Hop proanthocyanidins for the fining of beer

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Abstract

Fining agents are used in the clarification of beers; they help to reduce the time required to sediment suspended yeast cells and ensure the clarity and colloidal stability of beer. Following an adventitious observation during dry-hopping experiments, we identified a fining activity associated with Saaz hops. Extracts of hop cones were subsequently shown to have the capacity to flocculate yeast and result in their sedimentation. This activity has since been identified in extracts of many different hop varieties and, significantly in spent hops, the co-product resulting from commercial extraction of hops with either CO_2 or ethanol. Here we illustrate the activity of the novel finings extracted from spent hops following CO_2 extraction of Galena hops. The sediments formed on fining were compact, relative to those obtained when commercial isinglass was used to fine the same beers. The hop extracts were also effective in reducing 90° haze in beers under conditions designed to mimic both cask ale (12°C) and lager (4°C) type applications.

The compounds responsible for the fining activity appear to be large (30 to 100kDa, or more) polyphenols. Analysis of the polyphenols using colourimetric tests, indicated the presence of proanthocyanidins. On acidic hydrolysis these generated cyanidin, which would be derived from a polymer composed of catechin and epicatechin subunits. The presence of these materials in spent hops offers the possibility to develop commercial products, with desirable fining properties, from an existing co-product stream. Furthermore, the finings are derived from a traditional ingredient of the brewing process.

Key words. Fining agent, hop polyphenolics, proanthocyanidin, brewing, colloidal stability.

1. Introduction

The vast majority of beers consumed worldwide are intended to be served clear, bright and free from visible haze. It is important that clarity is achieved in fresh beer and that this is retained through the required shelf-life, such that beers are delivered to the consumer in optimal condition. Haze can be considered as the 'absence of clarity' and is caused by the presence of small insoluble particles, typically in the μm size range, which scatter light, leading to the perception of haziness (*1*). There are several different sources of haze in beers, ranging from sporadic negative factors such as microbial infection, through precipitates of relatively insoluble salts such as calcium oxalate, to the presence of colloidal materials (e.g. proteins, carbohydrate polymers) which are only sparingly soluble in the beer matrix and therefore have a tendency to form insoluble aggregates of material, leading to colloidal instability (*2*). Of particular relevance are the complexes formed between polyphenols and so-called haze-sensitive proteins, which are responsible for chill-haze in beer (*3; 4; 5*) (haze material which comes out of solution when beers are refrigerated but which dissolves when returned to 20°C). Ensuring the colloidal stability of beer involves control of factors across the brewing process from raw materials selection, through brewhouse processing and into finished beer (*6*). The maturation period, post-fermentation, is particularly significant in this regard. Green beers contain residual yeast cells in suspension, a factor which in itself can lead to haziness of beers if steps are not taken to remove them. Traditionally, the clarity of lager beers has been ensured by cold-conditioning them for periods of several weeks, during which the insoluble materials settle out to form 'tank bottoms', leaving behind bright beer. In the modern day industry it is not desirable to incur the costs of chilling and storing large quantities of beer, hence rapid maturation processes have been developed to ensure colloidal stability of beers over much shorter

time-frames. These usually involve the use of process-aids designed to selectively remove haze materials, or their precursors. Examples would be the use of PVPP to lower the polyphenolic precursors of haze, or of tannic acids or silica gels to remove portions of the haze-sensitive proteins in beer (*7*). Physical separation processes such as centrifugation and filtration are also used to remove particulates, however, a combination of approaches is often required in order to ensure that the loading of solids in rough beer does not lead to blinding of filters, or shortening of effective filtration run times.

Fining agents are used to accelerate the rate of separation of suspended particles from beers and in general work by cross-linking haze particles to generate larger aggregates of material which settle out more rapidly to the bottom of a vessel. Finings have traditionally been used most prevalently in the production of cask ales in the UK. Since cask ales contain live yeast in contact with the product and undergo secondary fermentation in the trade, it is necessary to clarify such beers by the addition of fining materials, such that yeast settles out efficiently on completion of the secondary fermentation, to form a compact sediment. Furthermore, the use of finings to treat brewery-conditioned beers has become more widespread, as part of the overall strategy of ensuring colloidal stability using shorter process times (*8*).

The most widely encountered finings material in brewing is isinglass, a purified protein preparation extracted with dilute acid from the swim-bladders of certain species of tropical or sub-tropical fish. The active ingredient is almost pure collagen (*9*). Isinglass acts by cross-linking suspended yeast cells, via a charge-interaction, leading to their aggregation and subsequent sedimentation. Isinglass carries a net positive charge at beer pH's, facilitating its interaction with the negatively charged surface of yeast cells (*8; 10*). The use of isinglass is well established in certain applications and regions of the world, mainly because isinglass

91 combines several features attractive to brewers. In addition to the flocculation of yeast
92 cells, isinglass is also active against chill-haze (*8*), forms sediments which are compact
93 (leading to minimal beer losses and easier run-off of beers from above the sediment),
94 improves subsequent filterability of beers and has been noted to improve beer foam (*8*),
95 most likely due to the removal of foam negative lipid materials. However, one aspect which
96 limits the usage of isinglass is the fact that it originates from fish swim bladders, meaning
97 that products manufactured using isinglass are not suitable for vegans and are not
98 considered kosher. At one time it was proposed that residues of isinglass in beer might pose
99 a threat to fish allergy sufferers and that products would need to be labelled accordingly
100 (*11; 12*); however, this requirement did not become law in the EU because it was possible to
101 prove that residual isinglass levels in treated beers were extremely low, hence did not pose
102 a threat (*12*). Due to the aforementioned concerns, researchers have attempted to identify
103 alternative fining materials with which to treat beers and wines. These have included
104 evaluation of avian collagen and pea protein extract (*10*), the use of plant pectins (*13*), or of
105 bovine collagen (*14*). To date none of these approaches have been exploited commercially,
106 probably because none of the materials individually match the performance of isinglass in
107 all of its beneficial features. Thus isinglass remains the only finings material in widespread
108 brewing usage. In spite of this, there are other aspects to the use of isinglass which might be
109 improved upon when developing novel fining agents; isinglass is not an easy material to
110 disperse and mix into water. The UK is the only region with a significant market in wet
111 isinglass products whereas the remainder of the world principally uses dry isinglass powder
112 which must first be dispersed in water to the appropriate strength, prior to dosing into the
113 process. Once these solutions have been prepared they have a limited shelf-life and need to

be stored refrigerated (4-10°C) to retain activity; at higher temperatures collagen rapidly denatures to inactive gelatine (*1*).

In this paper we describe the characterisation of a novel fining material which has the potential to compete with isinglass in brewing applications. The novel finings is sourced from hops, and can therefore be promoted as a natural ingredient of the brewing process; although with conventional usage of hops in brewing the compounds believed to confer fining activity would not typically persist into the product. Furthermore, the active material is shown to be extractable from spent hops, the co-product generated through the extraction of hop resins using liquid CO_2 or ethanol.

2. Materials and methods

Chemicals

Analytical grade acetic acid, ferric ammonium sulfate, butanol, and High Performance Liquid Chromatography (HPLC) grade acetone, acetonitrile, and ethyl acetate were purchased from Fisher Scientific (Loughborough, UK).

Materials

Hops (variety *Galena*) that had previously been extracted by CO_2 were provided by Barth Innovations Ltd (Paddock Wood, Kent, UK). Liquid Isinglass, AllKleer A, was purchased from Murphy and Sons Ltd (Nottingham, UK). Dry yeast (Youngs, Bilston, Uk) was purchased from the Hop Shop (Plymouth, UK).

Preparation of hop extracts

Hops were extracted using either water, or 70% acetone in water. Aqueous extracts were prepared by mixing hops with reverse osmosis purified water (15mL/g hop) on a rollerboard for 30min at room temperature. The extract was then crudely filtered using muslin cloth, centrifuged at 7500rpm, 4°C, for 20min (Beckman, J2-21M, High Wycombe, UK) and the supernatant sequentially filtered (Whatman No.1, 3, 5, 602, purchased from Fisher Scientific; 0.45µm hydrophilic syringe filters, Sartorius Stedim, Germany) and stored at -18°C prior to use.

Extraction into 70% acetone in water involved mixing the hops with solvent (15mL/g hop) on a rollerboard for 2h at room temperature. The extract was then filtered using Whatman No.1 filter paper and the acetone removed by rotary evaporation (Buchi, Rotavapor II, Labortechnik AG, Flavil, Switzerland). The aqueous solution was then adjusted to pH 4 (InoLab pH level 1, Wissenschaftlich Technische Workstätte, Weilheim, Germany) using HCl and partitioned against an equal volume of ethyl acetate. The aqueous phase extract was

retained, rotary evaporated to remove any residual ethyl acetate and stored at -18°C prior to use.

Preparation of green beer

Youngs Economy Pilsner kits (Young's Home Brew, Bilston, UK) were purchased from the Hop Shop (Plymouth, UK) and fermented following the instructions on the label for 96h at 22°C. The green beer was then syphoned into a separate container to leave behind yeast that had already sedimented.

Sedimentation studies

Clarity of green beer was determined at OD 600nm using a UV-Vis spectrophotometer (Jenway, 6315, Stone, UK). Sedimentation volumes, were determined by mixing the hop extract with green beer in Imhoff cones (1L, VWR, Lutterworth, UK) and leaving them for 24h at 4°C. The beer was partially de-gassed by stirring before the application of the hop extract, or, Isinglass.

Size filtration studies

The extracts were sequentially size fractionated using reconstituted cellulose, molecular weight cut off filters, at 100, 50, 30, 10, and 3kDa, Amicon, Ultra-15 centrifugal filter units (Millipore, Watford, UK).

HPLC fractionation

Hop extract (aqueous, 1mL) was injected onto a 250 x 4.6mm cyano (CN) column (Phenomenex, Macclesfield, UK) and eluted isocractically with a binary solvent mixture of 30% acetonitrile and 70% 0.1% acetic acid at 0.6mL/min. The eluent was collected as separate 0.9mL fractions. The activity of each fraction was determined by the addition of 100µl of each fraction to 10mL of green beer and observing the sedimentation of yeast.

Acidic Butanol hydrolysis of the active hop extract

The active fraction obtained from HPLC fractionation of the hop extract (200µl) was added to 3mL of 5%HCl in butanol and 0.1mL of 2% ferric ammonium sulfate in 2N HCl. The mixture was subsequently heated to 100°C for 20min.

Mass spectral analysis of the acidic butanol hydrolysate

The butanol-HCl hydrolysed extract was introduced into the electrospray source of a Micromass LCZ mass spectrometer (Manchester, UK) operated in positive ion mode at 10µL/min using a syringe pump (Harvard Apparatus, Edenbridge, UK). The source temperature was 300°C, the desolvation gas was nitrogen at 350L/h with a cone voltage of 60V. Mass spectra were recorded over the mass range *m/z* 250-350 with a scan rate of 0.5Hz.

Haze analysis

Haze in beer was evaluated as total haze. Beer treated with finings was allowed to clear and was then transferred into 50mm dia. glass cuvettes and the amount of light scattering at a measuring angle of 90° determined using a turbidimeter (Norit Haffmans Vos Rota 90/25, Germany).

2 protocols were adopted, one at 12°C where the fining agents were applied and the haze measured (at 12°C) after 72h, with no further treatment. In the second, fining agents were added at 4°C and the haze was determined (at 4°C) before and during filtration through sequential 11, 3 and 0.45µm filters.

3. Results

Preliminary studies found that aqueous extracts of whole cone hop samples (variety Saaz) could induce the flocculation and sedimentation of yeast cells in green beer. Subsequently, the same activity was observed for extracts prepared from spent hops, a by-product resulting from the commercial extraction of hops with liquid CO_2. These latter extracts were prepared and used in further studies.

Sedimentation studies

The addition of the hop extract to green beer caused yeast to flocculate and sediment. This resulted in a decrease in the OD 600nm of the beer (Figure 1). As little as 5mL/L of the hop extract (equivalent to 0.33g of original hop material/L) was sufficient to induce a reduction in the OD 600nm. Dose rates beyond 20mL/L had little further impact on OD 600nm reduction. This level (20mL/L) was thus identified as the optimum dosage for the hop extract in this beer for use in sedimentation trials.

Sediment volumes produced by the hop extract were compared with the corresponding sediments resulting from the use of commercial Isinglass. Both solutions were added to green beer in proportion to their optimum dose (20mL/L as determined by OD 600nm studies). After 24h, all beers had clarified and the yeast sedimented. The sediment volumes were more compact at 22°C, for both the Isinglass and the hop extract, than at 4°C (Figure 2). The hop extract produced smaller sediment volumes than Isinglass, at comparable dose rates, at both temperatures. Addition of larger volumes of isinglass and the hop extract both resulted in larger aggregates of yeast and hence sediment volumes.

In addition to the flocculation and sedimentation of yeast in green beer, it was found that the hop extract could be used to sediment suspensions (1% w/v) of hydrated (75min) dry

yeast (data not shown). This was used as an assay to determine the presence or absence of flocculation activity in further studies.

Characterisation of the active compound

Aqueous and 70% acetone (aq) hop extracts were size-fractionated using molecular weight cut off filters mounted in centrifuge tubes. These separate the filtrates from the retentate, with molecules passing through the filters or not, depending on their molecular weight. A series of filters were used sequentially to profile the size range of compounds in the extract; the presence of active compounds in the various fractions generated was detected by observing the flocculation and sedimentation of re-hydrated yeast. The smaller the volume of an extract required to flocculate the re-suspended yeast the greater the activity.

The activity of the aqueous extract was not retained by 100, 50 or 30kDa filters, but, was detected in the 10kDa retentate. No activity passed through this filter into subsequent fractions. The active component in the aqueous extracts thus appears to be in the molecular weight range from 10 to 30kDa.

The acetone extract produced a range of active fractions. The 100kDa retentate induced yeast flocculation with 4mL of the retentate, indicating the presence of higher molecular weight material than the aqueous extract. The 50-100kDa and 30-50kDa fractions both required 8mL of the retentate to induce the flocculation response, whereas the 10-30kDa fraction required 15mL. No activity was detected in the 3-10kDa fraction, but, additional activity was detected in the <3kDa fraction, equivalent to that observed for the 100kDa retentate. The acetone extract appeared to mostly contain higher molecular weight polymers, with less and less activity in lower molecular weight fractions. The activity observed in the <3kDa fraction could indicate that there were 2 active components in the extract, one in the high molecular weight range and one at low molecular weights.

239 Alternatively, the <3kDa activity could be due to fragments of the higher molecular weight
240 material from the hop itself, or, formed during extraction.
241 An extract was also fractionated by HPLC using a cyano column. The active compound
242 eluted in a fraction with a retention time of between 7.5 to 9min. An active extract from
243 cyano fractionation was hydrolysed in acidic butanol, which is a test for the presence of
244 proanthocyanidins (*15*). The solution turned red, indicative of the presence of
245 proanthocyanidins in the active fraction. The visible spectrum of the solution showed a
246 maximum absorption at 552nm (Figure 3) typical of anthocyanidin formation from
247 proanthocyanidins during hydrolysis (*15*).
248 Visible spectra can help with the identification of anthocyanidins. There are however a
249 number of different anthocyanidins that could be produced by the breakdown of
250 proanthocyanidins and the observed spectra can also be affected by the solvent, or the pH
251 of the solvent, in which the spectra are recorded. To help with identification of the
252 anthocyanidin the extract hydrolysed with butanol/HCl was analysed by direct infusion mass
253 spectrometry. The resulting spectrum showed a major ion at m/z 287 (Figure 4), which is
254 consistent with the presence of either cyanidin or robinetinidin. Cyanidin and robinetinidin
255 have visible absorption maxima of 535 and 525nm respectively (*16*) which differ from those
256 observed, but, this may be a solvent-related difference. Robinetinidin can be produced by
257 the hydrolysis of quebracho tannin. Proanthocyanidins producing cyanidin are more
258 common (*17*), and derive from proanthocyanidins containing catechin and epicatechin as
259 the polymer sub units (*15*). Catechin and epicatechin are optical isomers of one another.
260 Based on these results, the active compound in our hop extracts appears to be a large
261 polymeric proanthocyanidin, comprised of catechin and epicatechin subunits.
262

Activity of hop extracts against haze in beer

Two protocols were used for the beer haze studies, designed to mimic the two major applications of isinglass finings. The first involved treating beer with either hop extracts or isinglass at 12°C and maintaining the sample at that temperature, to mimic a cask ale process. The haze was then measured at 12°C, 72h after treatment, to determine the maximum amount of haze that could be formed under these conditions. Relative to the unfined control, all treatments reduced the level of haze in the beer sample (Figure 5). The lowest hop extract treatment resulted in the lowest level of haze, with haze increasing with increasing doses of the extract. However, even when added at 8 times the optimal dose-rate the level of haze was not as high as that observed for the unfined sample.

The second protocol was designed to mimic a lager beer application. In these experiments the finings were applied at 4°C and the samples maintained at 4°C thereafter. The samples were analysed unfiltered (Table 1), and results showed that both isinglass and the hop extract had substantially reduced the level of haze in the sample. The levels of haze were greater for the samples with the hop extract, relative to those fined with isinglass and as with the cask style experiment the level of haze increased at doses of the hop extract in excess of the optimum. It was however clear from the data, that the levels of haze obtained with the lowest doses of isinglass and hop extract were only marginally different.

To further evaluate the extracts under lager-style process conditions the beers were filtered sequentially. The unfined control beer was typically more hazy than the fined samples throughout the filtration process. Following the 0.45µm filtration step, there were few real

differences between the fining treatments, with substantial reductions in haze for both isinglass and the hop extract.

4. Discussion

Proanthocyanidins have been reported in hops, but, typically the size is significantly smaller than those found in the current study. Proanthocyanidins reported by Li and Deinzer (*18*) contain only a few polymer subunits. Those reported by Taylor et al. (*19*) were larger proanthocyanidins with up to 20 sub-units (average 7.8) which would equate to a molecular weight of around 6000Da. These are still significantly smaller than the proanthocyanidin molecular weight ranges suggested by our molecular weight fractionation studies, which imply the presence of polymers 10 times that size, or, even larger.

Rodrigues et al. (*20*) reported that proanthocyanidins are readily absorbed onto yeast lees in wine. The size of the polymers observed in the hop extracts appear to be sufficiently large to stick to not just one yeast cell but to join cells together and flocculate them. This results in the observed fining activity. Proanthocyanidins are also known to act as antioxidants, chelate metal ions and bind with proteins (*3; 15; 21*), further activities that may be beneficial as brewing processing aids and would also be consistent with the reduction in haze observed during the fining experiments. Considering the likely proanthocyanidin nature of the active material, it is also apparent that over-addition of the finings has the potential to induce haze in samples. This will depend on the levels of haze-sensitive proteins present, and thus on the stabilisation regime a beer has been subjected to. In this particular example (Figure 5), it was possible to dose the extract at up to 8 times the determined optimal dose, without increasing haze relative to the unfined control. Thus, by adopting customary procedures for optimising the dose rates of finings the potential negative consequenses of over-dosing would easily be avoided.

The use of polyphenol-rich extracts in the brewhouse has been reported as one potential route to improve the colloidal and flavour stability of beers (*22; 23*). Jelinek et al. (*22*)

312 reported a reduction in haze-active prolamines in beer when brewing (kettle addition) with
313 the addition of residual material from the processing of Saaz hops into T45 pellets. This
314 fraction contained almost 10% w/w total polyphenols. However, the possibility of using such
315 spent residues as a source of fining activity has not previously been reported, presumably
316 because addition in the brewhouse results in the degradation and removal (as trub) of large
317 polymeric proanthocyanidins.
318 Experiments using successive filtration post-fining (data in Table 5) were designed to
319 evaluate whether an aqueous extract of spent hops could match the performance of
320 isinglass in terms of haze reduction in a lager-type application (where beers would typically
321 be filtered post-fining). We have identified that acetone extracts of spent hops match and
322 even exceed isinglass in this regard (*24*). However, aqueous conditions appear to extract a
323 wider range of material from spent hops and are more prone to inducing additional haze if
324 not used at the optimal dose rate. Here it was shown that filtration post-fining enabled the
325 aqueous hop extract to broadly match the performance of isinglass in terms of total haze of
326 the filtered beers at 4°C.
327 Many of the attributes of the hop extracts make them suitable for use in the brewing
328 industry. The sediments formed following fining action are compact and not fluffy, the
329 sedimentation rate is fast (hence the dose sediment curve in Figure 1 was determined 2h
330 after extract addition) and they are of plant origin without need of chemical modification.
331 The results presented in this paper were obtained with extracts derived from the hop
332 variety Galena. However, hop extracts of other varieties have also been shown to be active
333 flocculants (*24*). The original activity was observed during dry hopping experiments where
334 the levels of hop addition were within typical brewing ranges. The use of a hop extract,
335 allows for an efficient use of previously extracted material, at less than 0.5g of CO_2-

336 extracted spent hops per litre. This may ultimately enable a wider use of these extracts
337 within the brewing industry.
338

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Table 1: Total haze (90° scatter) at 4°C for beers treated with isinglass or aqueous hop extract (fold dosage relative to optimum) and sequentially filtered.

Treatment	Unfiltered	11µm filter	3µm filter	0.45µm filter
Unfined	15.06	9.48	1.80	1.04
0.5x Isinglass	2.86	1.85	1.09	0.27
1x Isinglass	1.65	1.04	0.71	0.20
0.5x Hop Extract	3.11	2.88	1.15	0.22
1x Hop Extract	3.77	2.95	1.12	0.89
4x Hop extract	4.50	3.70	2.15	0.23

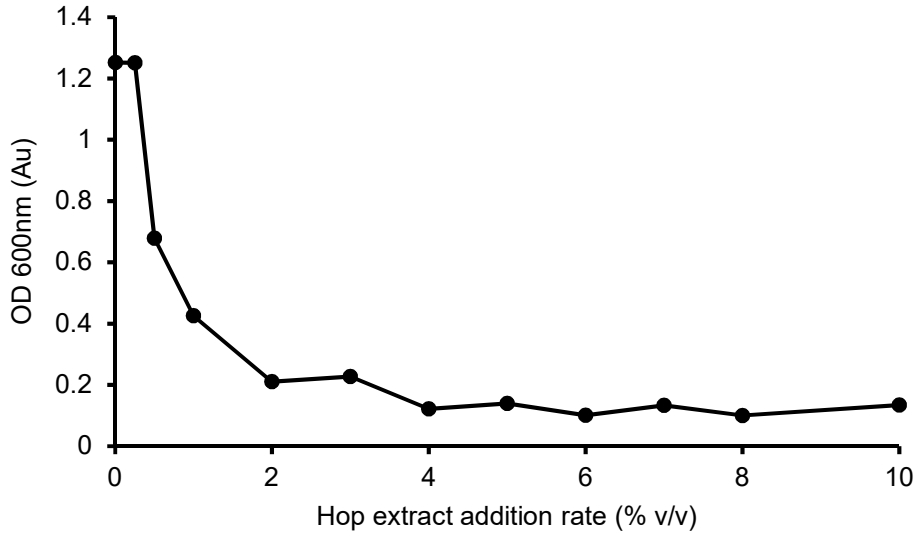

Figure 1: OD 600nm of green beer 2h after treatment with varying amounts of hop extract at 4°C.

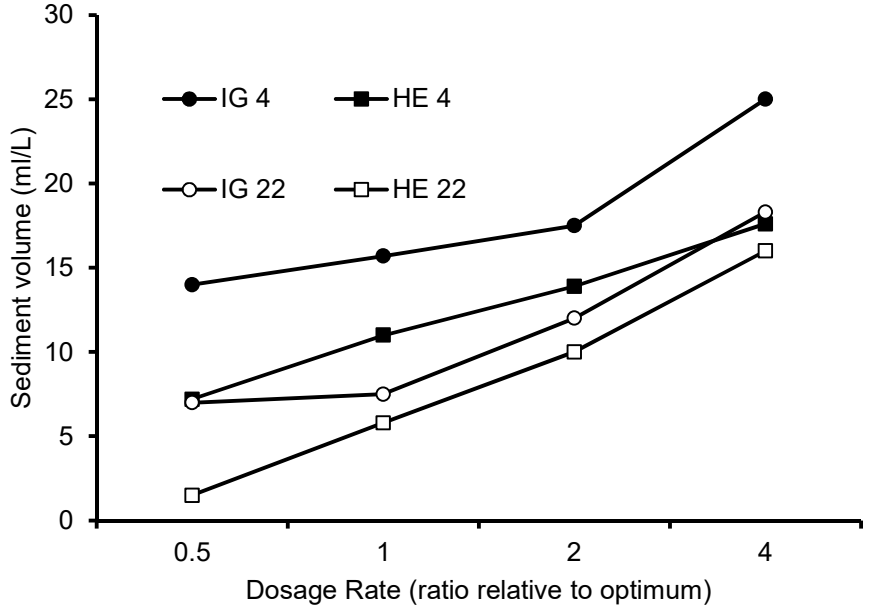

Figure 2: Sediment volumes (ml/L of beer) formed by the addition of varying amounts of isinglass (IG), or hop extract (HE) to green beer at 4°C (solid markers), or 22°C (open markers).

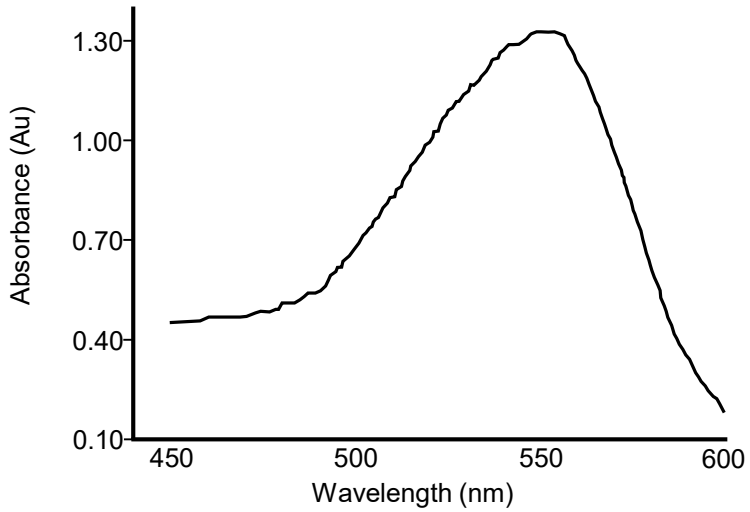

Figure 3: Visible absorbance spectrum of the butanol/HCl-hydrolysed hop extract

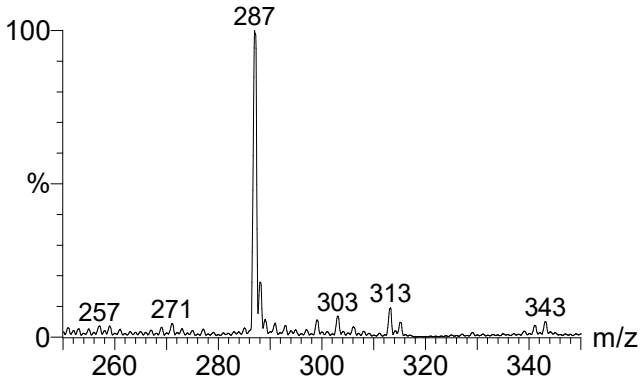

Figure 4: Mass spectrum of the butanol/HCl-hydrolysed hop extract.

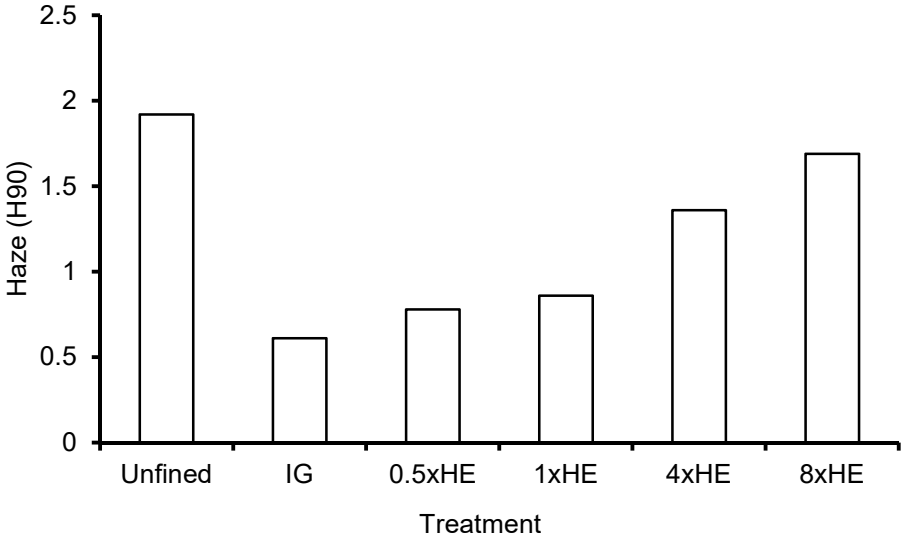

Figure 5: Total haze (90° scatter) at 12°C for green beer treated with isinglass (IG), varying amount of aqueous hop extract (HE, fold dosage relative to optimum), or unfined 72h after treatment.