Enzymatic release of odourant polyfunctional thiols from cysteine conjugates in hop

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Polyfunctional thiols are contributors to the hop varietal aroma of beer. Besides free thiols, a cysteine-*S*-conjugate has recently been shown to be an additional component of the thiol potential of hop. Such cysteine adducts investigated here in four hop cultivars and in different hop forms. Hop hydroalcoholic extracts were purified on a cation exchanger and subjected to apotryptophanase β-lyase activity. The Cascade hop variety exhibited the highest bound 3-sulphanylhexan-1-ol (grapefruit-like) potential, while both Tomahawk and Nelson Sauvin cultivars were confirmed to be important sources of bound 3-methyl-2-butene-1-thiol (skunky-like), 3-sulphanylpentan-1-ol and 4-sulphanyl-4-methylpentan-2-one (box-tree-like). Surprisingly, hop CO_2 extracts proved to contain cysteine conjugates. Although related, the concentrations of cysteine-bound thiols in hop are not strictly correlated to the amounts of free volatiles found in the derived beers. Copyright © 2013 The Institute of Brewing & Distilling

Keywords: polyfunctional thiols; odour; hop (*Humulus lupulus*); cysteine adduct; apotyptophanase; β-lyase

Introduction

Polyfunctional thiols are powerful odourants in many foods *(1)*. The compounds 3-sulphanylhexan-1-ol and 4-sulphanyl-4-methylpentan-2-one are known to impart varietal aromas to wines made from Sauvignon blanc, Muscat, Scheurebe and Riesling grapes, and Petite Arvine *(2–6)*. Yeast β-lyase activity has been implicated in catalysing grape non-volatile cysteine-*S*-conjugates catabolism through wine fermentation *(7–9)*. Several papers have reported conversion rates of *S*-3-(1-hydroxyhexyl)-cysteine and 4-*S*-(4-methylpentan-2-one)-cysteine to free thiols below 1%, explaining only 3–7% of the total 3-sulphanylhexan-1-ol as resulting from the action of yeast *(4,10–13)*. Recently, Roland *et al.* *(14)* demonstrated the ability of fermenting yeast also to convert *S*-3-(1-hydroxyhexyl)-glutathione to a volatile thiol (conversion rate of 4.5%). This pathway might contribute to 20% of the free polyfunctional thiols found in wine.

Cysteine- and glutathione-*S*-conjugates have also been found in passion fruit juice, *Poncirus trifoliata*, onion and bell pepper *(15–20)*. By means of β-lyase activity, Starkenmann *et al.* *(17)* were able to release 3-sulphanyl-2-methylpentan-1-ol from a fresh onion *S*-cysteine conjugate fraction.

Hop is the main contributor of thiols to beer *(21–25)*. The compounds 3-sulphanyl-4-methylpentan-1-ol, 3-sulphanyloctan-1-ol, and 3-sulphanylhexan-1-ol distinguish Nelson Sauvin-, Tomahawk-, and Cascade-hopped beers, respectively *(21,25,26)*. Although already found in hop, significant amounts of free thiols continue to be produced through wort boiling and fermentation *(21,23,25)*.

Very recently, a cysteine-*S*-conjugate [*S*-3-(1-hydroxyhexyl)-cysteine] has been characterized by HPLC-HRMS/MS in the Cascade hop variety *(26)*. The presence of other cysteine adducts is therefore suspected in hop. Better knowledge of this potential might help brewers to predict the thiol flavour of the derived beer.

The aim of the present work was to quantify the thiol potential hidden in the cysteine conjugate form in four different hop cultivars and two different hop forms. Hop extracts were incubated with a commercial enzyme exhibiting a β-lyase activity (apotryptophanase) with a view to releasing volatile thiols from any *S*-cysteine conjugates that might be present. Free thiols were then extracted and quantified by GC-PFPD.

Experimental

Chemicals

3-Sulphanylpropan-1-ol **14**, benzylthiol, (*E*)-hexen-2-al, *N*-boc-L-cysteine (98.5%), *S*-benzyl-L-cysteine (**IST**), apotryptophanase, citric acid and the Amberlite IR-120 resin were purchased from Sigma-Aldrich (Bornem, Belgium). Ammonia (28%) was from VWR (Leuven, Belgium). 3-Sulpha-nylhexan-1-ol **23** and 3-methyl-2-buten-1-thiol **37** were obtained from Oxford Chemicals (Oxford, UK). 4-Sulphanyl-4-methylpentan-2-one **29** was from Frutarom (Hartlepool, UK). Cesium carbonate, sodium borohy-dride and trifluoroacetic acid were sourced from Acros Organics (Geel, Belgium). Dichloromethane (99.9%) obtained from Romil (Cambridge, UK) was distilled before use. Milli-Q water was used (Millipore, Bedford, MA, USA). Sodium hydroxide and sodium sulphate (99%) were supplied by Janssen (Geel, Belgium). Anhydrous sodium sulphate was obtained from Merck (Darmstadt, Germany). Formic acid was provided by Fischer (Loughborough, UK). Polyvinylpyrrolidone was supplied by Spindal (Gretz-Armainvilliers, France).

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Reference compounds synthesized in our laboratory

3-Sulphanylbutyl acetate **3** had been synthesized previously [reagents and complete procedure described by Vermeulen *et al. (27)*]. 3-Sulphanylpentan-1-ol **21** was obtained with the reagents and procedure described in Vermeulen *et al. (28)*.

Hop samples

The investigated varieties were from crop 2011. Saaz bred in the Czech Republic were provided by Hopsteiner (Mainburg, Hallertau, Germany). Cascade and Tomahawk, bred in the USA, were provided by Yakima Chief (Louvain-la-Neuve, Belgium). Nelson Sauvin, bred in New Zealand, were provided by Hops Limited (Richmond, Nelson, New Zealand).

Synthesis of *S*-3-(1-hydroxyhexyl)-cysteine

The synthesis was carried out according to the procedure described by Thibon *et al. (29)* [Michael addition of N-Boc-L-cysteine on (*E*)-hexen-2-al in acetonitrile in the presence of cesium carbonate, reduction of the obtained aldehyde with sodium borohydride in methanol *(26)*]. Amine deprotection was achieved with trifluoroacetic acid, according to Pardon *et al. (30)*.

Determination of the conversion rates of cysteine-*S*-conjugates into free thiols

Amounts of 6.6, 0.4, or 0.1 mg of *S*-benzyl-cysteine and 3.2, 0.4 or 0.1 mg of *S*-3-(1-hydroxyhexyl)-cysteine (prepared in EtOH:H$_2$O:HCOOH, 49.5:49.5:1, v/v) were spiked into the 2 mL reaction mix [pyridoxal 5-phosphate (0.1 mm) and ethylenediaminetetraacetic acid (1 mm) in potassium phosphate (100 mm, pH 7.7)] in the presence or not of apotryptophanase. Free thiols were extracted as for hop fractions (5 mL of bi-distilled dichloromethane for 30 min, dried over sodium sulphate prior to concentration to 500 μL in a Danish-Kuderna) and quantified by GC-PFPD (see below) using complete calibration curves.

Extraction of cysteine-*S*-conjugates from hop

The extraction was conducted on the hop, according to a procedure adapted from Starkenmann *et al. (18–20)*. Milled pellets (100 g), or CO$_2$ extracts (31 g), spiked with 1 mL of 1.6 mg/L *S*-benzyl-L-cysteine, were extracted with 1 L of a hydroalcoholic solution (EtOH:H$_2$O:HCOOH, 49.5:49.5:1, v/v) for 2 h. After centrifugation for 30 min at 1200 **g**, the polyphenols present in the supernatant were partially removed by addition of 15 g of polyvinylpyrrolidone and stirring for 30 min. After a second centrifugation, the extract was purified on the IR-120 (H$^+$) cation exchange resin (100 g) conditioned with ultrapure water. After washing with 200 mL water, sequential fractions were recovered by eluting with aqueous ammonia solutions at the following concentrations: 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 m. The 1.2 and 1.5 m fractions were pooled, vacuum evaporated, and lyophilized.

Enzymatic assay on hop extract

The dried fraction 1.2–1.5 m (20 mg) was suspended with 2 mL of a potassium phosphate buffered solution (100 mm, pH 7.7) containing pyridoxal 5-phosphate (0.1 mm), and ethylenediaminetetraacetic acid [1 mm; adapted from Tominaga *et al. (8)*]. Five hundred microlitres of a solution containing the commercial apotryptophanase from *Escherichia coli* (75–150 units/mg) freshly prepared (1 mg in 0.5 mL of buffer) was added. Incubation was at 28 °C for 30 min. A control without enzyme was conducted in parallel. The mixture was then stirred with 5 mL of bi-distilled dichloromethane for 30 min. The organic phase was dried over sodium sulphate prior to concentration to 500 μL in a Danish-Kuderna and to 70 μL in a Dufton column.

Gas chromatography hyphenated to a pulsed-flame photometric detector (GC-PFPD)

A 2 μL aliquot of extract was analysed on a ThermoFinnignan Trace GC 2000 gas chromatograph equipped with a splitless injector. The injections were carried out in the splitless mode at 250 °C, the split being turned on after 0.5 min. Compounds were separated on the FFAP column (25 m × 0.32 mm i.d., 0.3 μm film thickness). The carrier gas was helium at a pressure of 45 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. At the OI Analytical PFPD detector (model 5380, internal diameter combustor = 2 mm), the following parameters were selected: 220 °C as the temperature, 600 V as the voltage, 18 ms as the gate width, 6 ms as the gate delay, 580 mV as the trigger level and 3.70 Hz as the pulse frequency.

Gas chromatography hyphenated to an electronic impact mass spectrometer (GC-MS)

Mass spectra (*m/z* = 40–380) were recorded at 70 eV on a ThermoFinnigan Trace MS mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector and an apolar CP-Sil 5 CB MS capillary column (50 m × 0.32 mm i.d., 1.2 μm film thickness) or a polar FFAP column (25 m × 0.32 mm i.d., 0.3 μm film thickness). The carrier gas was helium, and the pressure was set at 100 kPa. The oven temperature programme was the same as that described for GC-O. Spectral recording was automatic throughout elution, Xcalibur software was used.

Identifications

MS identifications were performed by comparing the mass spectra obtained from each sample with those obtained with pure or synthesized compounds *(27,28,31)* injected under the same conditions and/or present in the NIST library. The retention indices (retention times normalized with respect to adjacently eluting *n*-alkanes) were determined by injection onto two capillary columns (CP-Sil 5 CB and FFAP-CB) connected to the MS detector (identification checked by co-injection). In the case of PFPD detection, injection of thioesters allowed translation into the alkane-related decimal numeral system.

Quantification of free thiols (X) released by enzymatic treatment

For quantification in internal standard (IST) equivalents (16 mg/kg IST in the sample; recovery and conversion rates roughly identical), the following equation was used:

Concentration of X (mg/kg IST equivalent) = (peak area of X/peak area of free IST) × 16 × (molecular weight of free IST/molecular weight of bound IST) × (response coefficient of free IST/response coefficient of X)

RP-HPLC-ESI(+)-MS/MS determination of *S*-3-(1-hydroxyhexyl)-cysteine for selecting the most concentrated IR-120 eluted fractions

Ten microlitres of each ammonia-eluted fraction (concentrated 200 times after elution) was injected onto the C$_{18}$ Prevail column. RP-HPLC analysis was performed on a SpectraSystem equipped with an SCM degasser, an AS3000 autosampler and a P4000 quaternary pump. The system was controlled with the Xcalibur software version 1.2 (Thermo Fisher). Mass spectra were acquired using a LCQ mass spectrometer equipped with an ESI source. Standard solutions of *S*-benzyl-L-cysteine and the *S*-3-(1-hydroxyhexyl)-cysteine at 0.1, 1, 5 and 10 mg/L were injected into the RP-HPLC-ESI(+)-MS/MS for calibration.

Results and discussion

Hop is a complex matrix containing bitter acids and high concentrarions of polyphenols. A PVPP treatment was revealed as necessary before the cation exchange chromatography to yield a purified extract of *S*-cysteine conjugates. The most promising ammonia-eluted fractions were selected by RP-HPLC-MS/MS analysis on the basis of the recovery factor of the commercial standard *S*-benzyl-L-cysteine (**IST**) and the previously synthesized *S*-3-(1-hydroxyhexyl)-cysteine (3-cystHol) *(26)*.

Enzymatic assays were carried out on the lyophilized 1.2 and 1.5 M pooled fractions with commercial apotryptophanase previously used by many groups for its β-lyase activity *(18,19,30,32,33)*. The apotryptophanase activity requires a free cysteine amine group *(19)*. Therefore, *S*-glutathione conjugates (also evidenced as precursors of thiols in wine) were not degraded in our experiments *(14,34)*. The released volatile thiols were quantified by GC-PFPD. As depicted in Table 1, the efficiency of the conversion was strongly related to the substrate concentration. For both IST and 3-cystHol, a conversion rate of 30% was reached when concentrations of conjugates <50 mg/L were investigated (most probably the case in hop extracts). This conversion rate is relatively high compared with yeast activity either in musts *(4,10–12)* or in model media *(13,33)*. In our spiking experiments, the IST emerged as a good candidate for mimicking other cysteine-bound conjugates such as 3-cystHol. Therefore, all the concentrations in hop are given on a scale relative to the IST, even though complete calibration curves were calculated at the PFPD detector to take into account the true response coefficients.

The PFPD chromatograms obtained for four hop varieties and two conditionings, with and without the apotryptophanase treatment, are given in Fig. 1. In all samples, many polyfunctional thiols were released by the enzymatic treatment, although major discrepancies were found between them (Table 2).

The Cascade variety exhibited the highest potential, with 1641 µg/kg of 3-sulphanylhexan-1-ol (grapefruit, 3SHol, **23**) released (2706 µg/kg of cysteine conjugate in IST equivalents). This value is 14–22 times the amount of free thiols previously found in this variety (73–117 µg/kg) *(21)*. By comparison, the treatment of Tomahawk, Nelson Sauvin and Saaz extracts released only 276, 261 and 316 µg/kg of **23**, respectively [although still 29-, 23-, and 126-fold higher than, respectively, 7–12, 6–17, and 2–3 µg/kg of free thiol **23** previously reported *(21,25)*].

Adducts of 4-sulphanyl-4-methylpentan-2-one (catty, blackcurrant, 4S4M2Pone, **29**) were found in Nelson Sauvin and Tomahawk varieties, with up to 598 µg/kg in the former [79 times the concentration of the corresponding free thiol *(21,25)*]. The compound 4S4M2Pone was previously proposed as a marker for authentication of the Nelson Sauvin-derived beers. By comparison, the treatment of Tomahawk extracts released only 122 µg/kg of 4S4M2Pone [although still 87-fold higher than 1.4 µg/kg of free corresponding thiol **29** previously reported *(21)*].

All these values can be compared with the 25–48 µg/kg of **23** and 3–4 µg/kg of **29** bound to cysteine in Sauvignon blanc grapes *(34,35)*. Only a small part of the precursors found in grape are converted into free thiols through wine fermentation (0.2–10%) *(4,11,12,36)*. Between 29 and 90% of the precursors are taken in by yeast and not detected under the converted form in the fermented must *(7,37)*. In beer, if *S*-cysteine conjugates are considered as sole source of **23**, 8% of the 2706 µg/kg of 3-cyst-Hol evidenced here in Cascade hop would be necessary to explain the 243 ng/L of 3SHol found in the Cascade-derived beer [hopping rate of 1.78 g/L *(21)*; Fig. 2]. This ratio was comparable for the Saaz- and Nelson Sauvin-derived beers (5 and 13%, respectively) and higher for the Tomahawk-derived beer (41 %).

Another interesting thiol released by the enzymatic treatment applied to Tomahawk and Nelson Sauvin fractions was the skunky-like 3-methyl-2-buten-1-thiol (MBT, **37**). The mass spectrum (ions 61, 136, 69) and the retention time of the odourant zone on the two capillary columns (FFAP and CP-Sil 5 CB) confirmed the identification. The free compound has been quantified in the Tomahawk and Nelson Sauvin varieties at 5–11 µg/kg *(21)*, while the apotryptophanase treatment released 454–584 µg/kg (Fig. 2). To our knowledge, this is the first time MBT has been reported as released from a non-volatile fraction by the action of β-lyase. It has been known for a long time that MBT can arise through isohumulone degradation in beer exposed to light *(38–40)*. Recently, Gros *et al.* *(31)* evidenced an additional minor pathway starting with addition of yeast hydrogen sulphide onto the hop allylic alcohol 3-methyl-2-buten-1-ol. Quantitatively, the huge amount of cysteine-bound MBT demonstrated here leads us to reconsider the different sources of this skunky flavour. The free MBT found in the Tomahawk hopped beer *(21)* (addition of 1.78 g/L of pellets in the boiling kettle) represents 71% of the cysteine-*S*-conjugate potential evidenced here. For Nelson Sauvin variety, a ratio of 19% was found.

Bound 3-sulphanylpentan-1-ol (grapefruit, 3SPol, **21**) was evidenced in Tomahawk and Nelson Sauvin hops (82 and 724 µg/kg released, only 0.4 and 1.8-3.2 µg/kg unbound, respectively). The concentrations of free **21**, only found in the Nelson Sauvin-derived beer [39 ng/L = 22 µg/kg in hop equivalents *(26)*], could be related to the high potential of bound **21** evidenced here in hop (Fig. 2).

Table 1. Apotryptophanase-catalysed conversion rates of *S*-3-(1-hydroxyhexyl)-cysteine and *S*-benzyl-L-cysteine into free thiols. Assays variation coefficient below 10%.

	Concentration of the spiked conjugate		Concentration of apotryptophanase released thiol		Conversion rate (%)
	nmol/L	mg/L	nmol/L	mg/L	
S-3-(1-Hydroxyhexyl)-cysteine (3-cystHol)	7335	1621	612	82	8
	887	196	149	20	17
	222	49	68	9	31
S-Benzylcysteine	15,640	3300	1138	141	7
	948	200	195	24	20
	245	52	69	8	28

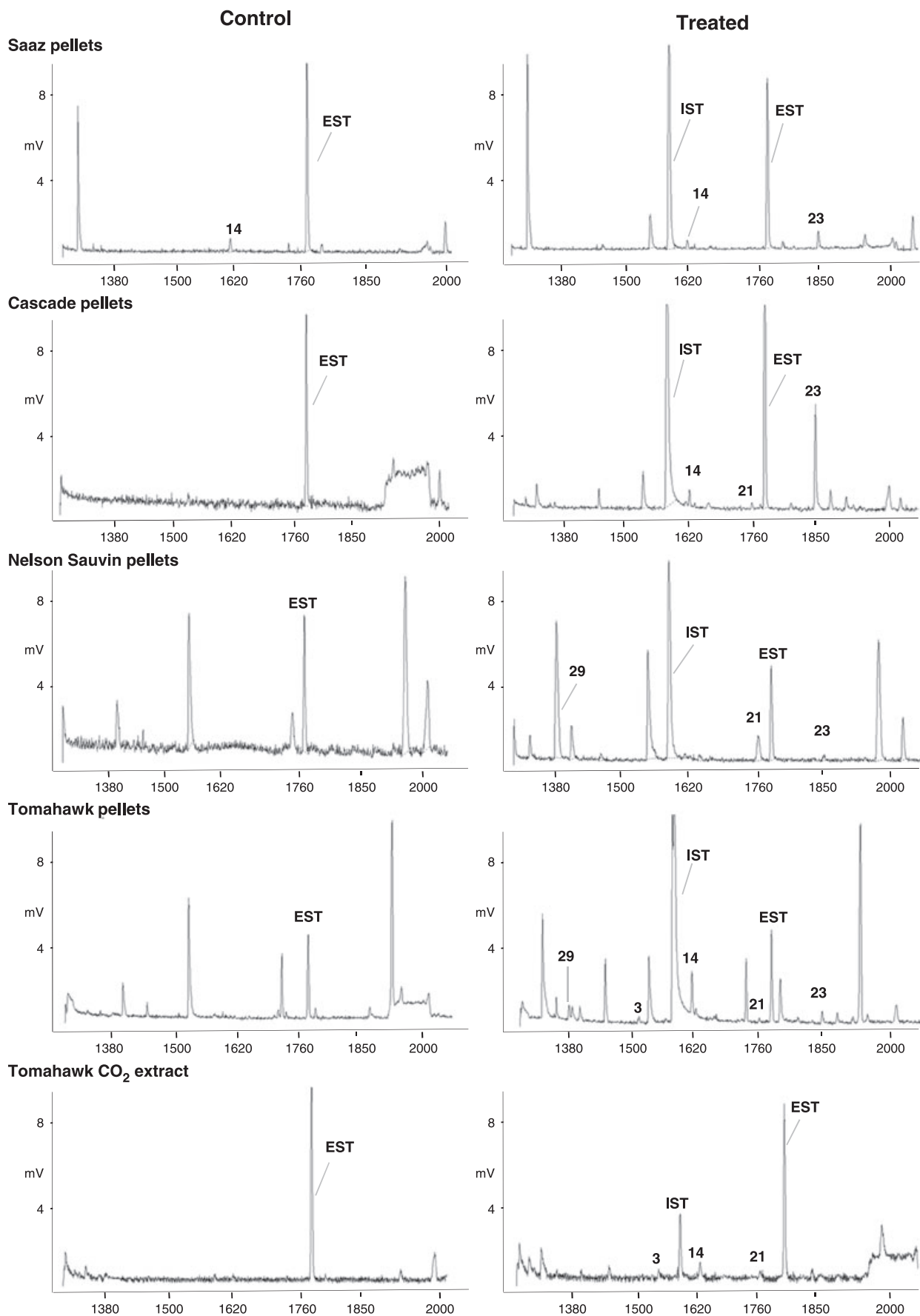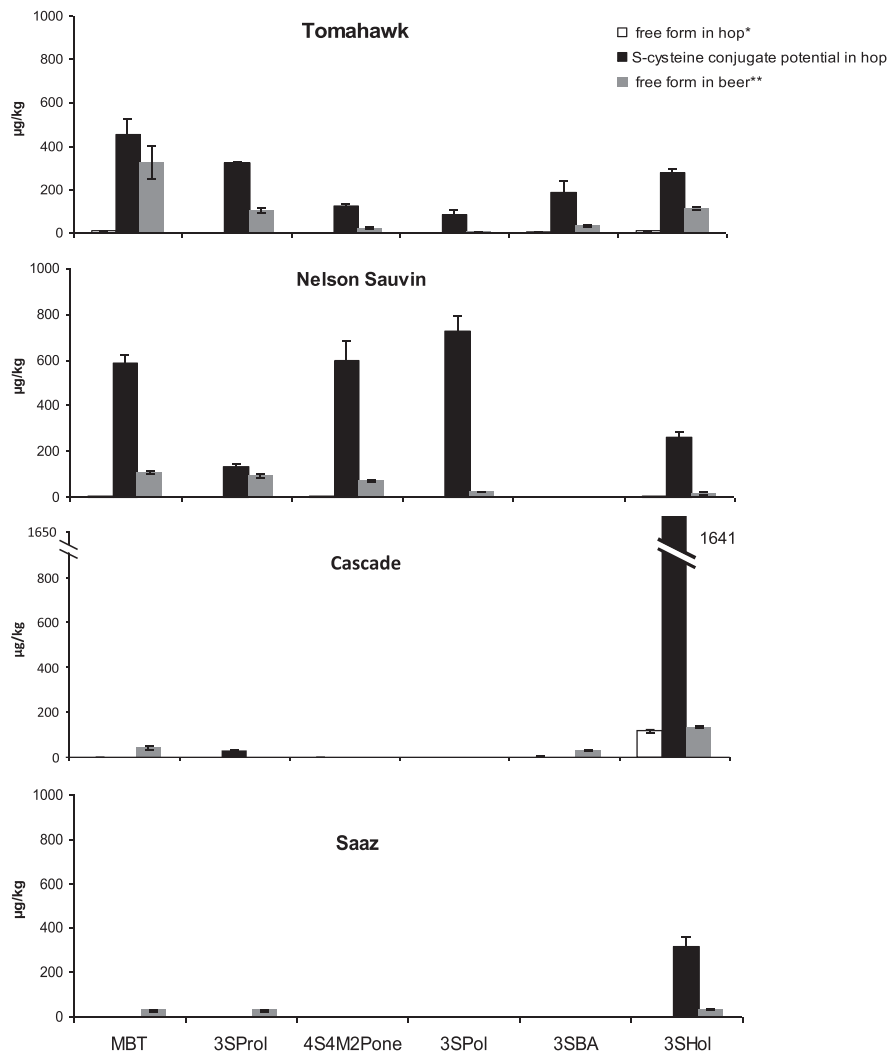wileyonlinelibrary.com/journal/jib

Figure 1. GC-PFPD chromatograms of hop extracts treated (right) or not treated (left – control) with apotryptophanase (IST: 16 mg/L).

Figure 2. Comparison between the concentrations of free thiols in pellets with those released by apotryptophanase or found in the derived beers (given in hop weight equivalents). *Gros *et al.* (21); **Gros *et al.* (26).

Table 2. Amounts of bound thiols (μg/kg, IST equivalents) released by apotryptophanase. Concentration determined by GC-PFPD. Values converted into pellet equivalents for CO_2 extract. Assays in duplicate

Retention Index						Concentration of thiols released by enzymatic treatment (μg/kg, pellet equivalents)				
							Pellets			CO_2 extract
CP-Sil5	FFAP	Number[a]	Acronym	Substance name	Odour (GC-O)	Saaz	Cascade	Nelson Sauvin	Tomahawk	Tomahawk
810	1112	37	MBT	3-Methyl-2-buten-1-thiol	Coffee, skunky	—	—	584	454	363
849	1620	14	3SProl	3-Sulphanylpropan-1-ol	Potatoes, pop corn	—	26	135	322	336
915	1381	29	4S4M2 Pone	4-Sulphanyl-4-methylpentan-2-one	Catty, blackcurrant	—	—	598	122	-
1009	1760	21	3SPol	3-Sulphanylpentan-1-ol	Catty, citrus	—	—	724	82	-
1033	1536	3	3SBA	3-Sulphanylbutyl acetate	Cheese, onion	—	—	—	157	120
1094	1858	23	3SHol	3-Sulphanylhexan-1-ol	Grapefruit	316	1641	261	276	87

[a]Numbering from Gros *et al.* (21,26).

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Several unknown compounds generated by the treatment, previously not found in hop in free form, remain to be identified (Fig. 1). In contrast, after the apotryptophanase assay, we failed to detect a large number of thiols previously quantified in free volatile form in hop and in beer [e.g. no cysteine adduct of 3-sulphanyl-4-methylpentan-1-ol in Nelson Sauvin (21,26)]. Other forms of bound precursors might be present in hop, including *S*-glutathione conjugates. An enzymatic treatment conducted either with yeast cell extract or commercial γ-glutamyltranspeptidase could provide interesting complementary data on these potential conjugates (14,34,41).

The enzymatic treatment was also applied to Tomahawk CO_2 extracts (Fig. 1). For the CO_2 extracts, the revealed free thiols are given in pellet equivalents (Table 2). Surprisingly, although very apolar, the CO_2 extract proved able to solubilize some polyfunctional thiol adducts. Despite the presence of the amino acid moiety, the cysteine adducts are probably less polar than expected. MBT, 3-sulphanylpropan-1-ol, the cheesy 3-sulphanylbutyl acetate and 3SHol were found after the apotryptophanase treatment.

In conclusion, hop pellets and hop CO_2 extracts emerge as two very interesting sources of cysteine conjugates, compared with the potential of grapes. Their concentrations are often related to the levels of free thiols that are found in the derived beers. Yet, no strict correlation can be established, suggesting that other bound forms could exist.

Acknowledgements

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