deuterated conjugate peak area)) + the Y-intercept) × (response coefficient of deuterated conjugate/response coefficient of natural conjugate).

Concentrations are given here in µg/kg of malt (accurate amount found in the injected water-methanol extract multiplied by the dilution factor of malt = 10).

Statistical analyses

All malt sample preparations were carried out in triplicate for *S*-conjugate analyses. Multiple comparisons of means were

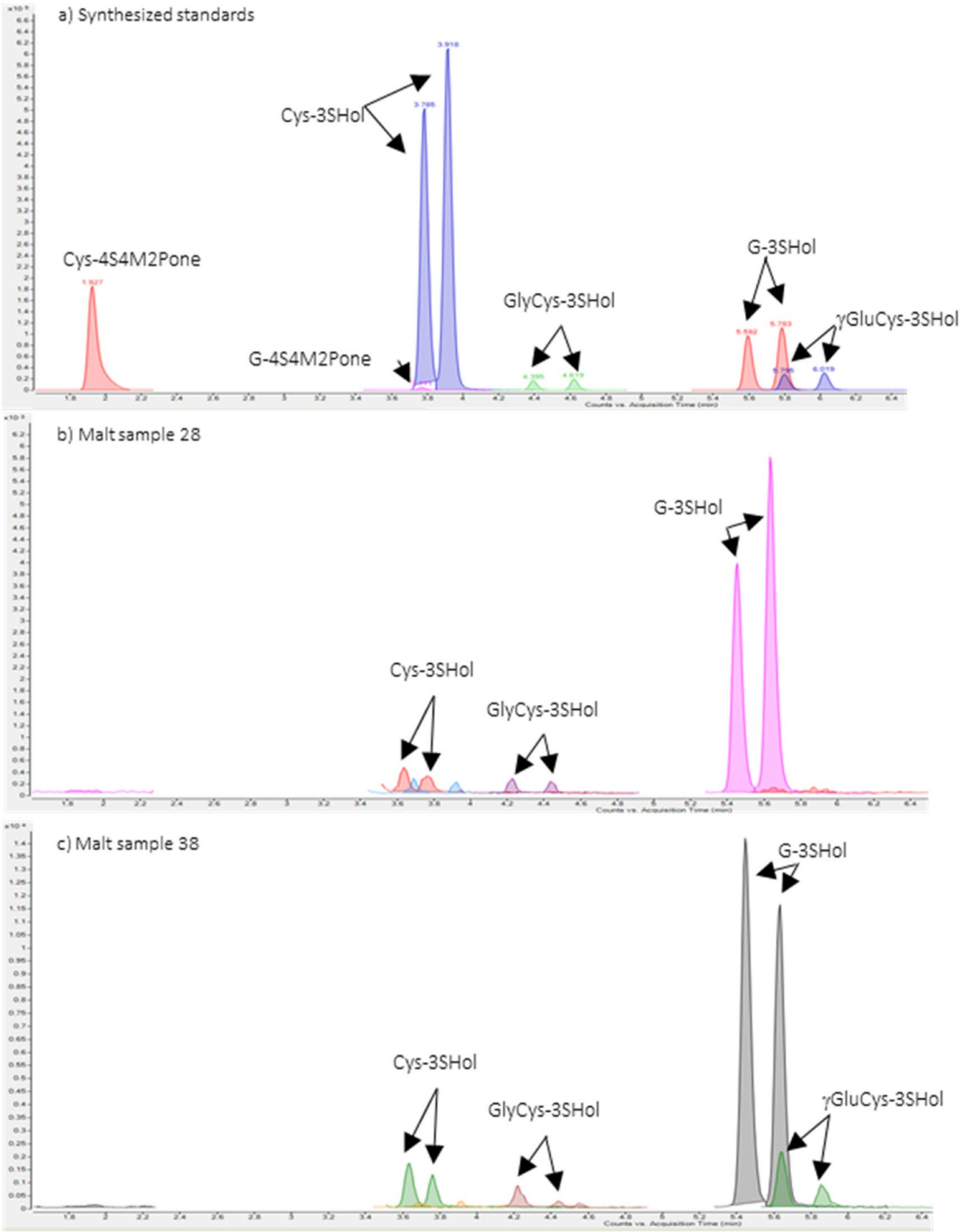

Figure 2. Chromatograms obtained for (a) synthesized S-conjugates, (b) pale malt 28, (c) special malt 38 (MRM transitions are summed as TIC for each analyte).

performed on special malt samples with Student–Newman–Keuls tests. Values that do not share a common letter for a same compound in Figure 5 are significantly different ($p > 0.05$).

Results and discussion

The *S*-conjugate quantitation in malt, as in hops, requires a first solid-liquid extraction. Yet malt contains many enzymes (amylolytic, proteolytic, etc.) that could be activated once in solution (principle of the mashing process). A 50:50 water:methanol mixture was here chosen to minimize enzymatic activity through the extraction step (already published methodology[22]).

Characterization of PFT precursors profiles in malts

The pale malts

Thirty pale malts arbitrarily numbered from 1 to 30 (2-4°EBC, Table 1) were extracted and analyzed by SIDA-LC-MS/MS (obtained chromatogram of sample 28

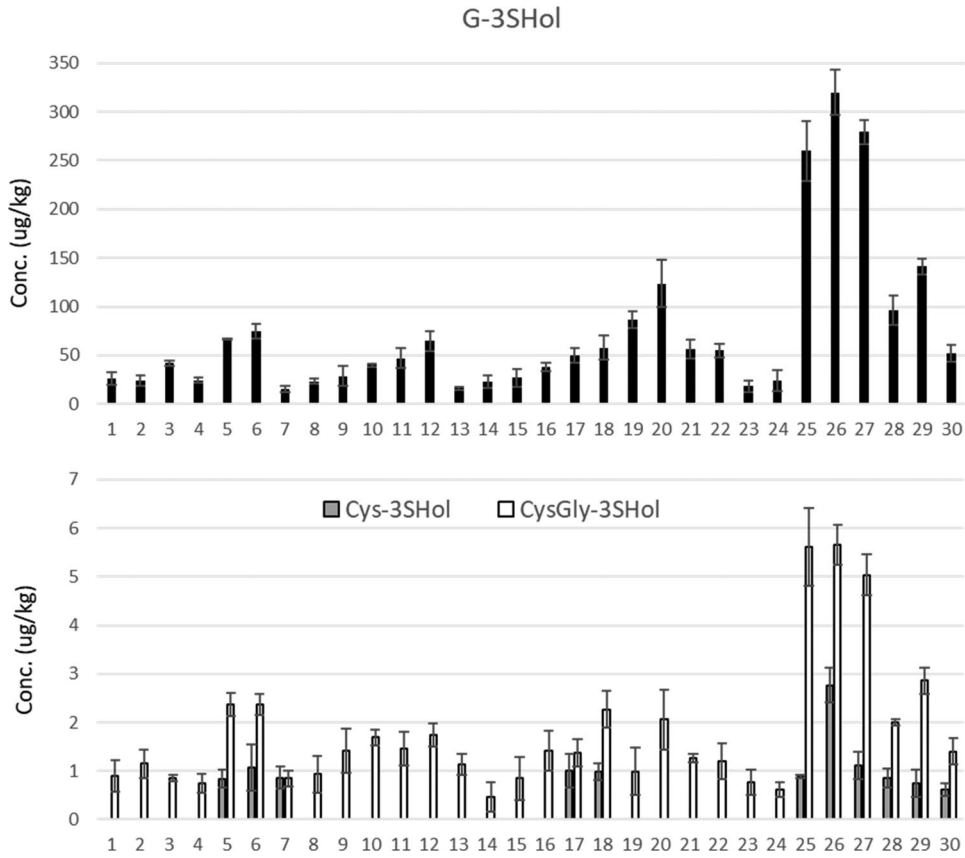

Figure 3. S-conjugate concentrations in 30 pale malts, given in µg/kg (G-3SHol in black; Cys-3SHol in grey; CysGly-3SHol in white). Standard deviations of triplicates are illustrated as error bars.

illustrated in Figure 2b, complete data in Table C in online Supplemental material). As depicted in Figure 3, G-3SHol was the major form in all samples, with amounts ranging from 15 to 320 µg/kg. CysGly-3SHol ranged from 0.5 to 5.6 µg/kg and Cys-3SHol from 0 to 2.8 µg/kg. It is interesting to mention that the dipeptide γGluCys-3SHol was evidenced in only one of the thirty pale malts investigated (9.1 µg/kg in sample 26). In agreement with previous studies, no trace of 4S4M2Pone precursors was detected.[22]

As expected from previous studies, the *S*-conjugate contents of malt were found to be far below the amounts found in hop (mg/kg levels)[19, 24] but quite similar to those usually found in grape musts.[36] Yet the distribution of *S*-conjugates appeared quite different from that of grapes, with the CysGly-3SHol content exceeding the Cys-3SHol level. Bonnaffoux et al.[31] showed the opposite in hundreds of samples of Sauvignon B. musts: Cys-3SHol was always more concentrated than the dipeptide conjugates (γGluCys-3SHol being present in slightly lower amount than CysGly-3SHol).

Values of CysGly-3SHol proved to correlate very well (R^2= 0.91) with G-3SHol levels (Figure 4), suggesting an *in situ* γGT activity during the germination step (as illustrated in Figure 1). On the other hand, a lower correlation (R^2= 0.57) was observed between CysGly-3SHol and Cys-3SHol (low carboxypeptidase activity).

The special malts

Twelve special malts, either kilned at higher temperature, caramelized, or roasted (numbered 31 to 42 by increasing °EBC color in Table 1), were further investigated by the same SIDA-LC-MS/MS method in water-methanol extracts (Figures 2c and 5).

The range of G-3SHol concentrations found in special malts was similar to that found in pale malts (up to 300 µg/kg). As in the pale malts 25, 26, and 27, the G-3SHol level in the three least-colored special malts, 31 (Carapils), 32 (Vienna), and 33 (Pale Ale), peaked at 225-280 µg/kg. CysGly-3SHol was also similar, with values close to 6 µg/kg, but in contrast to Pilsen malts, almost as much γGluCys-3SHol was found (3-6 µg/kg). This suggests chemical synthesis of this dipeptide from G-3SHol during the kilning process.

In some samples, higher levels of CysGly conjugate (up to 10 µg/kg in samples 35 Munich, 40 Abbaye, and 41 Biscuit) and Cys conjugate (up to 13 µg/kg in sample 41 Biscuit) were also evidenced.

Color appeared not to be the sole determinant, as depicted by very different levels of *S*-conjugates in the two Munich 15°EBC malts (precursor levels twice as high in sample 35 as in sample 36). Lipoxygenase activity (related both to variety and to the germination process) most probably influences levels of alkenals and hence the amounts of their glutathione nucleophilic addition products (non-limiting glutathione

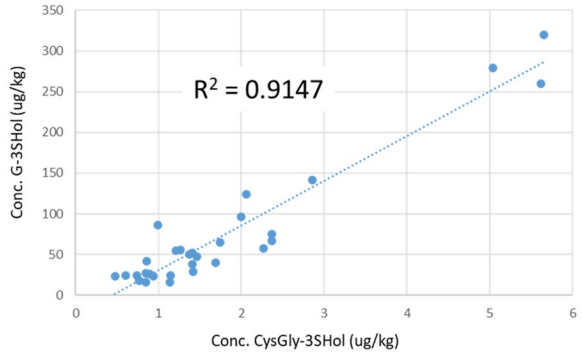

Figure 4. Correlation between amounts of G-3SHol and CysGly-3SHol (both given in µg/kg) found in 30 pale malt samples.

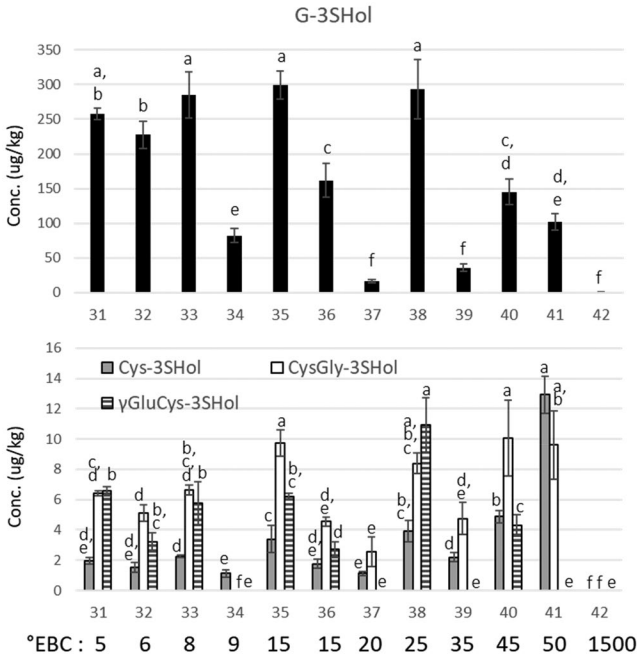

Figure 5. *S*-conjugate concentrations in 12 special malts, given in µg/kg (G-3SHol in black; Cys-3SHol in grey; CysGly-3SHol in white; γGluCys-3SHol: hatched). Standard deviations of triplicates are illustrated as error bars. Letters "a,b,c,d,e,f" illustrate statistical results of the Student-Newman-Keuls test. Samples that do not share a same letter for a same compound are significantly different ($p>0.05$).

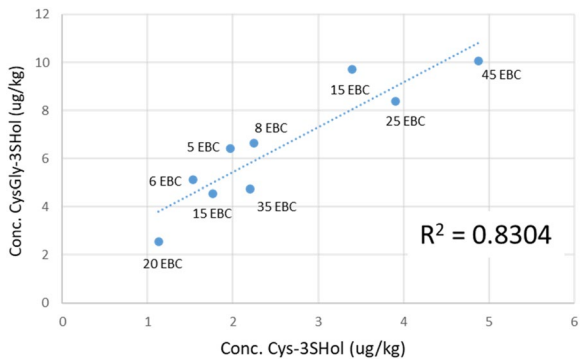

Figure 6. Correlation between the amounts of Cys-3SHol and CysGly-3SHol (both given in µg/kg) found in nine special malts ranging from 5 to 45°EBC.

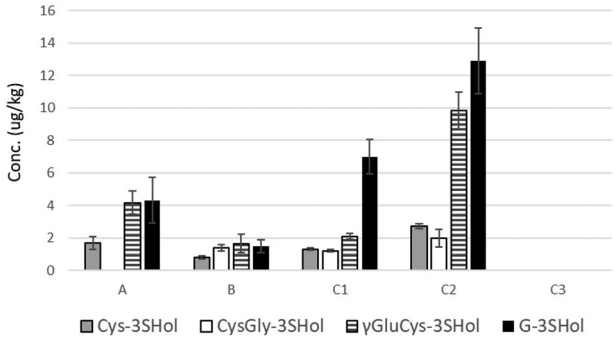

Figure 7. *S*-conjugate concentrations in five other malted cereals, given in μg/kg (G-3SHol in black; Cys-3SHol in grey; CysGly-3SHol in white; γGluCys-3SHol: hatched). Standard deviations of triplicates are illustrated as error bars.

concentration, close to 4800 mg/kg).[37] Similar conclusions were drawn from the comparison of samples 36 and 38. Compared to its lighter counterpart 36, from the same producer, the darker, 25°EBC Munich malt (38) showed higher concentrations of all four investigated *S*-conjugates.

On the other hand, as shown by Roland et al.,[22] the strong roasting process applied to sample 42 proved to completely destroy its PFT precursors (undetected levels in all four 3SHol conjugates). Very interesting also are the low amounts of *S*-conjugates (< 50 µg/kg) found in both the Carahell (37) and the Carabelge (39) samples, for which the traditional kilning step was bypassed (roasting applied to humid grains).

Another unexpected result was the total absence of CysGly-3SHol in sample 34 (acidic malt - congress mash pH = 3.4 vs 5.5 on average for the other samples). Low pH could have drastically reduced *in situ* G-, CysGly-, and Cys-3SHol formation during germination by impacting the *trans*-2-hexenal synthesis by lipoxygenase.

No clear relationship between color and *S*-conjugate content appeared among special malts (the R^2 values were only 0.54, 0.09, and 0.30, for the correlations between °EBC and Cys-3SHol, CysGly-3SHol, and G-3SHol, respectively). On the other hand, a linear relationship was evidenced between the Cys-3SHol and CysGly-3SHol levels (R^2=0.83) for the nine samples ranging from 5 to 45°EBC (acid malt 34 being left aside) (Figure 6). This indicates a similar and low chemical conversion ratio of CysGly- into Cys-3SHol. However, for more colored Biscuit malt (sample 41), a higher heat treatment has led to Cys-3SHol > CysGly-3SHol (conversion rate increased in that case).

Other malted cereals

PFT precursors quantitated in samples of malted rye (A), spelt (B), and wheat (C1 to C3) are depicted in Figure 7.

Malted rye, spelt, and wheat, compared to barley malt, contained much less G-3SHol (0 to 13 µg/kg). A similar trend was previously observed by Roland et al. in rice and sorghum.[22] Yet, as in barley malts, hundreds of mg/L (ppm) of reduced glutathione (GSH) occur in these cereals,[38] indicating again that the lipoxygenase activity could be the limiting factor.

On the other hand, our present data evidences the occurrence of γGluCys-3SHol in these malted cereals (up to 10 µg/kg). These samples also contained CysGly-3SHol (up to 2 µg/kg), except for malted rye (A).

Among the three malted wheat samples (C1, C2, C3), increasing the color (from 4 to 16°EBC) resulted in more *S*-conjugates (C1 vs. C2). Yet as in torrefied barley malt (42), no *S*-conjugate was found in the torrefied malted wheat (C3, 1000°EBC).

Conclusion

Among pale barley malts, sample origin and procedure strongly impact the occurrence of PFT precursors, G-3SHol and CysGly-3SHol levels being correlated. This is no longer true for darker malts up to 45°EBC, in which the dipeptide conjugate level becomes proportional to that of Cys-3SHol, possibly because of a slight but significant chemical conversion of the dipeptide conjugate to its cysteine counterpart, which does not occur in pale malts. The presence of γGluCys-3SHol in the darker malts indicates another conversion starting from G-3SHol. No precursors were found in the roasted malts (thermal degradation increased in that case). Kilning of acid or humid grains emerged as another factor leading to low levels of thiol conjugates. Malted rye, spelt, and wheat, although containing the unusual γGluCys-3SHol, emerged as poor sources of G-3SHol.

ORCID

Cécile Chenot http://orcid.org/0000-0003-4071-2523
Sonia Collin http://orcid.org/0000-0002-1929-098X
Aurélie Roland http://orcid.org/0000-0003-2673-3969

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