

A novel beer fining agent extracted from hops

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Abstract

On a shelf or in a bar, beers are intended to be served clear, bright and free from visible haze. Industrialization and novel brewing technologies have pushed brewers to develop beers that have the required shelf-life in terms of product quality, flavour, and appearance. During beer brewing, clarification post-fermentation is an important step to remove the majority of haze material prior to further steps such as filtration and bottling.

Fining agents are used to accelerate sedimentation of yeast and haze particles within beer, in order to reduce the time required for this haze material to settle. The focus of this study was towards the development of a novel fining agent extracted from spent hops. Spent hops contain significant amounts of polyphenols. Because of the high affinity of some phenolics with haze active proteins, they represent a good candidate for the flocculation of beer haze particles. As it is also sourced from hops, this product has great potential to replace other animal-based products such as isinglass finings.

The extraction procedure was firstly assessed in order to define the optimal protocol to extract the active component from the plant material. Extraction using 70% acetone(aq.) yielded the highest amount of proanthocyanidin polyphenols and also produced the best performance in beer fining. Then, the application of this extract to a broad variety of beers from laboratory to brewery scale was investigated. The hop extract has been found to effectively fine beers produced with a range of yeast strains and was able to remove efficiently haze from ales and lager beers when introduced at the individually determined optimal dose range. Beneficial impacts in fining performance were observed when using the hop extract in combination with silicate

auxiliary finings. An opposite effect was found when using the novel finings with stout beers, as haze was created during the fining reaction. Post-fining laboratory filtration trials using cellulose filters showed that the hop extract, when combined with auxiliary finings, improved the filterability of finished beer relative to isinglass fined beer.

The characterisation of the active material was then conducted, with a particular focus on the molecular weight and size range. Earlier research by Linforth and colleagues demonstrated the presence of polyphenolic material, more specifically proanthocyanidins. These compounds can be present as small monomers and dimers or as long polymeric assemblies of catechin and epicatechin. The lack of reference standards and the complexity of the molecular distribution present analytical challenges to define the structure and the molecular weight of these compounds. A novel method was developed using Analytical Ultracentrifugation to identify the molecular weight distribution of hop proanthocyanidins in the active fining fractions. Sedimentation velocity experiments revealed a heterogeneous fraction with discontinuous peaks. Sedimentation equilibrium experiments allowed the assignments of molecular weights, with compounds found in the hop extracts, up to 100 kDa. Major differences were observed comparing hop extracts from different varieties in both proanthocyanidin content and beer fining efficiency. The presence of high molecular weight polymers was shown to strongly correlate with superior fining activity (assessed in terms of beer haze removal and sediment formation).

Impacts of the hop finings addition on finished beer parameters were also investigated. No major differences were identified using hop extract finings compared to other fining strategies when observing the beer volatile profile through

gas chromatography after an accelerated ageing study. Significant reduction ($p < 0.05$) in iron concentrations was found when using the hop extract in lager beers compared to isinglass treated beer and an unfinned control. Improved oxidative stability was assessed by electron spin resonance spectroscopy for lager beers treated with the hop extract. Introducing this fining agent during the brewing process may thus also benefit the brewer for desirable aspects such as shelf-life and quality.

Acknowledgements

**To my mom, I would never
have been where I am right
now without you.**

List of abbreviations and Symbols

α	Alpha
ACN	Acetonitrile
AUC	Analytical Ultracentrifuge
aq.	Aqueous
Aux	Auxiliary finings
A₆₀₀	Optical density measured at 600 nanometres
β	Beta
β-CD	Beta cyclodextrin
C	Control
CN	Cyano
CO₂	Carbon dioxide
D	Diffusion coefficient
Da	Dalton(s)
DP	Degree of polymerisation
DMAC	Dimethylacetamide
ESR	Electron spin resonance
G	Relative centrifugal force
γ	Gamma
GA	Gallic acid
GAE	Gallic acid equivalent
GC	Gas Chromatography
GPC	Gel permeation chromatography
HCl	Hydrochloric acid
HE	Hop extract
HPLC	High performance liquid chromatography
H₂₅	Haze measured at an angle of 25 degrees
H₉₀	Haze measured at an angle of 90 degrees
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IG	Isinglass
LC	Liquid chromatography
LM_w	Low molecular weight
MALDI	Matrix Assisted Laser Desorption Ionisation
MD	Maltodextrin
MeOH	Methanol
MS	Mass spectrophotometer
M_w	Molecular weight
Na	Avogadro's number
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
P	Peak (numbered)
PA	Proanthocyanidin(s)
ppb	Parts per billion
PVPP	Polyvinylpolypyrrolidone
PVP	Polyvinylpyrrolidone

ρ	Density
R	Gas constant
rpm	Revolutions per minute
S	Sedimentation coefficient
SE	Sedimentation equilibrium
SPE	Solid phase extraction
SV	Sedimentation velocity
SiOH	Silanol
TOF	Time of flight
T45	Type 45
T90	Type 90
T150	Intensity measured at 150 minutes
UV	Ultraviolet
μ	Dynamic viscosity
%wt	Weight ratio

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Chapter 1: Introduction

Beer is nowadays the world's most widely consumed and probably the oldest alcoholic beverage, with global production in the region of 2 billion hL annually. Its production consists of a fermentation with yeast of carbohydrates derived from cereals, which also result in a natural carbonation of the beverage (Briggs et al., 2004). The majority of the beers consumed worldwide are intended to be served clear, bright and free from visible haze. Fining agents are used during the brewing process to decrease the time required to settle yeast cells in suspension and improves the colloidal stability of beer (Linforth et al., 2015). The current and most-known fining agent is isinglass: a by-product from fish swim bladders. As it is an animal-based product, there can be some health concerns for the consumer market for allergies or labelling beer as a vegan product. Alternatives to isinglass are investigated for the entire beverage market (Walker et al., 2007).

Hops in beer are mainly used in brewing to give beer its pleasant flavour and aroma (Briggs et al., 2004). Novel technologies, industrialization and the demand of brewers for more stable and consistent products have pushed hop producers to sell hops as concentrated extracts, or oils. After the chemical extraction of hop acids, oils, waxes and lipids, the plant material is left as spent hops, usually sold for the animal food market. Early work by Linforth et al., (2015) described the use of the spent hop material for green beer clarification. These materials are often left with a high polyphenolic content, which can be easily extracted in a liquid phase. Linforth et al., (2015) described the material with the ability to flocculate suspended yeast cells and beer proteins, which involved a clearer beer after fining reaction. The extract from spent hops has also the potential to capture the current market for tannic

acid and isinglass finings, with the obvious advantage in brewing applications that it is sourced from hops, a natural ingredient of the brewing process.

1.1 Beer brewing

Brewing consists of the conversion of sugars from cereals solubilised into water, into alcohol and carbon dioxide. This conversion occurs with the addition of yeast, to perform the fermentation reaction. Beer is typically flavoured with hops for their ability to offer bitterness and enjoyable flavour and to prolong shelf-life (Briggs et al., 2004). Five main steps represents beer brewing. The solubilisation of the cereal starches into water called mashing, the wort boiling, the fermentation, the beer clarification and beer conditioning.

1.1.1 Mashing and lautering

Mashing represents the process where the ground grains (usually malted barley, with auxiliary adjuncts such as rice, corn, wheat, rye...) are mixed with a fixed amount of hot water or so-called brewing liquor. In large modern breweries the process usually takes place in a mash conversion vessel, which is typically steam jacketed (for temperature control) and equipped with an agitator for continuous stirring during the conversion of the cereal starch units into soluble sugars through enzymatic degradation. The aim is to bring the mash to certain temperatures for malt enzymes to perform the enzymatic degradation of starches. The malt naturally contains diastatic enzymes (α -amylase and β -amylase, limit dextrinase and amyloglucosidase) which are able to break down the starch into sugar units depending on the temperature. Three main enzymatic rests are defined as specific temperature ranges where the maximum enzymatic activity is recorded. The β -glucanase rest (33-55°C) is where the relevant enzyme systems breaks down β -linkages in β -glucans, allowing the starch to be efficiently extracted from the

endosperm cell walls. The proteolytic rest (50-59°C) degrades proteins, releasing small proteins and free amino acids to the water. Amylase rests (63-71°C) are the most important steps of the mashing process. It represents the part where both α and β -amylase are breaking down starch entities into sugars. The β -amylase rest (63-66°C) cleaves two glucose units at a time from the non-reducing chain end of starches, producing maltose. The α -amylase rest (66-71°C) takes the larger starch units ('dextrins') and cuts them into smaller pieces. Lower temperature will favour the β -amylase for a crisper and less malty beer, and higher temperatures will allow a more efficient α -amylase rest for a fuller body and more malty beer. The temperature is then raised to (75-78°C) for the mashing out step, in order to decrease the wort viscosity for an easier transfer to further steps (Hornsey, 1999). Wort is usually characterised by its strength, or the amount of solids that are dissolved relative to the volume they are dispersed in. The specific gravity is used as a measure to determine the concentration of sugars present in the wort (Briggs et al., 2004).

The mash is then transferred into another vessel called the lauter tun. The liquid from the mash is separated from the solid husk material during this process by recirculation of the wort inside the vessel, creating a natural filter bed from the spent grains (Willaert, 2006). Extra 'sparge' water is also introduced at the top of the vessel which dilutes the original sweet wort to a lower gravity, but also extracts remaining sugars from the husk bed and thus improves extract recovery efficiency.

1.1.2 Boiling and hop addition

The sweet wort is then brought to boiling temperature in a kettle. The boiling process is performed to sterilize the wort in order to eliminate any bacteria or wild yeast present in the wort. It also denatures all enzymes to stop any enzymatic activity. A vigorous boil also coagulates the proteins solubilised earlier during the mashing

process. They form agglomerates which settle down at the end of the boil (hot trub). At varying stages of the boil, the hops are added to the wort. The addition of hops into beer is responsible for giving the beverage its bitter taste and aroma (Anioł et al., 2008). They add bitter resins and antibiotic resins to counteract the malty flavour from the grains. Bittering hops will be added during kettle-up or at the start of the boil, whereas aroma hops are added 10-15 min before the end of the boil, to retain the volatile aromatic oils. Wort is then cooled down to fermentation temperature (7 to 23°C) using heat exchangers to perform a quick decrease in the liquid temperature. Wort cooling also causes further insoluble material to separate out, called cold break. As for trub, cold break consists of proteins agglomerates combined with other sparingly soluble material including polyphenols, lipid, hop resins and β -glucans. It is important to perform sufficient protein agglomeration during the boil to improve beer stability and to reduce haziness of the final product. The sterilized wort is then transferred to the fermentation vessel, with the addition of yeast and some air or oxygen to assist with yeast cell growth at the start of fermentation

1.1.3 Fermentation and maturation

Fermentation can be performed in many different types of vessel (Boulton et al., 2001). Fermentation converts the sweet sugary wort into beer, using yeast (Lodolo et al., 2008). Fermentation is initiated by addition (yeast pitching) of a selected yeast strain to the cooled hopped wort (Briggs et al., 2004). The cooled wort has now the perfect environment for the yeast to grow and metabolise. Oxygen is rapidly consumed and gives way to anaerobic fermentation, forming ethanol and carbon dioxide. Brewing yeast strains are divided into two major classes, dependent on their fermentative characteristics, particularly whether the yeast rise to the top during fermentation or settle to the bottom of the fermenter. Ale yeast (*Saccharomyces*

cerevisiae) is a yeast species performing top fermentation, and lager yeast (*Saccharomyces pastorianus*) will perform a bottom fermentation (Russel, 2006). Ale yeasts are usually pitched at about 16°C and fermentation is performed between 15 and 20°C. Lager yeasts are pitched at cooler temperatures (7 to 10°C) and fermentations are usually carried out between 10 and 15°C (Boulton, 2010).

Yeast added will use the oxygen present in the wort to perform aerobic respiration. This reaction will form carbon dioxide and pyruvates. Once the oxygen has been consumed in the wort, anaerobic respiration will be carried out by the yeast to transform the pyruvates into ethanol. After fermentation, the suspended particles in beer (yeast particles and proteinaceous) need to be removed (Morris, 1986).

1.1.4 Clarification

Beer can be either matured or clarified at this stage, depending on the brewer specifications. Fermented beer can mature from several days, to several years (Russel, 2006), in order to clarify naturally, but also to allow the removal of undesirable sulfur compounds and aldehydes in beer by yeast. Lagering is a typical example of maturation where lager beer is stored at cellar temperature for a period varying from 1 to 6 months (Briggs et al., 2004). Yeast can also sediment naturally through gravitational force. The yeast sedimentation rate can also be promoted by the addition of clarification aids (fining agents), to green beer (Briggs et al., 2004). They are also added to unfiltered beer to remove all haze-active particles derived from the raw materials (Linforth et al., 2015). Beer can also be centrifuged, at high speeds in order to eliminate any traces of raw materials, debris and haze-active material. High-capacity breweries used the combination of centrifugation and high-grade filtration prior to beer conditioning. Beer filtration can be carried out using sheet filters, but can be supplemented with filter aids, such as slurried powders which

form incompressible and highly porous filter beds (Freeman, 2006). The most common examples are kieselguhr or diatomaceous earth (Martinovic et al., 2006). It is composed of siliceous sedimentary rock, or fossilized remains of freshwater life. Cross-flow membrane filtration is now a commonly employed alternative to kieselguhr filters in beer clarification. Whilst the capital cost is higher, such systems avoid the generation of spent kieselguhr. Novel technologies using rotating and vibrating filters have also been developed for beer filtration (Fillaudeau et al., 2007).

1.1.5 Conditioning and bottling

The filtered beer will then be transferred to the conditioning tank (bright beer tank) prior to packaging. A force-carbonation is a common practice to re-inject CO₂ usually performed in the sealed conditioning tank. In home-brewing and traditional breweries, a secondary fermentation is performed in the bottle by the addition of sugar in the beer prior to bottling. This second fermentation will create carbon dioxide, which will be trapped and solubilised in the beer prior to consumption. This addition of gas is performed to achieve two main characteristics: the fizziness and mouthfeel of the desired beverage, but also to minimise the oxygen content within the beer. The concentration of CO₂ is carefully manipulated to ensure that beer consumers can drink a consistent product (Briggs et al., 2004).

Beer is usually sold in various packages such as kegs, cans, casks, barrels, or bottles. Isinglass can also be introduced prior to keg filling in order to achieve better clarification within the keg, leaving the flocs formed at the bottom (Baxter et al., 2007). The main challenge for packaging operation is the prevention of air getting into beer. As it is filled from the bottling line pump into the container, it is important that minimal air contact should occur between beer and oxygen once again. Because the temperature of beer on filling is usually around 0°C, the beer temperature rise

during filling must be also controlled to keep carbon dioxide in solution (Briggs et al., 2004).

Pasteurization is also a common practice post packaging to ensure minimal presence of viable micro-organisms that will deteriorate the beverage. This is usually performed by passing beer through heat (53 to 67°C) for a short period of time (56 to 0.56 min respectively) (Briggs et al., 2004).

1.2 Beer haze

The occurrence of turbidity, or haze in a beverage takes place when a beam of light passing through a sample is scattered or deflected by particulate matter in suspension (Siebert, 2009). The scattered light is visible, and consequently, the sample appears hazy.

1.2.1 Context

The majority of beers produced worldwide are intended to be sold at a global market, and produced according to the principles of economy of scale (Bamforth, 1999; Freeman, 2006). This has pushed brewers to produce products with improved and longer shelf lives. When commercial brewing operations grew during the latter part of the 18th century, factors such as clarity and stability became more important, for wine as well as beer. In this market, a beer, and even a fresh beer must be served bright and clear. Haze has a negative impact on product appearance. Colloidal stability is achieved when beer can be stored for several months at 25°C, without displaying any signs of precipitation or change in composition. (Mastanjević et al., 2018)

1.2.2 Types of beer haze

By definition the term chill haze is the haze which forms when beer is cooled to low temperatures (0 to 4°C) and which redissolves when the beer is heated up to 20°C or more. Particles are pulled out of solution because of the temperature change, which creates a more turbid liquid environment (Asano et al., 1984; Ye et al., 2015). Permanent haze is described by haze which remains stable in the drink at 20°C or more (Moll, 1987). This type of haze can be also related to ageing and storage time. Beer haze was categorised by Glenister (1975) dividing haze into three major groups. The native particles, which originated from the beer by chemical precipitation or flocculation. These are mainly represented by all chemicals present in beer. The second group is represented by process particles which came from materials (filter aids, fining agents, and other added particles during brewing process) and thirdly foreign particles, which came into beer as accidental contaminants such as paper labels or glass. Bamforth (1999) proposed that beer haze is composed of visible haze as bits that contains proteins and polysaccharides. He also described an invisible haze (or called pseudo-haze) for small particles less than 0.1 micron in size that can cause levels of light scatter.

The majority of beer haze is derived from proteins and peptides present from raw materials of brewing such as grains or adjuncts. They display molecular weights from 1000 Da to 40 kDa. These proteins are mostly composed of proline amino acids, which display a high affinity to polyphenols (Asano et al., 1984). Residual starches, polysaccharides from cereal adjuncts, oxalates from calcium deficient worts, carbohydrates, metal ions and polyphenols have also been responsible for the formation of beer haze (Aron et al., 2010; Jurić et al., 2015; Siebert, 2009).

1.2.3 Beer haze analysis

Microscopic haze identification can be used to detect particles responsible for haze with specific shapes differentiated from yeast and other bacterial cells (Glenister, 1971). Steiner et al., (2010) used different microscopic techniques (light, fluorescence, and polarisation) to characterize different origins of beer haze. The use of stains is also a common practice used in microscopy to show the presence of specific compounds and has been very useful in brewing and haze studies (Siebert, 2009). Methylene Blue is used for staining dead yeast cells in beer, yielding useful information on yeast viability. Eosin Yellow is able to stain in bright pink any proteinaceous material. Other staining agents such as thionine and iodine can be used to detect the presence of residual starches, precipitated material out of polysaccharides and also PVPP (Steiner et al., 2010). These agents have been proven very useful as a quick identification of haze characterisation in beverages.

Nephelometry is an analytical technique based on measuring the light intensity response distributed by hazy solutions (Chapon, 1993). Haze can also be measured through analytical instruments. A turbidimeter consists of a source of light, a lens, a sample cell and one or several detectors. As a hazy sample scatters light, the light detectors are measuring the resulting light transferred at some angle to the incident light beam. In beer, the resulting light is usually measured at two different angles. H25 evaluates the light observed at an angle of 25 degrees, representing large particles such as yeast and micro-organisms whereas H90 represents the haze measured for the scattered light at an angle of 90 degrees for smaller particles and high molecular substances. Beer haze can be expressed, for example, using ASBC or EBC units within ranges described in Table 1.1.

Table 1.1 Beer haze scale expressed using EBC (European brewery convention) or ASBC (American Society of Brewing Chemist) units.

Visual Description	EBC units	ASBC units
Star Bright	0 to 0.5	0 to 34.5
Bright	0.5 to 1.0	34.5 to 69
Slightly opalescent	1.0 to 2.0	69 to 138
Opalescent	2.0 to 4.0	138 to 276
hazy	4.0 to 8.0	276 to 552
Very Hazy	8.0 and more	552 and more

1.2.4 Sedimentation of particles

A non-charged spherical particle will sediments downward if its density is greater than the liquid matrix density, and cream if the density is lower (Spasic, 2018). During sedimentation, the particle will reach a constant velocity, expressed through the Stokes law relationship:

$$v = \frac{2r^2 g (\rho_2 - \rho_1)}{9\mu}$$

Equation 1.1 Sedimentation velocity of a particle due to the gravitational force.

In this equation, r represents the particle diameter (m), ρ_2 the particle density, ρ_1 the density of the liquid (kg/m³), g is the acceleration due to gravity (m/s²) and μ the dynamic viscosity of the fluid containing the particles (Pa.s). This law indicates two viable strategies to improve beer clarification. The gravitational term can be increased by using centrifugal force, and diameter of particles can also be increased by the use of fining agents (Ward, 1983). Centrifuges are very efficient for yeast removal but less performant on the fine haze-active particles that fining agents are able to clarify (Ward, 1983).

The process of cold maturation represents the traditional process to clarify beer. Cold temperature boosts the formation and sedimentation of insoluble complexes. It also

decreases the solubility of some hazy materials present and reduces the energy from ambient heat that keeps the particles in suspension (Siebert, 2009). The effect of cold maturation can also be improved by the use of fining agents (Siebert, 2009). They assist the haze formation by precipitation of substances that, if not taken out of the beer matrix, can form a later haze in the beverage. Part of beer fining strategy is a post-fermentation clarification process used in brewing to increase green beer colloidal stability, removing microbiological particles, i.e. yeast cells, and non-microbiological particles, e.g. proteins in wort (Leather et al., 1997). Fining agents are generally electrostatically charged molecules, which can interact with charged particles and allow them to precipitate and to settle out. A large number of compounds can be used as fining agents, including proteins, polyphenols, synthetic polymers and others (Siebert, 2009).

Benefits of finings for brewers would be an improved yeast quality, rapid tank turn around, shorter filtration time and longer filter-bed life, reduced requirement for capital investment, and obviously a more consistent, reliable and lower overall production cost (Hornsey, 1999).

1.3 Fining agents

Finings are widely used in brewing, winemaking and the spirits industry as a clarification aid. The diversity of fining agents available offers choice and selectivity between different product origin, efficiency, and results in haze removal.

1.3.1 Interaction between finings and particles

Currently, the mechanism of the fining reaction is quite complex and not well-known. Ribereau-Gayon (1977) stated that the mechanism of fining for wine could be divided into two steps. First, the flocculation, mainly caused by the interaction

between wine tannins and the fining proteins. These tannins-proteins are able to create macrostructures such as visible flocs because of their high affinity. Secondly, the clarification process, where the formed aggregates can sediment due to their weight, resulting a clearer beverage.

In 1983, Salgues et al., went further to explain the fining mechanism. The colloidal stability will be dependent on the electrical charge of the selected fining agent and the affinity with the solvent, i.e. water. The process would be a combination of many successive reactions between the colloids of the solution and the fining agent: attraction, repulsion, and dehydration of particles that could have a dimension less than 0.1 μm .

Siebert in 1996 modelled the fining mechanism between proteins and polyphenols. The study demonstrated the dependence of both compounds and their stoichiometry for an efficient clarification, as well as an undesirable increased haze for an excess in concentration of one of those substances. In 1997, Kawamoto et al., thought that haze proteins would be denatured. The formation of tannins-proteins complexes would be achieved by a simple adsorption of the tannins without any dehydration. These complexes would be negatively charged hydrophobic stable colloids, which in the presence of cations, could then bind and form flocs. Ghanem et al., (2017) also stated that electrostatic interaction, chemical bond formation and absorption/adsorption are the main mechanisms of the fining reaction. Particles of opposite charge to the fining agent introduced are induced to coalesce, and form larger aggregates.

1.3.2 Classification

1.3.2.1 Protein based finings

These typically represent the class of fining agents made from animal proteins. Currently, the most commonly used protein-based fining agent is isinglass. It is well known for its ability to flocculate yeast and proteins, producing a much clearer beer after the fermentation (Leiper et al., 2002). Studies on the residual concentration of isinglass in finished beer has been performed (Baxter et al., 2007) and shown that only traces were present in packaged beer. This has raised important concerns as isinglass can cause potential human allergies and also will confer the beer to be not fully-vegetarian. It is currently sold under various forms such as paste, fine powder or liquid solution. Isinglass collagen is present as a rod-like triple helical structured molecule. Light scattering and physico-chemical analysis has shown that the molecular weight of isinglass is about 300 kDa (Leach, 1967). Isinglass denatures at about 29°C to create a random form of gelatin, not as effective at fining as the original isinglass (Hickman et al., 2000). Gelatin from skins of bovines, fish and pigs can also be used traditionally as a fining agent in small wineries, to reduce polyphenol concentration in red wine (Maury et al., 2001).

Albumins from egg white are also a common fining agent, mostly used in wine making (Boulton et al., 1999). It is used in highly tannic wines and barrel aged wines in order to remove harsh tannins for their excessive astringency and bitterness (Yokotsuka et al., 1995). As they are also animal collagen protein they may also be allergenic and methods are now proposed to detect residual egg white proteins in fined red wines (Tolin et al., 2012).

Casein represents a wide class of low molecular weight proteins derived from milk. It is often used as potassium caseinate in wine fining to prevent oxidation and remove phenolic compounds (Cosme et al., 2008).

1.3.2.2 Kettle finings

Kettle (or copper) finings are added during wort boiling to enhance agglomeration of haze-active proteins present (Prajapati et al., 2014). This class of finings are composed of carrageenans (typically kappa carrageenan), a long polysaccharide composed of sulphated galactose monomers refined from red seaweed (Necas et al., 2013). These molecules have a negative charge when solubilised in wort, which allow them to bind to positively charged proteins to form macroscopic flocs (Morris, 1986). It can be sold as semi refined (Irish moss, *Chondrus crispus*) or pure carrageenan (Ranken, 1929).

1.3.2.3 Mineral based finings

Auxiliary finings are also widely used in the beer and wine fining industry (Mitchell et al., 2005; Morris, 1986; Vincenzi et al., 2015). They can be present as an acidified polysaccharide or a polysilicate solution. Isinglass and silica gel in combination have been used for many years to improve beer clarity and beer stability (Leiper et al., 2002; Freeman, 2006). Silica gels can be available in various forms depending on their water content. Hydrogels contains 70% water whereas hydrated xerogel contains 35% and xerogel only 5%. All products have a similar porous structure and their surface is covered with silanol groups (SiOH) (Leiper et al., 2002). These units have a strong affinity for proline proteins, responsible for haze in beer when bound to polyphenols.

Bentonite, clay composed of hydrated alumina silicates has also been employed for the prevention of white wine protein hazing (Vincenzi et al., 2015). Bentonite acts

the same way as auxiliary finings does; the net negative charge from the silicates are highly reacting with positive charged wine proteins. However, Vincenzi et al., (2015) also demonstrated the loss of aroma in wine using bentonite finings with a significant removal of compounds such as ethyl esters and fatty acids.

1.3.2.4 Synthetic finings

Polyvinylpolypyrrolidone, (PVPP), is a synthetic polymer fining agent that is insoluble in water. It derives from a cross linked modification of polyvinylpyrrolidone (PVP), a polymer composed of N-vinylpyrrolidone units (Dadic et al., 1983). It is presented as a white powder and is used in beverages, spirits and beer as a clarification agent to improve beer clarity or filtration. This compound has a strong affinity for complexing polyphenols (Doner et al., 1993; Laborde et al., 2006; Magalhães et al., 2010; Mitchell et al., 2005). PVPP is mainly used to eliminate tannins, cinnamic acids or quinones. It is acting through hydrogen-bond formation between its carbonyl groups and hydrogen atoms from the hydroxyl group of polyphenols (Markovic et al., 2003). It is necessary to prepare a slurry of the material in warm water prior to beer introduction for the material to swell. Mitchell et al., (2005) has compared the proanthocyanidins level of ales treated with PVPP, silica xerogel and PVP-silica based product and concluded that PVPP was most efficient at binding polyphenols such as proanthocyanidins. It is mainly used as a slurry introduced in beer to create a filter bed during filtration for polyphenol removal (Freeman, 2006).

1.3.2.5 Vegan alternatives

Alternatives from vegetables and plants are expanding in order to replace animal-based fining products (Walker et al., 2007). Pea proteins can be an efficient replacement to caseinate proteins in white wine fining. They showed an efficient

removal of phenolic compounds as well as no sensorial differences were found in wine when using this extract (Cosme et al., 2012). Other plant alternatives have been developed from wheat, lentil, grape seed and soybean (Gazzola et al., 2017; Granato et al., 2014). The use of enzymes has also been researched in beer or wine for their ability to remove haze precursors. Papain is a protease enzyme extracted from papaya. It is able to digest residual proteins after fermentation to release free amino acids (Fukal, 1984), which would improve final beer colloidal stability.

Polyphenols such as tannic acid can also be added in multiple steps of the brewing process to improve beer colloidal stability. Extracted from gallnuts or sumac leaves, it can also enhance shelf-life and flavour stability of finished beer (Freeman, 2006; Formanek, 2015).

1.4 Hops and spent hops

Because of the high nutritional value of spent malt grains and hop, different food applications have been proposed to manufacture food products for animal or the human consumption (Davies et al., 1927; Lynch et al., 2016). Hop processing industries are nowadays offering the opportunity to brewers to perform the hop addition with processed products such as hop oils and extracts. They are sold as a concentrated liquid form of the main aroma and bitterness components of hops, avoiding the introduction of solid material into green beer and a more consistent finished product.

1.4.1 Plant properties

The hop plant, *Humulus lupulus*, belongs to the Cannabaceae family. It is recognized as a climbing plant which grows wild along hedgerows or is cultivated using field wirework. (De Clerck, 1957). The plant is grown in moderate climate regions, and

harvested between late summer and early autumn. Key plant property is the light dependency and also the fact they are dioecious. The hop cone of the female species are cultivated for its brewing active features, and are grown almost exclusively for the brewing industry (Almaguer et al., 2014). The hop cone is composed of a central axis called strig, surrounded by a petal-like shaped network called bracts and bracteoles. The lupulin glands, containing the brewing essential oils and bitter resins (Stevens, 1967), are located at the base of the bracts (Briggs et al., 2004). They are formed as the hop ripens, and only the cones of the female hop have lupulin glands (Almaguer et al., 2014). Hops can also form seeds when fertilized with a male hop. However, male hops are removed from the field to promote the cultivation of seedless hops as the seeds have no use and are undesirable by brewers (De Clerck, 1957; Almaguer et al., 2014).

Hops are bred around the world with specific genotypes and varieties. Each hop will present a typical essential oil pattern, and a characteristic aroma (Kovačević et al., 2002). For each hop variety their chemical composition differs and a classification is required for brewers to select their variety according to their final beer product. Bittering hops are used to bring bitterness into beer. They usually have a high content in alpha acids. Aroma hops are used for their specific flavour and aroma smell. They have a high oil content, and bring to beer characteristic sensory notes such as fruity, flowery, or spicy. (Briggs et al., 2004). Content in alpha acids or oil are linked due to the plant metabolism. Dual purpose hops are good balanced varieties which can be used as either an aroma or a bittering hop. Noble hops like Saaz refers to most traditional hop varieties grown in history. The soil they grow are giving them unique features and they are currently prized for their unique flavour profile and aroma. There is approximately 80 different varieties commercially available however; hop-

breeding programmes are developed to enrich the hop panel variety using hybridization between hop males and females.

The difficult chemistry related with hop compounds and their application in brewing has been subjected to extended research over a century (Almaguer et al., 2014). Female hop cones contain various compounds, such as essential oils, resins, polyphenols, lipids, proteins, cellulose, waxes and amino acids (Stevens, 1967). Hops are mainly valued in the brewing industry for their low water solubility compounds: resins and essential oils. The global chemical composition of a dry hop cone is described in Table 1.2.

Table 1.2 Global chemical composition of a female dried hop cone. (Verzele, 1986; Almaguer et al., 2014; Stevens, 1967).

Compound	Amount (%)
<i>Total resins</i>	15-30
<i>Proteins</i>	15
<i>Essential oil</i>	0.5-3.5
<i>Polyphenols</i>	4
<i>Monosaccharides</i>	2
<i>Amino acids</i>	0.1
<i>Pectins</i>	2
<i>Waxes</i>	0.5-25
<i>Cellulose</i>	43
<i>Ash</i>	8
<i>Moisture</i>	10

Hop oils are mainly composed of terpenes and terpenoids. The three main terpenes characteristic to hop aroma are myrcene, humulene and caryophyllene (Stevens, 1967). Hop resin is mainly composed of bitter compounds also called hop acids. These can be composed of alpha-acids (humulones, cohumulones) or beta-acids (lupulones) (Verzele, 1986). Both contribute to give beer its flavour and improves its conservation. Hop varieties will often be described with their specific content of alpha-acids. Hop also contains a large variety of polyphenols from small monomers

to large polymers. They contribute to the astringency, colloidal stability, foam and colour stability of beer (Moll, 1987; Briggs et al., 2004; Li et al., 2008). Xanthohumol is the main flavonoid found in the hop cone, with a high interest for medical research as it is involved in inhibitory mechanisms in the initiation and progression phase of carcinogenesis (Bartmańska et al., 2018). Hop is also composed of tannins, especially proanthocyanidins (Linthorpe et al., 2015). Except for xanthohumol, in different hop varieties, there are at least 30 other flavonoids present from 10 to 100-fold lower.

1.4.2 Hop products

Traditionally, hops were sold after drying in loose cloth bags. At time of harvest, the whole cone is displaying a moisture content between 75 and 80%. They are then dried in oast houses (65-75°C kilning temperatures) to reduce the moisture content close to 10%. In order to reduce transportation costs, dried hops were sold into more compressed package also called bale. This bale would usually weigh 200 lb for the American model. European packages may vary depending on the bag size (German land sack from 3-4 lb/ft³ to the export ballot, 15-22 lb/ft³) (Russel, 2006).

Improving the storage and the transport costs has pushed hop product industrialization. Using hop pellets and hop extracts facilitate the brewer to make beer flavours that are more consistent and easier to handle. More than 95% of the worldwide grown hops are transformed for their homogeneity and higher efficiency (Schönberger, 2006). The main processed hop products are pellets, extracts and oil (Briggs et al., 2004). There are two main kind of hop pellet, Type 90 (T90) and Type 45 (T45). Type 90 pellets are just hops processed mechanically into pellets. The name was given assuming that only 90% of the original plant material was transferred into the pellets due to processing losses. Type 45 are lupulin-enriched

pellets; the hop powder after chill drying is sieved to facilitate the separation of the lupulin glands from the other hop parts. The lupulin part can be then mixed with hops to reach the desired α -acid concentration. The appellation T45 was given assuming that those pellets would have at least the twice as much of the brewing principles than T90 pellets (Briggs et al., 2004; Schönberger, 2006).

Hops can also be sold in the brewing market as extracts. They are able to decrease the volume of hops compared to pellets (Bamforth, 2006). Many organic solvents were utilized for the manufacture of hop extracts (hexane, dichloromethane, carbon dioxide, methanol, and ethanol). Only two (carbon dioxide and ethanol) are still used due to safety, cost and environmental considerations (Schönberger, 2006). The two extraction processes yield hop extracts with two different composition. Ethanol extraction is performed by first immersing the hops in ethanol for the solvent to be enriched in hop compounds (Daoudand et al., 1992). Once the solid has been separated from the liquid, the hop solution was then evaporated under vacuum to remove any traces of organic solvent. This raw extract contains a broad variety of compounds such as hop oils, resins and other water soluble components (proteins, polyphenols, and salts). This extract can be then separated into two different products (tannin or resin extracts) through separators (Schönberger, 2006). The advantages of using ethanol extraction is that it is very stable even at cold temperatures and also that extract has a comparable profile to the original hop, with a slight lower concentration in polyphenols.

Liquid and supercritical CO₂ extraction has been used for obtaining hop extracts (King, 1993) and is widely employed in Australia, the United States, the United Kingdom, China, Poland, and Germany (Anioł et al., 2008; Gardner, 1993). Compared to the ethanol extraction this process can pull out more specific hop resins

and aroma compounds (Schönberger, 2006). Although both processes can be performed, nowadays the extraction using supercritical carbon dioxide has been preferred because of its lower extraction time and its higher flexibility of manufacturing. Typical conditions for liquid extraction of hops is 60 bar and 10°C whereas supercritical carbon dioxide extraction varies between 150 and 300 bar and between 50°C and 60°C. The pressure is then lowered down in the separation tank allowing the solvent to evaporate naturally and leaving all the hop compounds as a purified extract (Schönberger, 2006). Compared to the ethanol extraction, all polyphenol compounds remain in the spent hop material left after extraction (Biendl, 2007). Pressure and temperature have to be carefully manipulated to avoid the extraction of non-desired-compounds from the hops. Del Valle et al., (2003) showed that applying pressures higher than 200 bar leads to the extraction of undesired chlorophyll pigments and hard resins in some Chilean hops. The stability of the extract is considered as excellent as a shelf life up to 8 years is guaranteed for a cold storage (Schönberger, 2006).

Hop desirable volatiles can also be extracted as hop oils using steam distillation (Briggs et al., 2004). Steam is introduced through a hop bed under pressure, freeing essential oil components and turning it into vapour. When cooled, the steam can condenses into water and oil, and, due to its lighter density water can be separated from the oil. Using this method, the oil is free of any bittering components (Lam et al., 1986). Myrcene and linalool were evaluated as the most odour-active and of prime interest for brewers in the majority of hop varieties (Aberl et al., 2012; Steinhaus et al., 2000). Sulphur molecules have a high impact on hop oil condensation as they can give non desired off-flavors such as rubber or vegetables (Rettberg et al., 2017). This technique represents an efficient way to extract only

volatiles components responsible for the hoppy character of hops, without adding any bittering or astringency attributes into beer. The flow diagram of hop processing and their derived products is resumed in Figure 1.1.

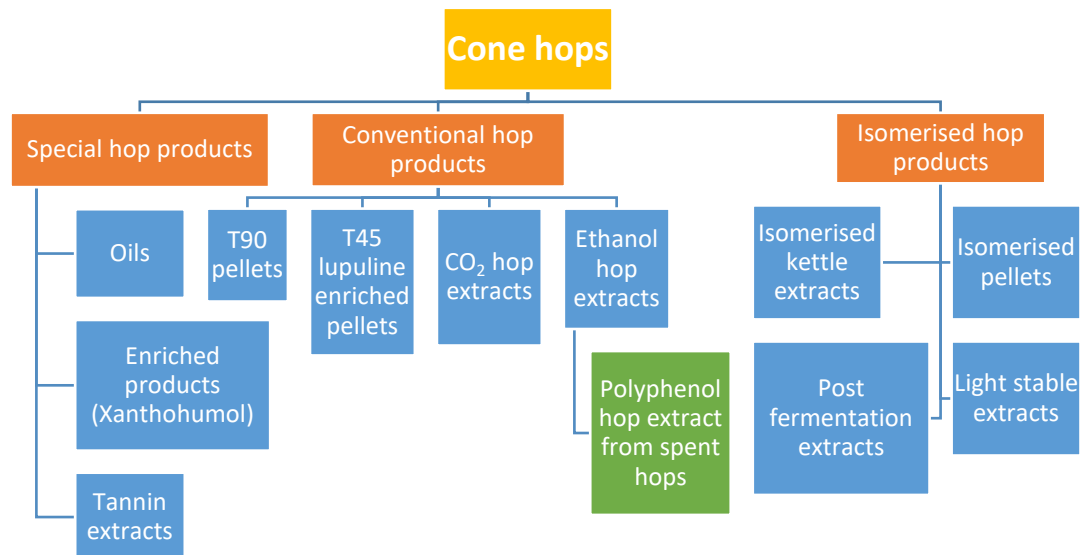


Figure 1.1 Flow diagram of manufactured hop products from cone hops

1.4.3 Spent hops

Spent hops are represented as two different products derived from the hop plant. It can represent the plant material left after the extraction of its main flavourful and odour-active components in the brewery (Wallen et al., 1979). Unlike malt grains, it does not own any high nutritional value, and is usually sold as a fertilizer or a pet feed product (Anioł et al., 2008) with the spent grains. However, Davies et al., (1927) has shown that spent hops were not willingly eaten by animals and could only be a portion of the total animal ration. Novel methods of waste management are emerging around the world, especially the use of the food industry waste (Russ et al., 2004) to produce higher quality products for animal and human consumption.

Spent hops can also represent the material resulting from the extraction processes used to extract hop oils and resins to manufacture advanced hop products. Spent hop

material after carbon dioxide extraction still contain a diverse range of compounds : polyphenols, minerals, essential oils, sugars, free amino acids and proteins (Anioł et al., 2008; Bedini et al., 2015; Davies et al., 1927). They can be conveniently extracted, affordable and manifest a low toxicity for the human consumption (Ray et al., 2001). Spent hops have been recently highly valued for different applications for their high polyphenol content (Bedini et al., 2015; Jaskula-Goiris et al., 2014; Karabín et al., 2018). Hop polyphenols represent over 100 different compounds, and can be classified into different categories : flavonols (kaempferol, quercetin, flavonol glycosides, rutin), flavan-3-ols (catechin, epicatechin, dimers and oligomeric flavanols, tannin polymers), phenolic acids (ferulic acid), and other polyphenolic compounds (xanthohumol, resveratrol, prenylflavonoids) (Almaguer et al., 2014). It has been found that proanthocyanidin polyphenols, polymers of catechin and epicatechin subunits are found in abundance in all the spent hop parts (bract, cone, and leaves) (Yamamoto et al., 2006; Linforth et al., 2015). These polymers also known as condensed tannins, can be present in various sizes from small oligomers (procyanidins) (Hümmer et al., 2008; Monagas et al., 2010) to large polymers (proanthocyanidins) (Neilson et al., 2016; Tanaka et al., 2014). These polymers display a high affinity for proteins.

The extraction of polyphenols is usually performed using a liquid-liquid extraction, using different solvents including water, ethanol, hexane, methanol, acetone, ethyl acetate, etc. (Anioł et al., 2008; Bosso et al., 2016; Hellström et al., 2009; Kim et al., 2010) and other parameters such as temperature or ultrasonic extraction. Due to the different properties of polyphenols and their interaction with other compounds, no individual organic solvent can extract all polyphenols from a botanical source. The presence of water and an organic solvent within the solvent matrix is preferred for

the global extraction of polyphenols, due to their polarity and their hydrophilic properties (Salem et al., 2011; Sun et al., 2005). Different studies have evaluated different solvent to extract proanthocyanidins from grapes and hops (Anioł et al., 2008; Pekić et al., 1998). As the degree of polymerisation increases, these polymers become more hydrophilic due to their structure. The optimal extraction of the proanthocyanidin content from spent hops will be reviewed differentiating solvents and their proportions in order to produce the most efficient extract in beer clarification.

1.5 Tannins and proanthocyanidins

1.5.1 Definition

Tannins are defined as water-soluble, high molecular weight polyphenols, naturally present in many vegetables, cereals, fruits, and wood (Smeriglio et al., 2017). They are represented in two main categories : the first group are the hydrolysable tannins such as gallotannins (Hagerman, 2002) or ellagitannins. These class of molecules can be hydrolysed by weak acids to form gallic acid, sugars, and ellagic acid for ellagitannins (Smeriglio et al., 2017). Gallotannins are used for beer stabilisation (Withouck et al., 2010). They are defined by a polymer assembly of gallic acid linked to a central glucose unit to create pentagalloyl glucose (Hagerman, 2002). They can be extracted from different plants and vegetables such as wood, gallnuts and sumac leaves (Zalacain et al., 2003).

The second group are the condensed tannins, also known as proanthocyanidins. They are the most abundant plant-derived polyphenols. These are represented as polymers and oligomers of flavan-3-ol units. Recent studies have demonstrated their dietary antioxidant activity, and beneficial impact on some health conditions, such as urinary

tract infections and cystitis (Howell, 2002; Polewski et al., 2016). They are present in various sizes, and may differ by the nature of the constitutive units, or by their degree of polymerization (between 2 and more than 100), and by the type of linkage (Labarbe et al., 1999). They are defined as polymers with a molecular skeleton of catechin and epicatechin monomer units.

They can be separated between two groups : these monomer units can be doubly linked by a C4-C8 bond and/or with an additional ether bond between O7-C2 for the proanthocyanidin A-group, or a linkage between the C4-C6 or C4-C8 for the B group (Neilson et al., 2016). It is supposed that A type PA's polymers would have a more branched shape then the B-type, due to the different bonds that differentiate the two groups.

Their molecular size depends on various parameters such as the type of organic compounds used for the extraction, the polarity of the extraction solvent, or the pH of the solution.

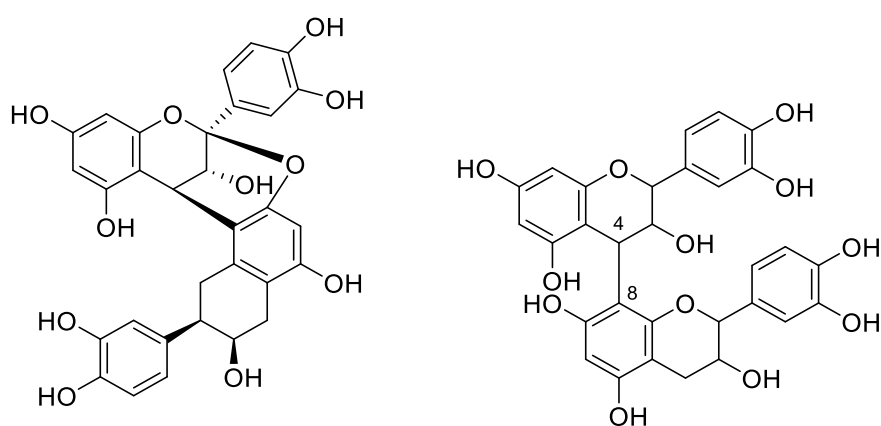


Figure 1.2 Chemical representation of proanthocyanidins, left, A-type (branched) and right, B-type (linear).

1.5.2 Abundance

Proanthocyanidins are present in a wide range of fruits, vegetables, berries, and their relative processed products (Hellström et al., 2009). They are part of the daily human diet, estimated to an average of 57.7 mg/person daily in the United States (Gu et al., 2004). They represent the major polyphenolic compound in commercial cinnamon, and are known to be present extensively in food products such as black and green tea, apples, grape seeds, cranberry, broccoli, grapefruit, chocolate, cocoa, white and red wine. (Howell, 2002; Ostroschi et al., 2018; Pedan et al., 2016; Watson et al., 2013). They are also the most widely spread tannins in woody plants, where they accumulate in tissues such as leaves, roots and bark (Porter, 2017). A reference proanthocyanidin database has been created by Gu et al., (2004) and Hellström et al., (2008) to evaluate the proanthocyanidin concentration in various food products. Proanthocyanidin in foods cover a large range of concentrations, distribution of oligomers and polymers. Highest contents per fresh weight were detected in chokeberries, blackcurrant, rowanberry, rose hips and cocoa products. Berries and fruits were generally the best sources of compounds, whereas roots, cereals and the majority of cereals lacked them entirely (Hellström et al., 2009).

Condensed tannins have been found in larger amounts than hydrolysable tannins in hops (Schönberger, 2006). Proanthocyanidin content in hops can range from 0.5 to 4% dry weight, depending on the geographic origin variety and horticultural practices (Callemien et al., 2005; Stevens, 1967). Previous studies have shown the presence of oligomeric and monomeric flavan-3-ols in hops (Appeldoorn et al., 2009; Neilson et al., 2016; Tanaka et al., 2014). They included catechin, epicatechin, procyanidins and trimeric procyanidins. (Li et al., 2006). However, other studies have also shown the presence of large proanthocyanidins polymers in their sample.

Taylor et al., (2003) used time-of-flight mass spectrometry coupled to gel permeation chromatography to separate proanthocyanidins from hops. They revealed the presence of heptamers through their mass spectra. Polymers as large as 20-mers were also detected by MALDI-TOF-MS. In the case of hop plants, it was shown that hop proanthocyanidins are represented as a complex mixture of monomeric, oligomeric and polymeric states of catechin or epicatechin.

1.5.3 Methods in tannins analysis

All phenol content (hydrolysable and condensed tannins) can be evaluated through the gallic acid assay (or Folin-Ciocalteu assay). The reagent is composed of a mixture of phosphomolybdate and phosphotungstate that can form complexes with polyphenols in acid solution. Molybdates are able to detect oxidizing monophenols and vicinal diphenols whereas tungstates complexes with ortho-dihydric phenols (Singleton, 1999). This assay is however not distinguishing polyphenol between their classes (Neilson et al., 2016). All compounds composed with phenol sub-units will produce blue complexes which can be quantified through a spectrophotometer. This assay is very useful to estimate the amount of polyphenol material within a sample. The concentration found in a sample can be plotted against a calibration curve of gallic acid standards after reaction with the Folin reagent to determine an estimate of the sample polyphenol concentration, expressed as gallic acid equivalent or GAE.

UV-Visible spectroscopy can be a suitable technique to evaluate polyphenols. Polyphenols compounds can be coloured (and absorb light at a certain wavelength), and the ability of the phenolic ring to absorb UV light gives spectroscopy a highly viable way to estimate phenolic compounds in plant or fruits samples (Tudo et al., 2019). Phenolic acids, flavanol monomers, oligomers and polymers can be observed

at 280 nm, 360 nm for flavonols and 520 for anthocyanins. UV-Visible spectroscopy is often coupled to liquid chromatography separation techniques allowing both separation and quantification of polyphenols.

The quickest and most frequent assay used to quantify proanthocyanidins is the acid butanol hydrolysis assay (Porter et al., 1985; Swain et al., 1959). It results in the depolymerisation of all polymers units in hot acidic conditions, allowing the conversion of the polymers to anthocyanidin, red-pigmented and easily quantifiable compounds using spectrophotometric analysis. A purified standard of proanthocyanidin isolated is essential if absolute quantitation is required. However, there is no pure proanthocyanidin reference available currently (Shay et al., 2017). This assay is typically used to compare different sources of condensed tannins, but does not give any information of the size of the polymer or their distribution within a sample origin.

Other quantitative assays include the 4-dimethylaminocinnamaldehyde (DMAC) and vanillin assay, which quantify condensation products. The reaction between vanillin and flavanols produces coloured compounds that can be measured using spectroscopy at 500 nm. This assay has been criticized because of the differential reaction rates between monomeric and polymeric forms of PA present, which can require a fractionation prior to analysis (Sun et al., 1998). The DMAC assay is based also on the condensation reaction between proanthocyanidins and DMAC, observed through spectroscopy of the products at 630 nm. Wang et al., (2016) demonstrated however the imperfections of this assay, as variation in the interflavan linkage in dimers or trimers induces differential absorbance response. These quantitative assays are not perfect, but represent a viable way to compare PA from different sources (Neilson et al., 2016).

As proanthocyanidins are usually present in various molecular sizes, this problem challenges research with current chemical analyses available (Taylor et al., 2003). Different analytical methods are based on the depolymerisation of all oligomers and polymers into monomeric subunits, which can give information about the average subunit composition and the average molecular weight of a sample (Shay et al., 2017; Tarascou et al., 2010). The known stoichiometry of the depolymerisation reaction allows the calculation of the mean degree of polymerisation of the PA solution, in consideration of the measurement of the total of monomers created (Neilson et al., 2016). These methods use phloroglucinolysis (Downey et al., 2003; Li et al., 2006), and thiolysis (Labarbe et al., 1999; Ramirez-Coronel et al., 2004; Hellström et al., 2008).

Phloroglucinolysis is based on the reaction between proanthocyanidins and an excess of phloroglucinol in acid conditions. This reaction yields one underivatized monomer per PA molecule (the terminal unit, indicating the number of PA molecules present in a sample) and the remaining sub-units as phloroglucinol monomeric adduct. These compounds can be further quantified through liquid chromatography. Thiolysis used the same chemical principle, as the reaction between proanthocyanidin and benzyl mercaptan yields benzyl thioether derivatives, and the unmodified end-unit for each proanthocyanidin molecule. These methods also have limitations. They demand the detection of both the released end unit monomers and the derivatized adducts products, which are not available commercially (Neilson et al., 2016). The mean degree of polymerisation value obtained is also an average which does not fully express the complexity of the proanthocyanidin molecular distribution in a sample. A-type proanthocyanidins ether bonds are also resistant to

thiolysis and phloroglucinolysis, which limits the global analysis of the proanthocyanidin content in polyphenolic samples (Esatbeyoglu et al., 2015).

As polymer size increases, the possible number of isomers increases as well, making chromatographic resolution into pure compounds increasingly difficult. As proanthocyanidins usually occur in complex mixtures, normal-phase HPLC can be used to evaluate proanthocyanidins. The retention time is generally proportional to the molecular weight of the PA present. However, high molecular weight polymers with DP higher than 10 and more elute as a single and wide peak (Gu, 2012).

Mass spectrometry has also been used to evaluate proanthocyanidins and resolve its molecular complexity (Hümmer et al., 2008). The analysis can be performed in MS, MS² or MSⁿ modes. Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy has been extensively used for the analysis of large molecules such as peptides, proteins and tannins in plants (María Monagas et al., 2010). Because of its unlimited mass range and its high mass accuracy for molecular weights, it represents a good candidate for the analysis of proanthocyanidins. The choice of an appropriate matrix is crucial for MALDI-TOF analysis. Ohnishi-Kameyama et al., (1997) described the use of 2,5-dihydroxylbenzoic acid for the study of proanthocyanidin oligomers up to decamers present in Fuji apple extracts. Polymers with DP up to 23 were also observed in cranberry press cake (Reed et al., 2005).

Despite its great advantages over the study of large molecular weight compounds, modes of operation, prior fractionation and clean-up, and operational parameters can directly affect the quality of the data, and must be picked on a case-by-case basis (Neilson et al., 2016). Another limitation of the application of MALDI-TOF to PA research is the poor ionization of large polymers. This can induce in bias towards the

smaller fraction of proanthocyanidin present because of poor or no detection of large compounds. The sensitivity can also be lowered for high molecular weight molecules.

Proanthocyanidin research especially for polymers is somewhat limited by existing techniques which are not suitable to provide qualitative and quantitative results for the polymeric fraction (Neilson et al., 2016). Novel methodologies have to be employed in order to analyse with accuracy and selectivity proanthocyanidin with a high degree of polymerisation.

1.6 Analytical ultracentrifugation

Analytical Ultracentrifugation (AUC) is a technique that is applied in this thesis to characterise the polymer size distribution of polyphenolic hop extracts. Analytical ultracentrifugation is a hydrodynamic method that monitors the transport of protein and other macromolecules in solution with optical technologies (Cole et al., 2008; Williams et al., 1958). Three different optical systems are currently available for the AUC to observe the sedimentation of particles through centrifugal force within a cell (absorbance, fluorescence and interference). AUC has different broad applications and can be used over a large range of solvents and solute concentrations (Cole et al., 2008). The transport of compounds and the response signal monitored depends on the molecular weight of the sample studied and to a lesser extent its molecular conformation. The molecular weights can be calculated from hydrodynamic principles and equations, independently of the need for mass calibration standards. Two main experiments can be used in Analytical Ultracentrifugation, sedimentation velocity and sedimentation equilibrium. Sedimentation velocity is helpful to determine sample heterogeneity/purity (Graceffa et al., 1988; Van Holde et al., 1978), because the sample is examined in a free solution and in a pre-defined solvent.

Sedimentation equilibrium allows the determination of molecular weights, solution non-ideality (Howlett et al., 2006) and average molecular weight for a heterogeneous sample. It is particularly useful to perform equilibrium experimentations in combination with sedimentation velocity runs. The analytical ultracentrifuge is able to measure directly molecular weights of solutes in their native state, without the need of any calibration, and for a very large molecular weight range (several hundred to many millions). It requires very low amount of sample (20-500 μL) and low concentrations (as low as 0.01 mg/mL for sedimentation velocity and 0.4 mg/mL for sedimentation equilibrium). This method is applicable to any sample with the fundamental requirements : any substance has to have a different refractive index from the solvent they are contained; that the particles sediment or float at a convenient rate at a technically feasible gravitational field and that the sample must be chemically non-destructive towards the cell material (Cole et al., 2008).

1.6.1 AUC – Sedimentation velocity

$m_p\omega^2r$ represents the force submitted to a particle due to the gravitational field, where m_p is the mass of the particle, r is the distance from the centre of the rotor, and ω is the rotor speed in radians per second ($\omega = 2\pi*\text{rpm}/60$). As a consequence, a counterforce will be applied on the particle due to the solvent motion expressed as $m_s\omega^2r$ where m_s is the mass of the solvent displaced. This mass of solvent displaced is equivalent to m_p times the partial specific volume of the particle $\bar{v}$ (cm^3/g), time the density of the solvent ρ (g/cm^3). The resultant force can be asserted as $(m_p - m_s)\omega^2r$. The buoyant mass, or effective mass of the particle is showed by $m_b = m_p(1 - \bar{v}\rho)$. By the motion of the particle in the solvent, the frictional force is also present, represented by $f v$, where f is the frictional coefficient and v the velocity of the particle. Equilibrating these force we can express the sedimentation coefficient (in

Svedbergs, 1S = 10⁻¹³s) using the following equation (Williams et al., 1958; Schwenke, 1977) :

$$s \equiv \frac{v}{\omega^2 r} = \frac{m_b}{f}$$

Equation 1.2 Expression of the sedimentation coefficient for a spherical single particle.

The buoyant mass $m_b = M_b/N_A$ where M_b is the buoyant molecular weight (in g/mol or Da) and N_A is Avogadro's number. Diffusion causes the sedimenting boundary to spread with time. Recording the shape and the motion of a boundary allows calculating both the translational diffusion coefficient, D , and the sedimentation coefficient. The diffusion coefficient can be expressed using the Stokes-Einstein equation as follows $D = RT/N_A f$, where N_A is the Avogadro's number, T represents the absolute temperature and R is the gas constant.

The Lamm equation shows the time evolution of the radial concentration distribution $c(r,t)$ of a species associated with a diffusion coefficient D and a sedimentation coefficient s during sedimentation in a centrifugal field inside a sector-shaped defined volume as follows (Williams et al., 1958; Schwenke, 1977; Cole et al., 2008)

$$\frac{\partial c}{\partial t} = D \left[\frac{\partial^2 c}{\partial r^2} + \frac{1}{r} \frac{\partial c}{\partial r} \right] - s\omega^2 \left[r \frac{\partial c}{\partial r} + 2c \right]$$

Equation 1.3 Radial concentration distribution of particles in a centrifugal field.

In this equation, c represents the weight concentration of macromolecules at radial position r studied and t is the time. The ultracentrifuge analysis using the optical equipment registers the radial concentration distribution $c(r,t)$ at different set times during the experiment and the instrument provides the rotor speed ω . This equation

can be solved numerically with the solution given as a distribution of sedimentation coefficients $g(s)$ vs s , which can be further corrected for diffusion effects to yield what is known as a $c(s)$ vs s plot. (Dam et al., 2004). Non-ideality effects (through molecular co-exclusion and back-flow phenomena) can be minimised by operating at low concentrations, otherwise measurements need to be made at a range of concentrations and extrapolated back to zero.

1.6.2 AUC – Sedimentation equilibrium

Sedimentation equilibrium happens at lower speeds than sedimentation velocity experiments. When the flux due to the sedimentation of the particles is exactly equal to the flux due to diffusion, the equilibrium state is reached (Cole et al., 2008). The main interest of this experiment is that the concentration distribution when the equilibrium state is reached is only dependant on the molecular weight since at equilibrium frictional effects do not come into consideration. This molecular weight estimation is absolute, i.e. entirely independent of the shape of the molecule. For an ideal single non-interacting compound, the equilibrium radial concentration gradient $c(r)$ can be express by the equation:

$$c(r) = c_0 \exp \left[\sigma \left(\frac{r^2 - r_0^2}{2} \right) \right]$$

Equation 1.4 Expression of the radial concentration of particles at equilibrium state.

In this equation, c_0 represents the concentration at an arbitrary radial reference distance r_0 . The parameter σ is related to the molecular weight of the solute by $\sigma = M(1 - \bar{v}\rho) \cdot \omega^2/RT$ where M is the molecular weight (in Da or g/mol) of the solute. Deviations from the exponential term can demonstrate the presence of non-ideality state or interacting molecular species (Cole et al., 2008). The drawback delayed wide

application was the procedure for the data analysis. A particularly useful programme for the analysis for systems which are polydispersed is the SEDFIT-MSTAR algorithm (Schuck et al., 2014), which yields an estimate for the molecular weight distribution $c(M)$ vs M .

1.7 Introduction to the Thesis

1.7.1 Objectives

The main objective of this thesis was to develop a novel beer fining agent extracted from spent hops. This project has been studied and scoped previously by Dr. Robert Linforth and co-workers, evaluating the potential to extract polyphenolic rich extracts from hop waste. These extracts have been showing efficient haze removal when applied to green beer post fermentation. As an industrial partnership has been settled with Barth Innovations Ltd., this product has to be evaluated and characterised prior to a future commercialization. As it is extracted from hops, and from spent hop waste this product can be highly valued for brewers in their marketing scheme as it can also confer the beer to become fully vegan.

As beer comprises a very large range of styles and aspects, it is important to evaluate the performance of this extract in a large range of beers. As this product will be delivered commercially as a food grade product, it is also necessary to review the fining agent through different analytical techniques in order to describe this product with scientific and accurate specifications.

Itemised project objectives can be summarised as:

- Selection of an optimal extraction process of the active compound present.
- Evaluation of the fining efficiency compared to the current products in a large range of beers.

- Understand and evaluate the molecular composition of the spent hop extract.
- Study of the impacts of the hop extract on other beer quality factors.

1.7.2 Structure

The beginning of the thesis has described an overview of beer brewing, with a special attention on beer haze. The second part introduced the range of fining present already present in the market, and their composition. The last part concerned hops, the main material to develop the novel fining agent, their differences in varieties and composition, but also a review on all current analytic techniques used to define hop polyphenols. Results chapters have been written using a research paper format, each section containing introduction, materials, methods, results and discussion statements. The second chapter will contain universal methods that will be used throughout all chapters. It will be focused on the evaluation at first of the optimal extraction process to extract the active compound present in spent hops. It will also describe the general application of the extract into green beer, from the microscopic to pilot scale. This chapter will also cover the beneficial use of silicates as auxiliary finings in combination with the hop extract, and the resulting haze removal efficiency in a large range of beers.

The third chapter will cover the characterisation of the hop extract and its active proanthocyanidins. Current analytical methodologies will be employed as well as the use of analytical ultracentrifugation as a novel technique to resolve the complexity of proanthocyanidin plant samples. Chapter 4 will describe the possible other outcomes of the use of hop polyphenols for finished beer properties. Concluding remarks can be found in chapter 5, as well as suggestions for further work in this area.

Chapter 2: Hop extract application into fermented beer

Preliminary experiments used the extraction protocol proposed earlier by Linforth et al., (2015) to develop and standardise methods of fining application that could be used in a range of studies. For the assessment of fining action it is important to understand the impact of fining addition and mixing strategies within the beer. Key assessment criteria are the amount and the compactness of sediment formed, reduction of suspended yeast cell counts and the residual haze of the fined beer. These criteria can be assessed by putting fined samples into Imhoff cones and allowing the finings to act for fixed periods. This allows sediment depth to be measured against a graduated scale and the fined beer can be sampled from the cones and the optical density measured as an indication of yeast cell counts. For haze analysis suspended yeast needs to be removed and this then allows haze analysis using light scattering techniques.

Following these preliminary experiments, modifications to the extraction procedure were investigated. Linforth et al., (2015) used a basic method commonly applied in PA analysis. However, these extraction conditions may not be optimal for long chain proanthocyanidins, which may be more hydrophilic relative to smaller ones. The optimised extraction method and the methods of fining assessment were then applied in a series of studies to determine the efficacy of the hop extract under a range of different beer styles, with or without an extra addition of auxiliary finings. This was further evaluated at the pilot scale.

2.1 Materials and Methods

2.1.1 Materials

Hop samples

Barth Innovations Ltd. (Tonbridge, UK) kindly supplied spent Target hops from 2015 harvest in powdered and pellet forms. They also supplied a prototype fining laboratory hop extract representative of the future commercial product project (Commercial hop extract, unknown variety, batches produced from 2016 to 2017). Saaz hop variety was purchased from the HopShop (Plymouth, UK), in whole cone dried form from the 2015 crop.

Brewing materials

Beers for fining assays were prepared using Young's Harvest Pilsner Lager home-brew kit or Young's Harvest Stout home-brew kit, together with dried light malt extract (The Hop Shop, Plymouth, UK, 6-7% protein), and granulated sugar (Sainsbury's, UK). Nottingham ale yeast (*Saccharomyces cerevisiae*), were purchased from Lallemmand (Burton-upon-Trent, UK). Beers produced with malt were brewed using extra pale malt supplied from Crisp Malt (Norfolk, UK) and using Mosaic hops in pellet form from Yakima Chief Hops, crop 2015 (Yakima, USA).

Fining materials

Fining Adjunct (auxiliary finings) and isinglass paste were purchased from Murphy & Son (Nottingham, UK). Isinglass was used in all finings trial by dissolving 10 g of isinglass paste in 100 mL of cold water, with 1 g of citric acid. Technical data sheet for Fining Adjunct is shown in Appendix 1.

Chemicals

Acetone, citric acid, hydrochloric acid, gallic acid, maltodextrin, n-butanol, eosin yellow and Folin and Ciocalteu's phenol reagent were purchased from Sigma-Aldrich in analytical reagent grade (Dorset, UK). HPLC grade methanol was also purchased from the same supplier. Ferric ammonium sulfate was supplied from Fisher-Scientific (Loughborough, UK) in analytical reagent grade. Sodium chloride, Arabic gum and β -cyclodextrin were purchased from Thermo Fisher Scientific, all analytical reagent grade (Hemel Hempstead, UK).

2.1.2 Universal methods (used across all thesis chapters)

2.1.2.1 Brewing beer for fining trials

Green beer for fining was produced in the Brewing Science laboratories of the University of Nottingham. A beer kit (Young's Pilsner or Stout) was poured into a 25 L fermentation bin adding boiling water to dissolve any residue left in the tin. Further boiling water was added to a total volume of 4 L to dissolve the beer kit. At this point sugar (1 kg) or a blend of sugar and malt extract (500 g of each) were added and stirred until dissolved. Tap water was then added to the fermentation bin to produce a final volume of 23 L. Yeast (11 g) was added to the wort to initiate fermentation. After 4 or 7 days aliquots of the green beer were removed using a siphon for fining trials.

Beer was also produced from grain (Crisp Malt, Norfolk, UK) using the Grainfather G30 system (The Grainfather, UK). Typical beers used in the study were produced using 5 kg of extra pale malt milled with a Roppi 250 mill (Zirc, Hungary) with a roller gap of 0.85 mm. This was added to 15 L of water for the mashing step.

Mashing started at 67°C for 1 h, then 72°C for 5 min, and finally at 78°C for 5 min using continuous recirculation of the liquid. Once the mashing was finished, the grains were suspended at the top of the vessel to run off the wort from the spent grains. Sparging was carried out using 18 L of water at 78°C, introduced manually at the top of the grain bed. The hops (10 g of Mosaic pellet hops) were then introduced and the mix was boiled for 1 h. The wort was then stirred to induce a whirlpool type effect and the trub allowed to settle. The final wort was passed through a chiller before introduction into the fermenter. A sachet (11 g) of dried yeast was then introduced and the beer was used for fining experiments after 7 days of fermentation at room temperature.

2.1.2.2 Sediment depth analysis

100 µL of hop extract was mixed with 10 mL of beer in 15 mL glass conical tubes, mixed on a Thermo Denley Spiramix roller-board (Loughborough, UK) for 15 min. Sedimentation depth (mm) was recorded by measuring the sediment formed after 1 h of reaction in the 15 mL glass conical tubes.

Sedimentation depth (mL) in 500 mL volumes of beer was measured in 1 L polycarbonate graduated Imhoff cones from Nalgene (13 mm closure size, 106 mm diameter, Sigma-Aldrich, UK). Sediment volume was recorded after 3, 6 and 24 h of sedimentation, and at a temperature of 4°C or 12°C.

2.1.2.3 Clarity measurements

OD₆₀₀ (A_{600}) is the optical density of a sample measured at a wavelength of 600 nm. It is measured as an index of the suspended yeast cell count. A Genesys 10S Vis spectrophotometer (Thermo Scientific, UK) was used to measure the absorbance of the beer samples in polystyrene cuvettes (Sarstedt, UK) with dimensions of 10 x 10

x 45 mm. Absorbance was measured at the same time intervals as for the sediment depth measurements, taking a sample from the top 2 cm layer of the Imhoff cone.

2.1.2.4 Residual Yeast removal prior to haze analysis

After 24 h of sedimentation, 350 mL of beer from the top section of the Imhoff cones was centrifuged using a Beckman J2-21 floor centrifuge (High Wycombe, UK) and a Fiberite F10-6 fixed angle rotor. The time of centrifugation was 15 min and 500 rpm for the centrifugation speed (Appendice 1). 200 mL of the supernatant was then poured in a 250 mL Duran glass bottle before haze analysis in order to remove any residual yeast cells within the beer.

2.1.2.5 Haze analysis

Total beer haze was measured using a turbidity meter pre-calibrated with formazine solutions (0 to 20 EBC) (Haffmans Vos Rota 90/25 from Pentair, Germany). The chamber was filled with deionised water at the same temperature as the beer studied. Beer was transferred into a 200 mL crystal bottle placed in the measuring chamber and the amount of light scattering at measuring angles of 90° and 25° was recorded. H25 (EBC units) is representative of the micro-organisms and larger particulate haze (larger than 1 µm) and H90 (EBC units) indicates finer particulate matter (smaller than 1 µm) such as chill haze. In order to study very hazy solutions, beer was first diluted with deionized water prior to haze analysis.

2.1.2.6 Yeast cell count measurements

Yeast cell counts were measured using an Aber Countstar Yeast instrument (Aberystwyth, Wales). This instrument used disposable slides each containing five 20 µL sample chambers. The instrument performs auto-focusing cell image analysis, providing yeast cell counts with or without staining.

2.1.2.7 Gallic acid assay for total phenolic content of extracts

A stock solution of 0.5 g/L was prepared weighing 50 mg of gallic acid diluted in a 100 mL flask with methanol. Five references solutions (100 μ L volumes, 0 to 0.5 g/L) were then prepared diluting the stock solution in deionized water and introduced into spectrophotometric cuvettes as well as diluted solutions of hop extracts (50-fold dilution in water, 100 μ L volume). Each cuvette was then filled with 1 mL of Folin & Ciocalteu phenol reagent solution (10-fold dilution, in water) and left for 20 min for reaction. All absorbances (references and samples) were measured at 750 nm against a deionized water blank. The sample absorbance was then compared to the plotted calibration curve obtained from the references results and expressed as absorbance values or gallic acid equivalent (GAE, in g/L)

2.1.2.8 Acid butanol assay for proanthocyanidin content of extracts

An acid butanol solution was made using 95 mL of n-butanol with 5 mL of concentrated HCl. A 2% ferric ammonium sulfate ($\text{FeNH}_4(\text{SO}_4)_2 \times 12 \text{H}_2\text{O}$) solution was prepared by adding 1 g of ferric ammonium sulfate into 50 mL of 2N HCl. For the assay, 3 mL of the acid butanol reagent was combined with 25 μ L of the sample and 100 μ L of the iron reagent solution in a screw culture tube. Tubes were heated at 90°C for 30 min. Once cooled, absorbance was measured at 550 nm using a Genesys 10S Vis spectrophotometer (Thermo Scientific, UK). Readings were corrected for the absorbance of a blank containing only sample solvent, acid butanol and iron reagent solution.

2.2 Experimental methods

2.2.1 Optimisation of the extraction method for preparation of hop finings

Target hop in cone and pellet form were milled using a Perten 3600 lab mill (Hägersten, Sweden) to produce hop powder. DesignExpert 7 (Stat-Ease, Minneapolis, USA) was used for a mixture design with different proportions of three different extraction solvents (acetone/ethanol/water, 30 design points) for extraction method optimisation. Initial extracts were produced using a Jasco PU-980 solvent delivery system to pump solvent (acetone/ethanol/water, variable ratio, v/v) at 2.5 mL/min, through a stainless steel cylindrical cell filled with 1 g of hop powder. The hop extract obtained was stored at 4°C. Samples were subsequently tested for total phenolic (Gallic acid equivalents) and PA contents. The activity of the extracts was assessed in a fining experiment measuring the sediment formed in 15 mL conical glass tubes. (Section 2.1.2.2, sediment depth analysis)

A second extraction method used 10 g of hop powder mixed with 100 mL of acetone/water (variable ratio, v/v), in a 250 mL Duran bottle on a Thermo Denley Spiramix roller-board (Loughborough, UK) for 15 min, with subsequent filtration through Whatman No1 filter paper using a Buchner flask. The acetone was then evaporated under vacuum using a Buchi R-II rotary evaporator (Flawil, Switzerland) at 45°C. The extract was then chilled overnight at 4°C, before centrifugation at 4000 rpm using a Thermo Jouan CR3i centrifuge (Loughborough, UK) for 10 min, after which the pellet was discarded. The extract was then topped up to 30 mL with deionised water. Samples were tested for PA content and fining activity using Imhoff cone experiment (Section 2.1.2.2, sediment depth analysis, Section 2.1.2.5 haze

analysis and Section 2.1.2.8 acid butanol assay for proanthocyanidin content of extracts).

2.2.2 Impact of mixing protocol on fining action in green beer

Barth Innovations also supplied a prototype fining laboratory hop extract representative of the future commercial product project (Commercial hop extract, no variety, batches produced from 2016 to 2017).

A series of different mixing regimes were studied to evaluate the impact of shear rate mixing on the fining reaction. Aliquots of 500 mL of kit green beer were mixed with 2 mL of commercial hop extract in a 600 mL beaker then blended (30 s) using different mixing strategies. After mixing, the beer was then poured in Imhoff cones for fining efficiency evaluation. A Yellow line OST basic 52 mixer from IKA (Oxford, UK), using a double paddle stirrer (3.5 cm diameter) was used as well as a Silverson L5M laboratory high shear mixer (Chesham, UK) and an Ultraturrax T10 basic from IKA (Oxford, UK) to produce a higher degree of mixing within beer. The Silverson mixer is a high shear rate laboratory mixer used to perform emulsification, dissolution and homogenisation. The Ultraturrax is a high performance-dispersing machine for the formulation of stable microfinished emulsions and dispersions. A second experiment will describe the effect of time of mixing and mixing velocity using the paddle stirrer only at lower speed, on fining efficiency. In addition to looking at sediment depth over time, haze was measured as a key criterion for beer clarity after 24 h of sedimentation. (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5 haze analysis).

2.2.3 Microscopy of beer fining

Commercial Budweiser beer was purchased (Sainsbury's supermarket, Nottingham, UK) for microscopy studies. A Leica DM microscope fitted with ICC50W camera, full Köhler illumination with phase contrast (Cambridge, UK) was used for the visualization of fining agents and beer haze particles. Haze particle visualisation was performed at various magnifications (x40, x100, and x400). Eosin yellow stain was used to show the presence of proteins within liquid samples.

10 mg of dye was mixed with 15 mL of distilled water prior to use. The dye solution and the liquid sample were then mixed together on the microscope slide. Saaz hop extract was made using the second routine extraction procedure detailed in the 2.2.1. extraction method section. A drop of Saaz hop extract was mixed with five drops of green beer on the slide and left to react for 2 min. The solution was then observed using light microscopy to study the yeast aggregation using hop extract finings. A drop of eosin yellow dye was then added onto a mix of five drops of Budweiser and a drop of Saaz hop extract to stain proteins after the fining reaction. The protein flocculation was observed using light microscopy in a yeast free beer matrix after 2 min of reaction. A drop of isinglass finings solution was also added to five drops of green beer and stained using a drop of eosin yellow and left for 2 min. The content was mixed then observed through light microscopy to determine the flocs created by isinglass finings into beer. Auxiliary silicates finings were also observed in its own liquid form.

2.2.4 Finings optimal dosage evaluation in a beer brewed from 100% barley malt

Six different dosages of the commercial hop extract (0.5 to 16 mL/L) were introduced into 500 mL of grain beer (7 d post-pitching) at 4°C and transferred into

Imhoff cones for sediment depth and haze analysis (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5, haze analysis). All samples were performed in triplicates. Three samples of beer without any finings were used as a control in order to compare the fining efficiency with that of the addition of the hop extract.

2.2.5 Using the hop extract for fining with an auxiliary finings

2.2.5.1 Dosage optimisation trial

20 L of kit green beer was brewed with 500 g of dried malt extract replacing 500 g of sugar in the recipe. Once chilled at 12°C and racked from the fermentation sediment, commercial hop extract was introduced to 500 mL aliquots of the beer at three different dose rates (between 1 and 4 mL/L). Three other fining treatments were conducted using these same dose rates of hop finings but with addition of an equivalent volume of silica based auxiliary finings, added at the same time as the commercial hop extract introduction. The green beer was then transferred to Imhoff cones which were then stored at 12°C and a record of the sediment depth was made after 24 h, along with residual haze analysis. (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5, haze analysis)

Following these initial trials, an experiment was designed to better understand the effect of the interaction of auxiliary finings and the commercial hop extract. Combinations of auxiliary finings and commercial hop extract were introduced simultaneously into a kit beer (12°C), and a grain beer (4°C) at different dosages using a response surface design. Two response surfaces (generated with Design Expert 7) evaluated the fining response of combinations of commercial hop extract and auxiliary finings on a kit beer (17 data points, from 0.66 to 4 mL/L) and a grain green beer (20 data points, from 1 to 8 mL/L). Sediment depth was recorded during

fining and the beer was analysed for haze after 24 h. (Section 2.1.2, sediment depth analysis, haze analysis)

2.2.5.2 Impact of time of addition of Auxiliary finings

This experiment involved a kit beer produced with an extra addition of dried malt extract and three different times (30 min, 6 h and 24 h) for the delay between the addition of either auxiliary finings or commercial hop extract in the beer (2 mL/L for each fining). A sample with commercial hop extract solely, plus a control were also included and the experiment was performed at 12°C. Haze analysis were performed after 36 h from when the first fining agent was introduced in the beer. (Section 2.1.2, haze analysis)

2.2.6 Evaluation of optimal fining conditions for a range of different beers

2.2.6.1 Variation of the protein content and suspended yeast cell counts in a model beer

A range of different beers was treated with 9 different fining regimes (variable dosages) along with an unfining control. The beers included a commercial Vixen ale sample (Charnwood Brewery, Loughborough, UK) and beers produced at Sutton Bonington to represent a range of yeast cell counts and protein contents. The samples were fined and placed in Imhoff cones at 4 and 12°C, to evaluate the impact of temperature on the process. The sediment depth was recorded after 24 h of clarification and haze analysis was then measured. (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5, haze analysis)

2.2.6.2 Impact of yeast strain on fining performance

Five different yeasts were used to cover the range from non-flocculent yeast to highly flocculent. Bry 97, Munich, Munich classic, Nottingham and Belle Saison yeasts were supplied from Lallemant (Burton-upon-Trent, UK). Green beer was made using the kit, and then divided into 6 aliquots of 3 L each before yeast pitching.

These were fermented (7 d) using 1.5 g of yeast, a different strain for each sample. All beers were then chilled to 12°C before fining into Imhoff cones. All samples were treated using three different fining strategies, commercial hop extract, isinglass, and a control without any finings. Measurement of the sediment depth and A_{600} were recorded after 6 and 24 h of reaction. (Section 2.1.2, sediment depth analysis, clarity measurements).

2.2.6.3 Performance of the hop finings in dark beer styles

Diageo also kindly supplied a sample of freshly fermented Guinness beer from their Saint James's Gate brewery (Dublin, UK). Four different dosages of commercial hop extract (0.5 to 4 mL/L) were tested as well as isinglass treatment (5 and 10 mL/L) and two combinations of commercial hop extract and auxiliary finings (2 mL/L or 4 mL/L of each) in 500 mL of beer in a fining experiment using Imhoff cones. Sediment depth, A_{600} , was recorded as well as haze analysis after 24 h of clarification (Section 2.1.2, sediment depth analysis, clarity measurements, haze analysis).

Another sample of Guinness was split into 16 500 mL Imhoff cones, with different fining combinations, at 4°C. A response surface using Design Expert 7 (12 data points) was used to design the fining map response of different combination of dosages of commercial hop extract and auxiliary finings into Guinness beer. Haze and sediment depth were recorded after 24 h of clarification (Section 2.1.2, sediment depth analysis, haze analysis).

2.2.6.4 Removal of dark beer haze using PVPP

The addition of PVPP was tested in combination with the commercial hop extract in fermented stout beer. PVPP (Polyclar™) was supplied from the HopShop (Plymouth, UK) and was prepared in warm water before use for the material to swell

and be fully functional. 5 g of PVPP was diluted with 200 mL of boiled water and stirred for 30 min.

A home-brew stout kit was produced using an addition of dried malt extract, racked and chilled to 4°C, followed by fining using combinations of PVPP and commercial hop extract. Different dosages of PVPP slurry (2 to 20 mL) with commercial hop extract (fixed addition of 4 mL/L) were studied, as well as a 6 h delayed introduction between the two fining agents in order to evaluate the best strategy to clear haze particles in stout. Sediment depth and haze analyses were performed after 24 h of clarification (Section 2.1.2, sediment depth analysis, haze analysis).

2.2.6.5 Pilot scale brewing trials using the hop derived finings

Brewing of beer at a pilot scale has been performed using the research brewery facility of AB InBev / University of Nottingham to evaluate fining activity with the commercial hop extract at a brewery scale. 7 hL of high gravity lager was prepared using 100% extra pale malt and fermentation with W34/70 dried yeast supplied from Fermentis (Bilston, UK) to reach 14.8 plato. After 10 days of fermentation, 20 L were sampled, then chilled to 4°C for a preliminary fining trial.

The dose rate of the commercial hop extract versus fining response was investigated by adding varying amounts of the commercial hop extract to 500 mL aliquots of the fermented beer in Imhoff cones. Sediment depths and 90° haze were measured at 4°C using the methods described in Section 2.1.2. For comparative purposes an unfinned control and isinglass fined beers were also included in the trial.

After determination of the optimal dose rate, the green fermented beer (pH = 4.26) was racked from the fermentation sediment and homogenised using recirculation. The beer was then separated into three stainless steel fermentation vessels, each

containing 100 L, and chilled at -1°C. Finings were added and mixed for 5 min using the agitators (paddle-stirrer) in each vessel to ensure homogeneity and left to react for 5 consecutive days. Suspended yeast cell count was measured after 24 h of clarification by pipetting beer from the top of the vessel. Haze was recorded at different stages of the maturation step (24 to 120 h post fining), and the sediment cropped volume was measured after 5 days of sedimentation in Imhoff cones. (Section 2.1.2, suspended yeast cell count, haze analysis).

In a second pilot scale trial, 10 hL of high-gravity lager beer was produced using the same recipe used in the previous experiment and then split into two 4 hL volumes after 12 days of fermentation. After being crash cooled at -1°C, one vessel was treated with a 4 mL/L dose of commercial hop extract (introduced at the bottom of the vessel prior to beer introduction) and the other left to sediment without any fining. Beer samples were taken every 24 h for two days to evaluate the haze removal action of the commercial hop extract. After 48 h of reaction the beer was then centrifuged through a Seital SPX flow disk centrifuge (Santoso, Italy) at 10000 rpm and filtered (0.6 µm pore size) prior to finished beer haze analysis (Section 2.1.2, haze analysis).

2.2.7 Impact of fining agents on beer filterability

Three batches of high gravity lager were brewed using the Grainfather brewing procedure (100% pale malt grain) and fermented for 7 days. They were then blended to give approximately 65 L of fermented beer and chilled to 4 °C. The beer sample was then split into 7 fermenters with 7 L of beer each, prior to fining treatments. Four different fining were employed to study the effect of fining in beer filterability: control (no fining), commercial hop extract (variable dose rate, mL/L), commercial hop extract + auxiliary finings (variable dose rate, mL/L, and same time addition),

isinglass (5 mL/L, reference dosage). After 24 h of reaction, 3 L was taken from the top of the fermenter, to perform filtration studies. Beer filterability was evaluated by pumping beer (1 L per run) through a filter sheet in a stainless steel filter housing. A Masterflex L/S easy-load peristaltic pump (Illinois, USA) set at 200 mL/min was used to deliver beer from a reservoir (1 litre) into a sealed stainless steel filter housing (10 cm diameter) containing a Carlson cellulose XE400 Filter sheet (0.5 to 2.5 microns pore size). The filtered mass over time was recorded every 30 s for 10 min. Pressure over the filtration time was 1 to 1.2 bar for all trials. Each fined sample was filtered in triplicates. Haze recordings were taken before filtration (24 h finings), and after filtration, with yeast cell counts taken before filtration (after 24 h of fining). (Section 2.1.2.5, haze analysis and Section 2.1.2.6, yeast cell count measurements).

2.2.8 Formulation optimisation of the hop extract

2.2.8.1 *Salt precipitation of proanthocyanidins*

A 200 mL sample of commercial hop extract was added to 30 g of sodium chloride, with continuous stirring. Once the salt was dissolved and the precipitate formed, the solid was then separated. Another 30 g of sodium chloride was added to the extract in order to form further precipitates. Once all the solids were collected, they were dried and milled using a coffee blender. The solid formed was then redissolved in 200 mL of pure methanol in order to compare the commercial hop extract, before and after precipitation for fining performance and PA concentration (Section 2.1.2.2, sediment depth analysis, Section 2.1.2.5, haze analysis and Section 2.1.2.8, acid butanol assay for proanthocyanidin content of extracts). Methanol was used instead of water to fully disperse the solid material as it was very difficult to fully solubilise in water.

2.2.8.2 Spray drying of the hop extract

Laboratory spray drying of hop extracts was investigated to see if drying the product first had an impact on the fining action. Commercial hop extract (100 mL) was added to a carrier (either Maltodextrin, β -cyclodextrin or Gum arabic at different mass ratios (%wt)). Each mix was then atomized by spray-drying at 4.5 bar of pressure using a Büchi B-191 mini-spray dryer (Chadderton, UK) at 130°C using a flow of introduction of 3 mL/min (controlled with a peristaltic pump). The yielded powders were then assayed into an Imhoff cone fining experiment with addition of solid powder into kit green beer and the powder redissolved into water prior to beer introduction. Haze and sediment depth were recorded after 24 h of clarification (Section 2.1.2. sediment depth analysis, haze analysis).

2.3 Results and Discussion

2.3.1 Optimisation of the extraction method for preparation of hop finings

Early work of Linforth et al., (2015) used aqueous hop extracts in their first beer clarification investigations. In order to optimise the fining activity of hop extracts, several approaches were taken to determine the best solvent mixture with which to extract the active compounds. Different extraction solvent and mixtures were cited for the extraction of proanthocyanidins in the literature such as acetone, water, ethyl acetate and alcohols (Bosso et al., 2016; Kennedy, 2005; Kim et al., 2010). Moreover, Hellström et al., (2008) indicated the evidence that mixtures of acetone and water were the most effective overall extraction solvent for proanthocyanidins.

In the first experiment, the extraction solvent was a trinary mixture of acetone ethanol and water in various proportions from 0 to 100% using an experimental

mixture design (30 data points in randomized extraction sequence). The results indicated that the presence of ethanol in the extraction mixture was having a negative effect on extraction efficiency as well as fining performance. Water/acetone extracts were more efficient (data not shown).

Another sequence of extracts (prepared from 2.2.1 method section) was produced covering the range from 0 to 100% for only acetone in water as the extracting solvent (10 different samples, in triplicates). All extracts varied in colour from orange-brown for the most aqueous ones, to a greenish colour for the highest concentrations of acetone. All extracts reacted in the gallic acid assay, indicating the presence of polyphenolics. They also gave the red-magenta colour in the acid butanol assay which confirmed the presence of proanthocyanidins in the hop extracts. A range of absorbance values were produced from the acid butanol assay, ranging between 0.6-1.02 (Figure 2.1). Absorbance values broadly correlate with the amount of proanthocyanidins present but do not yield any information on their molecular weight range.

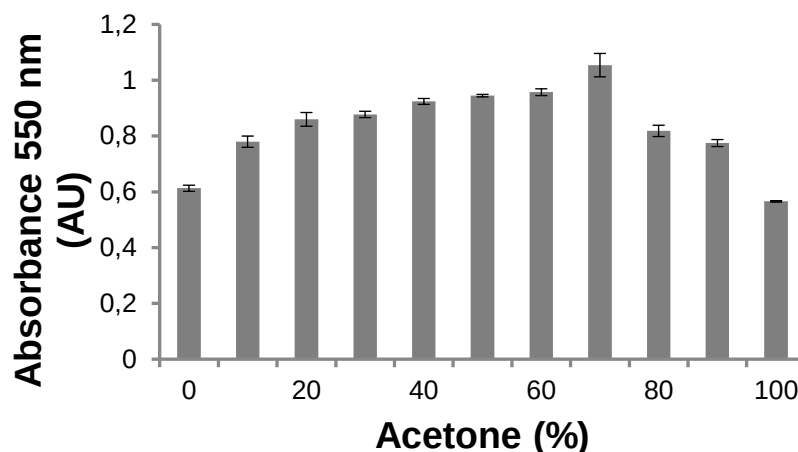


Figure 2.1 Absorbance values at 550 nm after hydrolysis of Target hop extracts extracted using different proportions of acetone in water. Values are expressed as the mean of triplicates with their respective standard deviation.

Extraction of hops with 70% acetone_(aq.) yielded the highest level of proanthocyanidins. Furthermore, fining performance in green beer was likewise found to be optimal at this solvent ratio (Table 2.1). A volume of 2 mL of Target hop extract (prepared from 2.2.1 method section) was added to a beaker containing 500 mL of beer after fermentation, at 4°C. After mixing, they were each poured into an Imhoff cone for fining performance evaluation. The two extremes samples (100% water and 100% acetone) gave the highest haze measurements after fining reaction. However, the lowest haze was observed for extracts using 70% acetone, suggesting that this extraction solvent is able to extract largest and most active polymers.

Table 2.1 Flocculation activity and haze results of fined green beer (4°C) using Target hop extracts from different solvent extractions at 4 mL/L. Haze analysis was performed after 24 h of clarification.

% Acetone	Sediment depth (mL)		Haze measurement (EBC)	
	3 h	24 h	H90	H25
0	6.25	6	17.3	27.8
10	6	6	12.7	21.7
20	6.25	6	15.7	25.7
30	6.25	6.5	12.2	20.2
40	6.5	6.5	10.4	18.3
50	6.5	6.25	10.4	18.1
60	6.5	6.5	10.9	19.3
70	6.5	7.25	9.4	17.3
80	6	6	10.9	19.0
90	6	7.25	12.1	21.9
100	6.25	7.5	18.0	28.8

H90 = 90° scatter; H25 = 25° scatter;

The hop finings extraction procedure has been evaluated and compared to the current literature relevant to proanthocyanidin extraction. Liquid/liquid extraction is the main method of extracting proanthocyanidins from plant material (Liu, 2012). Historically, Masquelier, who discovered proanthocyanidins, patented the extraction using only hot water as the solvent extraction (1987). Pekić et al., (1998) showed that using ethyl acetate in the absence of water proanthocyanidins could not be efficiently extract PA from grape seeds. PA from plant materials have been extracted with different solvents, including ethanol (Hernández-Jiménez et al., 2012; Tanaka et al., 2014), acetone (Monagas et al., 2009; Taylor et al., 2003), methanol (Appeldoorn et al., 2009; Gupta et al., 1981), and their respective water mixtures in different concentrations. The effectiveness of extraction in ternary mixtures including a combination of two solvents (acetone and ethanol) and water have also been studied for condensed tannins extraction in grape pomace seeds (Bosso et al.,

2016). They reported that the water acetone extracts obtained higher yields and higher mean degree of polymerization than the aqueous mixtures of ethanol and ternary mixtures. The solvent extraction efficiency relies on different parameters such as solvent polarity, extraction time, temperature of extraction, or the solid-liquid ratio. A report by Gu, (2012) shows as well that mixtures of water and acetone are more efficient than aqueous methanol for the general extraction of proanthocyanidins.

In the present extraction evaluation, the most concentrated proanthocyanidin fraction was found to be extracted from hops using 70% acetone _(aq.). As the degree of polymerisation of polymers increases, they become more hydrophilic (Tanaka et al., 2014). Consequently, it was thought that a lower proportion (less than 50%) of acetone in water might improve the extraction concentration as previous studies had confirmed fining activity in 100% aqueous extracts (Linforth et al., 2015). However, 70% acetone _(aq.) correlates with the solvent composition typically recommended for the extraction of proanthocyanidins (Magalhães et al., 2010). This extract also gave the most efficient results in terms of beer fining activity. Thus the use of 70% acetone _(aq.) was applied to the extraction of all proanthocyanidin extracts from hops used in subsequent studies.

2.3.2 Impact of mixing protocol on fining action in green beer

The hop extract has the ability to flocculate yeast and proteins in green beer (Linforth et al., 2015). However, dynamic effects such as mixing and shear rate have to be taken with real care for flocculants (Gregory, 1988). Mixing can improve and increase particle collisions, inducing an enhanced flocculation. Higher regimes can however disrupt the flocculation by diminishing the particle size of the flocs created. Two experiments were designed, one covering different shear rate mixing regimes

for the mixing of the prototype commercial hop extract into beer and a second experiment with a paddle stirrer only with lower mixing regimes. Sediment depths and haze measurements at 4°C are presented in Table 2.2.

Measurements on the unfined control beer were performed in triplicate and showed no sediment after 3 h of reaction, compared to 8 mL of compact sediment produced for the beer sample fined with mixing at 500 rpm for 30 s. A change in residual haze was noticeable between the unfined beer (4.7 EBC average) and the fined beer with the lowest mixing regime (2.4 EBC). The sediment also compacted over time, decreasing in volume from 8 mL after 3 h to 6 mL after 24 h of reaction. The haze values and the sediment depths for all paddle stirrer mixed samples were broadly similar. Similar results were observed for the fined beer samples using the Silverson mixer up to 4000 rpm, with compact sediment ranging from 6.5 mL to 8 mL after 3 h of reaction, and a final haze between 3 and 4 EBC.

Table 2.2 Flocculation activity and haze results of kit green beer fined with 2 mL/L of prototype commercial hop extract (4°C) using different mixing strategies. Haze analysis was performed after 24 h of clarification.

		Sediment depth (mL)		Haze 4°C (EBC)	
		3 h	24 h	H90	H25
Paddle stirrer	Control (no fining)	0	2.5	4.28	7.09
		0	2.5	4.84	9.79
		0	2.5	5.1	9.92
	Mixing velocity (rpm, 30 s)				
	500	8	6	2.35	4.53
	1000	9	6	1.93	2.78
	1500	9	5.5	2.44	4.28
	2000	8.5	6	2.37	5.09

Silverson	2000	7	6	3.01	5.47
	3000	8	7.5	4.78	10.21
	4000	6.5	6	3.16	7.26
	5000	4	5.5	4.34	10.63
	6000	3	6	12.04	25.87
	7000	1	5.5	8.55	15.18
	8000	2	4.5	12.81	26.98
Ultraturrax	11000	1	5	6.95	26.38
	16000	0	6	9.14	16.47
	22000	0	5.5	7.07	14.09

Increasing the mixing regime over 4000 rpm (Silverson and Ultraturrax) caused a breakdown of the fining mechanism. A significant decrease in sediment production was observed for all samples after 3 h of reaction, with no sediment produced for the highest mixing regime samples with the Ultraturrax at 16000 or 22000 rpm. A sediment was however produced after 24 h of reaction. However, the residual haze values were substantially increased at high mixing rates (> 3000 rpm) relative to lower ones. The development for a routine fining experiment needs a standard mixing procedure for the hop extract into beer.

In the second experiment, aliquots of the green beer kit (500 mL) were mixed in a beaker with 1 mL of commercial hop extract using only the paddle stirrer, with a range of time and speed settings. Samples were then transferred to Imhoff cones at 4°C. There was no significant effect ($p > 0.05$, ANOVA) on the sediment depth or A_{600} value due to mixing rate over a range of 50 to 500 rpm for mixing velocity, and 10 s to 2 min for mixing duration. This showed the fining reaction was very consistent and reproducible with a range of mixing conditions.

First experiment showed that high shear mixing regimes have the potential to negatively impact on flocculation (Table 2.2). To prevent floc from tearing apart or shearing, the mixing velocity and energy are usually tapered off as the size of floc increases. Zhang et al., (2019) studied the effect of mixing shear rate of suspended microalgal cells on the mean size and fractal dimensions of microalgal cell flocs formed. They demonstrated that an optimal shear rate produced higher sized and compactness desirable Al^{3+} coagulated *Chlorella vulgaris* flocs than higher or lower shear rates. At a brewery scale it is important to understand how bulk mixing and the addition protocol influence the action of fining agents. The second experiment using the paddle stirrer showed that neither time of mixing nor speed for a low mixing regime changed the final haze of the fined beer. Based on these results, in all further fining trials using, hop extracts were introduced in green beer in using the paddle stirrer set on 500 rpm, for 30 s prior to Imhoff cone introduction.

2.3.3 Microscopy of beer fining

Proanthocyanidins are well known for their ability to bind and precipitate proteins (Hagerman et al., 1981; Rauf et al., 2019), leading to the creation of water soluble or insoluble complexes. The flocculation reaction is quick, allows the flocculated particles to sediment after time. The fining mechanism in beer using the hop extract involves three major components: yeast, beer proteins and the proanthocyanidins introduced. The flocs can grow to colloidal size (Papadopoulou et al., 2004) and either settle or scatter light. Linforth et al., (2015) thought the hop extract to be a yeast and protein flocculent. However, they did not present any microscopy studies to show the flocculation action.

Light microscopy was used to evaluate yeast flocculation in a kit green beer (7 d) with Saaz hop extract (prepared from 2.2.1 method section). This was performed at

low magnification (x100), to allow the visualisation of a large yeast population. The image of the untreated beer (Figure 2.2, picture A) shows yeast cells that are mostly dispersed individually or in small groups. In contrast, Figure 1B shows an image taken 2 min after the application of the Saaz hop extract and reveals large flocs of yeast cells. These large flocs of yeast formed by the hop extract would be faster to sediment in a fining experiment due to their size (Section 1.2.4, Sedimentation of particles).

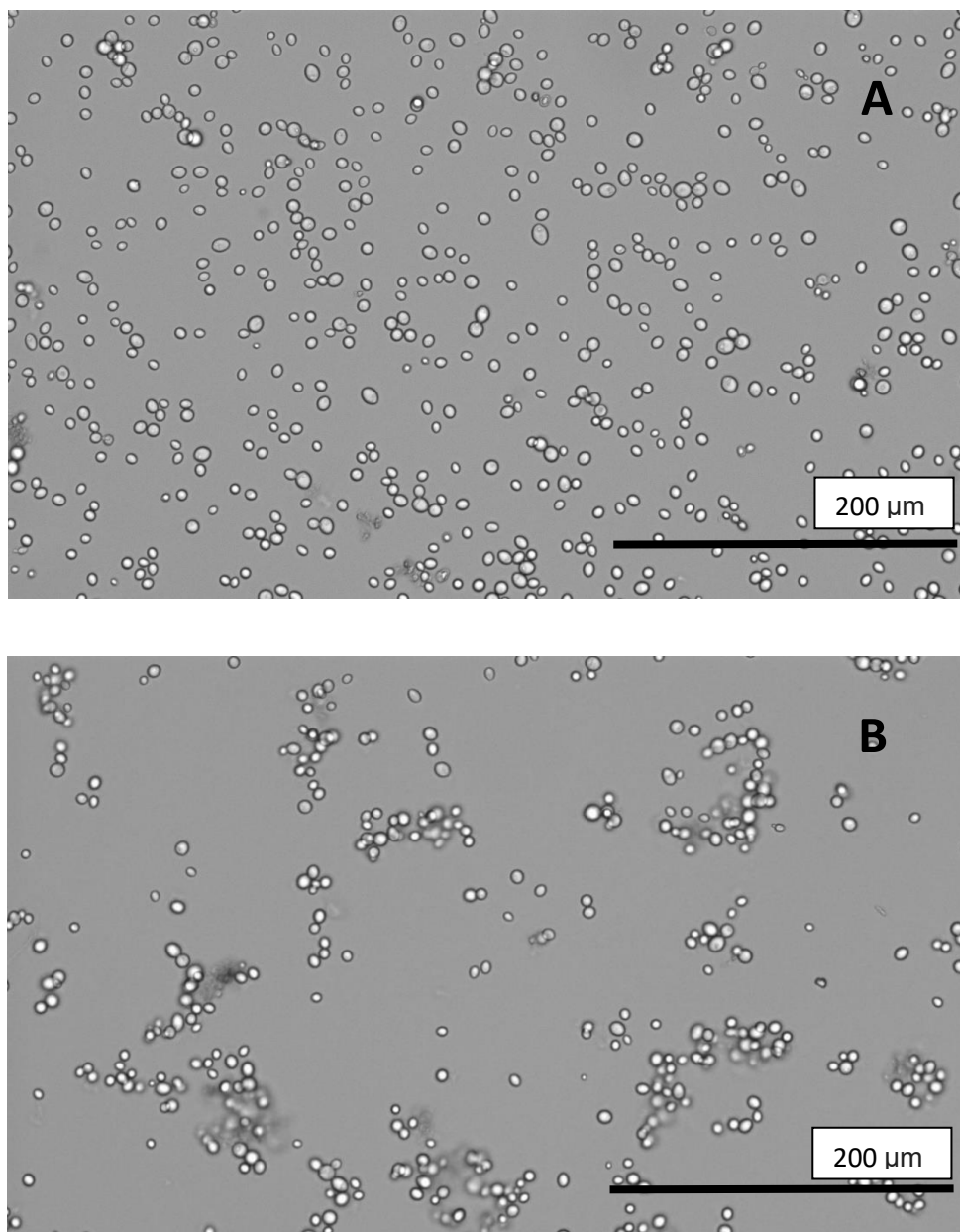


Figure 2.2 Light microscopy images of a kit green beer. A, before application of the hop extract and B, after hop extract application. The scale bar shown is 200 μm long.

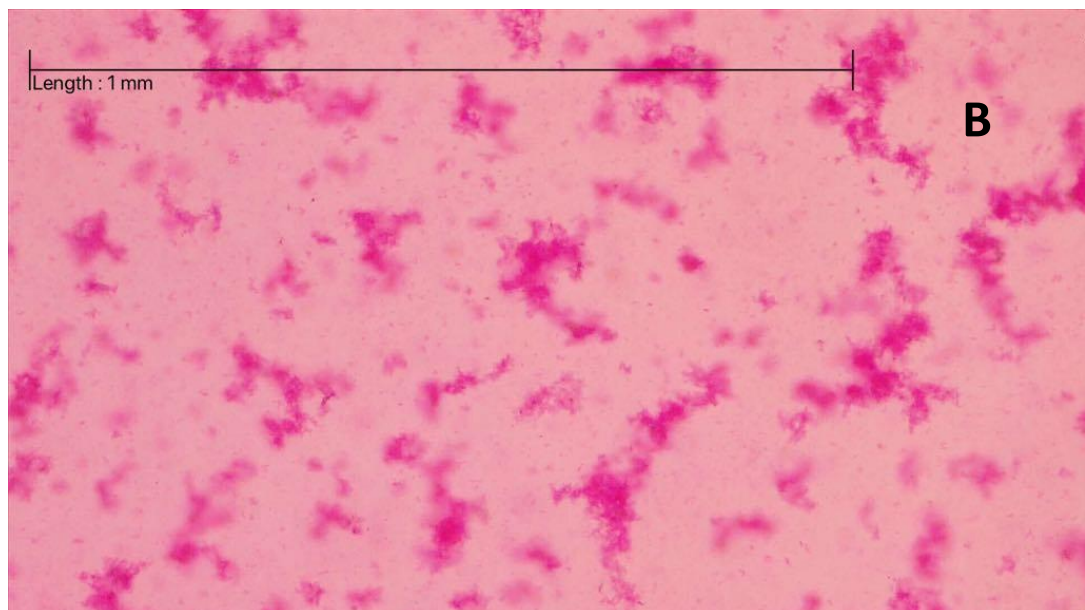
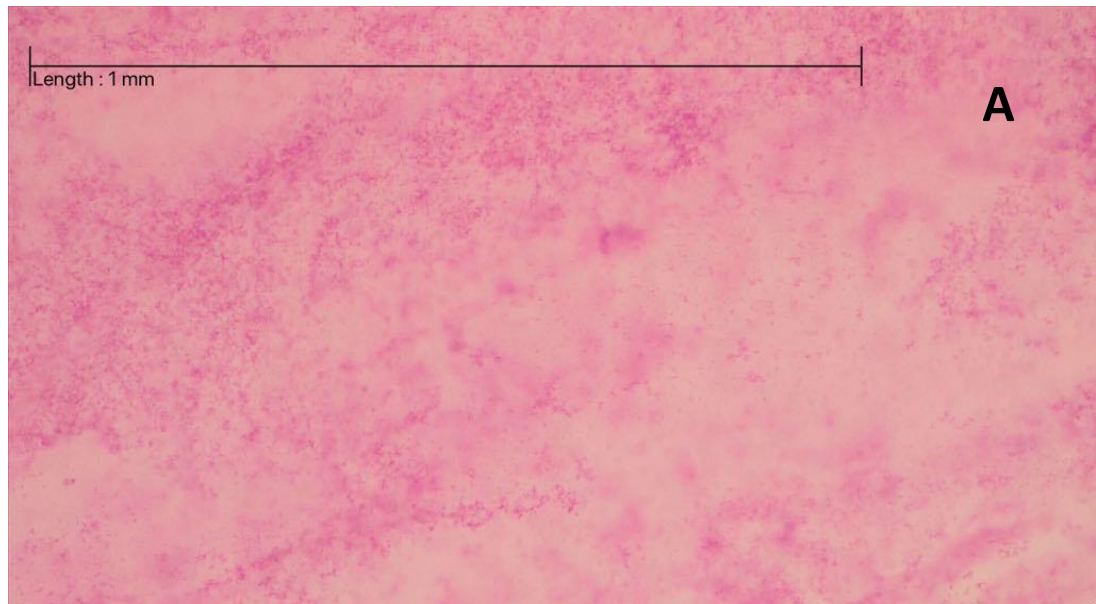


Figure 2.3 Light microscopy images of Budweiser beer with eosin yellow dye. A, before application of the hop extract and B, after hop extract application. The scale bar shown is 1mm long.

A commercial beer (Budweiser lager) was used to investigate protein flocculation in a yeast free beer matrix. Figure 2.3, picture A shows the beer dyed using eosin yellow (Steiner et al., 2010) to stain protein particles. A fine cloud of protein particles is

visible, formed by protein in the finished beer. These small particles are formed by the addition of the dye which tends to partially precipitate protein. In Figure 2B, a drop of Saaz hop extract was added to the slide prior to mixing. After 2 min, a clear set of aggregates are visible, with both small and large aggregates of protein. Yeast was not present in the sample, the beer was a filtered bright lager beer which indicates the potential of hop extracts to coagulate beer proteins.

The kit beer used in Figure 2.2 was similarly fined but this time using isinglass finings in the presence of eosin yellow dye. The dye stained large clumps of isinglass (Figure 2.4). Isinglass is comprised of collagen proteins (Hickman et al., 2000). The aggregates network shown in the picture were larger than when using the hop extract finings. The size of the isinglass lumps can also be due to the fact that isinglass is harder to disperse in water compared to the less viscous hop extract.

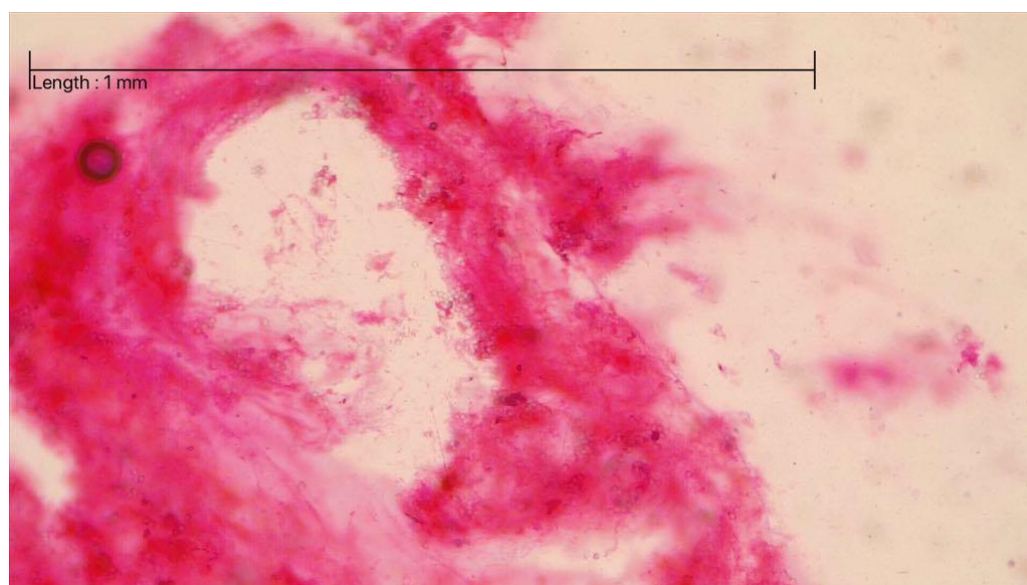


Figure 2.4 Light microscopy image of a kit green beer after application of isinglass finings dyed with eosin yellow. The scale bar is 1 mm long.

Light microscopy was also used to study the composition of silicates auxiliary finings. This fining agent (Fining adjunct, inorganic silica based acidified solution)

is mostly used in combination with isinglass fining to perform greater haze removal than isinglass on its own. The solution is showing small particles with clear-cut edges, almost like glass shaped particles. (Figure 2.5).

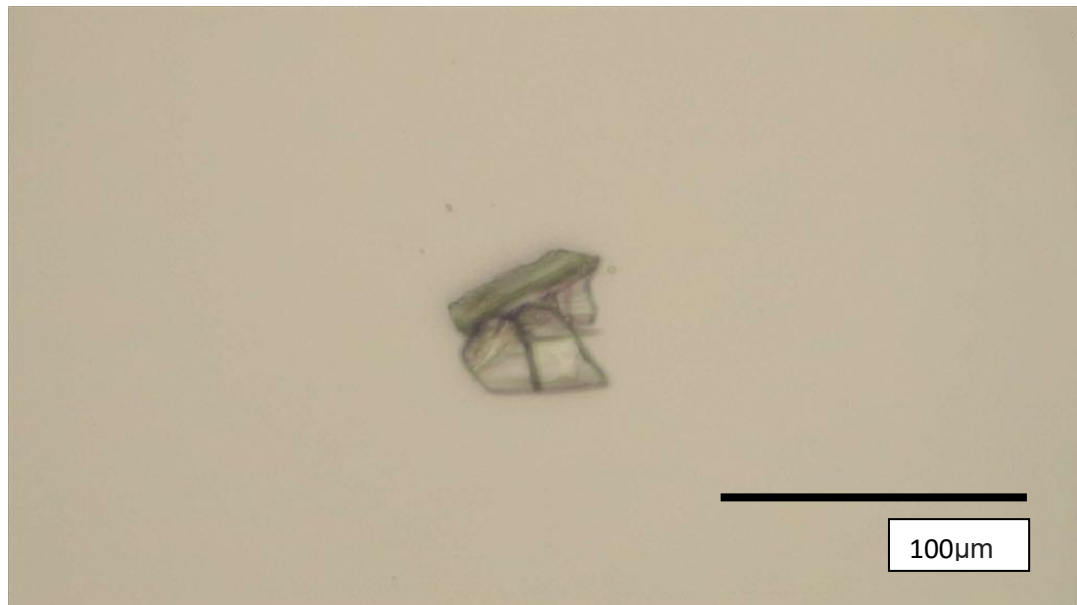


Figure 2.5 Light microscopy image of auxiliary (silicate) finings. The scale bar is 100 µm long.

Based on early microscopy experiments, hop extracts are easily dispersible into beer, and appear to be both a yeast and protein flocculent. Microscopy studies (Section 2.3.3) showed a clear aggregation between both proteins and yeast using the hop extract addition. Protein-polyphenols complexes have been shown previously to have the potential to form macro-aggregates (Steiner et al., 2010). Layers of different colours are usually found in the Imhoff cone sediments resulting from beer fining experiments; pale yellow being indicative of a yeast floc, and a darker colour with more transparent texture representative of haze-protein flocs.

2.3.4 Finings optimal dosage evaluation in a beer brewed from 100%

barley malt

Six different dosages of the prototype commercial hop extract were introduced into grain beer to compare the fining efficiency with different volumes of addition of the hop extract.

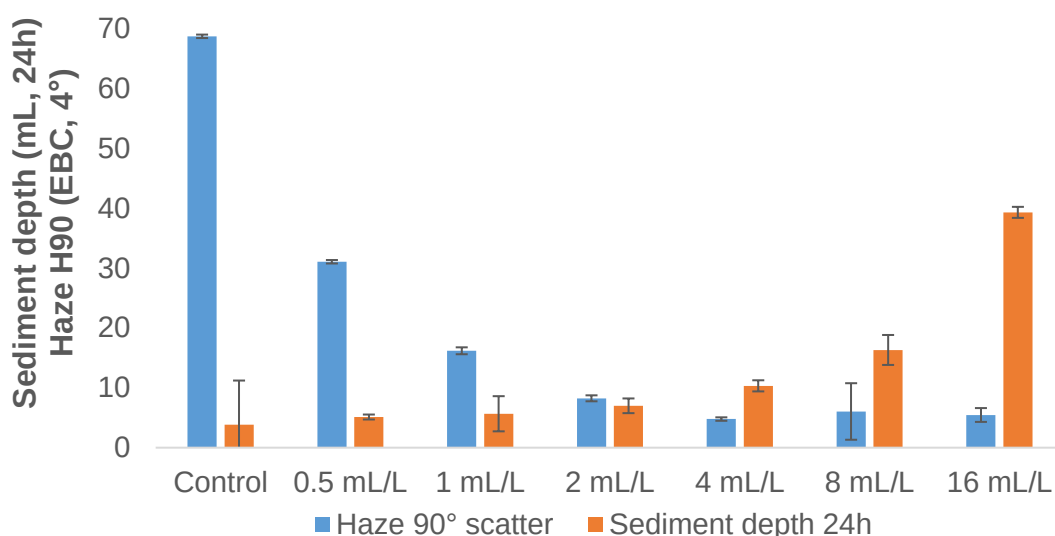


Figure 2.6 Sediment depths and haze recordings of grain beer (4°C, 7 d) fined using different doses of commercial hop extract. Haze analysis was performed after 24 h of clarification. Each result represented is the mean from triplicates, the errors bars show the standard deviation.

All beers with the commercial hop extract treatment formed a higher and more compacted sediment after 24 h compared to the control samples (Figure 2.6). Sediment height is increasing over dosage from 5.1 (0.5 mL/L dosage) to 39.4 mL (16 mL/L dosage). The control beer samples produced a sediment after 24 h, but, this was lower than all prototype commercial hop extract treated beers. It had a pale yeasty colour, whereas there were several layers of material in the hop extract treated samples, indicating the presence of protein-polyphenol aggregates. Haze measurements at 4°C were recorded after 24 h. Values ranged from 4.8 EBC for the 4 mL/L treatment to 69 EBC for the control sample. All treated beers had a lower

haze level compared to the control showing a positive fining action. It is important to note that the haze is decreased with increasing dosage until an optimum (4 mL/L). The two last dosages used in the series were over fined, with higher haze and fluffy sediments.

For all fining treatments used in this study, the sediment formed was significantly higher in size than the unfined control beer, showing an efficient yeast flocculation. As polyphenols are both able to bind with yeasts and beer proteins, this mechanism will produce the largest network between the flocculants, and the largest particles. Haze formation in beverages between polyphenol and proteins has been previously studied by Siebert et al., (1996). They reported that the formation of microscopic flocs depends on both concentration of protein or polyphenol added or present in solution. Moreover, the haze formation was different for the addition of either catechin or tannic acid with a fixed concentration of protein (Siebert et al., 1996). This shows first that different binding sites are available depending on the compound added, but also a possible difference due to size of the polyphenol added. As beer is already containing haze-active proteins, a low addition of polyphenols would only result in a poor flocculation. On the contrary, if polyphenols are introduced with a higher concentration, the sediment formed is not compact, but also beer haze is increased. This action will defend the principle found by Siebert, (1999), as a higher concentration of polyphenols added would only result in dimers or small flocs, resulting in an increase in haze due to their low molecular size. Voluminous sediments would also represent consequent beer losses for brewers as the sediment is usually cropped at the bottom of the vessel prior to further steps.

2.3.5 Using the hop extract for fining with an auxiliary finings

Typically, combinations of different fining agents are used in order to maximise haze removal. Currently, the most efficient beer fining strategy used by brewers is the combination of kettle finings during wort boiling, and the addition of isinglass finings with auxiliary finings post fermentation. It is used extensively in brewing to remove haze and improve filterability of beers (Walker, 2007). The next experiments determined if the commercial hop extract could be used in conjunction with auxiliary silicate finings added both to the beer simultaneously.

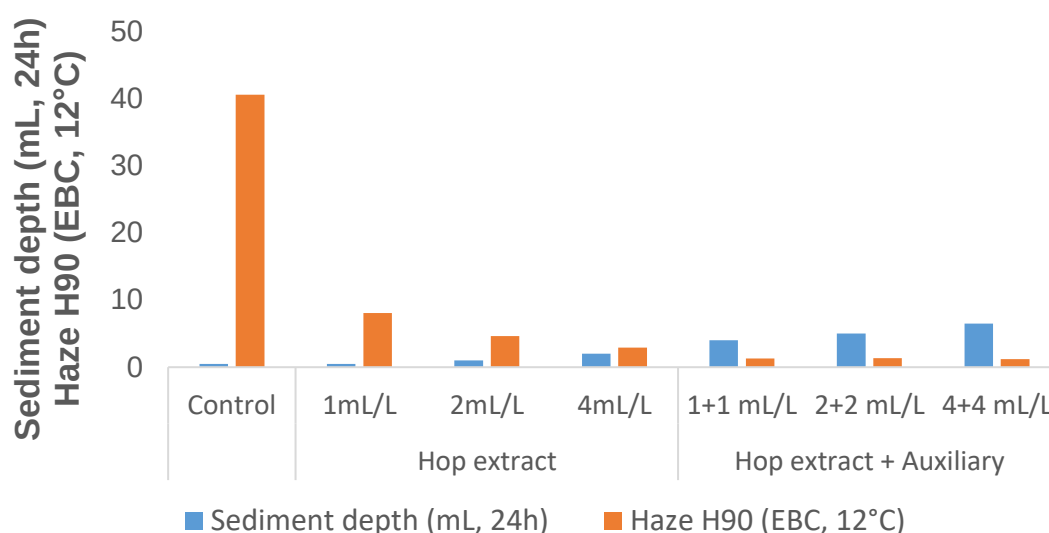


Figure 2.7 Sediment depths and haze recordings of kit green beer (12°C) fined with different dosages of commercial hop extract and a combination of hop extract and silicate auxiliary finings.

All treatments fined the beer successfully as compared to the control, with very low haze values and good sediment formation (Figure 2.7). The control had a small sediment after 24 h, with substantial haze material still in suspension. Haze decreased and sediment increased with dose rate for the commercial hop extract treated beers. The auxiliary/hop extract treated beers showed very low final haze values. They all produced a higher amount of sediment than the commercial hop extract treated beers. Interaction of the hop extract and the silicates was highly

effective for haze removal. The fact that the fining action was successful when hop extract and auxiliary finings were added at the same time is noteworthy. This is not the case when using isinglass, wherein the auxiliary must be added and allowed to act before the isinglass addition due to their opposite charge. A combination of the two finings introduced at the same time enhanced beer clarity. This result indicates that auxiliary finings and the commercial hop extract are composed of the same charge.

The commercial hop extract and auxiliary interaction was further investigated in a 2 factor response surface experiment where a range of addition levels of both finings agents varied. Both fining agents were added simultaneously to post-fermented beer. Figure 2.8 shows the sediment depth and the haze values recorded after 24 h of clarification using a kit beer. This beer has a much lower haze-active protein content as it is brewed with a simple malt extract. Sediment depth is increasing with a simple additive behaviour with increasing auxiliary and prototype hop extract finings ($R^2 = 0.97$). In terms of final haze, for the auxiliary, there was no significant effect ($p > 0.05$, ANOVA) of changing the dose rate between 0.66 to 4 mL/L of beer. There was however, a statistically significant impact of the prototype hop extract addition rate on haze, which decreased from 1.4 to 0.8 EBC ($p < 0.05$, ANOVA).

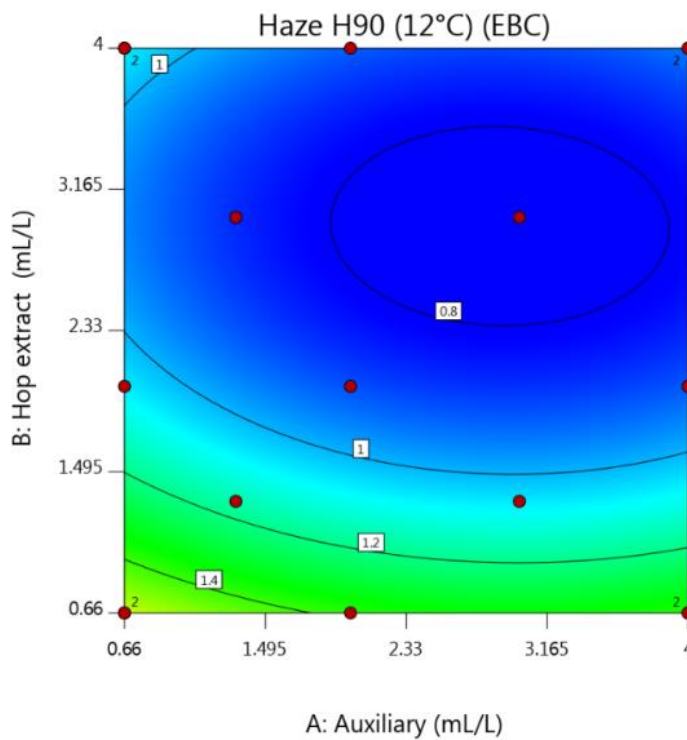
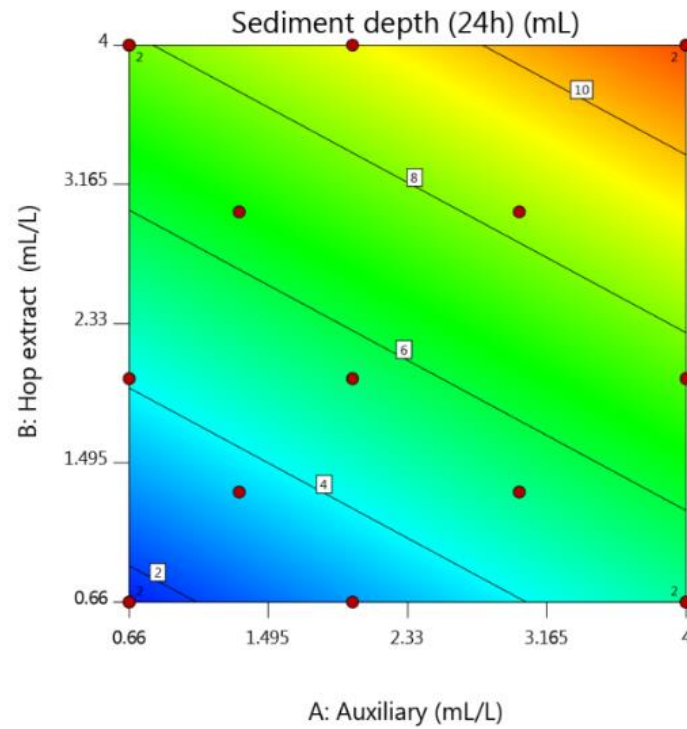


Figure 2.8 Sediment depth (mL) and haze H90 at 12°C with varying commercial Hop Extract / Auxiliary introduced (simultaneously) into kit green beer. The red dots show the experimental points and contour line join up the point of equal response. The colour shading is from blue, (low response), to red, maximum.

Figure 2.9 displays the same values recorded for a grain beer at 4°C using different volumes of auxiliary and prototype hop extract finings. The beer used has been brewed from grain, giving the matrix more complexity and a higher concentration of haze-active particles. The sediment response is as well increasing linearly with increasing dosages of both fining agents ($R^2 = 0.98$). Both finings have a significant effect ($p < 0.001$, ANOVA) on the sediment formation. However, the slope of the line shows that the amount of prototype commercial hop extract had a greater effect on sediment formation. Haze recordings are showing that the lowest value was observed for the highest dosages of auxiliary and around 3 mL/L of hop extract. With a clear interaction shown by the curved contour lines, haze is reduced from 26 to 8 EBC. These two sets of data show that depending on the beer, commercial hop extract or auxiliary may have a greater effect on sediment formation and haze. Overall the combination of the two seems generally effective, with one or the other fining agent dominating. Consequently, despite them appearing to have the same charge they appear to work in different ways and are not simply additive factors. As a result the dual addition of both fining agents results in an effective fining strategy.

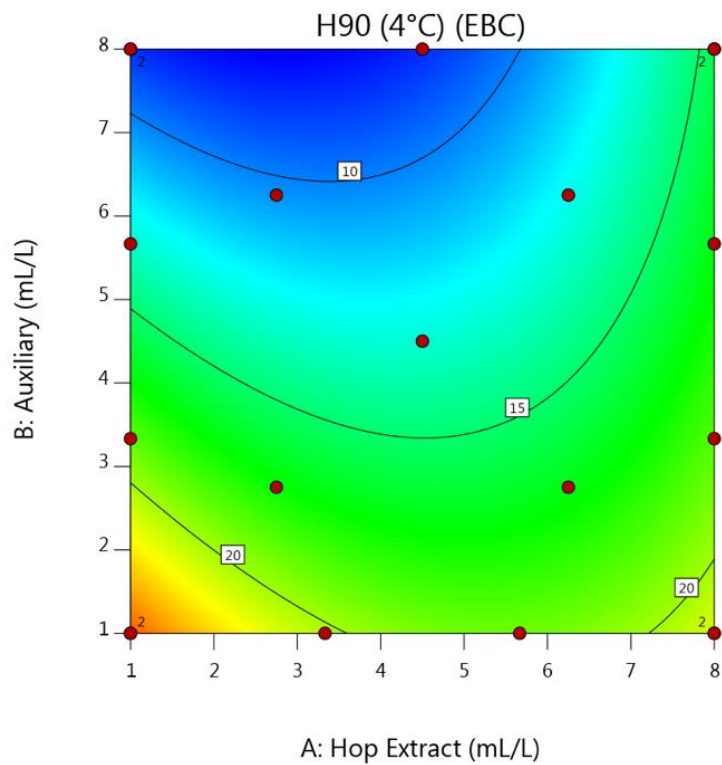
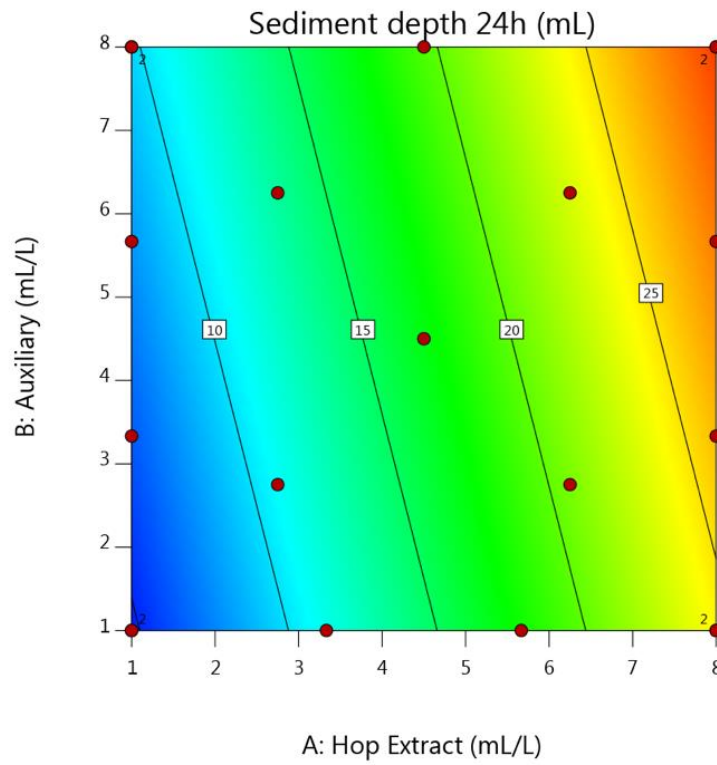


Figure 2.9 Sediment depth (mL) and haze H90 at 4°C with varying prototype commercial hop extract / auxiliary finings introduced (simultaneously) into grain beer (7 d). The red dots show the experimental points and contour line join up the point of equal response. The colour shading is from blue, (low response), to red, maximum.

A laboratory test was also performed to determine if the commercial hop extract would have a better efficiency if it was introduced at a different time to auxiliary finings. In this case one of the fining agents (auxiliary or hop extract) was added 30 min, 6 h, or 24h before the addition of the other one.

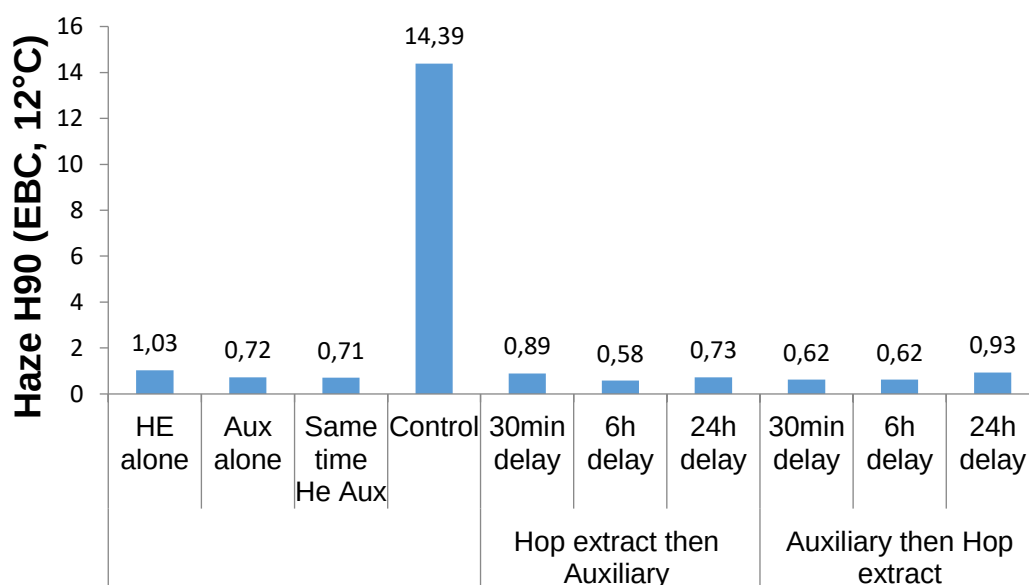


Figure 2.10 Haze measurements (H90) recordings for a kit green beer treated with combinations of commercial hop extract and auxiliary finings varying delay time between their introductions. Haze measurements were taken after 36h from the first fining agent introduced.

All treated beers showed a positive fining action relative to the control beer (Figure 2.10). Final beer hazes are ranging from 0.58 to 1.03 EBC for the treated samples compare to 14.39 EBC for the control sample. Samples with a combination of the two finings agents showed a lower haze than the beer sample treated with only the commercial hop extract. The optimum time delay between the introduction of the fining agents is 6 h, but it not does not seem to affect beer clarification. The introduction of both finings at the same time result in a final haze of 0.71 EBC (compared with 0.58 EBC for the delayed addition) and represent an easier way of fining introduction. Auxiliary finings is having a positive effect in combination with

the prototype commercial hop extract, resulting in beneficial haze removal interaction compared to the addition of the extract on its own. Two finings agents should have a better effect than one for fining efficiency and this clearly indicates that is the case.

Two response surface designs have been performed in order to understand the interaction of auxiliary finings and the prototype commercial hop extract on fining performance. In the kit beer, haze removal is mainly driven by the addition of the hop extract and is able to work out efficiently. Haze is reaching an optimum for various hop extract ranges, indicating that the majority of haze-particles proteins would have been removed efficiently. Using the grain beer, both fining agents are acting together towards haze removal. This time, auxiliary finings is having a more significant impact on haze removal, and best results are observed for high dose of fining agents. As seen in microscopy experiments, auxiliary finings can be observed as small particles of glass. Their bigger size compared to the commercial hop extract active components implies a bigger fining network formation and an efficient clarification. The difference between the two results validates the theory explained by Siebert et al., (1996) where haze formation can be explained through binding site stoichiometry. Higher would be the concentrations of particles to fine and higher dosages would be required to perform an efficient clarification.

Charge status is also an important parameter of beer clarification. Auxiliary finings is usually used in beer fining in combination with isinglass. However, those two finings cannot be introduced at the same time in the beer. Leiper et al., (2002) have shown that there is no benefit to pre-mix those two finings prior to beer introduction due to their opposite charge. A different result have been found when using the

commercial hop extract in combination with auxiliary finings, as the time of introduction of the two finings does not change significantly the haze removal mechanism. It seems that the polyphenols and the silicates particles are not counter-acting to each other. Because of their beneficial action together, it is possible that they form larger particles which would be able to attract more haze particles when added. Also interestingly, all delayed samples gave broadly the final haze value. That would imply that both finings agents would own a specific selectivity to proteins, and the combination of the two would have a larger selectivity to all haze-active particles into beer. A difference in the particles charge would also favour the specific binding of the finings agents to the haze-active beer proteins.

2.3.6 Evaluation of optimal fining conditions for a range of different beers

Beers are made using various raw materials, and various different systems of brewing (Briggs et al., 2004) from a few litres a week to thousands of hectolitres (hL) per brew. Beers may be classified into types on the basis of the nature of the raw materials used, the microbial agents used in the fermentation process, and the different steps to transform the starting materials into a drinkable beverage ready for consumption (Boulton et al., 2001). The next experiments will establish if the hop extract could be applied on a large range of different beers.

2.3.6.1 Variation of the protein content and suspended yeast cell counts in a model beer

It was then decided to evaluate a different range of fining agents on different beers, varying two main concentrations; yeast cell count and protein concentration. 3 model beers were produced varying the concentration of protein and their suspended yeast cell count and an additional commercial beer as a commercial reference was taken for the study (Table 2.3). Protein concentration was varied by the extra addition of dried malt extract (6% protein content) in the recipe relative to sugar. Suspended

yeast cell count was manipulated using different fermentation times. 9 different fining treatments were studied in Imhoff cone fining trials (both 4 and 12°C) in order to classify the best fining strategy for different beers (Table 2.4).

Table 2.3 Properties of the beers used in the fining study, pH, colour (EBC scale), yeast count (cells/mL), malt (additional malt added yes/no).

Beer ^a	pH	Colour	Suspended Yeast cells/mL	Additional malt extract?
CB	4.1	19.3	3.02 x 10 ⁶	N/A
MP	3.72	15.7	6.40 x 10 ⁶	Yes
Hp	3.67	15.1	3.09 x 10 ⁹	No
LP	3.68	16.1	1.92 x 10 ⁵	Yes

^a CB, Charnwood brewery beer, other codes refer to beers brewed at Sutton Bonington with high or low yeast (High; H, medium; M, low; L) or protein (high, P; Low, p) content. Malt extract was composed of 6% proteins.

Table 2.4 Fining treatments and codes used in the fining study, and method of application. Volumes are expressed in mL/L of green beer. Green beers were chilled at 4°C or 12°C for 12 h prior to fining.

Sample code	Fining treatment	Method of introduction
HE 2	Commercial hop extract from Barth Innovations Ltd. 2 mL/L	Instantly
Aux	Auxiliary Fining 2 mL/L	Instantly
C	Control	No fining
IG 10	Isinglass 10 mL/L	Instantly
IG 20	Isinglass 20 mL/L	Instantly
Aux + IG 2.5	Auxiliary 2 mL/L + Isinglass 2.5 mL/L	Aux first then IG after 15 min
Aux + IG 10	Auxiliary 2 mL/L + Isinglass 10 mL/L	Aux first then IG after 15 min
Aux + HE 2	Auxiliary 2 mL/L + Commercial Hop Extract 2mL/L	Aux first then HE after 15 min
HE 2 + Aux	Commercial Hop Extract 2 mL/L + Auxiliary 2mL/L	HE first then Aux after 15 min
HE 2 + Aux Same time	Commercial Hop Extract 2 mL/L + Auxiliary 2mL/L	Instantly HE and Aux at the same time

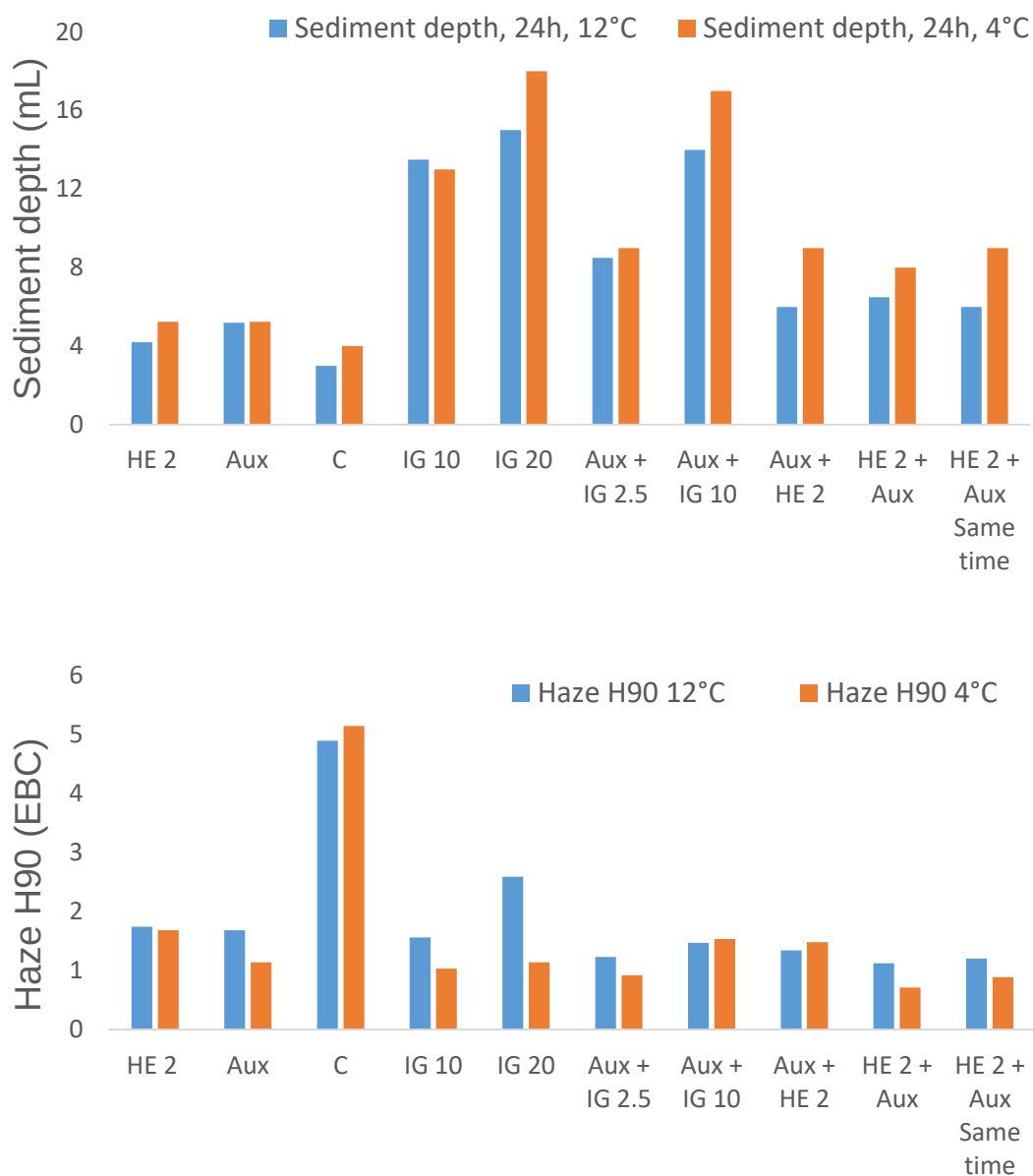


Figure 2.11 Haze recordings and sediment depth of Charnwood Brewery beer treated with a range of different fining strategies at 4°C and 12°C. Haze measurements has been recorded after 24h of clarification.

The data for the Charnwood Brewery beer (CB) is shown in Figure 2.11 (12°C and 4°C) as well as Appendices. All of the fining strategies resulted in larger sediment than the control treatment at both temperatures. There was a clear difference in sediment formation at the two temperatures. At 4°C the sediments showed a

maximum depth at 3 h and then decreased as the sediments compacted. At 12°C the maximum was typically at 6 h. This indicates that the finings were less active at 12 compared to 4°C resulting in slower sedimentation (Appendices).

Some of the fining strategies resulted in larger sediment volumes than others. Typically the isinglass (IG) based finings produced the larger sediments whilst those based on the commercial hop extract (HE) and auxiliary (Aux) were smaller (Figure 2.11). The significance of these differences is however difficult to evaluate. A smaller sediment volume may be the result of less efficient fining, or, it may be due to the production of a more compact sediment. Equally, larger sediment could be the product of more efficient fining or over fining resulting in less compact sediments. A comparison of the sediment data with the haze may help to distinguish between these 2 options.

The clarity of the treated beers was recorded at the same times as the sedimentation depth values. Figure 2.11 shows the haze measurements for the Charnwood brewery after 24 h at 4 and 12°C. All fining treatments generated clearer beers than the control, and data tend to follow the opposite trend as for the sediment depth trials, resulting in low haze for high sediment formed.

The 4°C treatment seems to be generally reducing haze more than at 12°C. Similar haze values have been found at 12°C between auxiliary finings and isinglass on their own. The hop extract showed slightly worst results, yet comparable to other fining agents, particularly at 12°C. The sequence of commercial hop extract and auxiliary was varied, such that HE was added before, after or at the same time as Aux. These combined application of auxiliary and commercial hop extract reduced haze with comparable efficiency to isinglass or isinglass and auxiliary. However, these small

differences need to be evaluated over a greater range of beers. Table 2.5 summarises haze results for a range of beers fined with the same protocols used in the Charnwood brewery experiment (Table 2.4). The beers were produced to have differing residual yeast cell counts and protein contents, as each of these factors impact on fining performance.

Table 2.5 Total haze (90° scatter) at 4°C and 12°C for beers treated with various amount of Isinglass (IG), Hop extract (HE) or Auxiliary (Aux)

Beer	Fining T°C	HE 2	Aux	C	IG 10	IG 20	Aux + IG 2.5	Aux + IG 10	Aux + HE 2	HE 2 + Aux	HE 2 + Aux Same time
CB	4°C	1.7	1.1	5.1	1.0	1.1	0.9	1.5	1.5	0.7	0.9
	12°C	1.7	1.7	4.9	1.6	2.6	1.2	1.5	1.3	1.1	1.2
MP	4°C	2.3	0.8	10.5	1.0	1.0	1.0	0.8	2.1	0.7	1.1
	12°C	1.6	1.2	8.9	1.2	1.1	0.9	0.7	1.1	0.8	0.9
Hp	4°C	0.8	0.6	12.2	0.4	0.4	0.5	0.3	0.8	0.5	0.7
	12°C	1.1	1.5	10.2	0.8	0.8	1.6	0.5	2.6	1.2	0.9
LP	4°C	2.9	1.8	6.9	1.3	0.8	0.8	0.7	0.8	0.8	1.0
	12°C	1.5	0.9	3.6	0.9	0.8	1.0	0.7	1.2	0.9	0.9

The data in Table 2.5 indicates that using a combination of the commercial hop extract and auxiliary was more effective than fining with either alone and comparable with a combination of auxiliary and isinglass with a range of beer properties for fined beer. Overall, the H90 values were much lower than that the control reference beer. The isinglass and auxiliary combinations were better than the isinglass alone. These regimes were comparable to the 2 regimes with commercial hop extract followed by or simultaneous with auxiliary. With these combined fining regimes working more effectively than either alone.

These experiments using different types of finings or sequences of addition were designed to evaluate whether the commercial hop extract could match the performance of isinglass or combinations in terms of haze reduction. The hop extract appears to be comparable; it helps to decrease haze as effectively as isinglass does. However, it is noticeable that auxiliary finings had a positive impact on haze reduction when used in combination with the prototype commercial hop extract as seen with isinglass.

All fining treatments have given a higher sediment formation and a decrease in final haze compared to an unfined beer. Isinglass works efficiently and gives some of the best results for fining performance in combination with auxiliary silicates finings, at both 4 and 12 °C. The commercial hop extract has also shown a beneficial fining action for beers with low and high initial yeast cells counts, with or without an extra protein addition. The commercial hop extract active polymers appears to be sufficiently large to bind to not just one yeast cell but to link cells together with protein (Linforth et al., 2015). Used in combination with auxiliary finings, it was clearly more effective. This combination of finings improved the flocculation mechanism and giving comparable results to isinglass finings. Temperature also significantly influences the fining reaction using the prototype commercial hop extract. Fining at 4°C with the commercial hop extract was more efficient as compared to 'cellar' temperatures (12°C).

Hickman et al., (2000) have studied the functionality of isinglass finings. They confirmed that higher molecular weight aggregates that increase the size of the collagen molecules (oligomers) increase the fining efficiency and the global clarification process. Higher performance results have been observed when the hop extract is combined with silicates finings, and a possible interaction between them

can be supposed. By gravitational force, the particles would be easier to settle when bind together then just suspended alone in solution. From this, the fining reaction would then be dependent on the size of the fining agents added, being able to bind to more particles and to form larger aggregates.

2.3.6.2 Impact of yeast strain on fining performance

Beers worldwide used in their recipe different yeasts for their production. Brewing yeast can be differentiated by their fermentation temperature, their brewing style (e.g. ale or lager yeast) or their degree of flocculence (Bokulich et al., 2013). Five different yeasts have been selected for their difference in degree of flocculence. They were then used for brewing aliquots of beer using the kit recipe which were treated with a fixed dose of prototype commercial hop extract and isinglass to determine if those fining agents can interact with a range of diverse yeasts. Table 2.6 showed the sediment volume and A_{600} recorded after 6 and 24 h of clarification.

Table 2.6 Sediment depth and A_{600} records of beers brewed using different yeast strains fined with different fining strategies at 12°C.

		Sediment depth (mL)		A_{600}	
		6 h	24 h	6 h	24 h
Bry 97 (flocculent) Cell count = 1.6×10^8 cells/mL	C	0	0.1	0.19	0.10
	HE 2mL/L	1.3	2	0.12	0.13
	IG 10mL/L	3.5	3.5	0.06	0.07
Munich (non-flocculent) Cell count = 1.34×10^8 cells/mL	C	0	0.2	0.29	0.07
	HE 2mL/L	0.7	1.7	0.17	0.09
	IG 10mL/L	3.8	2	0.09	0.06
Nottingham (highly flocculent) Cell count = 2.6×10^8 cells/mL	C	0	0.1	0.36	0.12
	HE 2mL/L	1.5	2	0.19	0.16
	IG 10mL/L	2.5	2.5	0.14	0.07
Munich classic (non-flocculent) Cell count = 1.78×10^8 cells/mL	C	0	0.2	0.33	0.06
	HE 2mL/L	0.7	2	0.21	0.06
	IG 10mL/L	1.8	2.5	0.13	0.06
Belle Saison (low flocculation) Cell count = 9.87×10^7 cells/mL	C	0.1	0.1	0.23	0.15
	HE 2mL/L	0.6	2	0.20	0.09
	IG 10mL/L	2	2.5	0.10	0.07

These two levels of finings (commercial hop extract 2 mL/L, isinglass 10 mL/L) were selected because they delivered a similar reduction in haze but showed a clear disparity in sediment formation (Figure 2.11). The data in Table 2.6 shows that isinglass had a greater and also quicker effect on the sediment depth of sediment and A_{600} than the hop extract. This could be due to the difference in the dose rate of the two finings affecting the speed of the process. The commercial hop extract had broadly the same sediment depth after 24 h of fining across the different yeast types. Various research studies have shown the interaction between polyphenolics and yeast cells (Jilani et al., 2015; León-González et al., 2018; Morata et al., 2003; Rizzo

et al., 2006). Both isinglass and the commercial hop extract interacted with a wide range of yeast whether they are considered as low or highly flocculent. This is demonstrating the broad fining ability of those fining agents to interact with a range of diverse yeasts.

2.3.6.3 Performance of the hop finings in dark beer styles.

The prototype commercial hop extract has also been tested for fining performance into dark beers. Generally, dark beers contained a more varied choice of malt for the mashing step. That often includes caramel malts, chocolate or coffee malts. These malts types are differentiated by their roasting profile, which is submitted to higher temperatures than pale malt. Dark beers are usually richer, with an intense burnt flavour and a very dark colour. Diageo kindly supplied a sample of Guinness beer after fermentation for a fining performance trial. The experiment used a range of isinglass, commercial hop extract and commercial hop extract/auxiliary finings evaluated in Imhoff cones at 4°C (Table 2.7)

Table 2.7 Sediment depth, A₆₀₀ and haze recording of Guinness treated beers with different fining strategies at 4°C. Haze measurements were taken after 24h of clarification. Beer properties (pH 4.03, Yeast Count = 1.26 e7 cells/mL ABV 7.26%).

	Sediment depth (24h, mL)	A₆₀₀ (24h)	Haze 90° scatter (EBC, 4°C)
<i>Control</i>	4.5	1.8	25.2
<i>HE 0.5mL/L</i>	6.5	1.9	25.8
<i>HE 1mL/L</i>	6.5	2.0	29.8
<i>HE 2mL/L</i>	5.5	2.2	49.0
<i>HE 4mL/L</i>	4.5	2.5	99.9
<i>IG 5mL/L</i>	16	1.4	6.0
<i>IG 10mL/L</i>	18.5	1.4	4.2
<i>HE 2mL/L + Aux 2mL/L</i>	11.5	1.8	21.7
<i>HE 4mL/L + Aux 4mL/L</i>	18	1.5	8.2

All treatments beers produced sediment equal or higher to the control beer after 24 h. The maximum obtained was for the two isinglass treatments, where large and compact sediment was observed. The commercial hop extract treated beers had a negative effect on haze reduction increasing commercial hop extract addition from 0.5 to 4 mL/L increased haze to values higher than the control, from 25.8 to the limits of the haze meter equipment (99.99 is out of scale). Isinglass treatment had a positive effect on the fined beers, as the haze was significantly reduced. A combination of auxiliary finings and the commercial hop extract is also having a positive result almost matching the isinglass performance reducing the haze to 8.2 EBC for the 4 mL/L dose. The negative impact of the commercial hop extract alone would involve formation of haze particles between the polyphenols and particles present in dark beers. These came out of solution, but, were not sedimenting resulting in increased haze. The addition of auxiliary would improve network size between the flocs, allowing them to settle and give better clarity. This is possibly due to interactions with melanoidins, which are polymeric compounds derived from the Maillard reaction from the malt roasting (Kuntcheva et al., 1996).

Figure 2.12 shows the maps of the sediment depth and the final beer haze after 24 h of reaction for the same beer sample treated with different dose combinations of commercial hop extract and auxiliary finings. Sediment depth like all fining trials, is increasing linearly with the two finings introduced ($R^2 = 0.96$). Both finings have a significant impact on the sediment formation ($p < 0.001$, ANOVA). The haze map showed a clear difference between the auxiliary and the commercial hop extract performance. Auxiliary is having the major significant effect on haze removal ($p < 0.0001$, ANOVA). Lowest haze was for a dose of 4 mL/L of auxiliary, and the

commercial hop extract had only a minor effect on the final beer haze, the opposite of that seen in Figure 2.12.

The use of hop extract in stout beers has an opposite effect on haze removal (Table 2.7, Figure 2.12). However, the addition of auxiliary finings in combination with the commercial hop extract significantly improves haze removal ($p < 0.0001$, ANOVA). Dosing polyphenol on its own in the beer, creates particulates which are able to scatter light and form more haze than the unfined control. It is possible that the particles formed can be filtered further to reach better clarity in subsequent steps of the brewing process. A process that may be aided by the prototype commercial hop extract forming particulates with them.

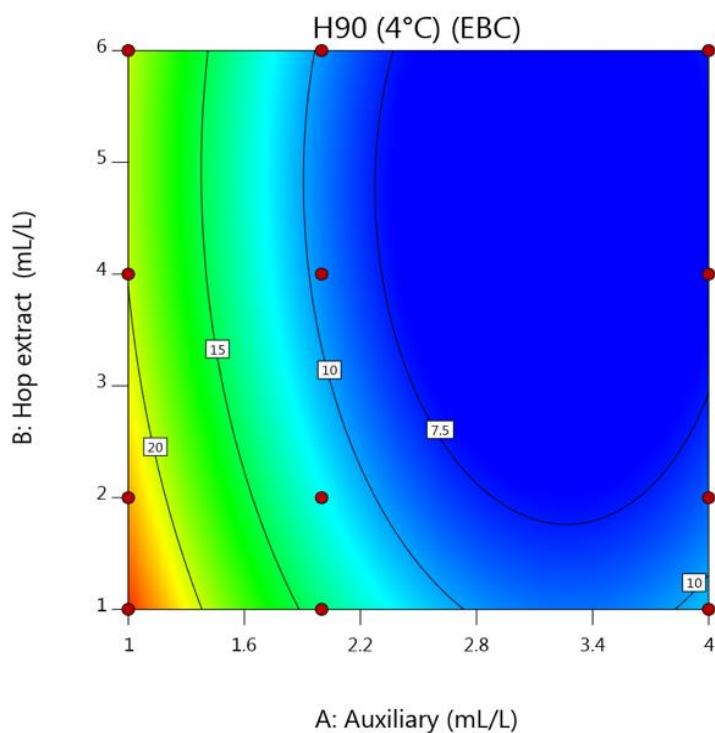
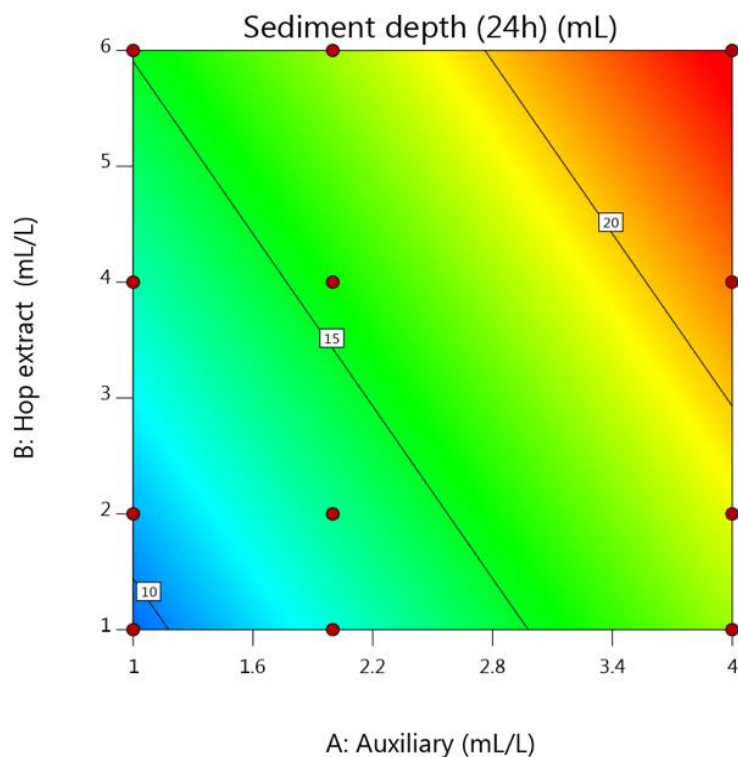


Figure 2.12 Sediment depth (mL) and haze H90 at 12°C according to the varying commercial Hop Extract / Auxiliary volume introduced (simultaneously) into Guinness green beer. (Control beer haze = 25 EBC). The red dots show the experimental points and contour line join up the point of equal response. The colour shading is from blue, (low response), to red, maximum.

2.3.6.4 Removal of dark beer haze using PVPP

Polyvinylpyrrolidone, (PVPP), is a fining agent that is insoluble in water. The use of impregnated polyvinylpyrrolidone (PVPP) filter beds has been extensively used in brewing industry to selectively remove chill and permanent haze polyphenols, and especially proanthocyanidins from beer (Doner et al., 1993; McMurrough et al., 1995; Mitchell et al., 2005; Rehmanji et al., 2002). As dosing the commercial hop extract into dark beers leaves haze, it is supposed that this suspended haze would contain in its majority polyphenols, which could be consequently removed by the addition of PVPP. Sediment depth after 24 h of reaction has been recorded, as well as haze at 90° scatter at 4°C (Table 2.8).

Table 2.8 Sediment depth and haze H90 recordings of kit stout beer treated with different fining strategies using prototype commercial hop extract and PVPP at 4°C.

		Sediment depth (24h, mL)	Haze 90° scatter (EBC, 4°C)
<i>Control</i>		2.25	23.3
<i>HE 4mL/L</i>		4	30.5
<i>HE 10mL/L</i>		4	67.4
<i>HE + Aux 4mL/L</i>		9	10.0
<i>IG 5mL/L</i>		5	3.2
<i>PVPP 4mL addition</i>		3.2	17.7
Commercial hop	<i>HE 4mL/L => PVPP 2mL</i>	3	44.4
Extract then PVPP (6h delay)	<i>HE 4mL/L => PVPP 4mL</i>	3	50.3
	<i>HE 4mL/L => PVPP 8mL</i>	4.5	45.4
	<i>HE 4mL/L => PVPP 20mL</i>	9	46.5
Commercial hop	<i>HE 4mL/L + PVPP 2mL</i>	4	30.5
extract and PVPP (same time)	<i>HE 4mL/L + PVPP 4mL</i>	4	32.1
	<i>HE 4mL/L + PVPP 8mL</i>	5	20.3
	<i>HE 4mL/L + PVPP 20mL</i>	7.5	5.1

Again, commercial hop extract alone is forming a higher sediment than the control but also increasing haze. No fining treatment (control) had a haze of 23 EBC, whereas dosing the commercial hop extract from 4 to 10 mL/L is increasing haze from 30 to 67 EBC. A combination of commercial hop extract and auxiliary fining decreased beer haze to 10 EBC. Isinglass was the most effective with a final beer haze of 3 EBC. Four different samples of commercial hop extract with different doses of PVPP were studied, with a delay of 6h between the addition of the commercial hop extract, and the slurry of PVPP. Sediment depth was increasing with the dose of PVPP, however, haze remained stable over the four different treatments, with a higher value than the control (40-50 EBC). It is still unsure if PVPP binds to the same sites of the polyphenol molecules to which the protein binds (McMurrough et al., 1997). Because PVPP links to tannic acid, it has to be introduced after any fermentation process in order to make them react with the haze formed components (Mastanjević et al., 2018).

The addition of both PVPP and the commercial hop extract simultaneously with four different PVPP dosage was also studied (4 mL/L of commercial hop extract and 2 mL to 20 mL of PVPP slurry). Haze clarity values decreased from 30 EBC for the 2 mL addition, to 5.19 EBC with 20 mL of PVPP addition again showing a combined fining approach can be used to aid beer clarity. It is also possible that PVPP could induce a bigger fining network by first binding to the polyphenols, in which could then react to the beer proteins.

2.3.6.5 Pilot scale brewing trials using the hop derived finings.

Scale-up trials are necessary to observe the fining action through a larger brewing process. The industrial process can change beer properties compared to a lab brewed

beer. All following trials have been performed at the research brewery at the University of Nottingham, Sutton Bonington campus.

An aliquot of high gravity lager beer brewed using the research brewery has been tested on a small scale using Imhoff cones to determine the best prototype commercial hop extract dosage to apply. Figure 2.13 showed the sediment depth and haze measurement for the beer treated with various amounts of commercial hop extract and isinglass. Haze values > 99.99 were off-scale for the haze meter. Based on these results, the finings addition rates for the main brewery trial were selected as 4 mL/L and 10 mL/L of commercial hop extract.

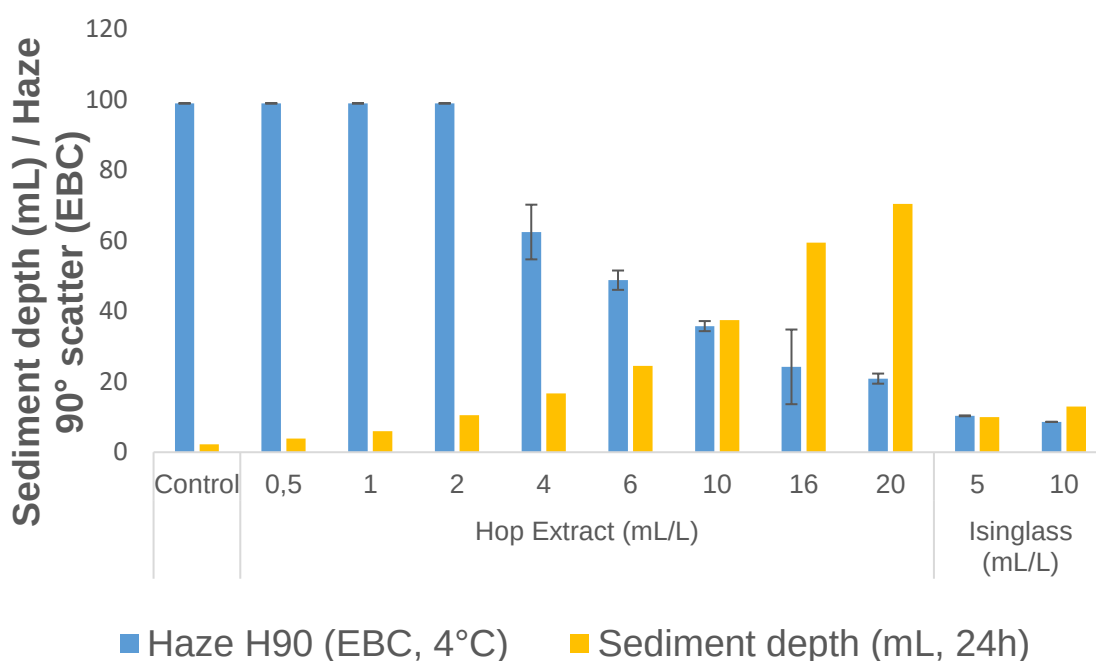


Figure 2.13 Sediment depth and haze recordings of an aliquot of brewery beer before end of fermentation using different doses of commercial hop extract and isinglass finings at 4°C. Haze measurements were done in triplicates with their standard deviation.

The choice was made as 4 mL/L is the first step of an efficient agglomeration and haze reduction, and 10 mL/L as more than double the dosages used. The two selected

dosages have then been applied to batches of 100 L of high gravity lager for fining evaluation. The introduction of the commercial hop extract has been performed through the top of the vessel, mixed using the paddle-stirrer inside the vessel (5 min) at low speed.

Table 2.9 Haze measurements, suspended yeast cell count and formed sediment volume of a brewery beer fined with doses of commercial hop extract and a control over maturation (4°C).

		Hop extract 10 mL/L	Hop extract 4 mL/L	Control
24 h post-fining	<i>Haze (H90)</i>	73.15	99.9	99.9
	<i>Yeast cell count (cells/mL)</i>	7.90×10^7	8.18×10^7	1.01×10^8
48 h post-fining	<i>Haze (H90)</i>	62.1	94.1	99.9
96 h post-fining	<i>Haze (H90)</i>	22.5	36.7	91.6
120 h post-fining	<i>Haze (H90)</i>	19.4	27.0	68.3
Immediately prior to filtration (5 days)	<i>Sediment crop volume (in mL)</i>	400	150	80

Table 2.9 shows the haze values of the two treated beers against an unfined control for a period of five consecutive days. The clarity of the control changed from 99 EBC to 68 EBC 120 h post fining, which is indicating that haze material did not sediment easily without any fining introduction. No real change occurred after 24 h of fining for haze and yeast cell count as 73 EBC, found for the beer with the high dose of commercial hop extract is still very high for clarity. Interestingly it was noticeable that relatively long periods (5 days) were required for the finings to work to full effect reducing haze from 73 to 19 EBC for the higher dose of commercial hop extract, and from 99 to 27 EBC for the lower dose. This reaction is also confirmed by the amount of sediment cropped at the bottom of the vessel, with 150

and 400 mL of sediment found compared to 80 mL for the control. This time effect is contrary to expectations from prior studies and may be simply a property of the scale of the sample. Such times would not be particularly problematic as brewery cold-conditioning times are still typically of this order (3-6 days) of a few breweries employing accelerated ageing techniques.

The second scale-up trial treated a volume of 4 hL. The finings were introduced at the bottom of the vessel prior to beer introduction. Table 2.10 is indicating that one day is not sufficient to demonstrate the performance of the commercial hop extract. Haze values were comparable between the control and the treated beer after a day of reaction (dilution of beer with water prior to analysis to reach haze-meter analysis scale). However, a clear change in haze was noticed after 48 h between the fined beer and the control sample. Control beer had a clarity of 53 EBC compared to 17 EBC for the commercial hop extract treated beer, showing a positive effect on haze removal. The two samples passed through the centrifuge at 10000 rpm and the filtration facilities are however giving a similar finished clarity of 1 EBC and no change was observed in haze for the final beers. An improvement in filtration was observed with this trial and will be reviewed in the next section.

Table 2.10 Haze measurements for a high gravity lager brewery beer fined with commercial hop extract over time prior and post filtration at 4°C.

		Haze 90° scatter (EBC, 4°C)	
		Control	HE 4mL/L
Post fining	Day+1 (4-fold dilution)	52.3	55.6
	Day+2 (no dilution)	52.8	17.4
Post centrifugation/filtration		1.0	0.96

2.3.7 Impact of fining agents on beer filterability

Fining post fermentation can have a consequent beneficial impact in filtration. One of the main priority for a brewer is to ensure that the process is configured to minimize the load of the filter (Bamforth, 1999). Linforth et al., (2015) showed that beer haze post filtration using hop extracts can match the performance of isinglass. However, no published work has been done to study the effect of the commercial hop extract fining agent on beer filtration. Different fining strategies have been tested using a laboratory beer filter using cellulose filters (Figure 2.14). This filter type has an initial coarse filtration before a finer final filter.

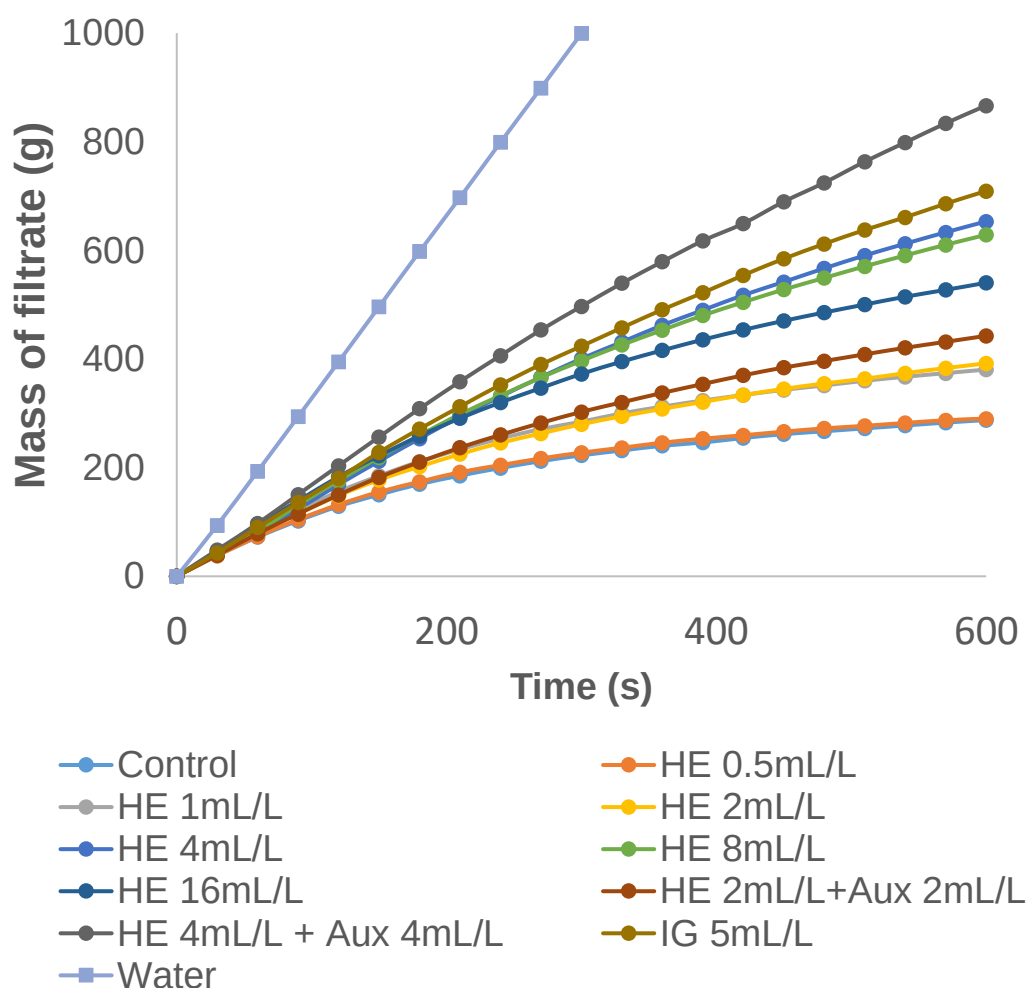


Figure 2.14 Recording of beer filterability for a high gravity lager fined using different fining strategies at 4°C. Data for each fining is the mean of triplicates samples.

The filtered average weights of filtered beer over time are displayed in Figure 2.14. The linearity and slope of the water curve is indicating the maximum potential filtration rate. All treated beers with 1mL or more of commercial hop extract added filtered faster than the control beer. Increasing the dosage of commercial hop extract increased beer filterability up to an optimal dosage of 4 mL/L, then decreased for beer samples treated with higher commercial hop extract dosages (8 and 16 mL/L). Isinglass on its own is having a greater effect on filterability than any beer treated with the commercial hop extract alone. Combinations of commercial hop extract and auxiliary finings are however giving the greatest results in terms of beer filtration, with the best results for the 4 mL dosage of both finings. All beer samples post fining (24 h) as well as post fining and filtration were analysed for haze measurements at 4°C (Table 2.11).

Table 2.11 Haze measurements of a high gravity grain lager fined with different fining strategies before and after filtration through cellulose paper filter (0.5 to 2.5 µm).

	H90 after fining (4°C, EBC)	H90 after fining + filtration (4°C, EBC)
<i>Control</i>	37.62	3.11
<i>HE 0.5mL/L</i>	20.26	2.92
<i>HE 1mL/L</i>	16.25	3.02
<i>HE 2mL/L</i>	11.87	2.92
<i>HE 4mL/L</i>	5.71	2.9
<i>HE 8mL/L</i>	7.16	3.17
<i>HE 16mL/L</i>	8.84	3.05
<i>HE 2mL/L + Aux 2mL/L</i>	9.63	3.1
<i>HE 4mL/L + Aux 4mL/L</i>	4.32	2.9
<i>IG 5mL/L</i>	5.07	2.55

Control beer had a haze value of 37 EBC at 4°C. Dosing the commercial hop extract decreased the haze of the fined beer from 20 to 5.7 EBC for the optimal dosage of 4 mL/L. As seen in the filtration results, dosing the prototype hop extract over 4 mL/L is increasing haze. Siebert et al., (1996) demonstrated that the amount of haze, material to further filter, depends both on the concentration of protein and polyphenol to a certain ratio. Overdosing the commercial hop extract would then allow a build-up of the filter and a non-efficient filtration. The combination of auxiliary and the commercial hop extract decreased haze with a greater effect than the hop extract alone. The minimal haze value was observed for the 4 mL/L dosage of both fining agents comparable to isinglass performance.

Once the beers have been passed through the cellulose filtration system, haze values have been recorded at 4°C. All treated beers with the control reference are giving haze between 2 and 3 EBC. In considering the similarity between the filtered beers for haze, it is important to remember the difference in filterability (Figure 2.14).

Another parameter to look for brewers during filtration process is the pressure build-up during the filtration run. This is an indication of the filter cake thickness during the filtration process. The two 4 hL batches of beer used in the up-scale trials (Table 2.12) were passed through the brewery centrifuge at 10000 rpm and a 0.6 micron filter system with a continuous flow of beer of 6 hL/h. The pressure was recorded for 30 min (Table 2.12). Although this has been only recorded once without replication, the commercial hop extract treated beer is having a maximum pressure of 1.6 bar compared to 2 bar for the control beer. This is indicating that the pressure at the filter is reduced by 20%. The commercial hop extract finings can allow more beer to be filtered for the same amount of pressure produced, and that would represent a significant benefit for brewers.

Table 2.3 Record of the pressure created from a high gravity lager filtered through a 0.6 µm filter with and without the addition of commercial hop extract finings at 1°C.

Time (min)	Pressure Control (bar)	Pressure HE 4mL/L (bar)
0	0.5	0.5
5	0.9	0.9
10	1.1	1.0
15	1.2	1.1
20	1.5	1.3
25	1.7	1.4
30	2	1.6

2.3.8 Formulation optimisation of the commercial hop extract

Different strategies were performed to see if the active material could be formulated as a solid powder, without changing its fining properties. These included salt precipitation and spray drying.

2.3.8.1 Salt precipitation of Proanthocyanidins

Laboratory experiments showed the formation of a precipitate when a large amount of sodium chloride was added to the commercial hop extract. The addition of NaCl, formed a brown solid at the top of the extract solution. A clear change of colour was also noticeable for the commercial hop extract, from an orange-brown colour to an orange-yellow. The addition of such a large amounts of ions, may change the properties and the solubility of polyphenols in water, forming precipitates, this may fractionate the extract. That reaction may represent an extra clean-up step for the extract to remove non-active or negative impact components which can affect haze.

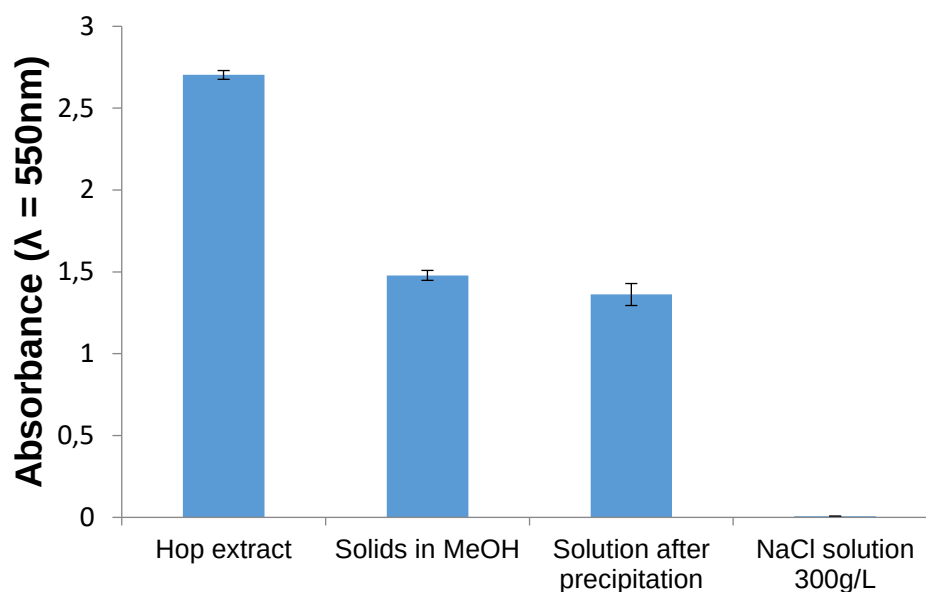


Figure 2.3 Absorbance values at a wavelength $\lambda = 550$ nm after acid butanol hydrolysis of commercial hop extracts samples and their co-products formed during the NaCl precipitation. Data is represented as the mean of triplicates with their standard deviation.

Once the PA precipitation was performed and the discarded solid was redissolved in methanol, the original commercial hop extract, the methanolic solution with the solid precipitated (2.58 yielded solid for 200 mL of extract) as well as the commercial hop extract after precipitation were studied through the acid butanol assay for PA content. The commercial hop extract had an absorbance of 2.68 as a reference of its PA concentration (Figure 2.15). The methanolic solution containing the solids has an absorbance of 1.47. Compared to the original commercial hop extract, we can consider that half of the PA compounds were transferred into the solid during precipitation. The hop extract solution after precipitation had a final absorbance of 1.3 after the assay, which shows that it still contain PA material after the precipitation reaction. All extracts were then tested in Imhoff cone beer fining experiments in order to evaluate their fining efficiency.

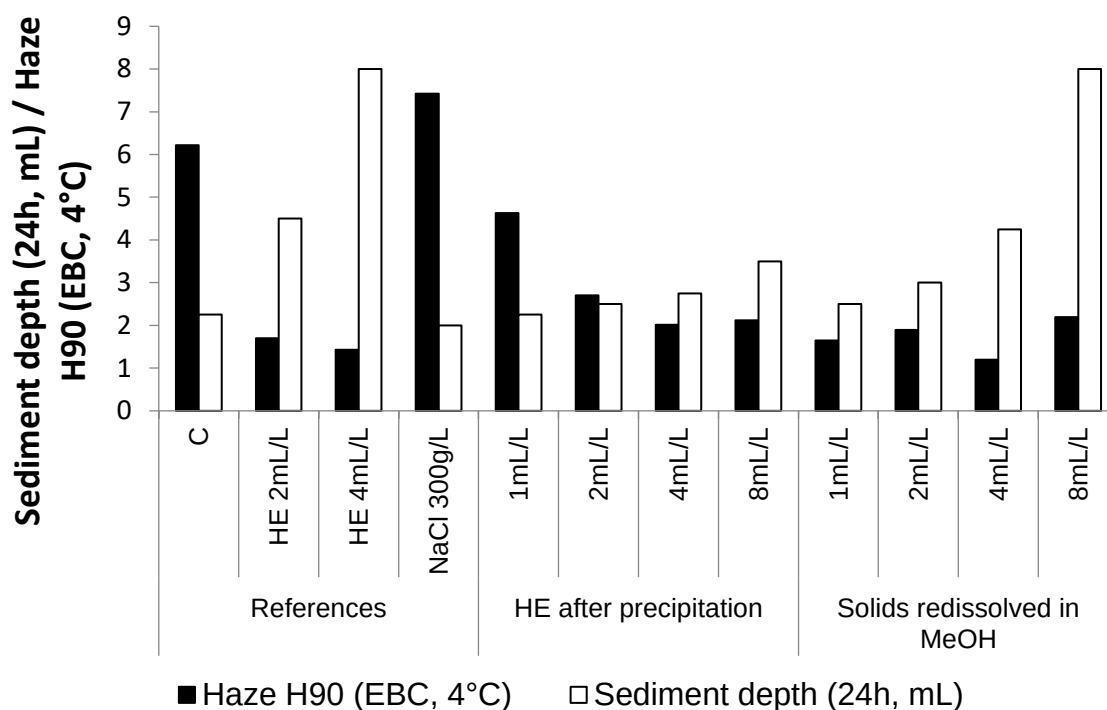


Figure 2.16 Sediment depths and haze recordings of green beer (4°C) using different hop extract samples before and after NaCl precipitation. Commercial hop extract and NaCl in solution were run as references. Haze analysis was performed after 24 h of clarification.

The unfined control beer produced a compact sediment after 24 h (2.25 mL) with a haze value of 6.22 EBC (Figure 2.16). The commercial hop extract at 2 or 4 mL/L had a sediment increasing from 4.5 to 8 mL. Haze decreased accordingly, with the lowest value observed for the 4 mL/L commercial hop extract sample (1.4 EBC). Salted water at 300 g/L did not show any flocculation activity or haze removal compared to the control. The commercial hop extract after precipitation showed activity, reducing beer haze with increasing dosage towards a minimum of 2.0 EBC (4 mL/L dosage). This was less effective compared to the non-fractionated extract. The redissolved solid gave better results than the original extract. Sediment depth value increased over the dosage range from 2.5 to 8 mL. Haze also decreased with dosage, with an optimal value observed for the 4 mL/L dose (1.2 EBC). Comparing the same dosage (4 mL/L) for the commercial hop extract on its own and the redissolved solid, the beer treated with the solid redissolved has a lower haze and is

producing less sediment. This can imply that the most active part of the extract was recovered in the precipitated solid, which had a cleaner fining reaction.

2.3.8.2 Spray drying of the hop extract

Spray drying is a technology used in the food industry, to dry and particulate food samples. This can allow better storage of the products, an increased shelf life and stability of the active compounds, but, also aids the transportation of the product. Three different matrixes (maltodextrin, β -cyclodextrin, and arabic gum) were selected for the production of commercial hop extract spray dried powders due to their application and spray drying into food products (Bertolini et al., 2001; Kim et al., 1996; Ostroschi et al., 2018; Van Den Hoven et al., 2012).

All samples were then tested in beer, introducing the same amount of hop extract active material in each Imhoff cone. For each experiment, each powder was tested in pair, one with the solid directly introduced in beer and the other with the solid sample redissolved in 2 mL of deionised water. Figure 2.17 showed the sediment depth and haze for beers treated with maltodextrin spray dried products.

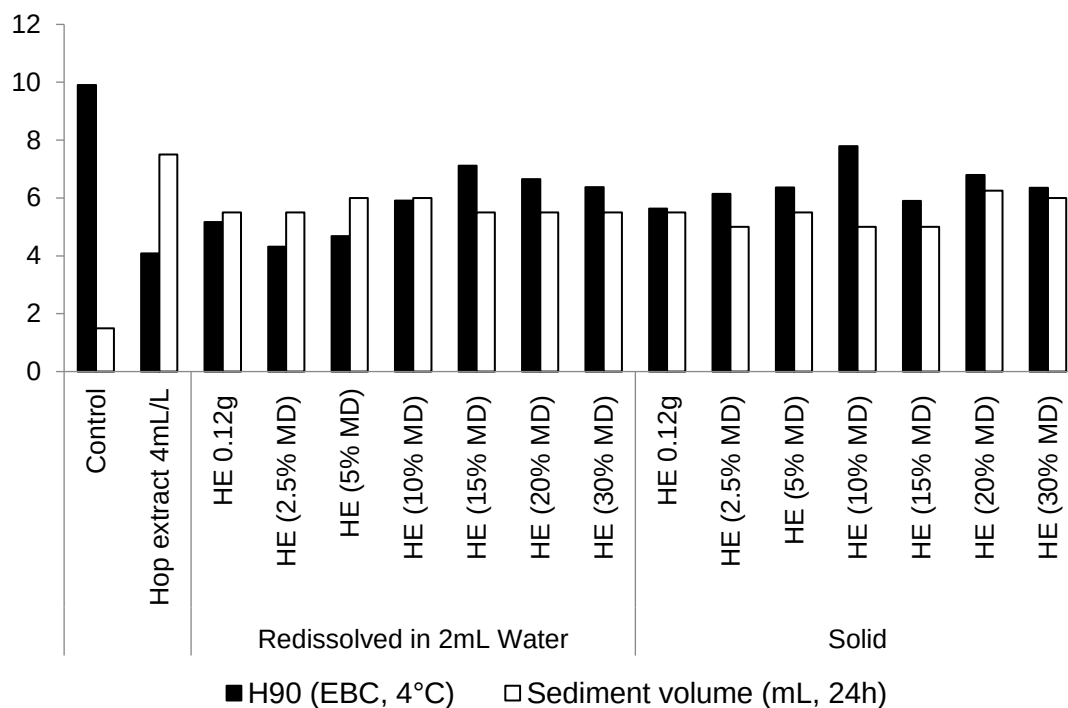


Figure 2.17 Sediment depths and haze recordings of green beer (4°C) using commercial hop extract samples from spray drying using maltodextrin carrier in different doses. Haze analysis was performed after 24 h of clarification.

All treated samples with spray dried products formed a compact sediment after 24 h of clarification. They also had decreased haze compared to the control beer (Figure 2.17). However, in the introduced solid and re-dissolved set of samples, the final beer haze is higher relative to the original hop extract treated beer. None of the sample produced through the spray dryer with maltodextrin improved fining activity compared to the original prototype hop extract.

β -cyclodextrin was spray dried in the same way as used for the maltodextrin. Figure 2.18 shows the haze and resulting sediment after 24 h of fining reaction for powders and re-dissolved samples spray dried with β -cyclodextrin as the carrier.

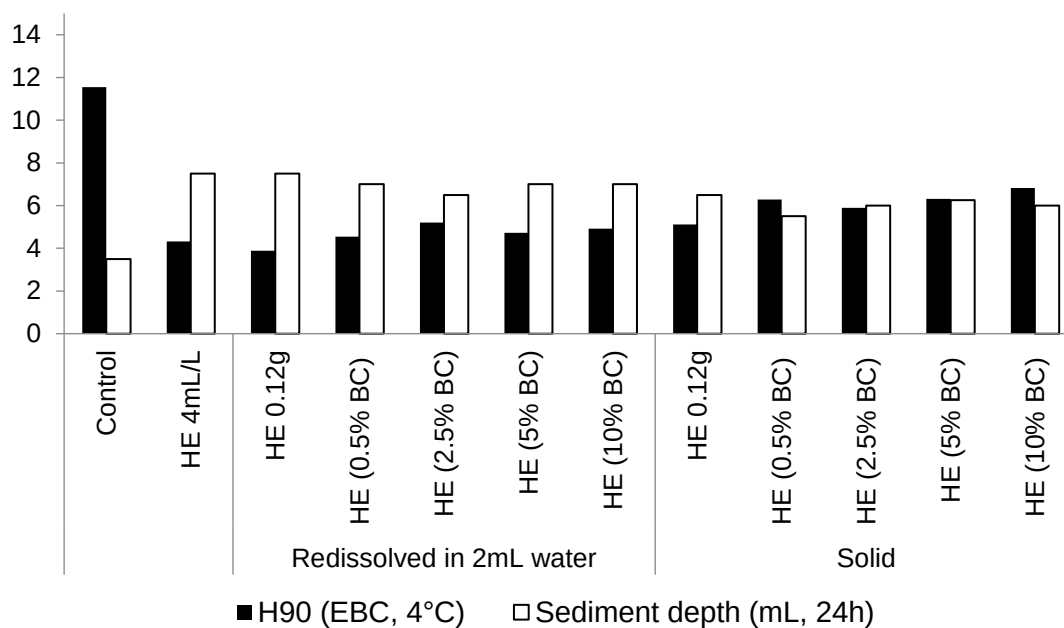


Figure 2.18 Sediment depths and haze recordings of green beer (4°C) using commercial hop extract samples from spray drying using β -cyclodextrin carrier in different doses. Haze analysis was performed after 24 h of clarification.

Both powders and re-dissolved samples show a positive effect compared to the control. Beer samples with the carrier addition have higher haze value than the commercial hop extract dosed beer. The addition of β -cyclodextrin as a carrier agent did not improve the fining mechanism. However, comparable results have been found between the original prototype hop extract and the re-dissolved sample of spray dried hop extract, without any carrier. This would induce a beneficial aspect for the manufacturer as it would reduce transportation and improve product storage.

Sediment depth and haze values for beers treated with gum arabic spray dried products have been displayed in Table 2.13.

Table 2.13 Sediment depths and haze recordings of green beer (4°C) using commercial hop extract samples from spray drying using gum arabic carrier in different doses. Haze analysis was performed after 24h of clarification.

		Sediment depth (mL, 24h)	Haze 90° scatter (EBC, 4°C)
	<i>Control</i>	3.25	12.23
	<i>HE 4mL/L</i>	7.5	5.75
	<i>IG 5mL/L</i>	6	3.47
Powder dissolved in 2mL of water prior to beer introduction	<i>HE 0.12g</i>	7.5	3.71
	<i>HE (0.25% GA)</i>	5.5	5.52
	<i>HE (0.5% GA)</i>	5.5	5.62
	<i>HE (1% GA)</i>	6.5	4.86
	<i>HE (2.5% GA)</i>	7	4.96
	<i>HE (5% GA)</i>	7	4.2
	<i>HE (10% GA)</i>	5	80.65
	<i>HE (15% GA)</i>	3	49.63
	<i>HE (20% GA)</i>	3	39.57
Solid powder introduced into beer	<i>HE 0.12g</i>	5	11.1
	<i>HE (0.25% GA)</i>	5.5	5.44
	<i>HE (0.5% GA)</i>	6	7.3
	<i>HE (1% GA)</i>	7	7.29
	<i>HE (2.5% GA)</i>	6.5	6.09
	<i>HE (5% GA)</i>	5	11.19
	<i>HE (10% GA)</i>	3.5	97.45
	<i>HE (15% GA)</i>	4	58.12
	<i>HE (20% GA)</i>	3.75	43.46

Gum Arabic is a polysaccharide often used in emulsification of food products. The addition of gum Arabic with the commercial hop extract clearly added haze at many doses (Table 2.13). This and the factors of additional time, effort and cost combined with the issue of introducing spray drying adjuncts into beer clearly show that this

approach is not viable. However, simply drying the hop extract to produce a powder did not add extra material, affect activity and may be beneficial for product transport.

2.4 Conclusions

The active fining component extracted from the hop plant material has been identified to belong to the proanthocyanidin group of polyphenol polymers (Linforth et al., 2015). The Folin-Ciocalteu assay method used here has been a rapid method to demonstrate and quantify the total phenolic concentration of plant extract. All hop extracts contained ‘gallic acid equivalents’ according to this assay, indicating the presence of polyphenols. Moreover, the acid butanol hydrolysis assay has been useful to prove that the active compound belong to the class of proanthocyanidins.

The optimal extraction solvent for the extraction of PA from hops has been studied using trinary mixtures of ethanol, acetone and water. First results indicated that ethanol was reducing PA content in some extracts. Further trials determined the optimal solvent extraction to be a mixture of acetone and water. 70% acetone has given the highest concentration of extracted proanthocyanidins and also the best fining efficiency. Many studies have shown that acetone and water were optimal for the extraction of proanthocyanidins from hops and other botanical sources (Mitchell, 2017; Kähkönen et al., 2001; Naczki et al., 2004). This process of extraction represents a simple and economical way to extract the active compound as the acetone could potentially be recovered for further extractions.

Polyphenols are well known to bind to proteins. The main goal of this product is to flocculate chill haze particles into post-fermented beer for an improved clarity before filtration and centrifugation steps. Microscopy studies were able to confirm the flocculation action between the hop extract polyphenolics and the beer proteins, as

seen in previous beer haze studies (Steiner et al., 2010). The reaction forms flocs of proteins and yeast in beer, which can then settle for efficient haze removal. Based on early experiments, the hop extract is easily dispersible into beer, and the mixing velocity and time (paddle stirrer) seemed to have little effect on the fining mechanism. For all fining treatments used in this study, the fined beer was significantly brighter relative to the relevant controls. Moreover, the fining reaction between hop extract and haze components did not seem to depend substantially of the type of yeast strain used for the fermentation. The model explained brilliantly in two studies by Siebert et al., (1996, 1999) indicated that optimal haze removal is occurring for a matching concentration of polyphenols and protein introduced. The hop extract like all fining agents needs to be dosed into beer carefully to avoid under or over dosing. A low dose resulted in poor fining results, i.e. low sediment formed and beer still hazy. A very high dose resulted in high volume fluffy sediments and increased haze.

This chapter confirmed the application of the hop extract to post-fermented beer as discovered by Linforth et al., (2015). Its liquid aqueous state gives the brewer an agent for yeast and chill haze removal prior to centrifugation/filtration. It is working efficiently in a broad range of blond beers (lagers and ales). Trials on dark beers shown that the introduction of the hop extract resulted in an opposite effect. Dosing polyphenols in these beers resulted in increased haze and poor flocculation. The hop extract was found to work more efficiently in combination with silicates auxiliary finings. The best hop extract treatment for each beer (with or without auxiliary finings) was able to closely match the fining performance of isinglass, although in some beers the hop extract alone was slightly less effective in terms of residual haze. Temperature significantly influences the fining reaction. Fining at 4°C with the hop

extract was more efficient as compared to 'cellar' temperatures (12°C). Using auxiliary finings in combination with the hop extract significantly improved clarification for all grain beers. This can imply synergetic reaction between polyphenol and silicates, assisting the formation of bigger aggregates, beneficial for the fining reaction.

It has also been confirmed that this product can be used on a larger scale through pilot plant trials. A major gain in filtration time has been found using the hop extract finings, which can represent a considerable advantage for brewers.

However, the chemical composition of the hop extract has still been a particular challenge as proanthocyanidins are present at various sizes across the plant kingdom. Straightforwardly, little is known about the ways in which these units are assembled to form polymers (Dixon et al., 2005). Novel, user-friendly methodologies for the quantification, analytical separation and structural characterisation of oligomeric and polymeric proanthocyanidins must be developed to enlarge the understanding of these biological compounds (Neilson et al., 2016).

The next chapter will proceed to the characterization of the hop extract, using current techniques and a novel use of analytical ultracentrifugation to determine the molecular distribution of the active compounds present into the hop extract. As most of the trials included the use of the prototype commercial extract without data on the hop variety used for the manufacturing of the product, the scientific approach will be guided by using different hop varieties and their difference in polyphenol composition as well as fining efficiency.

Chapter 3: Characterisation of the hop extract and its active fraction

The extract from spent hops has been proven to be composed of polyphenolics (Linforth et al., 2015). For the development and the commercialization of a new product, it is important to evaluate and characterise the composition of the material studied. The active material in the extract is believed to belong to the proanthocyanidin class of molecules. They are represented by assemblies of catechin and epicatechin monomers, and they can be present in various types and molecular sizes within a sample (Neilson et al., 2016). As a result, analytical techniques have to be developed in order to define the fining active material into the spent hop extract. Clean-up fractionation methods will be demonstrated first in order to remove non-active material in our sample for further analytical investigations. As hops are present in numerous botanical varieties, it is also important to take into account the varietal differences in terms of active material within our extract. This can assist with a better selection of varieties for optimum fining performance.

Different analytical methodologies for proanthocyanidins are based on the degradation of the proanthocyanidin polymer into sub-units (Gu, 2012; Kennedy et al., 2001). The quantification of the subunits allows to determine an average molecular weight of the sample, without any indication of the molecular distribution. This chemical reaction will be investigated to prove the presence of polymers into hop extracts of different varieties, and their mean degree of polymerisation (DP). Average DP have been recorded from 2-3 for apples and kiwis, whereas DP 50 have been evaluated for chokeberries (Hellström et al., 2009).

The analysis of proanthocyanidins presents characterisation challenges, particularly when it comes to the larger polymers (Okuda, 1991) . Small polymers, up to the tetramer ($DP = 4$), are easily fractionated by chromatography (Tanaka et al., 2014) and analysed by MALDI-TOF (Monagas et al., 2010). However, the analysis of large proanthocyanidins ($DP > 20$) by MALDI-TOF is challenging. María Monagas et al., (2010) commented that MALDI-TOF-MS is not well-suited to the analysis of highly polydisperse proanthocyanidins, due to the generation of multiply charged ions which makes spectral interpretation difficult. Furthermore they concluded that high molecular weight polymers were challenging with regard to suppression of ionisation, reduction of desorption and in-source fragmentation to form non-covalent ion clusters. Chromatographic methods are also limited in terms of accurate analysis of molecular weight due to the absence of appropriate calibration standards (Kelm, 2005). Hence there is a need for improved methodology for the analysis of large proanthocyanidin polymers, particularly those species with $DP > 4$. Improved analytical methodologies are needed to increase our knowledge on the proanthocyanidin analysis, their biological roles and the chemistry of these complex compounds (Neilson et al., 2016).

A novel method using analytical centrifugation will be detailed in order to determine polymer size distributions in hop extracts. The inherent separation and analytical capability of AUC – without the need for a separation matrix (column or membrane), and its absolute nature makes it extremely promising for characterising complex systems like the polyphenolic mixtures analysed here. The development of the analytical methodology using the analytical ultracentrifuge will answer the need for characterisation of the molecular size distribution of proanthocyanidin polymers in hops but also in other botanical sources of proanthocyanidins. Hop extracts obtained

a profile with discrete peaks, present from small monomers to high molecular weight polymers. Presence of polymer material for Saaz hop will be detected up to 90 kDa, and approximately 50 kDa for Magnum hop.

From that result and in order to produce a more efficient extract, two studies will involve the separation of the high molecular weight fraction of proanthocyanidins. Linforth et al., (2015) have referenced that the fining activity is spread across different molecular weight, and activity would be present on the 100 kDa region. Ultrafiltration and HPLC fractionation method will be employed to separate the high molecular weight PA fraction from the small monomer and oligomer part of the sample.

Research in wine have proven that proanthocyanidins accumulates during the berry development (Bogs et al., 2005). Climate conditions and time of harvest can represent important parameters to the proanthocyanidin content of a plant as well as beer flavour brewed with hops picked at different harvests time (Lafontaine et al., 2019). Different hop samples of the same variety will be picked at different time intervals to be analysed for their PA content, fining efficiency but also their molecular distribution. Other botanical sources of proanthocyanidins will be finally characterised for their polyphenolic content, fining efficiency in green beer and also in their molecular distribution through AUC in order to compare hops to other potential sources of proanthocyanidins (Hellström et al., 2009).

3.1 Materials and methods

Chemicals

Gallic acid, n-butanol, hydrochloric acid (37%) and Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Dorset, UK) in analytical reagent grade.

Acetone, acetonitrile, formic acid and methanol were also purchased from Sigma-Aldrich (Dorset, UK) in HPLC grade.

Hop samples

Twelve hop varieties Magnum, Cascade, Citra, Simcoe, Target, Hallertauer Hersbrucker, Saaz (Saaz whole cone 1), Summit, Pilgrim, Galena, East Kent Golding, Crystal were purchased from the HopShop (Plymouth, UK), in whole cone dried form, from the 2015 crop. Two further samples of Saaz hop were kindly supplied by Barth Innovations Ltd. (Tonbridge, UK), as pellet and whole dried cones (Saaz whole cone 2, Saaz pellets). Another variety of hops (Nugget) has been picked from an allotment in Kegworth, UK, from the start of flowering to the end of hop ripening (2018 crop). All extracts previously mentioned with the variety name were prepared by using the extraction method in the 2.2.1 section.

Other botanical PA sources

Dried cranberries, dried currants and cinnamon (powder) were purchased from Sainsbury's supermarket (Nottingham, UK). Grape seeds from Greenfield superfoods were purchased online at sous-chef.co.uk; aronia berries were purchased online from natureshealthbox.co.uk and Triple A sorghum seeds were purchased from Amazon.co.uk

3.1.1 Clean-up fractionation of the hop extract using solid-phase extraction

The hop extract (prepared from 2.2.1 method section, different varieties) was fractionated using solid-phase extraction in order to reduce the level of non-active material in the hop extract.

For Strata CN (3 mL, Phenomenex, Macclesfield, UK) cartridges, the column was conditioned with acetonitrile and then equilibrated with 0.1% acetic acid aqueous solution. Hop extract (5 mL) was then loaded onto the column and washed with 5% ACN in 0.1% acetic acid. The sample was then eluted with 5 mL of 30% ACN.

For Sep-pak C18 Vac 6cc solid-phase extraction cartridges (Waters, Elstree, UK), the column was conditioned with methanol and then equilibrated with deionised water. Hop extract (5 mL) was then loaded onto the column and washed twice with water and ethyl acetate (20 mL). The sample was then eluted with 5 mL of methanol. This methodology was based on Sun et al., (1998).

For LH-20 solid phase extraction, 5 g of Sephadex LH-20 was mixed with either methanol or acetone in order for the material to swell for 24 h. The mix was then introduced into a Thermo scientific (Loughborough, UK) blank cartridge. Hop extract (5 mL) was then loaded onto the column and washed with deionised water. The sample was then eluted with 5 mL of variable volumes of methanol/water, or acetone/water. This methodology was based on techniques reported by Cuevas-Rodríguez et al., (2010); Gu, (2012); Taylor et al., (2003).

3.1.2 Evaluation of different hop varieties for proanthocyanidin content and beer

The polyphenolic content of 12 hops (Magnum, Cascade, Citra, Simcoe, Target, Hallertauer Hersbrucker, Saaz, Summit, Pilgrim, Galena, East Kent Golding and

Crystals were extracted using 70% acetone_(aq.) using the second method described in Section 2.2.1, Optimisation of the extraction method for preparation of hop finings. They were then evaluated for their proanthocyanidin content and their fining efficiency (Section 2.1.2.2, sediment depth analysis, Section 2.1.2.5, haze analysis, Section 2.1.2.7, gallic acid assay and Section 2.1.2.8, acid butanol assay for proanthocyanidin content of extracts).

3.1.3 Molecular characterisation of hop proanthocyanidins

3.1.3.1 Phloroglucinol degradation

A 100 µL aliquot of methanolic fraction of hop extract (prepared from 2.2.1 method section, different varieties) from C18 SPE was added to a volume of 3 mL of 5 mg/mL phloroglucinol acidic ethanol solution (1% HCl in ethanol) and left to react overnight at 22°C. The solvents were removed after reaction using a Buchi R-II (Flavil, Switzerland) rotary evaporator and the sample re-dissolved in water (3 mL). This was partitioned twice, with equal volumes of ethyl acetate and the ethyl acetate fractions combined. The ethyl acetate was evaporated and the sample re-dissolved into aqueous methanol (MeOH / water; 70/30; v/v) prior to HPLC (1 mL).

Determination of the phloroglucinol adduct concentration (HPLC)

For HPLC, solvent A was a 1% aqueous acetic acid solution, and solvent B was a mix of methanol and 1% aqueous acetic acid (60:40 v/v). The column used was a Symmetry C18 (Waters, Elstree, UK) (3.5 µm; 4.6x150 mm).

HPLC mobile phase was delivered using a Jasco PU-980 Intelligent pump module (Dunmow, UK). The solvent gradient started at 100% solvent A at t=0 changing to 40% of solvent A, 60% solvent B at t= 60 min. Runs were performed at room temperature with a flow of 0.7 mL/min; the 20 µL injection was performed using a

Jasco AS-2055 Plus autosampler (Dunmow, UK). The compounds eluted were monitored at 280 nm using a Waters 490E (Elstree, UK) multiwavelength detector.

Determination of the monomer background and terminal units formed (LC-MS)

For LC-MS, solvent A was a 0.1% aqueous formic acid solution, and solvent B was a mix of methanol and 1% aqueous formic acid (60:40 v/v). The column used was a Symmetry C18 (Waters, Elstree, UK) 3.5 μm (4.6x150 mm). The solvent gradient started at 100% solvent A at $t=0$ changing to 40% of solvent A, 60% solvent B at $t=30$ min. Runs were performed at room temperature with a flow of 0.7 mL/min. The 20 μL injection was performed using an Agilent 1100 Capillary LC system composed of a micro-vacuum degasser, pump and a thermostatted autosampler coupled with a Quattro Ultima MS/MS mass spectrometer.

3.1.3.2 Analytical ultracentrifugation (AUC), Sedimentation velocity analysis

Sedimentation velocity experiments were performed using an Optima XL-I analytical ultracentrifuge (Beckman, Palo Alto, USA) employing Rayleigh interference optics. Methanolic fractions of the hop extract (prepared from 2.2.1 method section, different varieties) at either 6, 10, 12 or 24-fold dilution (395 μL of sample, and 405 μL methanol reference) were injected into the 12mm double sector titanium cells with sapphire windows. The 6-fold dilution was run twice in every AUC experiment. The cells were loaded into the rotor which was spun at 45000 rpm (20.0°C). Scans were taken at 5 min intervals for an acquisition of 200 scans minimum for each cell.

3.1.3.3 Sedimentation equilibrium analysis

Sedimentation equilibrium experiments were performed using the Beckman (Palo Alto, USA) Optima XL-I analytical ultracentrifuge and the Rayleigh interference system. Double-sector titanium cells of 20.0 mm optical path length with sapphire

windows were filled with 140 μL of hop extract (prepared from 2.2.1 method section, different varieties) at 40-fold dilution (sample sector) and 140 μL of reference solvent (methanol) in the reference sector cell. Experiments were performed starting with a low centrifuge speed of 20000 rpm before moving to higher speeds. Once equilibrium was reached at each speed the rotational speed was increased by increments of 5000 rpm, up to a maximum of 40000 rpm for the final scans. Scans were taken every hour, until the equilibrium state at a given speed was reached (the total run time for experiments was between 4-5 days due to the considerable time required to reach equilibrium at each speed). Samples were analysed at a low concentration (40-fold diluted in methanol) to reduce non-ideality effects (Schuck et al., 2014).

3.1.3.4 AUC data processing

Sedimentation velocity experimental data were analysed in SEDFIT (Schuck, 2000) using the $c(s)$ processing (Dam and Schuck, 2004) and expressed using the sedimentation coefficient distribution $c(s)$ vs s where s is the sedimentation coefficient in Svedberg units ($S = 10^{-13}$ sec). The sedimentation coefficient distribution is a measure of the size distribution but is influenced by shape. Sedimentation equilibrium data were analysed in SEDFIT-MSTAR (Schuck et al., 2014), particularly useful for heterogeneous systems. It is based on the M^* function of Creeth & Harding, (1982) and uses a smart-smooth algorithm for generating distributions of molar mass $c(M)$ vs M_w . Although less resolving (due to the lower speeds) distributions are absolute: the two methods (sedimentation velocity and equilibrium) provide a powerful combination for the analysis of heterogeneous systems such as the polymeric proanthocyanidin system under analysis here.

3.1.4 Separation of the hop proanthocyanidin high molecular weight fraction

3.1.3.1 Fractionation of the hop extract through high performance liquid chromatography (HPLC)

4 mL of dried Saaz hop extract (prepared using the 2.2.1 method section) after C18 fractionation was taken up in aqueous methanol (0.2mL MeOH and 0.8 mL of water). The mixture was then introduced into a 250 x 4.6 mm cyano column (Phenomenex, Macclesfield, UK) and eluted isocratically with a solvent mixture composed of 70% 0.1% acetic acid and 30% ACN at 1 mL/min. The eluent was then collected as separate 1 mL fractions. The PA content and the fining activity of each fraction was determined through the small scale beer bioassay and the PA assay referenced in (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.8, acid butanol assay for proanthocyanidin content of extracts). All active fractions were then eluted in methanol using C18 solid phase extraction cartridges prior to analytical ultracentrifugation analysis.

3.1.3.2 Ultrafiltration through molecular weight cut-off filtration

Ultrafiltration was performed on a sample of Saaz hop extract (2.2.1 method section, 70% acetone_(aq.)). 680 mL of the extract was introduced into an Armfield FT17 (Ringwood, UK) cross-flow filtration system, with a 50 kDa cut-off. 327 g of filtrate were obtained for 290 g of retentate. Three different extracts were compared for fining efficiency. The prototype industrial hop extract, another laboratory Saaz extract (2.2.1 method section, 70% acetone_(aq.)), and the 50K retentate from the filtration were assayed for PA content using the acid butanol hydrolysis assay (Section 2.1.2, acid butanol assay for proanthocyanidin content of extracts). They were then diluted in order for the three extracts to have the same absorbance for the

PA assay, i.e., the same amount of initial PA. The objective of the experiment was to see if the polymeric fraction after filtration would induce better results than an extract for the same amount of proanthocyanidins introduced. They were then dosed at 4 mL/L into a kit green beer and fining activity tested in Imhoff cone experiments (Section 2.1.2, sediment depth analysis, haze analysis). Sediment depth and haze were recorded after 24h of clarification.

3.1.5 Variation in proanthocyanidin profile with hop harvest time

First samples (Nugget small cones) were picked at the beginning of flowering (mid-July). Two other consecutive samples were picked each consecutive 20 days (Nugget medium cone, Nugget big cone). A sample of Nugget hop leaf were also picked when the big and grown cones were taken from the allotment (end of August). Another sample (Nugget brown cone) was picked a month after the regular harvest for the hop to be aged and oxidized. The polyphenol content were then extracted using the 70% acetone_(aq.) extraction shown in section 2.2.1 for all samples and then tested for polyphenol content, beer fining efficiency. They were then fractionated using C18 solid phase extraction prior to AUC sedimentation velocity $c(s)$ profile.

3.1.6 Proanthocyanidin from different botanical sources

Seven different extracts were prepared from cinnamon, sorghum, grape seed, freeze dried blackcurrant, aronia berries, currants, Saaz hop and cranberry using the normal extraction procedure with 70% acetone in water (Section 2.2.1). All extracts were tested for proanthocyanidin content using the acid-butanol assay (Section 2.1.2.8, acid butanol assay for proanthocyanidin content of extracts) and for their fining efficiency in green beer (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5, haze analysis). All extracts were fractionated using C18 cartridges and then profiled through AUC sedimentation velocity experiments at 45000 rpm. The

molecular distribution of aronia and cinnamon methanolic C18 fractions were also analysed through sedimentation equilibrium experiments.

3.2 Results and discussion

3.2.1 Clean-up fractionation of the hop extract through solid-phase extraction

Prior fractionation is necessary in order to separate the active compound from other plant material present in the sample. The polyphenolic extract from spent hops is composed of proanthocyanidins but also of others phenolics and compounds of similar polarity and extractability (Almaguer et al., 2014). Fractionation steps were developed in order to recover only the core active fraction of the sample for analytical experiments. Solid-phase extraction is a preparation method where compounds in a liquid phase are separated by others from selective adsorption on a solid material (Zhang et al., 2000). They can then be eluted using a specific solvent. This method is used in order to concentrate, isolate, or purify molecules of interest prior to analysis. Three different solid phase extraction media were tested, selected according to the literature on proanthocyanidin characterisation (Gabetta et al., 2000; Pedan et al., 2016; Sun et al., 1998). The objective was to only elute the active PA material from the spent hop sample with a minimum of contamination. 30 mL of aqueous extract from the Saaz and Target hop were produced for fractionation study using the second extraction method reported previously (Section 2.2.1). The separation protocols utilised are summarised in Table 3.1.

Table 3.1 Solid-phase extraction protocols used for laboratories hop extract fractionation.

		Solid-phase extraction cartridge type		
		CN	LH-20	C18
Solvent	Conditioning	ACN / 0.1% Acetic Acid	MeOH or Acetone	MeOH/Water
	Load	Hop extract aq.	Hop extract aq.	Hop extract aq.
	Wash 1	5% ACN in 0,1% Acetic acid	Water	Water
	Wash 2	N/A	N/A	Ethyl Acetate
	Elution	30% ACN	MeOH/W (v/v) or Acetone/W (v/v)	Methanol

Fractions of the load, washes and the eluted fraction were then bio-assayed by determining their fining activity in small tubes of freshly fermented beer (Section 2.1.2.2, sediment depth analysis) (100µL of fraction mixed into 10mL of green beer). Fractions were also assayed for gallic acid assay and the acid butanol hydrolysis for proanthocyanidins assay in order to evaluate their polyphenolic content (Section 2.1.2.7, Gallic acid assay and Section 2.1.2.8, acid butanol assay for the proanthocyanidin evaluation of extracts).

Table 3.2 Fining activity of an original Saaz hop extract and its multiple eluted fractions separated on a CN SPE cartridge.

CN Elution Saaz sample	Sediment depth (mm)		Activity recovery (%)	GA Assay (λ = 750 nm)	PA Assay (λ = 550 nm)
	30 min	1 h			
Saaz hop extract	18	18	N/A	1.91	2.70
Load CN	18	18	100%	1.64	1.85
Wash	3	3.5	5.5%	0.26	0.25
CN Elution 30% ACN	10	11	62.5%	0.34	0.49
Control (no fining)	2	4.5	N/A	N/A	N/A

Table 3.3 Activity of an original Target hop extract and its multiple eluted fractions separated on a Sephadex LH-20 cartridge (Methanol or acetone conditioned cartridge).

LH20 Target Sample (MeOH conditioned)	Sediment depth (mm)		Activity recovery (%)	GA Assay (λ = 750 nm)	PA Assay (λ = 550 nm)
	30 min	1 h			
Target hop extract	17	17	N/A	1.93	> 3
Load LH-20	12.5	12.5	83%	0.74	2.99
Wash Water	2	2	0%	0.31	0.44
Elution 30% Methanol	8.5	8.5	57%	0.55	1.17
Elution 80% Methanol	2	2	0%	0.28	0.36
Elution 100% methanol	4	4	13%	0.30	0.34

LH20 Target Sample (Acetone conditioned)	Sediment depth (mm)		Activity recovery (%)	GA Assay (λ = 750 nm)	PA Assay (λ = 550 nm)
	30 min	1 h			
Load LH-20	10.5	11	57%	0.63	1.17
Wash Water	7	7.5	46%	0.52	0.77
Elution 30% Acetone	9.5	9.5	50%	0.46	0.79
Elution 80% Acetone	9	9	46%	0.94	1.31
Elution 100% Acetone	2	2.5	0%	0.08	0.20
Control (no fining)	2	3	3	N/A	N/A

Table 3.4 Activity of an original Saaz hop extract and its multiple eluted fractions separated on C18 SPE cartridge.

C18 Saaz Sample	Sediment depth (mm)		Activity recovery (%)	GA Assay (λ = 750 nm)	PA Assay (λ = 550 nm)
	30 min	1 h			
Saaz hop extract	18	18.5	N/A	1.91	2.70
Load C18	1	1.5	0%	0.16	0.17
Wash Water	1	2	0%	0.12	0.10
Wash Ethyl Acetate	1	2	0%	0.21	0.49
Elution C18 (Methanol)	18	17	92%	0.95	1.79
Control (no fining)	1	2	N/A	N/A	N/A

Tables 3.2 to 3.4 show the sediment depth formed from 10 mL of green beer fined with the different fractions from the three different extraction steps after 30 and 60 min. The sediment value was then used as an index of activity recovery (with the unfined control beer taken as a reference). Tables 3.2-3.4 also show the general polyphenol concentration of each fraction analysed using the gallic acid assay, and the PA content measured using the acid butanol hydrolysis assay (Section 2.1.2, acid butanol assay for proanthocyanidin content of extracts).

The CN media (Table 3.2) did not help the fractionation of the PA active molecules in the hop sample. 100% of the fining action was recovered in the load fraction, which means that the active fraction did not bind to the solid-phase extraction material. The eluted fraction with 30% acetonitrile recovered 62% of the initial fining performance, but had a low absorbance (0.5) for the PA assay compared to 2.7 for the initial extract. Previous work on aqueous hop extract by Linforth et al., (2015) has shown that cyano column linked to HPLC gave an efficient elution of the active compound. However a poor recovery using this solid phase material was seen here with acetone and water hop extracts, because most of this activity was not retained through the cartridge material and passed straight through after loading the compounds. Probably the extraction is pulling out only specific PA which acetone and water are extracting in higher concentration.

LH-20 fractionation has been studied using both methanol and acetone as the main eluent as the resin can be used using a variety of solvents (Cuevas-Rodríguez et al., 2010; Taylor et al., 2003). The resin has to be prepared before elution for the material to swell and absorb the solvent. Most of the active material contained in the hop extract was also recovered in the load fraction, with a high concentration of proanthocyanidins detected. The LH-20 phase was able to spread the activity across

different eluted fractions. The methanol prepared column yielded three fractions; one with half the activity of the original extract and two with a poor activity and with a low PA concentration. The acetone-conditioned cartridge yielded two active fractions. Both 30% and 80% acetone eluted fractions recovered about half of the initial fining performance of the hop extract. Both fractions eluted in this fractionation-step had a noticeable concentration of proanthocyanidins from the PA assay. Literature sources used this resin for separation of PA with the size of the polymer (Taylor et al., 2003; Gu, 2012). The acetone-conditioned cartridge has been able to yield two different fractions with each of them having half of the original activity of the hop extract. Those fractions may be differentiated by the different sizes of PA polymer present. As the polymer chain length increases, it becomes more hydrophilic, and would be transferred easier into the first phase (30% acetone).

The C18 methanol eluted fraction contained the majority of active fining material. The fraction was a clear, stable and non-viscous solution with a colour from yellow to brown depending on the concentration. About 92% of the initial fining performance from the Saaz original extract was recovered in the eluted fraction from the C18 SPE. The C18 media has been able to bind the majority of the active compounds during the loading of the hop extract sample. C18 cartridges are well known to retain the most hydrophobic molecules and as a consequence the longest polymers. The two washes fractions also contained polyphenols which are not active. The two solutions are perceived as a bright to intense yellow, and are supposed to contain other phenolics not responsible for the fining activity. This technique answered the need of the isolation of the proanthocyanidins, as seen in previous work done by Sun et al., (1998). This fractionation step has been chosen in order to analyse the hop extract for all further chromatographic and analytic experiments.

3.2.2 Evaluation of different hop varieties for proanthocyanidin content and beer fining efficiency

The brewing industry nowadays uses multiple hop varieties which differ in content and composition of the resin and essential oil fractions (Krofta et al., 2008). Differences in aroma properties can be observed between hop varieties (Eyres et al., 2007). Lermusieau et al., (2001) determined the total polyphenol content of six different varieties and found significant differences, with Saaz variety containing the highest total polyphenol content and Nugget having the lowest. Krofta et al., (2008) found a clear difference in antioxidant activity comparing Saaz (high) to other varieties such as Magnum, Taurus, or Newport. They also compared this antioxidant activity with different Czech hops and found once again significant differences. Saaz had the highest antioxidant activity compared to the minimum found for Sládek. Earlier work by Linforth et al., (2015) has shown the ability of whole Saaz aqueous hop extracts and also spent Galena hops to induce flocculation and sedimentation of yeast cells in beers. With regard to the range of varieties used across the current brewing market, the question arises as to what differences in fining activity could be seen across a range of different hop varieties. The 12 different varieties were selected as representative of different applications in beer. Hops like Target, Galena and Magnum are high in bittering materials whereas Cascade, Crystal and Saaz are used for their low concentration in bittering compounds and good hoppy aroma (Parker, 2011). Furthermore, Simcoe and Citra were selected as exemplar dual purpose hops (used for either or both bittering and aroma).

Acetone-water extracts of 12 hop varieties were prepared using the extraction method in the section 2.2.1 and submitted to both gallic acid and the acid butanol

hydrolysis assay. The resulting absorbance was displayed in two different data sets (Figure 3.1, Table 3.5).

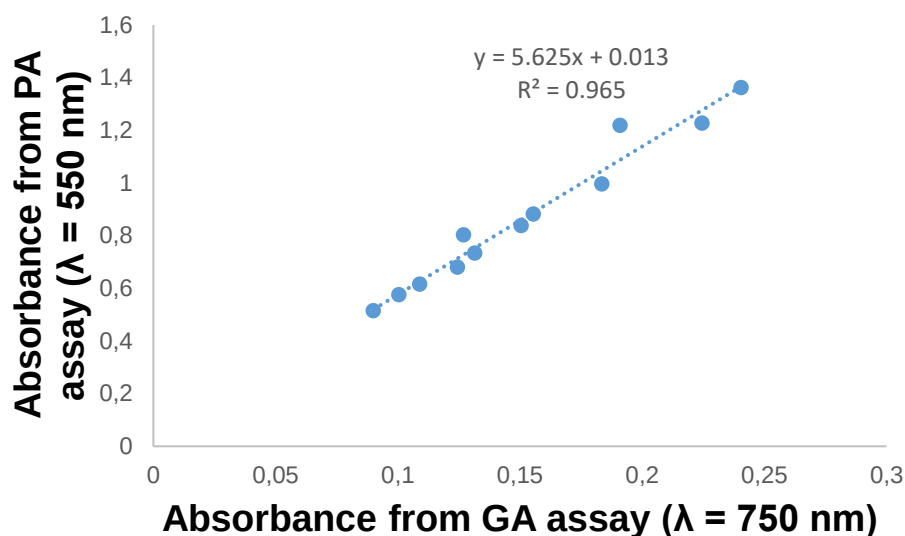


Figure 3.1 Correlation between general polyphenolic concentration (GA assay) with proanthocyanidin content (through PA assay) for 12 different hop samples.

Table 3.5 Absorbance values from the GA assay and the PA assay for 12 different hop varieties extracts.

Hop Variety	GA Assay (λ = 750 nm)	PA Assay (λ = 550 nm)	PA/GA
Magnum	0.09	0.51	5.7
Hallertauer	0.10	0.57	5.7
Hersbrucker	0.10	0.61	5.6
Galena	0.10	0.61	5.6
Crystal	0.12	0.68	5.5
Pilgrim	0.13	0.73	5.6
Cascade	0.12	0.80	6.3
East Kent Goldings	0.15	0.83	5.6
Target	0.15	0.88	5.7
Simcoe	0.18	0.99	5.4
Citra	0.19	1.21	6.4
Summit	0.22	1.22	5.5
Saaz	0.24	1.36	5.7

During acid hydrolysis all hop variety extracts generated the blue complex indicating the presence of phenol groups, and the red-magenta colour typical of proanthocyanidins cleavage to anthocyanidins. A range of absorbance values were produced from 0.53 to 2.64 during the PA acid hydrolysis (Figure 3.1). In the data

set, Saaz, Summit and Citra all produced a response which was more than twice that of Galena, Hallertauer Hersbrucker and Magnum hop extracts, which thus appeared to contain lower levels of PA. Results to the PA and GA assays were directly correlated (Figure 3.1) with a good linearity ($R^2=0.96$). In addition, the ratio PA/GA remains broadly constant for all hop varieties.

The fining action can be characterised by 2 factors; yeast sedimentation and haze removal. Both factors would be affected by the abundance of haze-active material to fine, or the abundance of PA from the hop source (Table 3.5). Sediment depth however can depend on the level of compaction, and measured haze post-fining may depend upon haze addition as well as removal since these are crude hop extracts.

Extracts (70% acetone_(aq.)) of the same 12 hop varieties (prepared using the extraction method described in 2.2.1) from the first experiment were used to fine a

kit beer (Section 2.1.2.2, sediment depth analysis and Section 2.1.2.5, haze analysis).

Sediment depth and haze records are shown in Figure 3.2.

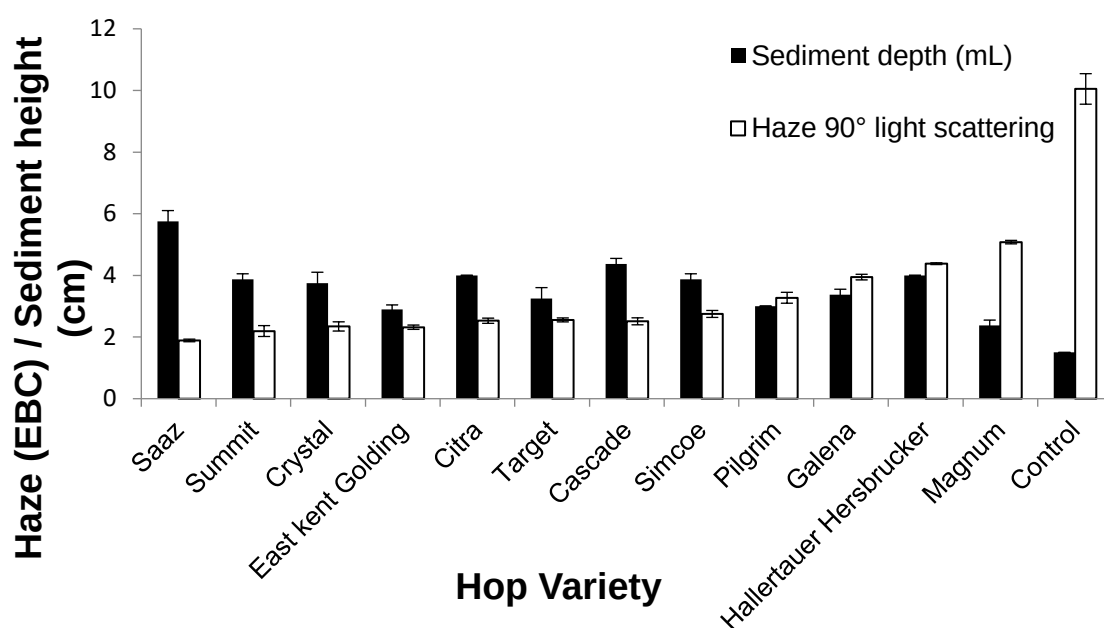


Figure 3.2 Sediment depth and haze H90 of a kit green beer fined with 12 different hop varieties extracts and a reference control (no fining) at 4°C. Measurements were recorded after 24 h of clarification and represented as a mean of duplicates with their standard deviation.

The twelve hop varieties have been studied through their PA concentration and their fining efficiency (Figure 3.1, Figure 3.2, and Table 3.5). All hop varieties showed fining activity by reducing beer haze and increasing sediment formation relative to the unfined control beer. However, not all hops showed the same activity. Linforth et al., (2015) previous work was mainly done on hop extract from Galena hop variety but stated as well the yeast flocculation ability of different hop extracts from different varieties. Residual haze (90° scatter) differed according to variety and was broadly inversely proportional to sediment depth formed. Magnum had the least fining activity, and Saaz/Citra hop extracts effected the greatest haze reduction of the varieties investigated. The trend in fining activity with hop variety was in broad

agreement with the trend observed in the PA assay (Figure 3.1, Table 3.5). Conversely, some varieties (e.g. Cascade, Crystal) showed efficient fining activity, but not a very high concentration of proanthocyanidins according to the PA assay (Figure 3.2, Table 3.5). This may reflect differences in the specific proanthocyanidins present and their molecular size distribution. Theoretically, the bigger the molecules involved, the more they can flocculate haze particles. It can be also supposed that a low content of high molecular weight polymers could involve a slight increase in haze after fining. Monomers and low molecular weight phenolics would not be able to form large aggregates, resulting in the product becoming cloudier.

Two regressions were fitted (one linear, one polynomial) between the haze recorded after fining at 4°C, and the PA concentration given by the acid butanol assay for the 12 different hop varieties (Figure 3.3). The linear correlation coefficient (0.72) indicates the general tendency for increasing PA content to flocculate more proteins and yeast cells in the fermented beer. However, the polynomial regression is showing with higher correlation (0.94) that fining performance tends towards an optimum at high PA levels in terms of haze removal.

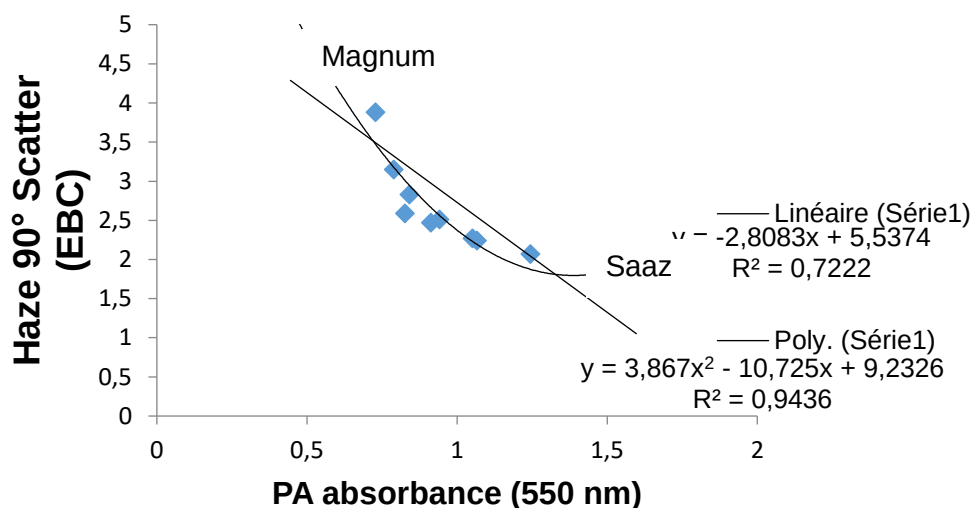


Figure 3.3 Relationship between proanthocyanidin content of hop extracts and final haze after fining a kit green beer at 4°C for 12 different hop varieties.

Interestingly alpha-acid content is not an indicator of the fining efficiency of the hop varieties presented. High alpha acids hops like Summit and Citra had a great clarification efficiency whereas Magnum and Galena did not perform as well, even though their alpha acid content is approximately equal (12 to 14%). Saaz hop has proved to be the most efficient of all the hops studied for beer clarification, and has been demonstrated to have a special effect on other beer properties. Lermusieau et al., (2001) found that Saaz contained the highest amounts of polyphenols and was the most efficient to reduce the formation of staling compounds during wort boiling, when comparing 6 different varieties. This hop variety has also been used as an efficient chill haze removing agent by Jelínek et al., (2014) and an accelerating tool for beer lautering (Karabín et al., 2018).

Differences in proanthocyanidin contents with variety have been reported for other polyphenol-rich botanical sources. Lv Q. et al., (2015) mentioned significant differences in the proanthocyanidin content across 32 different lychee cultivars. Asproudi et al., (2015) also showed varietal differences in PA content for different

wine grape varieties. In addition, differences between the proanthocyanidin content from the PA assay suggest that flavanol composition and complexity in hop extracts involves a varietal characteristic: further evaluation of the proanthocyanidin composition is needed. The two assays here are only crude analytical assays and yield no information about the size of the polymer present for each hop variety. Deeper analytical methods have to be employed in order to define each hop variety within their PA molecular size and distribution.

3.2.3 Molecular characterisation of hop proanthocyanidins

Proanthocyanidins are a subclass of flavonoids that can be present in either monomeric or polymerized forms. They are distinguished by their constituent units, interflavan linkages, and degree of polymerisation (Neilson et al., 2016). This complexity of structure induces a number of analytical challenges. Currently, proanthocyanidin research is somewhat limited by the existence of appropriate analytical methods, especially for compounds with DP > 2-3 (Neilson et al., 2016).

3.2.3.1 Polymer degradation using phloroglucinol

The degree of polymerisation defines the number of monomer units that form the chain of the polymer. This number is proportional to the length and the molecular weight of the polymer. Proanthocyanidin size can be expressed in terms of the average degree of polymerisation, which represents the average molecular weight in the sample. However, it does not give any information of the polymer size distribution. Tanaka et al., (2014) reported large amounts of highly oligomeric (> 20-mer) proanthocyanidins in their hop bract extract. The oligomers were separated using High-Speed Counter-Current Chromatography fractionation following ultrafiltration (cut-off > 3 kDa). Structural studies using ^1H and ^{13}C NMR indicated

that the proanthocyanidins were of the B-type, with flavan-3-ol units that are linked through C-4 to C-8 (or C-6) interflavan bonds.

It is possible to determine quantitative information about the subunit composition of a proanthocyanidin extract because of the relative ease with which the interflavanoid bond is cleaved (Kennedy et al., 2001). The proanthocyanidin polymer can be degraded using an excess of phloroglucinol, in acidic ethanol conditions. The reaction is based on the cleavage of the interflavan bond for every polymer into monomers. The monomers created would then attach to one unit of phloroglucinol to form an adduct. The terminal unit of each chain of monomers would then be released as an unmodified molecule of catechin or epicatechin. The adduct concentration can be determined using UV-Vis HPLC spectroscopy, and the terminal chain concentration using LC-MS.

LC-MS calibration showed good linearity ($R^2=0.988$) for standard solutions of catechin and epicatechin and the relation between peak area and concentration of compounds. The background concentration was also determined to see if the hop extracts contained a known concentration of monomers before the phloroglucinol degradation. Methanolic fractions of 11 hop samples (Citra data not shown) after C18 fractionation were analysed in duplicate on the LC-MS before the phloroglucinolysis to show that all hops contain a different background levels of the monomers (Table 3.6).

Table 3.6 Determination of the average degree of polymerisation (DP) and average molecular weight of 11 different hop varieties through phloroglucinolysis reaction. Each value is the mean of duplicates.

	Background concentration (mg/mL)		Terminal subunits (mg/mL, background subtracted)			Adducts (mg/mL)	DP	Av. Mwt (Da)
	Catechin	Epicat.	Catechin	Epicat.	Cat/Epi			
H. Hers.	3.6E-04	1.1E-04	4.9E-03	5.5E-04	9.9	0.03	6.3	1834
Galena	8.7E-05	9.3E-05	4.9E-03	7.0E-04	7.7	0.04	7.2	2075
Simcoe	2.0E-04	3.4E-04	3.9E-03	1.0E-03	3.8	0.04	8.8	2562
Pilgrim	1.5E-04	3.9E-04	3.5E-03	1.1E-03	3.2	0.04	9.7	2803
Crystal	2.7E-04	2.1E-04	6.7E-03	9.3E-04	7.2	0.08	10.5	3046
Magnum	2.2E-04	1.2E-04	4.7E-03	8.5E-04	5.5	0.04	10.6	3062
Target	7.5E-05	9.6E-05	7.4E-03	1.8E-03	4.2	0.10	11.3	3269
E.Kent	6.9E-05	2.2E-05	2.9E-03	4.9E-04	5.5	0.03	13.8	3991
Cascade	1.9E-04	1.4E-04	4.1E-03	7.3E-04	4.7	0.04	17.0	4919
Saaz	1.6E-04	2.1E-05	8.2E-03	1.1E-03	7.9	0.16	17.1	4972
Summit	3.5E-04	2.7E-04	2.4E-03	1.8E-03	1.8	0.07	19.3	5611

After degradation, all hop extracts produced catechin or epicatechin terminal subunits, or phloroglucinol adducts. All extracts following phloroglucinolysis contained more catechin end units than epicatechin. Degree of polymerisation was then determined using the ratio of adducts present compared to the concentration of terminal units formed. An average molecular weight was then calculated using the DP and the molar mass of catechin (290.26 Da).

Results demonstrated the presence of polymers in all samples, since their average molecular weight is much greater than the weight of a single unit. Hops like Hallertauer or Galena had an average molecular weight corresponding to approx. 6 monomeric units, and at the high end of the size range Saaz and Summit polymers contained on average at least 17 monomer units.

In addition, a regression plot between the absorbance obtained from the PA assay and the degree of polymerisation showed a relationship with statistical significance ($p = 0.01$, ANOVA; Figure 3.4).

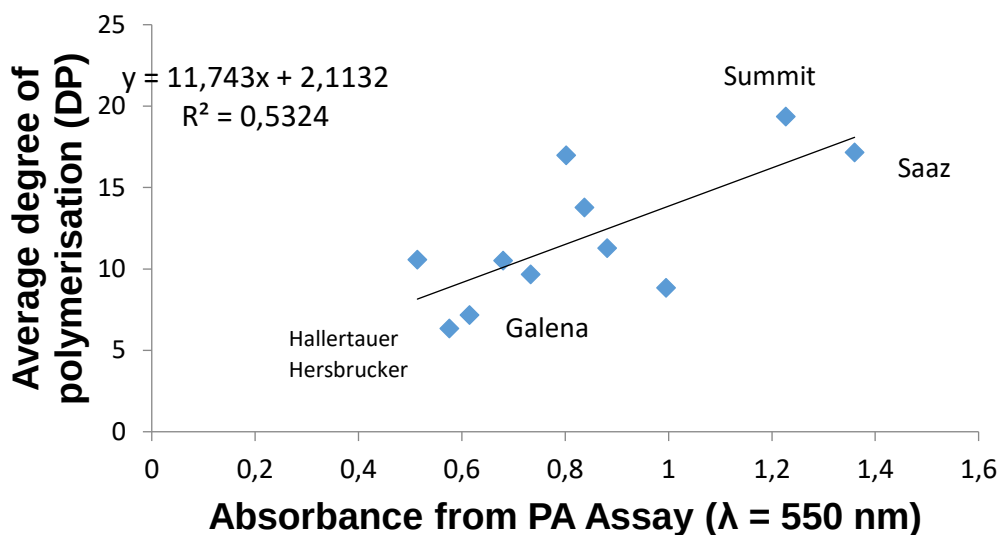


Figure 3.4 Proanthocyanidin content determined through acid butanol hydrolysis plotted against the average degree of polymerisation of 11 different hop varieties determined through phlorglucinolysis.

This assay clearly indicates the presence of PA polymers within the hop extracts. For the fining performance, it is supposed that the flocculation mechanism is dependent on the size of the aggregates formed. The bigger the PA polymer is, the bigger would be the flocs formed during fining. By the gravitational force they would tend to settle at the bottom of the fermenter more readily and result in better haze removal. On the other hand, if the polymer size tends to be small, the flocs formed would not be big enough to settle, which can result in the formation of additional haze in the beer sample. It is important to understand the polymer distribution of the hop samples, in order to better understand the fining mechanism but also to avoid the use of hop varieties which will not fine effectively. The current strategies to determine the polymer distribution are giving an approximate or an

average of the global molecular weight profile of proanthocyanidin extracts. The polymer degradation study using excess of phloroglucinol is unfortunately based on the fact that the reaction is total between the phloroglucinol and the polymer present in the samples. The data shows a good indication of the presence of polymers in the hop extracts, but only indicates average degree of polymerisation of materials present. Bearing in mind that low molecular weight phenolic material might predominate, the average molecular weight could substantially underestimate the size of large PA molecules present in hops. However, this could only be determined using a technique such as Analytical Ultracentrifugation (introduced in Section 1.6), capable of resolving the phenolic materials according to their size and shape. Both fining experiments and the chemical degradation are showing that Saaz is the most efficient hop variety for haze removal, and the variety with the highest degree of polymerisation.

3.2.3.2 AUC sedimentation velocity (SV) profiles of different hop varieties

Analytical ultracentrifugation is a well-known method for the determination of the molecular weight of molecules such as proteins and polysaccharides. The sedimentation velocity technique has the ability to profile solutions, to determine the complexity of a mixture, and to give a first indication of approximate molecular weight through the sedimentation coefficients obtained (Cole et al., 2008).

Sedimentation velocity is an analytical method which measures the rate at which molecules move in response to a high centrifugal force. This force depletes the compounds from the meniscus region (close to the centre of the rotor) ultimately forming a pellet at the outside of the cell. This transport rate is expressed in terms of sedimentation coefficient values, measured in Svedbergs. The aim of this experiment was to determine the size distribution profile of the PA polymers present in the

extracts of different hop varieties, and to attempt to correlate the results with their beer fining activity (Section 3.2.2).

Development of an AUC technique for the analysis of proanthocyanidin extracts from hops

Preliminary AUC experiments with crude acetone/water extracts did not yield data that could be processed by the AUC software, presumably due to the wide range of material present within the sample. The C18 Sep-Pak SPE clean-up method was developed (based on that reported by Sun et al., (1998) for the separation of grape and wine proanthocyanidins) in order to separate proanthocyanidins from other material. The cleaned-up fractions proved suitably pure for characterisation by AUC.

AUC data processing parameters: specific volume, solvent density and frictional ratio.

The partial specific volume of a substance is represented by the ratio of the substance's anhydrous volume to its anhydrous mass, and is a key parameter for the processing of the analytical ultracentrifuge data. Tanaka et al., (2014) and Linforth et al., (2015) each reported that the proanthocyanidins in hops are mainly comprised of catechin and epicatechin subunits. Based on this, the partial specific volume was calculated to be 0.706 mL/g according to the method of Durchschlag et al., (1997) to calculate the specific volume of organic molecules. The density of the solvent used for the processing was that of pure methanol at 20.0°C.

In the SEDFIT procedure (Dam et al., 2004), sedimentation coefficient distributions are generated from the evolution of the concentration distribution in the ultracentrifuge cell with time, to give the derived $g(s)$ as a function of s distribution. Although the sedimentation coefficients of discretely resolved components are unaffected, the distribution can be influenced by diffusive broadening. The $c(s)$ as a

function of s method in SEDFIT provides a correction for this by evaluating a parameter known as the frictional ratio. The frictional ratio could not be determined from the literature, because the sample studied is not composed of only one molecular species. Instead a (weight average) frictional ratio was thus determined by iterative fitting using the software and applied to all species. All experiments showed slightly different frictional ratio values between samples, but, a consistent value was typically obtained across a dilution series for a given hop extract.

The diffusion-deconvoluted model, $c(s)$ provides evidence of different oligomeric states for the hop proanthocyanidin fractions; the profiles have peaks at discrete size ranges and are not continuous. For Saaz, the profile (Figure 3.4) showed 4 main peaks with sedimentation coefficients of 0.15, 1.1, 2.7 and 4.4.

The peak with a coefficient of 0.15 S corresponds to smaller molecules; monomers and small oligomers. Proanthocyanidin-rich extracts of Saaz showed an additional high molecular weight peak (P3, at 4.4 S) which was not present in Magnum, as well as a higher signal response for polymerised higher molecular weight material (2.7 S). Conversely, the Magnum extract gave a higher signal in the lowest molecular weight band.

These sedimentation coefficient data correspond with a greater fining activity of Saaz compared to Magnum in beer clarification studies before, supporting the hypothesis that larger proanthocyanidin polymers would result in larger particles of sediment that settle more efficiently.

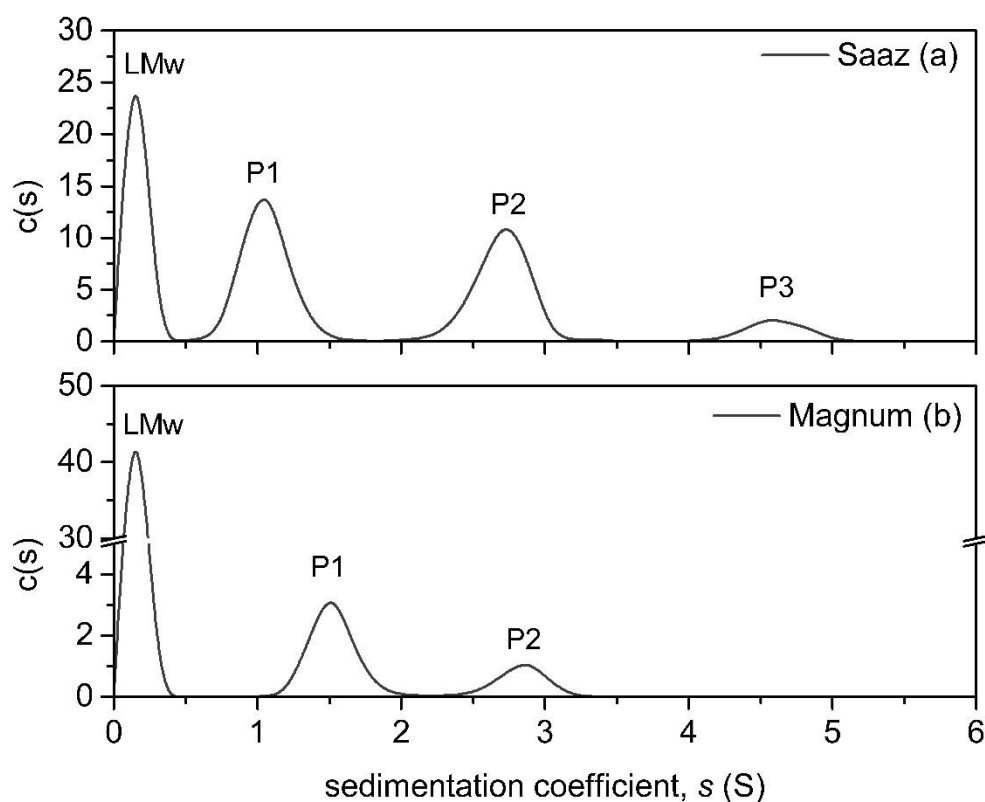


Figure 3.5 Sedimentation velocity $c(s)$ profiles for Saaz (A) and Magnum (B) hop extracts (in methanol, 6-fold dilution) after C18 fractionation. Centrifugation velocity 45000 rpm, temperature 20.0°C
Peaks are numbered LMw (low molecular weight), P1-P3 for cross-reference with corresponding data in Table 3.7 and Figure 3.7

A key feature of any analytical technique is the reproducibility of the data obtained. Each sample run consisted of a set of cells running simultaneously with different dilutions of an extract. The results from each cell were obtained and averaged to give the $c(s)$ values (Table 3.7). These showed minimal coefficient of variation (standard deviation relative to the average) for each $c(s)$ peak in the profile. In addition to this there may be day to day and botanical variation between samples. Three independently sourced samples of Saaz were profiled in separate AUC runs. These

showed similar profiles on each occasion, suggesting consistency of this molecular weight range fingerprint for particular hop varieties (Table 3.7).

Table 3.7 Sedimentation velocity c(s) profiles for Saaz and Magnum hop extracts. P1 to P3 are the different molecular size peaks on the c(s) profiles in Figure 3.5. Speed 45000 rpm, temperature 20.0°C

		c(s) sedimentation coefficient							
		values (S)*			% Area‡				
	LMw	P1	P2	P3	LMw	P1	P2	P3	
Saaz Pellets	0.15 ±0.003	1.04 ± 0.08	2.64 ± 0.08	4.46 ±0.13	33.4	32.5	27.7	6.4	
Saaz Whole	0.15								
Cones 1	±0.003	1.12 ± 0.07	2.65 ±0.06	4.22 ± 0.15	34.0	32.4	27.1	6.5	
Saaz Whole	0.15								
Cones 2	±0.005	1.09 ± 0.03	2.70 ± 0.06	4.41 ± 0.17	31.8	34.5	27.2	6.5	
Magnum	0.15								
Whole Cones	±0.004	1.56 ± 0.05	2.95 ± 0.08	-	84.9	11.5	3.6	0	

* Average peak in Svedbergs ± SD of five determinations based on the dilution series of each sample.

‡ Absolute peak areas were calculated using integration and then expressed as a percentage of total area of each sample to reflect the proportion of material present in each size range.

The sedimentation velocity technique appears to be highly reproducible, both within and between runs. There also appears to be varietal consistency. This consistency allows us to clearly demonstrate a difference in hop proanthocyanidin molecular weight profiles between different varieties of hops as can be seen in the Saaz and Magnum data (Table 3.7 and Figure 3.5). The same 12 hop varieties extracts

(prepared from 2.2.1) used elsewhere in this chapter have been analysed through sedimentation velocity experiments, at 45000 rpm. Table 3.8 displayed the 12 different sedimentation velocity profiles of each hop variety, with their proportion of each peak present in each sedimentation coefficient range.

Table 3.8 Sedimentation velocity $c(s)$ profiles for 12 different hop extracts from different hop varieties. Speed 45000 rpm, temperature 20.0°C.

	Sedimentation coefficient values (S)				%Area			
	LMw	P1	P2	P3	% LMw	% P1	% P2	% P3
<i>Saaz</i>	0.15 ± 0.01	1.09 ± 0.03	2.69 ± 0.01	4.44 ± 0.17	32	35	27	7
<i>Summit</i>	0.16 ± 0.01	1.20 ± 0.05	2.14 ± 0.07	3.57 ± 0.09	59	17	18	7
<i>Citra</i>	0.16 ± 0.01	1.25 ± 0.08	2.27 ± 0.08	3.81 ± 0.25	51	19	21	9
<i>Simcoe</i>	0.16 ± 0.01	1.24 ± 0.10	2.11 ± 0.08	3.30 ± 0.11	58	14	20	8
<i>East Kent Goldings</i>	0.16 ± 0.01	1.50 ± 0.03	2.80 ± 0.15	-	64	21	15	0
<i>Target</i>	0.23 ± 0.04	1.61 ± 0.04	2.94 ± 0.07	-	56	33	11	0
<i>Cascade</i>	0.15 ± 0.01	1.71 ± 0.06	2.91 ± 0.05	-	82	10	8	0
<i>Crystal</i>	0.17 ± 0.01	1.20 ± 0.09	2.29 ± 0.10	3.67 ± 0.12	51	19	22	8
<i>Pilgrim</i>	0.17 ± 0.01	1.04 ± 0.10	2.16 ± 0.10	3.48 ± 0.14	62	15	17	6
<i>Galena</i>	0.24 ± 0.01	1.36 ± 0.03	2.41 ± 0.01	-	57	29	14	0
<i>Hallertauer Hersbrucker</i>	0.16 ± 0.01	1.01 ± 0.02	2.03 ± 0.05	3.23 ± 0.14	53	21	17	9
<i>Magnum</i>	0.15 ± 0.01	1.56 ± 0.04	2.85 ± 0.08	-	85	12	4	0

Absolute area of the peaks has been integrated in order to give an indication of the distribution of the polymers within the samples. All hops, apart from Saaz, contained a majority of low molecular weight compounds (LMw), which is indicated by the greatest proportion of the total area being due to the lowest Svedberg peak (Table 3.8). Five hops varieties did not have a 4th sedimentation velocity peak at the high end of the sedimentation coefficient range, which indicates the presence of the highest molecular weight polymers. A major correlation was found between the sum of the absolute area of peaks 2 and 3, and the PA absorbance value given from the acid butanol hydrolysis assay. Highest molecular weights compounds appear to correlate with the PA concentration in the samples (Figure 3.6, Table 3.9).

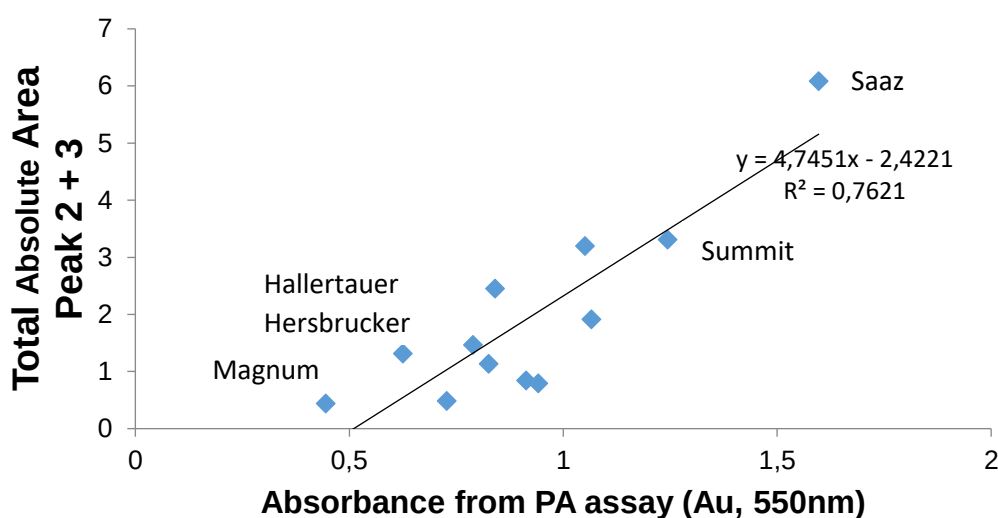


Figure 3.6 Plot of the sum of c(s) P2 and P3 absolute area for 12 different hops according to their PA absorbance (acid hydrolysis assay, 550nm), ($p = 0.0002$, ANOVA).

Table 3.9 Absolute area from sedimentation velocity experiments of P2 and P3 for 12 different hop varieties.

Hop variety	Absolute Area P2 + P3
<i>Magnum</i>	0.44
<i>Galena</i>	0.487
<i>Target</i>	0.791
<i>East Kent Golding</i>	0.943
<i>Cascade</i>	1.14
<i>Hallertauer Hersbrucker</i>	1.31
<i>Pilgrim</i>	1.47
<i>Citra</i>	1.91
<i>Crystal</i>	2.45
<i>Simcoe</i>	3.19
<i>Summit</i>	3.31
<i>Saaz</i>	6.08

In AUC, sedimentation velocity can define the profile of a mixture and shows the heterogeneous nature of the sample and its global composition. Although related to molecular size, information on molecular weight cannot be directly extracted from sedimentation velocity profiles. However, these experiments do clearly show a discrete set of peaks with distinct differences in molecular size rather than a continuous distribution. This implies that the hop plant produces a series of PA with particular degrees of polymerisation rather than one main PA with more or less sub units.

The Magnum hop has been shown as the least fining-active hop variety in the brewing application study. The AUC profile is showing a low amount of high molecular weight polymers, which confirms once again the requirement for high molecular weight polymers for a good fining efficiency.

3.2.3.3 Sedimentation equilibrium (SE) AUC analysis of proanthocyanidin hop extracts.

Sedimentation equilibrium studies can be used to measure the average molecular weight of material in a sample. Although not as resolving as sedimentation velocity, it can provide an absolute estimate of the molecular weights of the major components

present. Since the concentration distribution at equilibrium does not change with time, shape and frictional effects do not influence the pattern, which is solely a function of molecular weight. The molecular weight distribution of methanolic SPE fractions derived from Saaz or Magnum hop varieties were studied through sedimentation equilibrium experiments. Because of the complexity of the hop extracts showed by the sedimentation velocity experiments, the process used various centrifugal speeds for the experiments.

All SV profiles indicate the presence of at least 3 or 4 different groups of compounds. One which contains a majority of small molecules, monomers and ions, and others which could represent proanthocyanidin polymers.

Sedimentation equilibrium was used in this study to resolve and determine actual molecular weights for each band of proanthocyanidins in the hop extracts. Equilibrium experiments were starting with a low speed of 20000 rpm before moving to higher speeds. Once equilibrium was reached at each speed the rotational speed was increased by increments of 5000 rpm, up to a maximum of 40000 rpm for the final scans. The sedimentation equilibrium studies were consequently of longer duration, taking 4 to 5 days for each run, as compared to the sedimentation velocity experiments which were typically conducted in less than a day. This longer time period is necessary for the sedimentation and diffusive forces to reach equilibrium. Results for Magnum hop variety showed the presence of two distinct peaks, one with a molecular weight average of 5 kDa (97% of total area), and the second peak with a molecular weight of 52 kDa which only accounted for < 3% of total area (Figure 3.7). In contrast, the Saaz plot in Figure 3.7 A has 3 peaks. The first peak has an average molecular weight of 3 kDa (62% Area), the second is at about 12 kDa (26% Area), and lastly there is a higher molecular weight peak at about 85 kDa (12%

Area). Clearly the Saaz hop extract contained a greater proportion of higher molecular weight species, which is in agreement with the sedimentation velocity data (Figure 3.5).

The $c(M)$ distributions (Figure 3.7) indicated the presence of three different molecular weight groups in Saaz and two in Magnum hops. It is noticeable that in each case the fitting procedure has identified one less peak in the $c(M)$ fit (Figure 3.7), relative to the $c(s)$ fit from sedimentation velocity experiments (Figure 3.5).

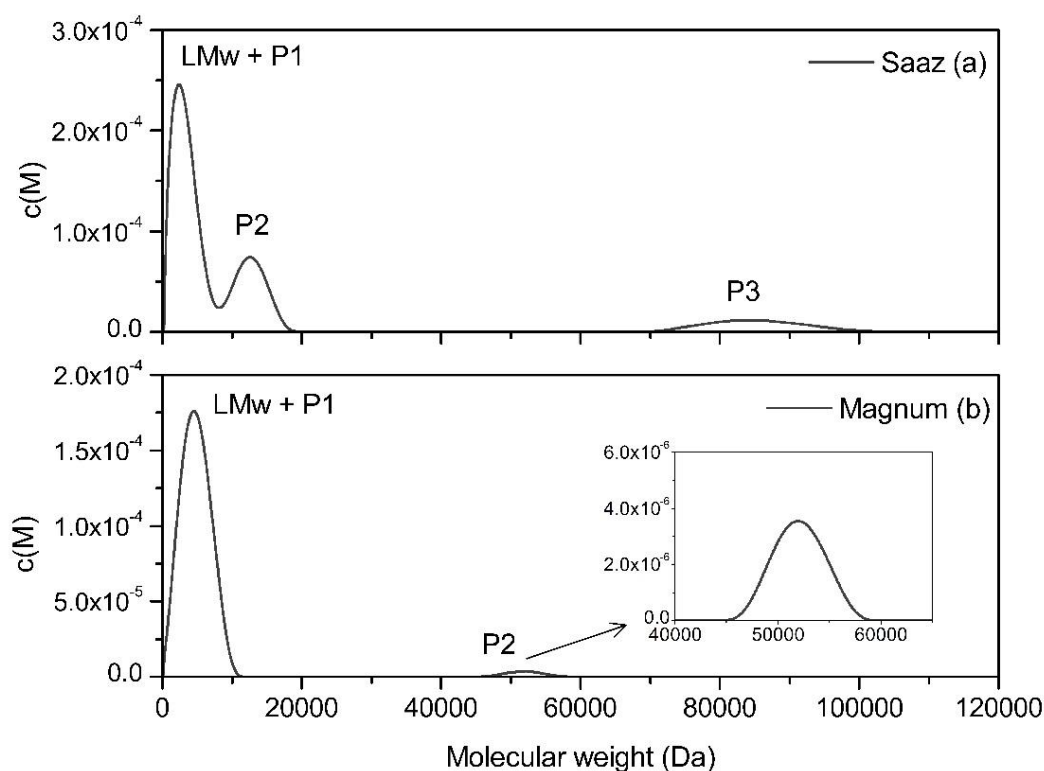


Figure 3.7 SEDFIT-MSTAR analysis of sedimentation equilibrium data, showing the $c(M)$ molecular weight distribution of A: Saaz and B: Magnum hop polyphenolic extracts (40-fold diluted, in methanol). Centrifuged to equilibrium at ascending speeds between 20000 and 40000 rpm at 20°C.

It is considered that the lowest mass peak on the $c(M)$ fits correlates with the two lowest sedimentation coefficient peaks on the $c(s)$ fits. This is in part because the

resolving power of sedimentation equilibrium experiments is less than that for sedimentation velocity experiments, due to the shorter cells/path lengths used to ensure that equilibrium can be achieved within a reasonable time frame. The development of this analytical technique using AUC has confirmed the presence of very high molecular weight polymers in hops. It is still not sure if these polymers are proanthocyanidins. Despite their low concentrations (approx. 20 mg/mL dry matter in the C18 purified methanolic fractions), AUC was able to detect this group of polymers. Li et al., (2006) have clearly shown the polymer distribution of hop proanthocyanidins with accurate molecular masses, up to hexamers using tandem mass spectrometry. However, due to the noted difficulties in analysing high molecular weight proanthocyanidins, the present results are the first indication that much larger polymers are present in hops. Significantly, AUC is a non-destructive method which measures the transport properties of molecules through a known distance under centrifugal force.

Different analytical approaches have been taken in order to characterise the molecular size distribution of proanthocyanidin hop extracts. The phloroglucinolysis reaction provided a mean degree of polymerisation for each sample. This number however does not fully reflect the complex molecular distribution of proanthocyanidins in polyphenolic extracts. (Kennedy et al., 2001). The data plotted in Table 3.6 indicated that hop extracts are composed of monomers of catechin and epicatechin, but also polymers. Although this reaction is preferred to the thiolysis degradation with benzyl mercaptan, the yield for the conversion of PA into their constitutive adduct subunit can vary (Kennedy et al., 2001). This inferred that the reaction is not considered as total, and that this assay might thus have some degree of inaccuracy. However, the experiments performed here provide an indication of

the presence of polymers within the hop extracts. Average molecular weights were found to be between 1800 and 5600 Da, demonstrating the complexity and heterogeneity of the samples studied. A broad correlation was detected between the average DP found in the experiment and the PA concentration found in all hop varieties studied. The highest DP found was for Saaz and Summit hops, which are as well the two highest PA concentration found and the best for fining efficiency.

The sedimentation velocity technique has the ability to profile solutions, to determine the complexity of a mixture, and to give a first indication of approximate molecular weight through the sedimentation coefficients obtained (Cole et al., 2008). The results in Table 3.8 indicated that all of the hop extracts analysed had a heterogeneous molecular weight distribution with peaks at discrete size ranges rather than forming a continuous size distribution. It should be noted that unlike other systems, such as aminocelluloses (Heinze et al., 2011), no simple single mathematical correlation was found between the sedimentation peaks using the power law relation ($s \propto M^b$) which would apply to species of different molecular weight of similar shape or conformation. In addition to the A-type branched and the B-type linear proanthocyanidins, Longo et al., (2018) have demonstrated the presence of novel cyclic B-type hexameric proanthocyanidins in red wine using Isotopic Exchange HPLC-HRMS/MS. The highest mass peak for Saaz was found during sedimentation velocity experiments at 4.4 S, which from the sedimentation equilibrium data in Figure 3.7 corresponded to a mass of around 90 kDa. In contrast, we have the presence of components at about 12 kDa, for a sedimentation peak at 2.6 S, suggesting the larger species are more compact structures. Magnum hop also revealed a small presence of polymers, around 50 kDa.

The possibility remained that high molecular weight compounds analysed using the AUC might be artefacts of smaller proanthocyanidin bound to other species such as proteins or lignins from the hop. To investigate this hypothesis, the protein content of the crude hop extract from aqueous acetone extraction was examined using a CB-X assay (G-Biosciences). This assay showed a low protein content of less than 1% of dry matter (0.5-0.6 g/L of protein for a total dry matter of 60 g/L). This concentration would not be enough to contribute to the high molecular weight peaks detected which represented about 30 to 35% in the Saaz hop sample (Table 3.7). Furthermore, a lignin standard material (Sigma-Aldrich lignin, alkali, low sulfonate) was exposed to the SPE clean-up procedure used to prepare the hop extract samples prior to analytical ultracentrifugation analysis. Virtually none of the lignin introduced was recovered after the procedure into the eluted fraction for analysis (using A_{280} for $\lambda = 280$ nm). It was then considered that lignin was unlikely to be a significant component of the polyphenolic hop extract fraction. However, only one type of lignin has been tested and replication using different types of lignin should be performed to confirm that lignin is not present into the hop extract. In addition, if binding between the PA and a heterogeneous polymer such as lignin was envisaged, it would be very unlikely that the sedimentation velocity profiles from 3 different samples of Saaz hop would have a much similar profiles as seen in Table 3.7. On the basis above and the selectivity of the extraction and fractionation procedures, it was acceptable to think that the high molecular weight compounds found in the hop extracts can be proanthocyanidins. It can be also noted that Tagashira et al., (1997) reported high molecular weight polyphenols in their hop bracts extracts (species not specified), might show between 36 and 40 kDa in molecular size.

3.2.4 Separation of the hop proanthocyanidin high molecular weight fraction

The preceding results showed the presence of polyphenolic compounds with a high molecular weight. Different separation strategies have been employed in order to separate the high molecular PA polymer fraction. From the AUC trials, it is estimated that hop extracts PA would be divided into two, or three component groups. Linforth et al., (2015) used spin-filters on the hop extract and have shown that the fining efficiency is spread across different fractions, some with a low molecular weight ($< 3\text{kDa}$) and an induced yeast flocculation found with the fraction for compounds higher than 100 kDa . In order to produce a more efficient and concentrated extract, another fractionation step could be employed during the manufacturing process in order to produce a more active finings material.

3.2.4.1 Ultrafiltration through molecular weight cut-off filtration (50 kDa)

Figure 3.8 shows the mean of the sediment depth and the resulting haze after 24 h of clarification for different fining treatments. All three extracts (retentate, Saaz (prepared using the 2.2.1 method section) and prototype industrial hop extract) were previously diluted to give the same final absorbance after a PA assay in order to introduce the same amount of PA in beer. Both control and the filtrate from the filtration using the 50 kDa cut-off filter showed similar results post-fining (average sediment depth of 4 mL , final haze 9 EBC). All three hop extracts treatments had a positive fining action relative to the reference control, reducing haze to between 2 and 3 EBC . The retentate from the ultrafiltration gave the best results in terms of clarity, with the ability to reduce haze to 2.1 EBC . Some differences are noticed in sediment depth for the same amount of PA introduced for the 3 extracts. However, there is no significant difference on haze between the industrial hop extract, the Saaz

hop extract and the retentate after filtration through a 50 kDa membrane on haze removal ($p > 0.05$, post-hoc Tukey HSD test). The filtrate displayed no activity, comparable results were obtained compared to the reference control.

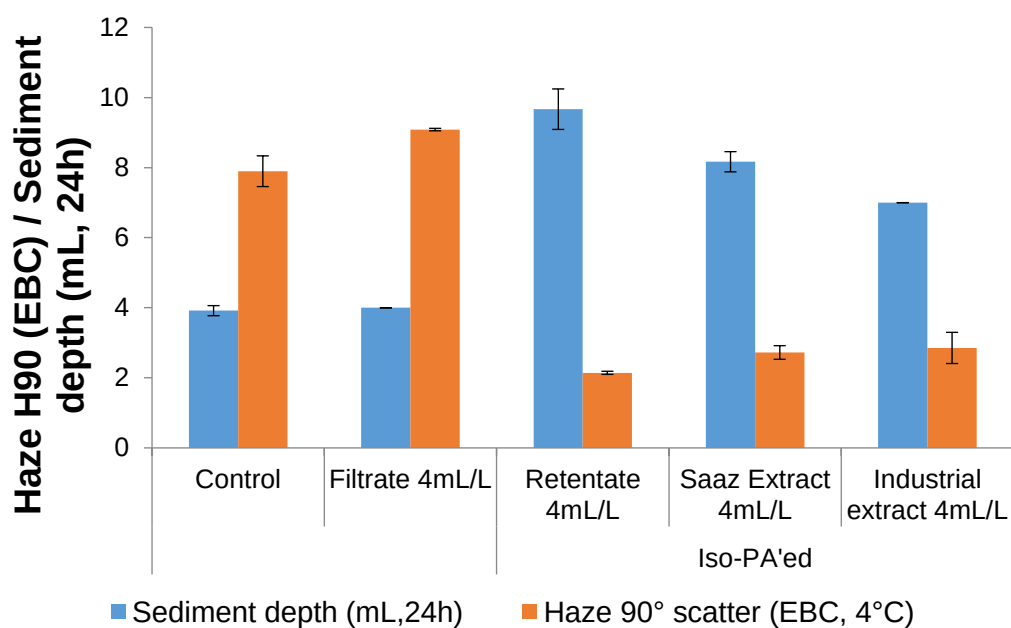


Figure 3.8 Sediment depth and haze H90 of a kit green beer fined with 3 different hop extracts with the same amount of PA introduced (Iso-PAed, from PA assay) at 4°C. Values are the mean of triplicates with their standard deviation.

3.2.4.2 Fractionation of the hop proanthocyanidins through high performance liquid chromatography (HPLC)

An extract of Saaz hop (prepared from section 2.2.1) after C18 SPE clean-up yielded 30 different fractions after HPLC fractionation using a cyano column. Of those fractions, 13 fractions contained activity and gave positive results in the PA assay (fractions 6 to 18). From those fraction samples, 7 different fractions has been passed through another C18 SPE in order to elute the active material in 100% methanol to facilitate the analysis using AUC in sedimentation velocity. Table 3.10 shows the

sedimentation velocity profiles for 7 different fractions from the HPLC fractionation of Saaz hop.

Table 3.10 Sedimentation velocity $c(s)$ profiles for C18 cleaned-up Saaz hop extract after HPLC fractionation using cyano column. Speed 45000 rpm, temperature 20.0°C.

	c(s) sedimentation coefficient values (S)				% Area[‡]			
	LMw	P1	P2	P3	%LMw	%P1	%P2	%P3
Fraction 6	0.51	1.73	2.58	-	53	39	8	-
Fraction 8	0.54	1.66	2.68	-	30	43	27	-
Fraction 10	0.54	1.85	3.05	-	27	44	29	-
Fraction 12	0.45	1.64	2.76	4.33	21	28	41	10
Fraction 14	0.44	1.71	3.05	4.27	17	23	46	14
Fraction 16	0.28	1.66	2.90	4.51	29	23	27	21
Fraction 18	0.36	1.52	2.87	4.71	40	32	23	5

[‡] Absolute peak areas were calculated using integration and then expressed as a percentage of total area of each sample to reflect the proportion of material present in each size range.

Fractions 6, 8 and 10 contained only three peaks in sedimentation velocity measurements. Amongst those three fractions, the proportion of low molecular weight compounds (%LMw) decreased with HPLC elution time. The presence of higher molecular weight compounds (%P2) increased for later eluting fractions. The 4th peak of high molecular weight polymers appeared between fractions 12 and 18. Fraction 16 contained the highest abundance of high molecular weight polymers (20% of P3 compounds). The wide molecular distribution of the hop extract induced a different polarity for each compound present in the hop extract. Cyano columns are defined to have a low capacity to accept H linkages, and no ability to give H linkages. The affinity between polyphenols and the CN material is weak. The main parameter which will define the separation here is the size of the molecules and steric hindrance in this experiment. Because of the large proportion of water present in the eluent phase, monomers and small dimers will easily be eluted in the first fractions

because of their high affinity with water. Longer polymers will have a different polarity compared to small molecules (Watson et al., 2013), and have a higher affinity to the column material.

As proanthocyanidins are mostly composed of aromatics, they can generate an affinity for large polymers to bind to the triple carbon bond of the CN column. However, because of the complexity of proanthocyanidins skeleton (branched and linear types), monomers and small oligomers can still be retained by the largest polymers within the column. This separation yielded fractions with different composition but has not been very performant in terms of size fractionation. Wilson, (1981) separated phenolic from apple juice using a cyano column. They also reported an easy elution and identification of small monomers such as catechin, epicatechin, chlorogenic acid, procyanidins dimers and other small phenolics in the first fractions, but also obtained a wide distribution of peaks identified as pentamers, hexamers and other polymers difficult to identify.

Two different methods have been employed to separate the high molecular weight fraction of proanthocyanidins from hop extract samples according to Linforth et al., (2015). Spin filtration experiments were not able to successfully improve the fining properties of the hop extract. Only a very small improvement in residual haze was reported using the retentate from a 50 kDa cut-off filter. This technique only allowed to concentrate the extract, removing water and potentially non-active component using the filtration. Other experiments have also found that the activity was spread across different molecular cut-off fractions, as revealed by Linforth et al., (2015). Activity was revealed below the 3 kDa cut-off as well as some were found up to 100 kDa.

Earlier research reported in a University of Nottingham MSc dissertation by Ren (2015) used the HPLC fractionation method described earlier with a Saaz hop extract (prepared using the same protocol detailed in 2.2.1) using cyano columns. It was observed that a set of 4 fractions (4 to 7) eluted answered positively to both gallic acid and the acid butanol hydrolysis assay, showing the presence of proanthocyanidins. They have also demonstrated the flocculating activity of those fractions, with the latest one having the highest yeast sediment formed after a quick beer fining bioassay. Fraction 5 had the highest absorbance observed at 280 nm (phenolics wavelength), but was not the most active in terms of beer clarification. In the experiment proposed earlier using the HPLC fractionation for the same hop extract variety, it was found that the maximum absorbance from the PA assay was found in fraction 10, which did not contain the additional 4th peak in sedimentation velocity mode. A maximum in absorbance does not effectively means that the fraction will contain the biggest size of molecules present. A quick bioassay using 10 mL of green beer was also performed to compare fractions in fining efficiency. Highest sediment was found equally for fractions 10 to 16. This result would imply that firstly both small oligomers and polymers are active during the fining reaction, but also a lower amount of polymers could induce a similar activity compared to higher amount of oligomers.

3.2.5 Variation in proanthocyanidin profile with hop harvest time

For berries, accumulation of proanthocyanidins occurs during the development of the fruit, and finishes when ripening starts (Bogs et al., 2005). If paralleled in hops, it would imply that the polymer development will happen when the flower starts to grow. This factor is also dependent on many factors such as geographic and climatic conditions, but also on the plant variety and vigour, and the horticultural practices.

Three different hop samples from the same variety were picked from the start of flowering until hop maturation, collected with time intervals (20 days, small, medium and big Nugget hop cone). A hop leaf sample was picked on the same day of the big hop cone collection. A last sample (brown hop cone) were collected after 30 days were left for hops to age and oxidise on the plant hedge. They were all dried straight after collection for 24h in a drying cabinet at around 60°C. Once dried, they were milled and the PA extracted following the standard extraction procedure (Section 2.2.1). In order to differentiate the PA polymer production, a dilution step before fining was developed in order to normalize all samples with their own proanthocyanidin concentration (from the acid butanol hydrolysis assay). All extracts from the five different samples gave the red-magenta colour typical of the presence of PA in an acid-butanol hydrolysis (Table 3.11).

Table 3.11 Absorbance reading from the acid butanol hydrolysis of Nugget hop cone and leaf samples at various growing stages. Values are the mean of triplicates with their standard deviation.

	Absorbance from PA Assay	Dilution factor for fining trial
Nugget small cone	1.26 ± 0.02	0.72
Nugget medium cone	2.13 ± 0.01	0.43
Nugget big cone	1.65 ± 0.01	0.55
Nugget brown cone	0.91 ± 0.05	1.00
Nugget leaf	1.01 ± 0.01	0.90

The highest concentration was found for the medium sized hop cone samples. Hop leaves also contained a significant amount of polyphenols. The lowest concentration was detected for the old brown form hop, where the plant is over-aged and actually degrading over time. The hop extracts were then tested for fining performance using a kit green beer. Haze and sediment depth after 24 h are shown in Figure 3.9.

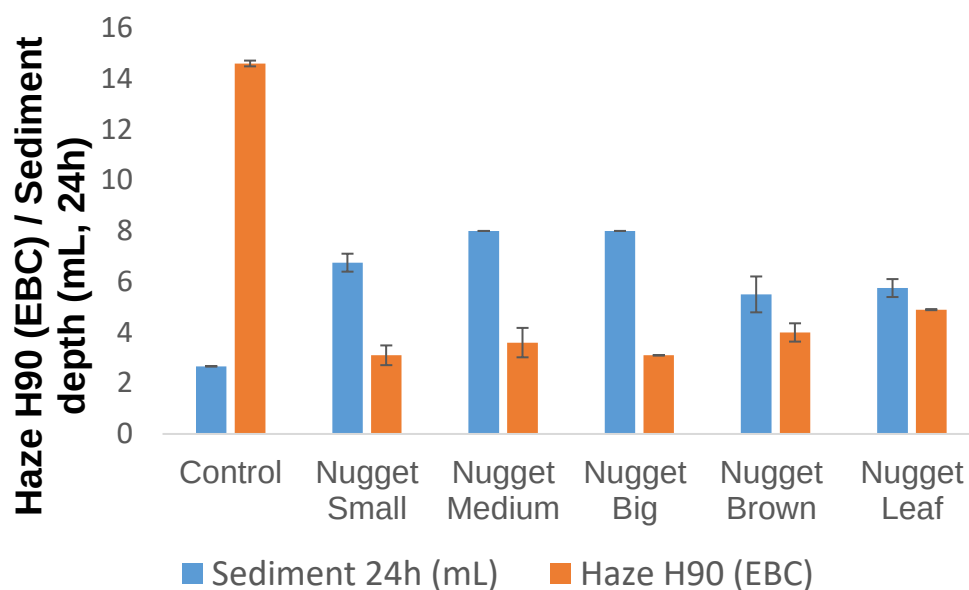


Figure 3.9 Sediment depth and haze H90 for a kit green beer (4°C) fined with Nugget hop extracts at various growing stages (4 mL/L). Data are the mean of triplicates with their standard deviation.

All five samples had a positive impact on beer clarification compared to the control beer. The highest sediment depth occurred with extracts of the medium and large size hop flowers. No statistical differences have been found in haze removal between small, medium, and big cone hop ($p > 0.1$, ANOVA). In addition, all extracts of hop flowers gave a better performance than the leaf PA sample. The beer fining assay is not able to provide any direct indication as to the size of the polymers present. Sedimentation velocity with AUC is necessary to determine if the plant will produce different polymer size ranges during the flowering cycle of the hop. Extracts were cleaned-up using C18 solid phase extraction, and the methanolic fractions was analysed through sedimentation velocity experiments using the same procedure as detailed during the hop variety screening. Table 3.12 reports the sedimentation velocity profiles of the 4 different Nugget hop cone extracts at different growing stages and the Nugget leaf extract.

Table 3.12 Sedimentation velocity *c(s)* profiles for Nugget hop extracts at different stages of the plant flowering. Speed 45000 rpm, temperature 20.0°C. Data are the mean of triplicates with their standard deviation

	c(s) sedimentation coefficient values (S)*				% Area [‡]			
	LMw	P1	P2	P3	LMw	P1	P2	P3
Nugget small	0.18 ± 0.01	1.51 ± 0.11	2.88 ± 0.09	N/A	56	26	18	N/A
Nugget medium	0.20 ± 0.04	1.73 ± 0.01	3.03 ± 0.05	N/A	52	33	15	N/A
Nugget big	0.17 ± 0.01	1.50 ± 0.09	2.53 ± 0.10	N/A	70	16	14	N/A
Nugget brown	0.19 ± 0.02	1.28 ± 0.02	2.94 ± 0.12	N/A	70	17	13	N/A
Nugget leaf	0.15 ± 0,01	1.04 ± 0,05	2.19 ± 0,11	3.83 ± 0.27	63	16	18	3,73

Sedimentation coefficient values ranged from 0.15 to 3.8 S. All the hop extracts from the hop flowers had 3 discrete sedimentation velocity peaks, whereas the leaf sample had an additional 4th peak at 3.8 S. This peak however represented a very small portion of the sample (approximately 4%). As found in sedimentation velocity experiments of hop cone extracts reported earlier in this chapter, the majority of the compounds analysed were low molecular weight compounds (LMw) between 0.15 and 0.20 S, representing between 52 and 70% of the sample composition. The proportion of high molecular weight polymers decreased for the big cone and the old-brown growing stage hop, with compounds represented by P1 and P2 decreasing in concentration, with a corresponding increase in low molecular weight compounds. Across the four cone samples, highest molecular weight compounds were found for the medium growth stage hop, with a peak at 3.0 S.

PA has been studied in berry development in wine studies to explain the change in astringency during wine ageing (Asproudi et al., 2015). They sampled berries every 15 days from before véraison until commercial maturity and determined the total proanthocyanidin content of each berry extract (using 60/40% acetone and water

extraction). In berry skins, they reported a high concentration prior to véraison which decreased during ripening. This trend has also been observed in red wine grapes by De Freitas et al., (2000) and also noticed for other cultivars (Downey et al., 2003). This trend has been also noticed in the Nugget hop picked samples in UK, with a decreasing proanthocyanidin concentration after the intermediate stage of development of the hop cone (Table 3.11).

Lafontaine et al., (2019) picked 5-6 weekly Cascade hop samples over three consecutive year of harvest. Humulones concentration did not change with the time of harvest, whereas total essential oil significantly increased over the three harvest years investigated. They also noticed that early harvested Cascade hops would be better as a bittering agent, and the later one best for dry-hopping or adding aroma in beer. The latest harvested samples attributed a more intense citrusy aroma and contained higher concentrations of free thiols, and terpene alcohols. Concerning polyphenols, Mikyška et al., (2012) studied polyphenol changes with time of storage. During the first two months of storage, a 5-15% reduction in total polyphenols was found. They reported a decrease of 30-40% in total polyphenol content and 20% decreased content of flavonoids for 12 months stored hops. It was also found in the present experiment that the brown old-aged hops contained a lower concentration of proanthocyanidins. The highest polyphenol concentration was found for the intermediate growth stage hop cone, and this sample also contained the highest sedimentation coefficient polymers amongst the 4 different hop cones samples. Polyphenols in hops vary with the time and year of harvest (Inui et al., 2017). A recent study by Zhou et al., (2019) also showed that different harvest time significantly changed the phenolic contents and physico-chemical parameters of Merlot and Pinot Noir red wines. Degradation processes are the main reason for PA

decrease. This decrease can be explained by the oxidation phenomena happening during ripening. This oxidation forms covalent bonds between quinones or other polyphenols and polysaccharidic or protein structures, making them more problematic to extract with organic solvents during ripening (Kennedy et al., 2000; Pourcel et al., 2005).

3.2.6 Proanthocyanidins from different botanical sources

Polyphenols are distributed among the plant kingdom and present in many vegetable sources such as fruits, nuts and berries (Hellström et al., 2009). They are an important part in the daily human diet, and are have a high antioxidant potential (Santos-Buelga et al., 2000). Cranberry extracts have been highly recommended for treating some medical conditions such as urinary tract infections. Hellström et al., (2009) have shown the diversity of plant sources containing proanthocyanidins. Citrus fruits did not contained proanthocyanidins. Processed products (apple cider or strawberry jams) contained less PA on average than the respective fruits and berry without any industrial processing. Smallest oligomers were dominant in raspberry, pear and kiwi fruit whereas highest concentrations and DP have been reported for blackcurrant or chokeberries. Ostroschi et al., (2018) found a significant concentration of proanthocyanidins in cinnamon extracts and tried to perform spray drying to manufacture more stable products. Grape seed extract has been recommended for its use as an antioxidant. Grape seed oils have been commercialized in the market as novel health foods because of the antioxidant activity provided by proanthocyanidins. (Nakamura et al., 2003). From the reference database made by Hellström et al., (2009) on proanthocyanidin sources, different polyphenol sources have been tested for PA content as well as fining efficiency to compare the hop extract with other polyphenolic sources across the plant kingdom.

3.2.6.1 PA concentration and fining efficiency

8 different extracts were produced from the routine extraction used for hops (section 2.2.1, 70% acetone_(aq.)). Figure 3.10 displayed the resulting absorbance from the acid butanol hydrolysis for 7 different polyphenolic sources and Saaz hop. Absorbance data shown in Figure 3.10 indicated a high variation in PA content between the different sources tested. Four sources (sorghum, grape seed, currant and cranberry) gave absorbance values (A_{550}) less than 0.2, indicating a low level of proanthocyanidins in the extract. On the other hand, 3 others sources (cinnamon, freeze-dried blackcurrant and aronia) showed a higher abundance of PA even compared to the Saaz hop. The blackcurrant sample had an absorbance at 550 nm of 2.75 which would indicate a high concentration of polyphenolic material within the extract. It is interesting to note that the cinnamon and Saaz extracts had a deep brown colour and that the blackcurrant and aronia extracts were an intense purple colour.

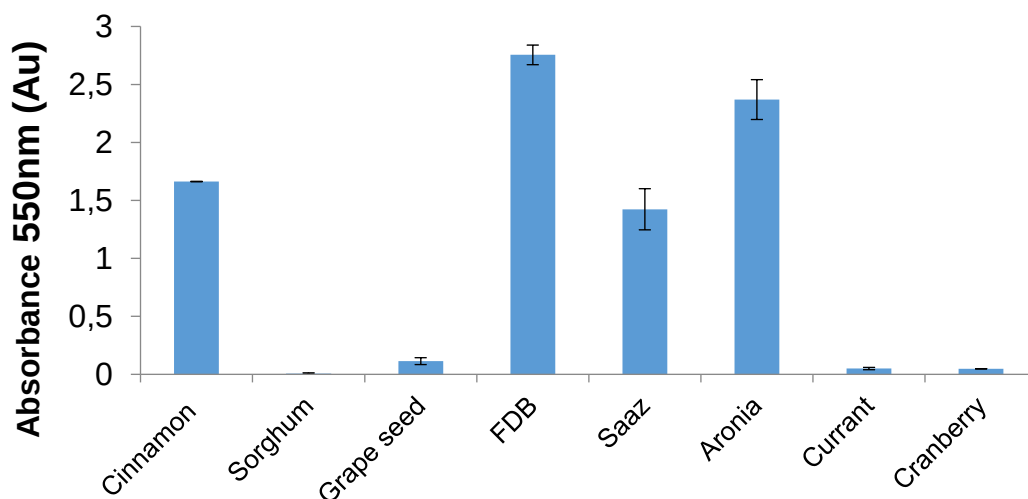


Figure 3.10 Absorbance readings from PA assay using different botanical sources of proanthocyanidins at 550 nm. Data is the mean of triplicate measurements $\pm$ standard deviation.

All botanical PA extracts were tested for fining performance in a kit green beer (after 7d of fermentation). They were introduced at a fixed dosage of 4mL/L. After 24 h of clarification, sediment depth and haze were recorded at 4°C. (Figure 3.11).

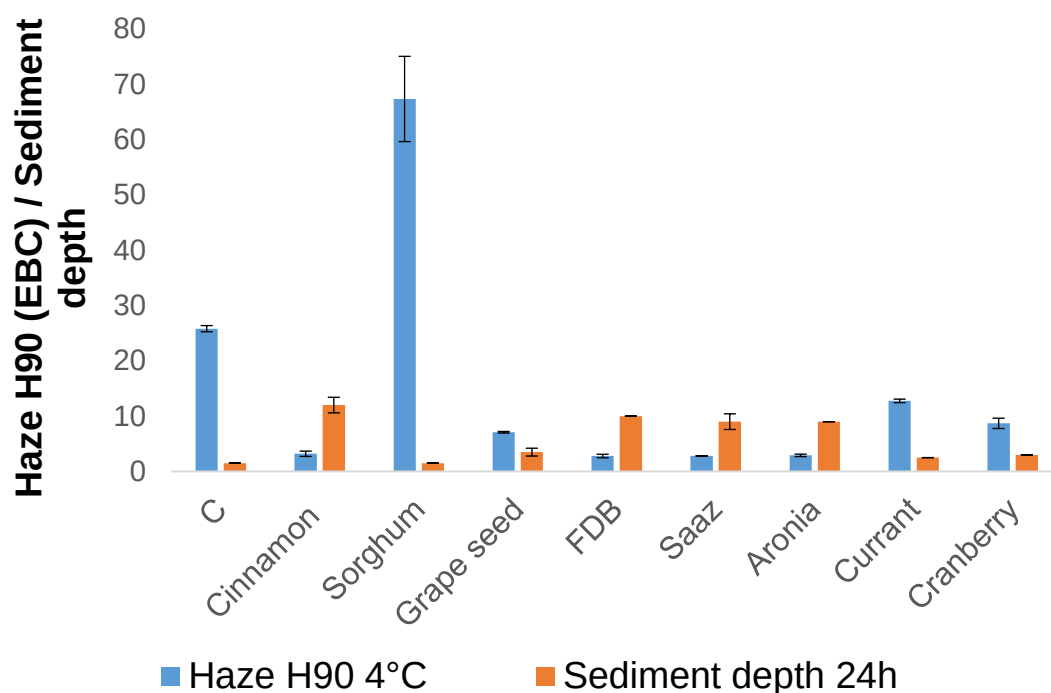


Figure 3.11 Sediment depth and haze H90 of a kit green beer fined with different botanical sources polyphenolic extracts at 4°C relative to an unfined reference control beer (C). Data is the mean of triplicates with their standard deviation.

The control beer after 24 h had a final haze of 26 EBC. All extracts except for sorghum had a positive effect on sediment formation and haze removal. Beers treated with polyphenolic extracts of sorghum had a much higher haze than the untreated sample (67 EBC). In relation with Figure 3.11, the low PA concentration samples (grape seed, currant, and cranberry) still showed a positive effect on beer clarification with final hazes between 7 and 13 EBC. Accordingly, the highest concentration of PA found during acid-butanol assay are giving best results in fining performance. Cinnamon, freeze-dried blackcurrant, Saaz hop and Aronia berry are clarifying the beer to a haze value between 2 and 3 EBC. This is a good indication that PA from any botanical sources would be able to perform beer clarification. It is

also important to notice that Saaz hop and freeze-dried blackcurrant have the same final haze value, even though freeze-dried blackcurrant extract had double the absorbance in the PA assay. All studied samples have been evaluated subtracting the first absorbance revealed at 550 nm prior to reaction. It is possible that other phenolics may have interfere the signal during the PA assay due to the close colour of the final red-magenta product issued from the acid butanol hydrolysis assay and the original colour of the blackcurrant extract. This result may indicate that different botanical sources can have different shape or size of proanthocyanidins.

3.2.6.2 Molecular profiling of different proanthocyanidin sources through AUC

Sedimentation velocity experiments were performed on 5 different botanical phenolic extracts after C18 fractionation, in methanol at 45000 rpm. Three high performance fining sources (Cinnamon, Aronia, and freeze-dried blackcurrant) were selected alongside a medium performance extract, (grape seed) and sorghum extract which increased beer haze post fining.

The $c(s)$ profile for cinnamon extract displayed 3 distinct peaks with an additional ‘tail’ of high sedimentation coefficient compounds between 4 and 8 S (Figure 3.12).

Average sedimentation coefficients peaks were measured at 0.44, 1.68 and 2.98 S. The majority of compounds present were represented by the second peak, with a relative concentration of 40% of the total sample. Interestingly, only 20% of the total extract was represented by the first peak (representing LMw compounds).

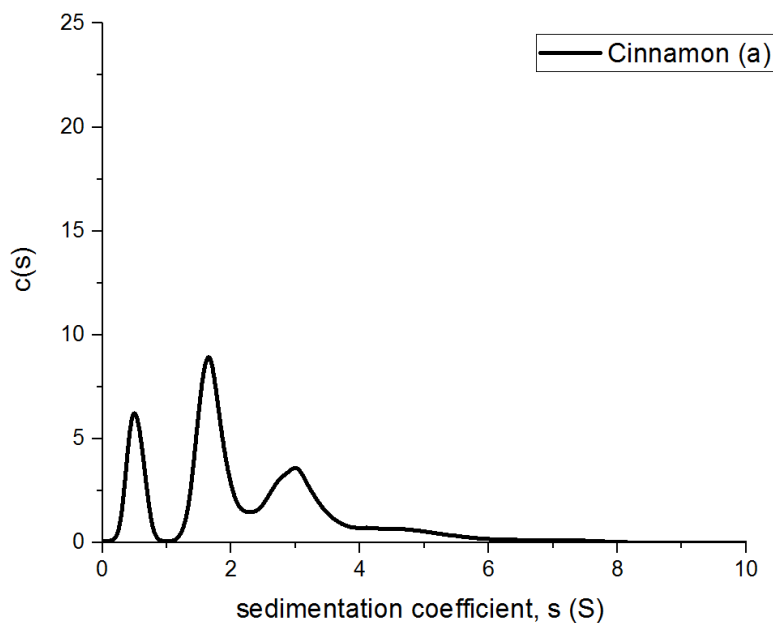


Figure 3.12 Sedimentation velocity $c(s)$ profile for cinnamon extract (a) (in methanol, 6-fold dilution) after C18 fractionation. Centrifugation velocity 45000 rpm, temperature 20.0°C.

The highest molecular weight compounds (between 4 and 8 S) represented on average 14% of the total sample. Highest molecular weights compounds in hops have been found at 4.4 S representing approximately 6% of the sample. However, the compounds present in the cinnamon are more dispersed in sedimentation coefficient. Saaz had a more distinct sedimentation velocity profile compared to aronia berry. Aronia displayed a wider distribution of compounds, with compounds with higher sedimentation coefficient compared to Saaz. The two first peaks found for Saaz were recorded at 0.15 and 1.1 S whereas higher sedimentation coefficient (0.44 and 1.68 S) are found for those peaks in Aronia.

Aronia berry displayed a set of 3 distinct peaks with an additional presence of compounds between 3.8 and 9.5 S (Figure 3.13).

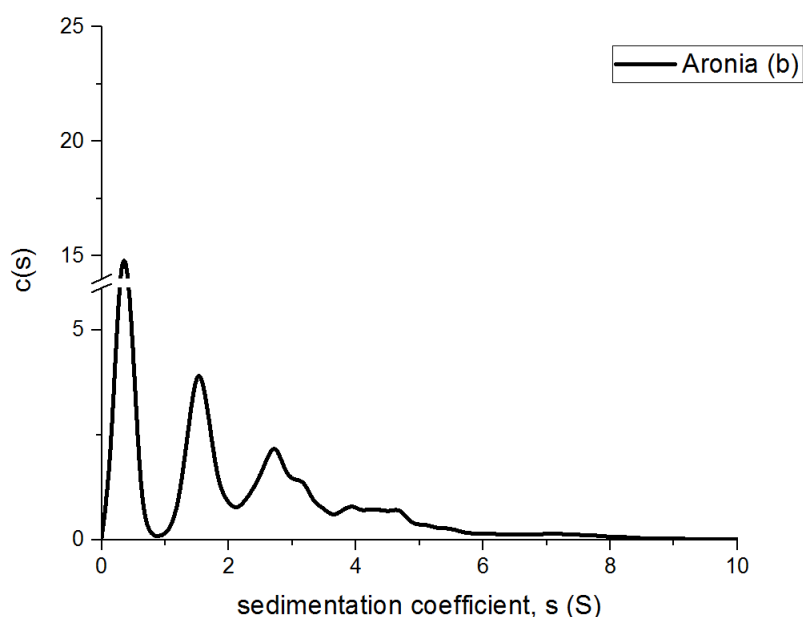


Figure 3.13 Sedimentation velocity $c(s)$ profile for Aronia berry extract (b) (in methanol, 6-fold dilution) after C18 fractionnation. Centrifugation velocity 45000 rpm, temperature 20.0°C.

The three peaks showed on average sedimentation coefficient recorded at 0.36, 1.58 and 2.87 S. The first peak represented by low molecular weight compounds showed a high intensity, representing 42% of the total concentration, and about 20% for each of the two other main peaks. Between 4 and 9.5 S, there is no clear peak represented as was the case in Saaz hop extracts. The distribution is wide and extended up to a maximum of 9.5 S which was not exceeded by any other botanical extract investigated here.

Figure 3.14 displayed the sedimentation velocity profile for freeze dried blackcurrant extract.

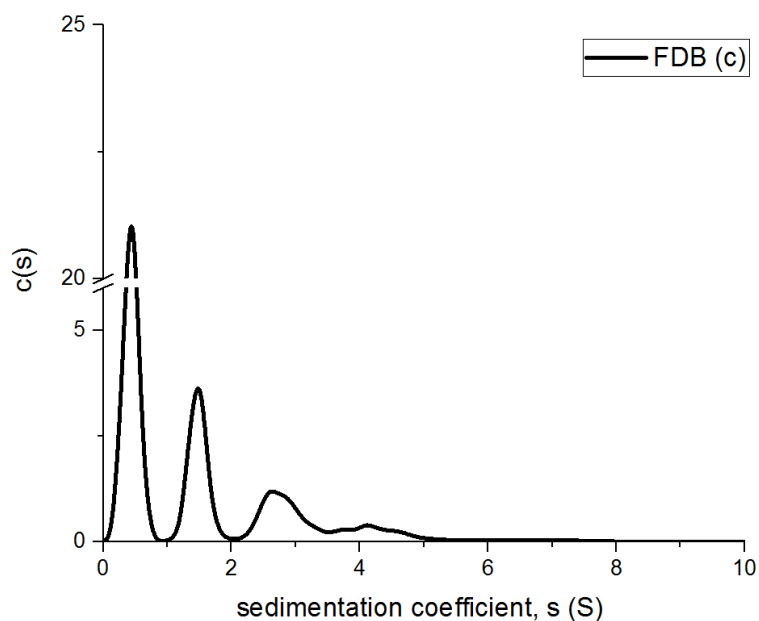


Figure 3.14 Sedimentation velocity $c(s)$ profile for freeze dried blackcurrant extract (c) (in methanol, 6-fold dilution) after C18 fractionation. Centrifugation velocity 45000 rpm, temperature 20.0°C.

This extract showed 4 distinct peaks with their respective sedimentation coefficient associated (0.44, 1.47, 2.86 and 4.49 S). As found in aronia berry, a very high intensity peak was present for low molecular weight compounds, containing 61% of the total material in the extract. A decreased proportion was found at the other higher molecular weight peaks (19% P1, 13% P2 and 7% P3 respectively). The highest sedimentation coefficient species in this extract were at up to 5.5 S.

Figure 3.15 shows the sedimentation velocity profile of grape seed extract. This extract was only diluted 2-fold prior to AUC analysis because of its low proanthocyanidin content. Although the concentration of this extract was very low, the profile is indicated 4 distinct peaks, much as seen in the Saaz hop extracts. Peaks were recorded at 0.17 S (63%), 0.93 S (15%), 1.82 S (18%) and 4.0 S (4%).

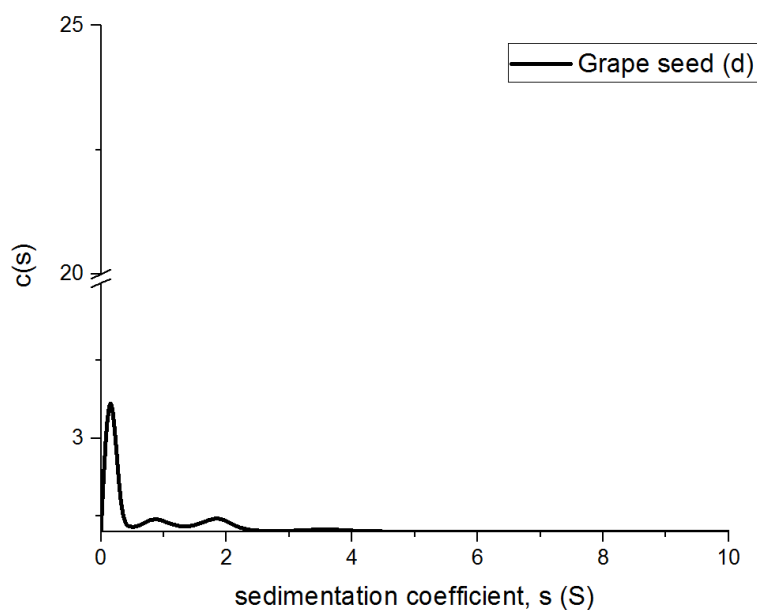


Figure 3.15 Sedimentation velocity $c(s)$ profile for grape seed extract (d) (in methanol, 2-fold dilution) after C18 fractionation. Centrifugation velocity 45000 rpm, temperature 20.0°C.

The sedimentation velocity profile of white sorghum extract is shown in Figure 3.16. This extract was analysed without any dilution as it contained a very low concentration of PA based on the acid butanol hydrolysis assay. The sedimentation velocity profile displayed three distinct peaks at 0.19 S (78%), 1.34 S (18%), 3.09 S (4%).

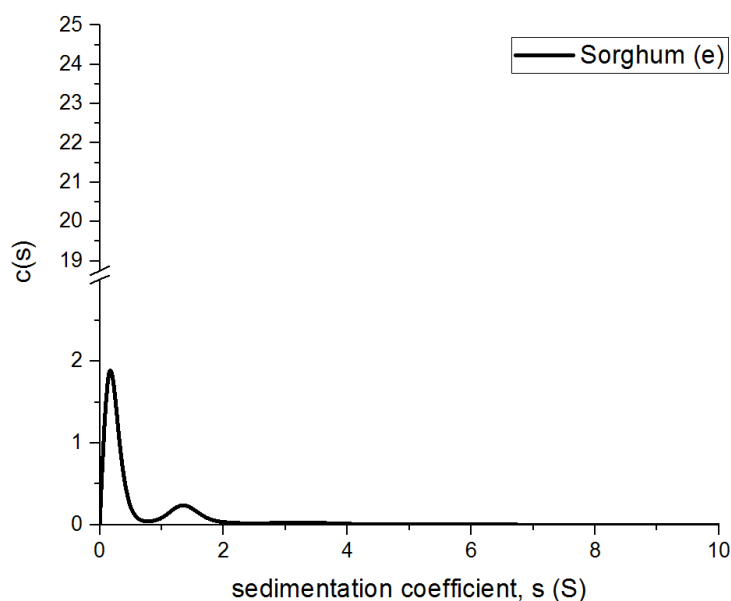


Figure 3.16 Sedimentation velocity $c(s)$ profile of white sorghum extract (e) (in methanol, no dilution) after C18 fractionation. Centrifugation velocity 45000 rpm, temperature 20.0°C.

It was then proposed to study two extracts of interest through longer (4-5 day) sedimentation equilibrium studies to obtain their molecular weight distribution. Because of their positive action in beer fining and their abundance of proanthocyanidins, cinnamon and aronia berry extracts were selected for the sedimentation equilibrium studies (using the same procedure detailed in section 3.2.3.3). Results for cinnamon extracts showed the presence of three distinct peaks, between 0 and 125 kDa (Figure 3.17).

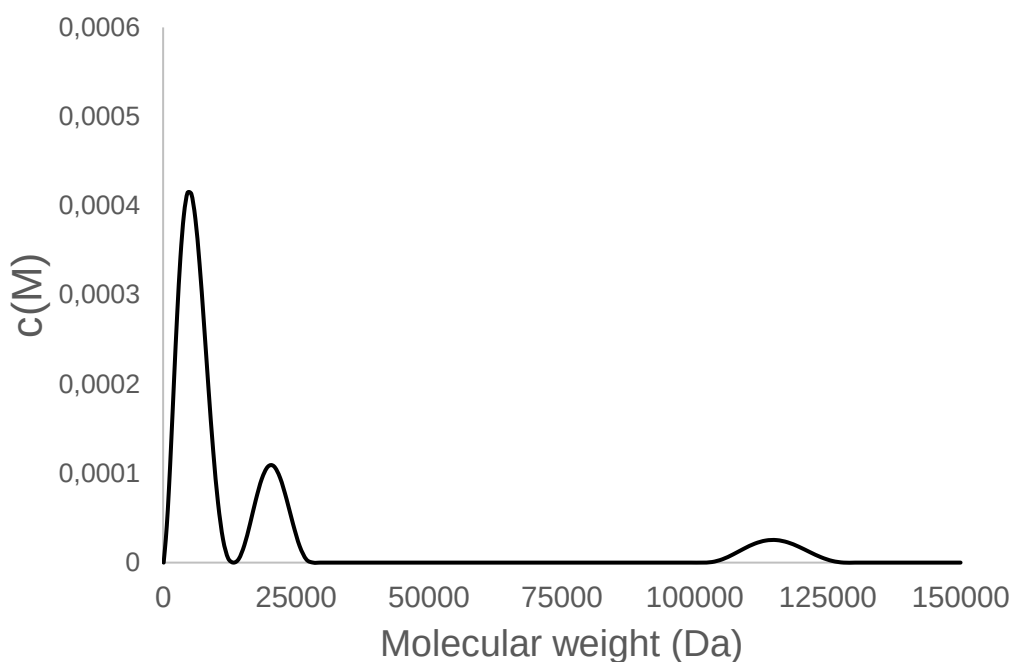


Figure 3.17 SEDFIT-MSTAR analysis of sedimentation equilibrium data, showing the $c(M)$ molecular weight distribution of cinnamon polyphenolic extracts (40-fold diluted, in methanol). Centrifuged to equilibrium at ascending speeds between 20000 and 40000 rpm at 20°C.

The first peak is composed of compounds with a molecular weight average of 5500 Da (70% of the total area). The second group is composed of polymers with an average of 20 kDa (21% of the total concentration) and the last group consisted of high molecular weight compounds and accounted for 9% of the total area (115 kDa).

The sedimentation equilibrium study revealed the molecular weight distribution of aronia berry extract as shown in Figure 3.18.

The aronia berry extract also showed three distinct molecular weight peaks. The first group of compounds with an average molecular weight of 3100 Da (62% of the total area). About 12% of the total sample is composed of molecules with an average molecular weight of 12500 Da. The last peak is corresponding to 26% of the total area, with a molecular weight of 85 kDa.

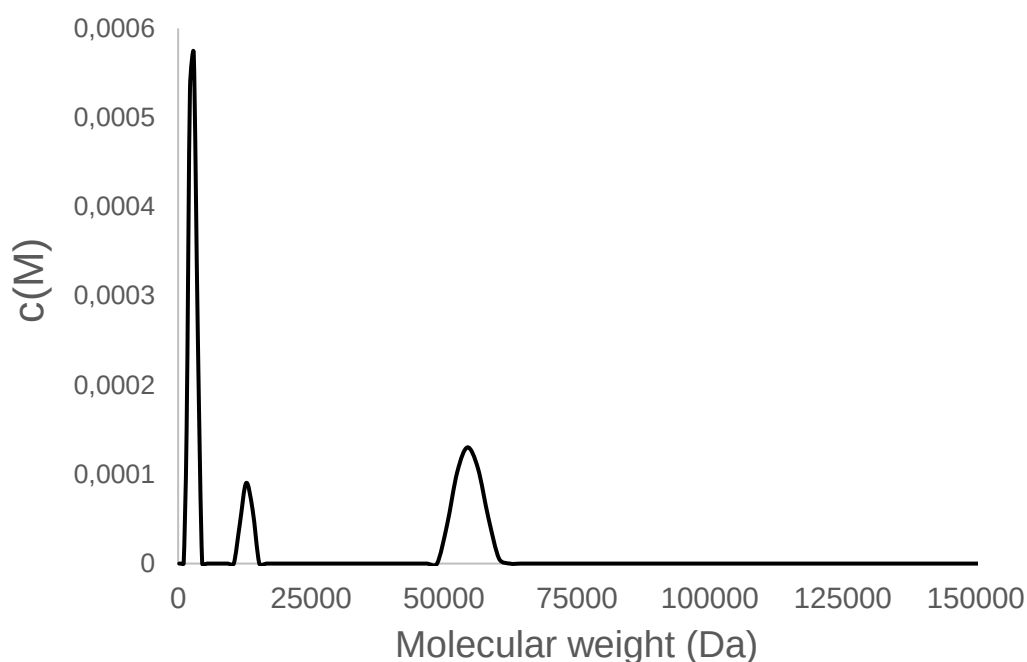


Figure 3.18 SEDFIT-MSTAR analysis of sedimentation equilibrium data, showing the $c(M)$ molecular weight distribution of aronia berry polyphenolic extracts (40-fold diluted, in methanol). Centrifuged to equilibrium at ascending speeds between 20000 and 40000 rpm at 20°C.

Proanthocyanidins are an abundant but complex class of polyphenols, found in foods and botanicals (Neilson et al., 2016). They can contain a wide variety of linkages and subunits. They can be present from dimers and trimers, found in some fruits (Hellström et al., 2009), to high molecular weight polymers (Santos-Buelga et al., 2000; Tanaka et al., 2014). Novel analytical methodologies have to be developed in order to improve the knowledge of those complex compounds. Eight different botanical sources of PA have been analysed for their respective concentrations of proanthocyanidins. Some samples contained a high concentrations of PA, in agreement with previous reports (Hellström et al., 2009). Dried fruits such as cranberry or currants displayed a small PA concentration, however, processed products such as freeze-dried blackcurrant showed much higher concentrations of PA. This is probably due to the manufacturing process, removing part of the non-

polyphenolic fraction in the respective processed fruit or berry. High concentrations of PA have been correlated respectively with a positive beer fining activity. Comparable results have been seen between Saaz hop, Aronia, cinnamon and freeze-dried blackcurrant extracts for beer haze removal. From the AUC profile of Saaz and the high concentrations of PA found in other extracts, it was shown that high molecular weight polymers can be also found in other botanical sources.

Five sedimentation velocity profiles have been reported (Figure 3.12 to Figure 3.16). A lower dilution factor was used for the low fining activity extracts (grape seed and sorghum) in order to obtain a cleaner resolution due to their low PA content. All extracts except cinnamon were mainly composed of low molecular weight units, with the lowest sedimentation coefficient peaks accounting for between 40 and 70% of the global content of the samples. Compared to sedimentation velocity experiments with hops, the presence of compounds with even higher sedimentation coefficients than for Saaz hops variety (P3 at 4.4 S) was identified. Presence of material has been observed between 4 and 8 S for aronia, freeze dried blackcurrant and cinnamon extracts. Hellström et al., (2009) have found high mean degree of polymerisation for chokeberries (aronia) and blackcurrant. The present sedimentation velocity experiments also demonstrate the presence of high sedimentation coefficient compounds. These compounds have been found for some other botanical sources (aronia, cinnamon and blackcurrant) and have higher coefficient compared to any hops. Aronia contained species up to 9.5 S which would correspond to a high mean degree of polymerisation found for this berry. Chemical degradation of sorghum grain done by Gupta et al., (1978) showed that it displayed a molecular weight between 1700 and 2000 Da. Another sample of sorghum tannins have been analysed by GPC by Brandon et al., (1980) and have determined that the

proanthocyanidin polymer was consisting in the main of 2,3-cis procyanidin units with an average molecular weight of 2500 Da. Those results found in the literature are in accordance with the sedimentation velocity profiles found for sorghum, as the majority has been found for low molecular weight compounds, with a very small presence of high molecular weights compounds.

Sedimentation equilibrium studies have also been applied for two high fining performance extracts, with a high respective PA concentration. Both extracts showed three distinct molecular weight bands, with a molecular weight range similar to Saaz hop. Although higher sedimentation coefficients were found for aronia berry extract, the highest molecular weight band was found for the cinnamon extract. This may indicate a different shape of the polymer between those two extracts. It is known that proanthocyanidins can be formed in different shapes and lengths (Maria Monagas et al., 2009; Li et al., 2008; Tanaka et al., 2014). A-type proanthocyanidins are described as branched polymers whereas B-type proanthocyanidins have a linear structure. Also it has been recently discovered that crown proanthocyanidin are present in wine (Longo et al., 2018). This potentially indicates that the high molecular weight peaks from sedimentation equilibrium studies differentiating Saaz, cinnamon and aronia berry extracts, would have a different distinct shapes. Currently, the proanthocyanidin research is somewhat hindered by the limitations of existing analytical methods. Novel assays have to be developed in order to advance the understanding of how proanthocyanidins are structured in each botanical source, and how they impact on the biological activity (Neilson et al., 2016). Methods are lacking for PA with DP > 2-3, with high complexity to define them because of the lack of standards, or precise methods to define their skeleton structure (Kelm et al., 2005).

3.3 Conclusions

This chapter has been dedicated to the characterisation of the novel hop extract finings using different analytical techniques. As shown in the previous section the active compound belongs to the proanthocyanidin class of compounds. Better known as condensed tannins, they are mainly described by their degree of polymerization (DP), due to their presence from small oligomers to large polymers (Gu et al., 2004).

Differences in proanthocyanidin content have been noticed for different hop varieties as well as their activity in beer fining. Saaz hop variety showed the highest concentration of PA as well as best results in fining efficiency compared to all other hops. However, problematic variety like Magnum resulted in a poor flocculation. This may indicate that although concentration of PA can be different, the difference in size distribution can also impact fining efficiency. Flocculation mechanism is mainly depending on the size of the reagents and it is possible that different varieties produce different size distribution of proanthocyanidins. However, there is no clear method to define the molecular distribution of proanthocyanidin extracts. Current analytical strategies involves the degradation of the polymers, which yields an average degree of polymerisation through the addition of phloroglucinol or benzyl mercaptan. The phloroglucinolysis reaction used in the thesis showed that all hop varieties presented an average degree of polymerisation suggesting the presence of rather large molecules but without any indication of the molecular size distribution. Saaz hop has shown the highest degree of polymerisation in this experiment as well as the lowest number has been observed for Magnum hop variety. This follows previous results found concerning PA concentration as well as fining experiments. However, this experiment is still limiting the understanding of the polymer distribution of extracts as the only data obtained is an average degree of

polymerisation. More information is needed to better understand the size of the polymer present and to confirm differences in fining efficiency for different hop varieties

A significant disadvantage of using chromatographic techniques in proanthocyanidin analysis is the lack of polymer reference standards in a high purity form. Progress has been made in the preparation of synthetic proanthocyanidins (Makabe, 2013), with Ohmori et al., (2011) reporting the assembly of catechin monomers to yield a 24-mer. However, this is currently the upward size-limit achieved through synthesis.

A novel analytical method had to be developed to fully characterise these high molecular weight polymers. Analytical ultracentrifugation has not previously been applied to the characterisation of proanthocyanidin polymers. This study thus represents a significant step forwards in developing methods for the analysis and characterisation of proanthocyanidins and may ultimately lead to an improved understanding of proanthocyanidin structures and molecular size distributions across the plant kingdom. Fractionation steps have to be previously performed in order to only elute the active material. The C18 cartridges have been showing an efficient elution, recovering up to 90% of the active material, and was chosen for further analysis with the AUC. Experiments using different sources of the same variety proved the reproducibility of the technique, and the evidence of different sedimentation coefficient peaks, proving the heterogeneity of proanthocyanidin extracts (Monagas et al., 2010). Best results in fining efficiency have been accorded to Saaz hop variety, which displayed highest sedimentation coefficient compounds from the AUC analysis. Fining efficiency would then be dependent on the size of the polymer introduced. Best results would be obtained for an extract containing a low

concentration of monomers and small oligomers, and with a high concentration of high molecular weight polymers.

Confirmed using the sedimentation velocity analysis, molecular distribution of proanthocyanidins would be different for each hop variety, as some would be more able to produce high molecular weights compounds during their flowering process. AUC technique is usually applied to the characterisation of purified macromolecules or simple polymer mixtures (Harding et al., 2010) and the present study represents the first attempt to apply this technique to analyse complex mixtures of polyphenolics such as are present in fractions extracted from plant materials, taking advantage of recent advances in analysis software such as SEDFIT-MSTAR. This technique has been able to detect presence of high molecular weight compounds with an upwards size limit in the region of 100 kDa for Saaz (or 56 kDa for Magnum hop). High molecular weight compounds have also been detected for other polyphenolic sources such as aronia berry or cinnamon extracts. However, no clear data has been produced concerning the shape of the actual PA studied. It is known that PA are present in different structures and shapes and it is likely that shape also varied depending on the source or the cultivar of the polyphenolic botanical source studied. It is however still unsure that the high molecular weight polymers shown in sedimentation equilibrium are with confidence proanthocyanidins. Working by elimination and scientific assumptions showed that proteins are not present into the C18 fractions. A type of lignin has also been tested through this fractionation step and indicated that the eluted fraction was not composed of lignin. However, it is very difficult to fully affirm that the hop extract fractions are only composed of proanthocyanidins. Limitation in chemical analysis as well as compound references inhibits the full characterisation of this extract. However, this novel method using

analytical ultracentrifugation has been able to show high molecular weight polymers, and a direct link between polymer size and fining efficiency.

In conclusion, analytical ultracentrifugation has been a suitable technique for the non-destructive analysis of polyphenolic extracts, and more precisely of high molecular weight proanthocyanidins. As these molecules are also of considerable interest for their beneficial health aspects (Cos et al., 2012), this analytical technique could have cross-disciplinary application in the characterisation of proanthocyanidins of different processed products or botanical sources. Novel methodologies and reference compounds have to be developed in order to fully understand and characterise proanthocyanidin from plant and food products.

Chapter 4: Impacts of the hop extract addition on finished beer

Proanthocyanidins, in addition to binding to proteins also have antioxidant properties (Masquelier, 1987; Rauf et al., 2019), and are metal ion chelators (Wu et al., 2010). Moreover, hop polyphenols have also shown beneficial impacts in terms of beer flavour stability and beer freshness (Mikyška et al., 2002). Early work by Linforth et al., (2015) has shown that the spent hop extract can act efficiently as a yeast flocculator and a haze removal agent. However, there is a need to understand what other impacts the addition of the hop extract can have on the finished beer after clarification. As beer is stored, it ages, and forms in its matrix staling compounds which can be responsible for some off-flavours (Vanderhaegen et al., 2006). In this chapter the impact of the hop extract and current fining strategies on the volatile flavour stability of finished beer is investigated through an accelerated forced ageing trial. The forced ageing generates staling compounds in beer and these are analysed by Gas Chromatography Mass Spectrometry to determine if the hop extract changes the volatile profile of beer after storage compared to other fining strategies.

Changes in beer flavour stability through storage can be observed due to beer oxygenation. Oxygen radical species can be generated through storage by chemical reaction, which negatively impact the beer stability (Uchida et al., 2000). This reaction can be also enhanced catalytically with the presence of metal ions such as iron or manganese (Jenkins et al., 2018). As an opposite, the presence of a metal chelator have also been proven to decrease the formation of beer radicals (Uchida et al., 1996). Uchida et al., (1996) demonstrated that this radical formation had a lag time. Different lag times for the same beer brand has been well correlated through

sensory analysis to the beer flavour deterioration on the shelf. The determination of this lag time can be performed using electron spin resonance spectroscopy (ESR), where beer is forced aged to produce radicals which can then be detected through a spin trap. This technique have become a powerful tool for brewers to evaluate the endogenous antioxidant activity of beer. The T150 value (indicator of how much staling can occur in a particular beer after 150 min of assay) has also been an important indicator of product shelf-life. Beer flavour stability as well as metal ion concentration will be determined for beers treated with the hop extract. As proanthocyanidins are well known to chelate metal ions, a decreased concentration of iron metals will be observed using hop extract finings into lager beer. A reduction in the T150 value have also been found for beers treated with the hop extract compared to other finings strategies.

4.1 Materials and Methods

Chemicals

2-methyl butanal, 2-methyl propanal, 3-methyl butanal, furfural, trans-2-nonenal, benzaldehyde, 3-heptanone and N-tert-Butyl- α -phenylnitrone were supplied by Sigma-Aldrich in analytical grade (Dorset, UK).

Isoamyl acetate, ethyl isovalerate, hexanal, and γ -nonalactone were supplied from Fisher Scientific in analytical grade (Loughborough, UK). Nitric acid (TraceMetal™ grade) and HPLC grade ethanol were also supplied from Fisher Scientific.

4.1.1 Beer volatile profile through force-aged trial using hop extract finings

Preparation of beer and forced ageing trial

A high-gravity lager beer (23 L) was produced using the Grainfather all grain method (Section 2.1.2.1, brewing of beer for fining trials). After fermentation, the beer was

split into four aliquots of 5 L each and chilled overnight at 4°C. Each aliquot received a different fining treatment (Saaz laboratory hop extract produced from section 2.2.1, 4 mL/L; commercial hop extract, 4 mL/L; isinglass solution, 5 mL/L, Section 2.1.1, Materials) as well as a control reference with no fining for 24 h at 4°C. For each aliquot, green beer (4.5 L) was then taken out after clarification using a siphon tube and centrifuged at 500 rpm for 15 min. The residual yeast was then discarded and the beer was poured into 500 mL brown opaque PET bottles, 8 bottles of each fining treatment, making a total of 32 bottles. In order to simulate ageing through shelf-life and generate measurable differences in staling compound concentrations, beer samples were subjected to an accelerated force ageing trial. Two beers of each fining treatment were stored at each of four different temperatures (4°C, 30°C, 40°C and 50°C) for one week using Sanyo MIR-153 incubators (Japan).

Analysis of beer volatiles using headspace solid-phase micro-extraction (SPME) and GC-MS

Volatiles analysis was performed using a Thermo Scientific Trace 1300 GC system equipped with a TriPlus SPME autosampler coupled to a Thermo Scientific ISQ QD single quadrupole mass spectrometer. Samples for SPME analysis were prepared in 20 mL brown glass vials with crimp caps and a PTFE/Silicon septum (Supelco, UK). For all beer samples, 10 g of beer was weighed into a vial and 100 µL of a 10 mg/L stock solution of 3-heptanone (internal standard) was added.

The fibre used for the analysis was 50/30 µm DVB/PDMS (Supelco, UK). The compounds were separated through a ZB-WAX column (30 m x 0.25 mm x 0.25 µm film thickness) from Phenomenex, UK. The temperature program was set to start at an initial temperature of 40°C for 2 min, then increased by 8°C/min to reach the final temperature of 240°C which was held for 1 min. The incubation time for the fibre

was set on 10 min, the desorption time was 1 min and the agitator unit from the GC system was held at 35°C. All samples were analysed in a randomized order with blank samples between each four sample runs.

Mass spectra were obtained in electron impact ionization mode with the ion source set up at 200°C and a measurement dwell time of 0.1 s. 10 compounds were selected according to their presence in the beer volatile profile with their ability to give flavour and/or produce off-flavours. (Olaniran et al., 2017; Saison et al., 2010). The detector was operated in selected ion mode (between m/z 58 and 106, Table 4.1). Calibration was obtained with authentic standard compounds introduced in pure methanol then diluted to selected concentrations with water. All references selected compounds were introduced at different concentrations (0.1, 1, 10, 100 µg/L) into aqueous solutions (5 mL of water) to form a calibration series. The peak areas were normalized against the internal standard, and standard calibration curves then used to obtain beer staling compound concentrations. Calculations and calibration were made using Xcalibur software. (Thermo Fisher, UK). Statistical analysis was performed using XLSTAT Student 2019 version (Addinsoft Inc, U.S.A.).

Table 4.1 Selected staling compounds indicators of beer ageing, retention time and the ions used to monitor the compounds by GC-MS

	RT (min)	Selected ions
<i>2methylpropanal</i>	3.06	41, 43, 72
<i>2-me-butanal</i>	4.67	41, 57, 58
<i>3-me-butanal</i>	4.76	41, 44, 57, 58, 71
<i>Ethyl isovalerate</i>	7.76	57, 85, 88
<i>hexanal</i>	8.16	56, 57, 82
<i>Isoamyl acetate</i>	8.97	43, 70, 87
<i>furfural</i>	16.13	95, 96
<i>E-2-nonenal</i>	17.3	70, 83, 122
<i>benzaldehyde</i>	17.4	77, 105, 106
<i>gamma-nonalactone</i>	25.37	85

All compounds displayed in Table 4.1 showed good linearity of response ($R^2 > 0.98$) across the concentrations studied (0.1 to 100 µg/L).

4.1.2 Metal ion concentrations in lager beer treated with hop extract finings

Metal ion analysis using inductively coupled plasma mass spectrometry (ICP-MS)

Metal ion concentration analysis in beer was performed using a Thermo-Fisher Scientific iCAP-Q equipped with CCTED (collision cell technology with energy discrimination). All beers samples (10 mL) were mixed with 200 µL of metal free HNO₃ and were filtered through 0.2 µm cellulose syringe filters. Multi-element analysis was undertaken using ICP- MS (Thermo-Fisher iCAP-Q) with a Flatopole collision cell (typically charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. External calibration standards were in the range 0 – 100 µg/L (ppb) and in the preferred matrix of 2% HNO₃ (Trace Metal grade). Samples were introduced via a covered autosampler (Cetac ASX-520) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific, UK). Sample processing was undertaken using Qtegra software (Thermo-Fisher Scientific, UK). Results are reported as gravimetric concentrations (µg/L or mg/L). Statistical analysis was performed using XLSTAT Student 2019 version (Addinsoft Inc. U.S.A.).

4.1.3 Beer flavour stability measurements for lager beers using hop extract finings

Electron spin resonance (ESR) spectroscopy measurements

452 mg N-tert-Butyl- α -phenylnitron (PBN) was dissolved first in 500 µL of ethanol and 500 µL of water. 280 µL of PBN solution was then added to 7 g of beer sample. Samples were placed in a 60°C heating block for incubation and aliquots were taken and injected into the ESR equipment periodically. ESR spectra were recorded with

a centre field of 3478 G and sweep width of 17 G. The microwave bridge had a power of 2.31 mW and frequency of 9.77. Receiver gain was 1261, modulation frequency 86 kHz, modulation amplitude 1.1 G, modulation phase 0.85°, time constant 20.48 ms. Scans were aggregated and the peak to peak height of the first derivative of the ESR spectra was recorded as the intensity value at a given time-point. Assays were run for 150 min with 15 samples taken during this time and samples were taken using an autosampler (Bruker, Billerica, U.S.A.). Statistical analysis was performed using XLSTAT Student 2019 version (Addinsoft Inc. U.S.A.).

4.2 Results and Discussion

4.2.1 Beer volatile profile through force-aged trial using hop extract finings

During beer storage, the fresh flavour profile is changing with the formation of off-flavour notes such as cardboard, solvent, or caramel (Saison et al., 2010b). We aimed to measure the development of beer staling volatile compounds according to time of storage using different fining strategies, to see if the novel hop finings has the potential to influence beer flavour stability. Force ageing was used in this study to mimic longer times of storage. Li et al., (2015) determined that 1 day of storage at 50°C was equivalent to a staling flavour development of 4 weeks at room temperature. A set of temperature (from 4 to 50°C) was selected for force ageing storage, with a fixed week period for storage time, to correlate to shelf storage from a week to approximately 6 months (50°C, 1 week equivalent). Table 4.2 summarises the average concentration of each staling compound found for each beer treated with different fining strategies and at each of the different force-ageing temperatures. Figure 4.1 and 4.2 displayed the main trends found for two staling compounds at 50°C.

Table 4.2 Concentrations of staling compounds in forced-aged green beer treated with different fining strategies according to varying temperatures for a week. Data are represented as average of 2 independent replicates and 4 technical replicates. Statistical difference has been detailed using post-hoc Tukey HSD test.

Force-ageing condition	Fining treatment	Staling compound concentration (µg/L)									
		2-methyl butanal	2-methyl propanal	3-methyl butanal	Ethyl isovalerate	hexanal	Isoamyl acetate	Furfural	t-2-nonenal	Benzaldehyde	γ-nonalactone
4°C 1 week	Control	1.35 ^a	1.97 ^{ab}	3.39 ^{bc}	6.47	0.51	1123	5.02	1.32 ^{ab}	1.22	367 ^b
	Isinglass (5mL/L)	0.95 ^b	1.50 ^b	2.39 ^c	7.92	0.35	1125	3.45	2.11 ^a	0.75	175 ^a
	Industrial hop extract (4mL/L)	1.22 ^{ab}	2.17 ^{ab}	4.39 ^b	6.42	0.64	992	6.42	1.19 ^b	2.06	198 ^a
	Saaz hop extract (4mL/L)	1.26 ^{ab}	2.30 ^a	7.44 ^a	6.32	0.46	1164	4.78	1.25 ^{ab}	1.29	378 ^b
	Statistical difference (Tukey HSD)	*	*	****	nsd	nsd	nsd	nsd	*	nsd	*
30°C 1 week	Control	2.82 ^a	11.54 ^{ab}	8.85 ^{bc}	8.97	0.41	1187	73 ^a	1.71	1.93	208 ^b
	Isinglass (5mL/L)	1.98 ^b	8.39 ^b	6.54 ^c	7.80	0.39	1113	66 ^{ab}	1.37	1.83	349 ^a
	Industrial hop extract (4mL/L)	2.43 ^{ab}	12.52 ^a	11.28 ^b	8.61	0.31	1168	62 ^{ab}	1.54	1.33	260 ^{ab}
	Saaz hop extract (4mL/L)	2.63 ^a	13.70 ^a	17.89 ^a	8.71	0.30	1100	52 ^b	1.27	1.58	254 ^{ab}
	Statistical difference (Tukey HSD)	***	**	****	nsd	nsd	nsd	*	nsd	nsd	**

40°C 1 week	Control	2.57	5.31	5.83 ^c	11.45	1.45	952	295	2.02	4.37	302
	Isinglass (5mL/L)	2.13	6.17	5.86 ^c	11.09	1.76	1254	295	1.52	3.98	266
	Industrial hop extract (4mL/L)	2.52	5.47	8.27 ^b	10.20	1.56	1040	288	1.44	4.33	216
	Saaz hop extract (4mL/L)	2.49	5.64	12.34 ^a	12.54	1.50	1062	272	1.41	3.60	230
	Statistical difference (Tukey HSD)	nsd	nsd	****	nsd	nsd	nsd	nsd	nsd	nsd	nsd
50°C 1 week	Control	3.73	9.01	8.61 ^{bc}	13.27	3.09	1059	888	1.55	9.44 ^a	184 ^b
	Isinglass (5mL/L)	3.23	8.23	7.86 ^c	14.77	2.76	1160	862	1.62	6.56 ^b	334 ^a
	Industrial hop extract (4mL/L)	3.67	9.05	10.14 ^b	15.27	2.78	1127	851	1.60	6.21 ^b	310 ^a
	Saaz hop extract (4mL/L)	3.38	8.90	14.49 ^a	13.17	2.77	1030	867	1.56	7.20 ^{ab}	267 ^{ab}
	Statistical difference (Tukey HSD)	nsd	nsd	****	nsd	nsd	nsd	nsd	nsd	**	**

**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, nsd $p > 0.05$

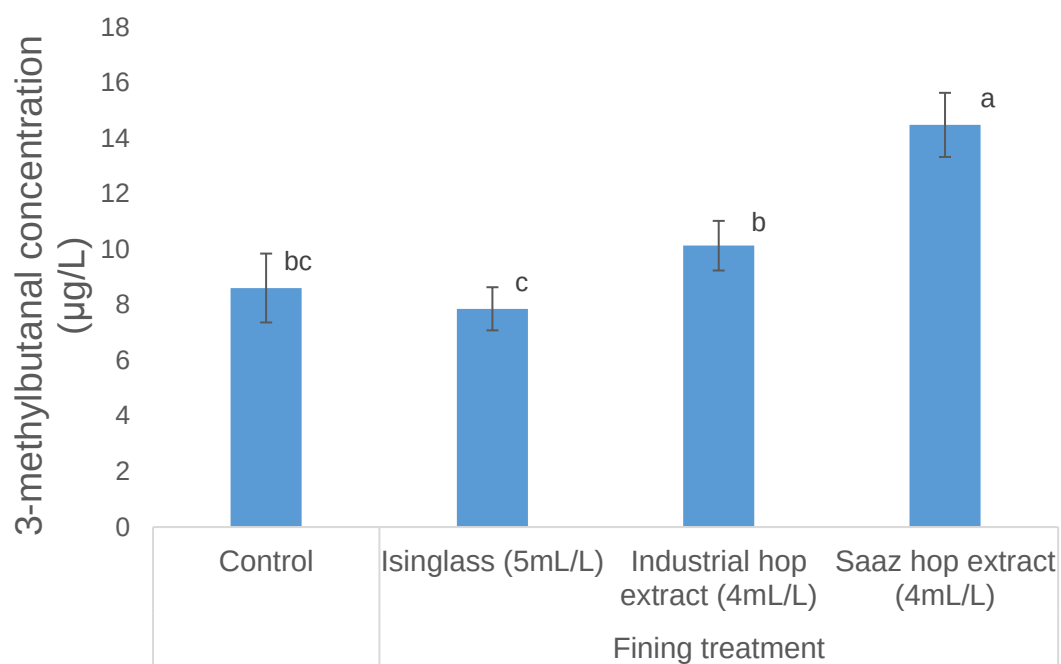


Figure 4.1 Average concentration of 3-methylbutanal in forced-aged green beer treated with different fining strategies at 50°C for a week with their standard deviation. Statistical grouping have been performed according to Tukey HSD test.

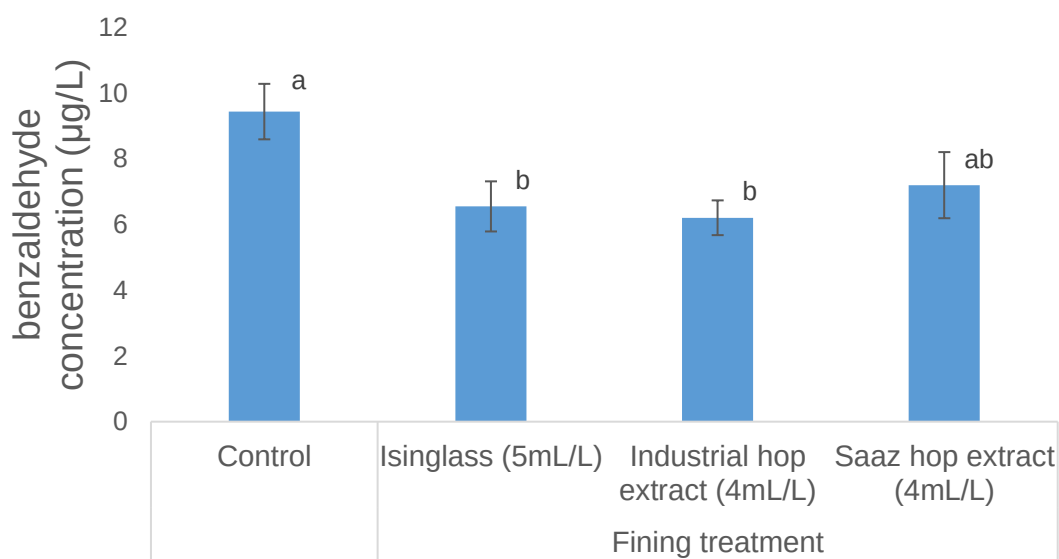


Figure 4.2 Average concentration of benzaldehyde in forced-aged green beer treated with different fining strategies at 50°C for a week with their standard deviation. Statistical grouping have been performed according to Tukey HSD test.

Concentrations of compounds such as 2-methylbutanal, furfural, and γ -nonalactone observed at 4°C (fresh beer) were in the concentration ranges typically reported in other lager beers (Saison et al., 2009; 2010). However, the concentration of t-2-nonenal found in the experiment was higher compared to concentrations found in other lager beers (from 0.05 to 0.5 $\mu\text{g/L}$) (Meilgaard, 1975; Saison et al., 2009). Changes in temperature caused an increased concentration for the majority of compounds, except for isoamyl acetate and γ -nonalactone. Different rates of increase were observed for the other staling compounds, with up to 100-fold increases in concentration between 4°C and 60°C for furfural. Between treatments and temperatures, three major trends were found with significant differences in volatile formation on ageing. This was observed for 3-methyl butanal, where the two hop extracts generated significantly higher concentrations compared to all other fining treatments ($p < 0.05$, post-hoc Tukey HSD test), at all storage temperatures (Figure 4.1). This compound typically yields to sweet, bread like off flavour (Huang et al., 2017). Regarding benzaldehyde, the control reference samples had a much higher concentration than all other treatments when stored at 50°C for a week (Figure 4.2). This can imply that all fining treatments are inhibiting the formation of benzaldehyde over time. Another statistical difference have been found for γ -nonalactone for different temperatures. However, this data has to be taken with care for this compound because of the high disparity of the measurements for all samples, and all temperatures. The majority of other staling compounds had no major statistical difference between treatments for all temperatures ($p > 0.05$, post-hoc Tukey HSD test). The hop extract did not diminish the staling volatile profile of lager beer, through storage. This trial has been done using 4 technical replicates per analysis, and with only two separate replicates per sample point. Greater replication might

have helped to discern whether other differences in mean values represent significant impacts, however, the overall conclusion was clear; there was very little direct influence of fining strategy on the development of staling compounds through forced ageing.

4.2.2 Metal ions concentration in lager beer treated with hop extract finings

The mineral content of beer is mainly contributed from the cereals, water, hops and other adjuncts used during the brewing process (Briggs et al., 2004). The main ions present in beer are the cations: calcium, magnesium, sodium and potassium, and the anions: sulfate, nitrate, phosphate chlorides and silicates. Minor ions present are copper, iron, manganese and zinc (Montanari et al., 2008). These metals offer a certain nutritional aspect for the consumer (Pohl, 2008), but some metals can affect beer quality (Jenkins et al., 2018). Transition metals like iron and copper are a real problem since these compounds affect beer ageing by catalysing oxidative reactions. These reactions forms free radicals in beer, which are likely to oxidize a large range of organic compounds present in beer. As a result, these free radicals are negatively contributing to the foaming quality and flavour stability of the beer (Pohl, 2008). The metal concentration of the commercial hop extract is displayed in Table 4.3.

Table 4.3 Metal concentration of the commercial hop extract obtained from ICP-MS.

<i>Metal ion</i>	<i>Concentration</i>
Na	3.47
Mg	0.72
P (mg/L)	0.65
K	20.69
Ca	1.73
Al	10.19
V	0.05
Cr	3.12
Mn	1.11
Fe (µg/L)	12.89
Co	0.04
Ni	1.97
Cu	19.92
Zn	45.49

The beers brewed using different fining treatments in the experiment in Section 4.1.1 were tested for metal ion concentration after 24 h of fining reaction using the commercial hop extract. Figure 4.3 displays the mean of the iron concentration following four different fining treatments. The unfined control had an iron concentration of 100 µg/L whereas all fining treatments resulted in lower concentrations of iron. Isinglass reduced the average concentration to 49 µg/L. There was however no statistical difference between isinglass and the control beer reference ($p > 0.1$, post-hoc Tukey HSD test) due to the high variability in data. The addition of the industrial commercial hop extract reduced significantly the concentration to 17 µg/L on average, with statistical difference compared to the control ($p < 0.05$, post-hoc Tukey HSD test). Saaz hop extract decreased the average iron concentration to 10 µg/L, as well with statistical difference compared to the control ($p < 0.05$, post-hoc Tukey HSD test). Both hop extracts showed a significant decrease in iron concentration, validating the iron chelating ability of polyphenols (Hider et al., 2001). All beers treated with fining agents have been found with a

higher concentration of manganese, with statistical difference ($p < 0.05$, post-hoc Tukey HSD test). This increased concentration is potentially problematic as manganese can negatively impact beer flavour stability (Jenkins et al., 2018). The industrial hop extract treated beers had a lower concentration of copper compared to all other beers, which can be a benefit for beer stability as copper is also negatively impacting on beer stability. Other metal ion concentrations were also significantly reduced by the addition of the hop extract; i.e. aluminium, vanadium, cobalt. (Table 4.4)

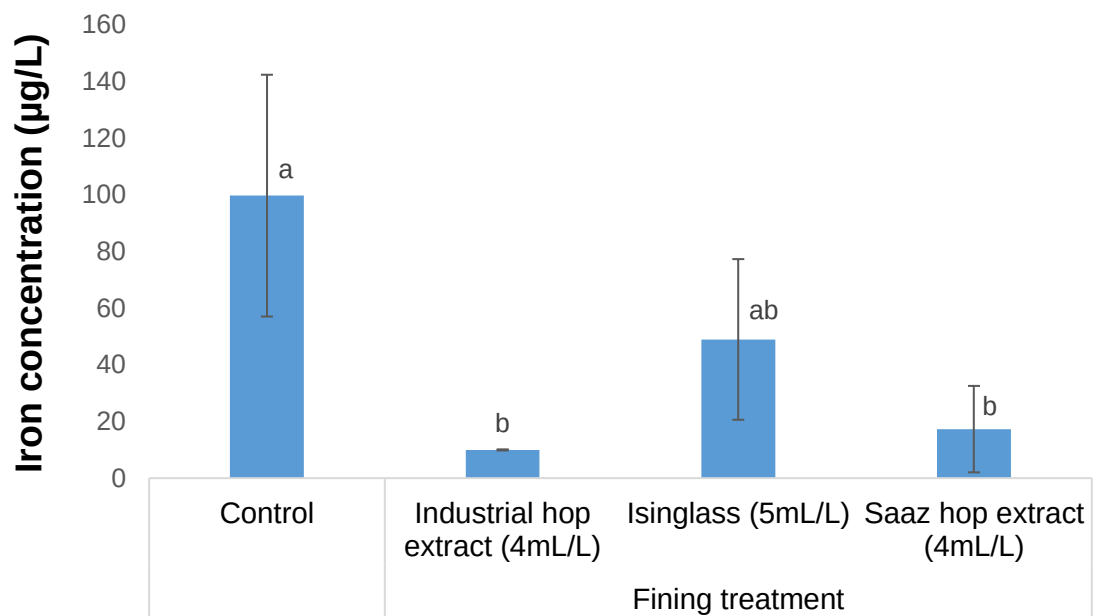


Figure 4.3 Iron concentration (in µg/L) of a kit lager beer treated with different fining strategies after 24h of clarification. Data is expressed as the mean of 4 replicates with their standard deviation. Statistical grouping has been performed according to Tukey HSD test.

Table 4.4 Metal ion concentration (in $\mu\text{g/L}$) of a kit lager beer treated with different fining strategies after 24 h of clarification. Data is expressed as the mean of 4 replicates with their standard deviation. Statistical grouping have been performed according to Tukey HSD test.

Fining treatment	Al ($\mu\text{g/L}$)	V ($\mu\text{g/L}$)	Fe ($\mu\text{g/L}$)	Co ($\mu\text{g/L}$)	Mn ($\mu\text{g/L}$)	Cu ($\mu\text{g/L}$)
Isinglass (5mL/L)	12.60 ^{ab} ± 2.61	0.142 ^b ± 0.09	48.81 ^{ab} ± 28.3	0.251 ^a ± 0.15	207.87 ^a ± 15.4	218.14 ^{ab} ± 6.33
Industrial HE (4mL/L)	8.20 ^b ± 0.55	0.082 ^b ± 0.01	9.96 ^b ± 0.19	0.061 ^a ± 0.01	211.88 ^a ± 1.62	203.71 ^c ± 0.68
Saaz hop extract (4mL/L)	7.44 ^b $\pm$ 0.95	0.110 ^b ± 0.07	17.30 ^b ± 15.3	0.066 ^a ± 0.02	212.23 ^a ± 2.23	223.46 ^a ± 2.08
Control	14.41 ^a ± 4.71	0.581 ^a ± 0.29	99.70 ^a ± 42.67	0.120 ^a ± 0.02	187.81 ^b ± 8.18	209.99 ^{bc} ± 9.46
P Value (Tukey HSD)	0.01	0.002	0.002	0.054	0.0003	0.002

The beer samples from the 10 hL brewing trial using the commercial hop derived finings (Section 2.3.6.5, Table 2.9) were also tested for metal ion concentration using the ICP-MS. Figure 4.4 displays the mean of the iron concentration for the unfined control and the two beers treated with different dosage (4 and 10 mL/L) of the hop extract, after fining and maturation time (5 d).

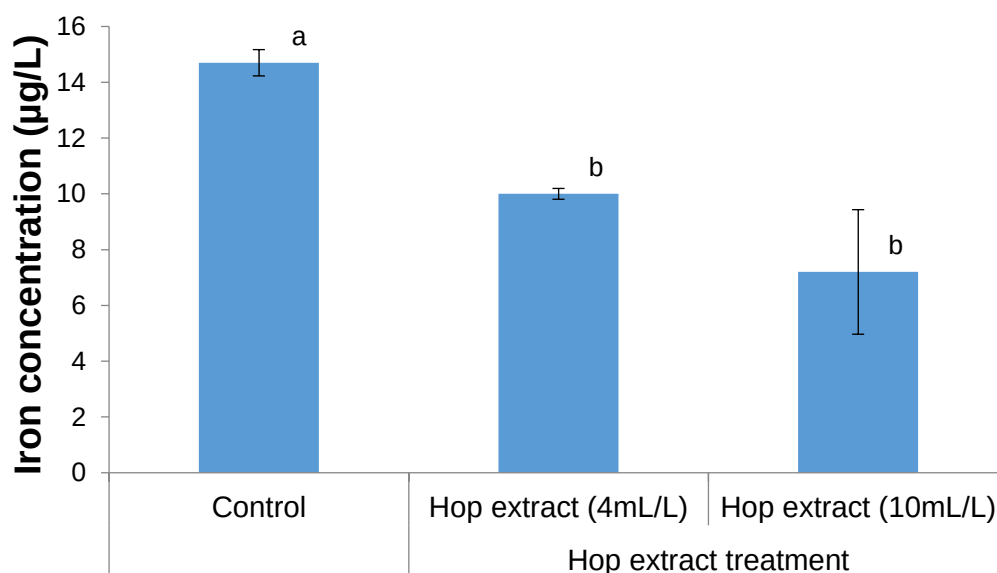


Figure 4.4 Iron concentration (in $\mu\text{g/L}$) of a 100% pale malt lager beer treated with different doses of commercial hop extract and a control (unfined) after 24h of clarification. Data is expressed as the mean of 4 replicate measurements with their standard deviation. Statistical grouping have been performed according to Tukey HSD test.

The control beer had an average iron concentration of 15 µg/L whereas the two hop extract treated beers had a decreased average concentration of 10 µg/L for the 4 mL/L dosage and 7 µg/L for the higher dosage (10 mL/L). Statistical difference was found between the control reference and the two hop extract dosages ($p < 0.05$, post-hoc Tukey HSD test). No major difference was found for manganese and copper concentrations between the four beers studied ($p > 0.05$, post-hoc Tukey HSD test). Furthermore, a decrease in nickel and zinc ion concentrations was also found for the two hop extracts (Table 4.5). High dosage of the commercial hop extract also increase concentrations of metals such as copper because of their natural composition in metals (Table 4.3).

Table 4.5 Metal ion concentration (in µg/L) of a 100% pale malt grain lager beer treated with different doses of hop extract and a control (unfined) after 24h of clarification. Data is expressed as the mean of 4 replicates with their standard deviation. Statistical grouping have been performed according to Tukey HSD test.

Fining treatment	Fe (µg/L)	Ni (µg/L)	Zn (µg/L)	Mn (µg/L)	Cu (µg/L)
<i>Hop extract 4mL/L</i>	10.03 ^b ± 0.19	3.17 ^b ± 0.06	17.07 ^b ± 1.38	96.37 ^a ± 2.04	34.94 ^a ± 0.94
<i>Hop extract 10mL/L</i>	7.22 ^b ± 0.47	3.50 ^b ± 0.10	15.47 ^b ± 4.50	127.64 ^a ± 26.45	56.53 ^a ± 21.68
<i>Control</i>	14.67 ^a ± 2.23	6.29 ^a ± 0.47	33.67 ^a ± 0.72	97.67 ^a ± 1.27	34.75 ^a ± 0.41
<i>P Value (Tukey HSD)</i>	0.001	< 0.0001	0.0003	0.079	0.125

Proanthocyanidins and other phenolics as well as antioxidants (Santos-Buelga et al., 2000) are also known in health care for their transition metal chelation activity (Cos et al., 2012), and especially towards iron (Guo et al., 2007; Hatcher et al., 2009; Wu et al., 2010). Proanthocyanidins bind metals through a complexation reaction involving their o-diphenol groups. The ortho-dihydroxyl group on the flavonoid B-ring of PA is the major unit responsible for metal complexation. Spectrophotometric and potentiometric experiments demonstrated that iron (III) created mono-, di-, and

tridentate complexes with tannins (Slabbert, 1992). Here, the metal ion concentrations of two different lager beers were measured (Figure 4.3, Figure 4.4, Table 4.4, and Table 4.5). In general, the metal ion concentration of the 100% malt beer brewed in the 10 hL pilot brewery was much lower than that of the kit beer, as the water used for brewing was stationer-mineralised by way of pre-treatment (almost ion free water). Of all of the ions measured using ICP-MS only iron was found to be significantly reduced by the hop finings addition across both studies reported here. The iron concentration found in the present experiments are in accordance with the study performed by Pohl (2008), where a range of between 15 to 1000 ppb was found depending on the beer style. The addition of hop finings has been shown to reduce iron concentration. This effect has been demonstrated in numerous studies showing the metal chelation potential of proanthocyanidins (Slabbert, 1992; Guo et al., 2007; Hatcher et al., 2009). A rapid laboratory test using a solution of iron (III) chloride and a drop of hop extract has shown an instant colorimetric complexation, changing the solution from yellow to dark green. Jenkins et al., (2018) affirmed that as little as 10 ppb transition metal ion addition can make a detectable difference to the oxidative stability of beers. They also reported that iron was consistently detrimental to beer flavour stability measurements using ESR, with due importance given to lowering concentrations of such transition metals in the promotion of beer flavour stability.

4.2.3 Beer oxidative stability measurements for lager beers treated with hop extract finings.

Alpha acids and numerous polyphenolic compounds demonstrate a positive effect in improving beer flavour stability (Schönberger, 2012). As the beer ages, it is exposed to heat, oxygen and light, which impact the flavour characteristics and cause a

decreased storage stability. This deterioration creates highly reactive radicals within the beer. The electron spin resonance (ESR) technology coupled with a spin trap has become a useful technique for the brewer to predict beer shelf life and beer flavour stability (Huvaere et al., 2008). Studies by Andersen et al., (2000; 1998) using ESR indicated that polyphenols (phenolic acids, catechin and proanthocyanidins) may not impact the formation of free radicals in wort brewing or during beer storage, with no significant impact on the beer flavour stability. However, Aron et al., (2010) reported the opposite effect, that the addition of 100 ppm of a galena hop polyphenol extract into beer produced an anti-staling effect, measured by ESR.

The ESR forcing test (lag-time and T150 measurement) was conducted on the freshly packaged beers produced for the forced ageing trial (Section 4.1.1). This assay is used to determine the oxidative stability of a beer, where T150 is the intensity of the ESR signal after 150 min of the assay. T150 is thought to be an indication of the oxidative stability of the beer studied which can be considered to predict the oxidative deterioration that may occur during storage or conditioning (Jenkins et al., 2018).

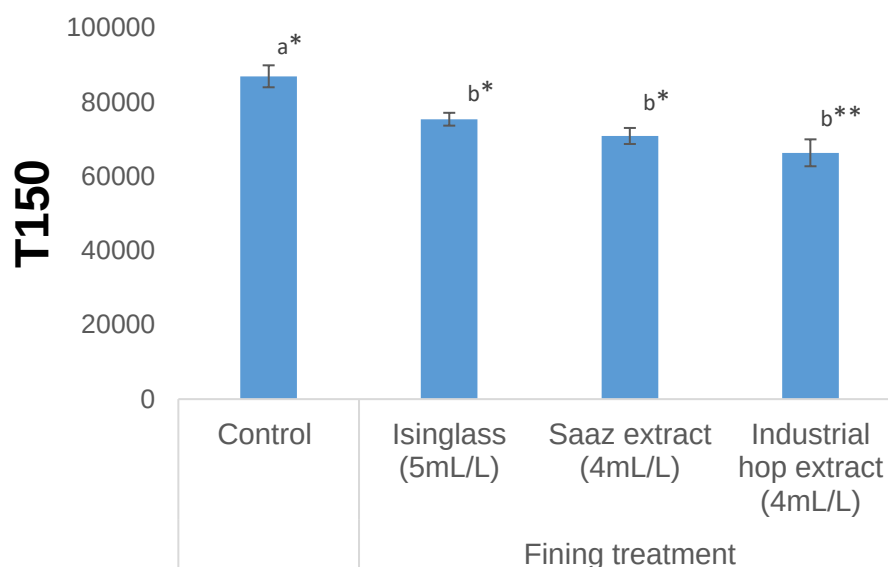


Figure 4.5 T150 intensity measurements for a kit beer treated with different fining strategies. Data is presented as the mean of duplicates with their standard deviation. Statistical grouping have been performed according to Tukey HSD test. (** $p < 0.01$, * $p < 0.05$)

Four different fining strategies were tested in duplicate during this experiment. Figure 4.5 shows the free radical formation at 150 min (T150) of trial beers in the ESR assay. The control unfined beer had the highest T150 value (87000), which indicates a lower oxidative stability compared to the other treatments. All fining treatments had a significant impact on the reduction of the T150 signal relative to the control ($p < 0.05$, post-hoc Tukey HSD test), with higher difference found for the industrial hop extract ($p < 0.01$). Isinglass reduced the signal by around 13% relative to control, and the two hop extract dosages tested (4 mL/L of a Saaz extract and the same dosage of a prototype hop finings manufactured by Barth Innovations Ltd. gave reductions of 18 and 24% respectively compared to the control. These data suggest a potential to improve the beer flavour stability and freshness using the hop extract fining material.

To further test this impact on oxidative stability over repeat brews, another pilot scale trial was conducted. 10 hL of high-gravity lager (100% malt) were produced using the AB InBev research brewery facilities with the recipe used in Section

2.2.6.5. The fermented green beer was split into two volumes of 3.5 hL and one of 1.5 hL. Each 3.5 hL aliquot received a fining treatment of 2 mL/L of industrial commercial hop extract (diluted in different amount of water initially to represent 2% or 3% of the total beer volume prior to beer introduction) and the other 1.5 hL left unfined as a control reference. After four days of clarification at -1°C, samples were taken for ESR analysis. The control reference beer once again had the higher radical formation after 150 min of forcing at 60°C (T150, Figure 4.6).

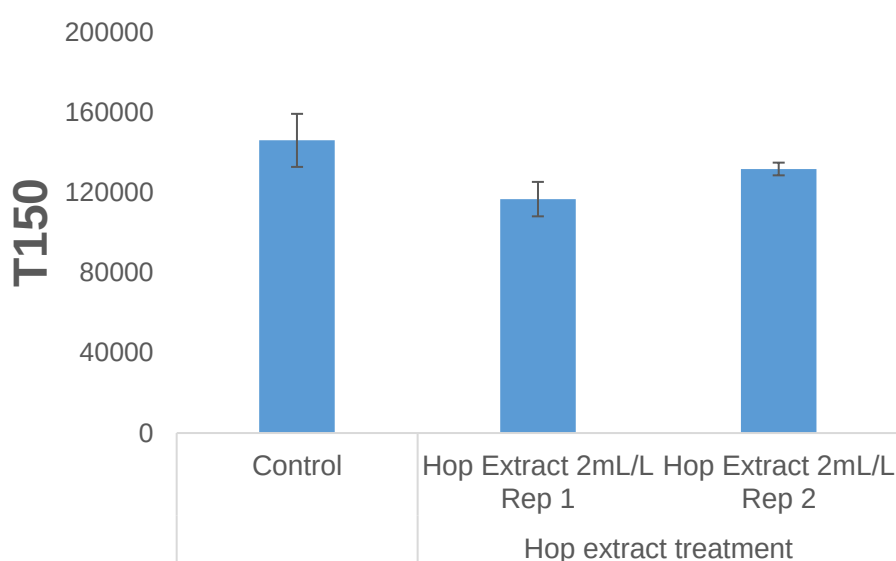


Figure 4.6 T150 intensity measurements for a 100% pale malt lager beer treated with 2 mL/L of commercial hop extract and an unfined reference control. Data is presented as the mean of duplicates with their standard deviation.

The two technical replicates showed a decrease in the signal due to the introduction of the commercial hop extract. The two hop extracts reduced the T150 signal by 15% on average compared to an unfined sample. However, no statistical differences have been found between those three samples ($p > 0.01$, ANOVA). More replication would be needed in this experiment to evaluate if the hop extract can effectively improve beer oxidative stability at a brewery pilot scale.

The addition of the commercial spent hop extract has thus been shown to favour the decrease of the T150 intensity signal for two separate lager brewing trials, showing that the addition of polyphenol was acting positively towards a better beer flavour stability (Figure 4.5, Figure 4.6). Forster et al., (2007) brewed two beers, one with the addition of type-45 pellets (polyphenol rich) and one with a CO₂ extract (low polyphenol). They showed a reduced signal using ESR instrumental methods for the beer using the pellets than with the CO₂ extract (Forster et al., 2007). They also reported that their polyphenol-rich brewed beers after 4 weeks of storage at 27°C were considered pleasant, and that the one without polyphenol addition was described as undrinkable and too aged. Mikyška et al., (2002) also demonstrated a positive effect from hop polyphenols on beer flavour stability using the thiobarbituric acid index assay. They reported by this assay that the addition of hop polyphenols slowed the flavour deterioration of beer during a storage trial. A study made by Jaskula-Goiris et al., (2014) showed that the use of polyphenols show a significant potential to enhance beer flavour quality and stability. Fresh beers with and without the addition of hop polyphenols (4 of each) were given to a panel of 6 trained panelists, asked to rank all samples according to their preference (scoring between 1 and 4). Beer without the addition of hop polyphenol during brewing was the least liked by the panel, with statistical preference. The most preferred was a sample of beer with a dosage of 50 mg/L of hop polyphenols in brewing and sparging liquor. The panel also tried the same beer samples passed through a force ageing step of 5 days at 40°C and concluded that all beers without the addition of hop polyphenols were the most able to develop off-notes and aged flavours. The beers with the addition of hop polyphenols have been both preferred in their fresh state, but also found to be more stable in flavour with force ageing conditions. In addition

to reduced wort filtration time, beers are having an improved stability and a longer beer fresh flavour. They concluded accordingly to their previous work that adding extra hop polyphenol during brewing in combination with colloidal clarification to remove haze sensitive proteins offer the brewer a very powerful tool to produce beers with better flavour and flavour stability.

4.3 Conclusions

Results reported in Chapters 2 and 3 have shown the ability of the hop extract to remove haze material from beer after fermentation. However, there was relatively little known about other impacts this fining addition might have on finished beer properties. The research reported in this chapter involved the evaluation of the introduction of hop extract finings on the volatile profile of beer (selected staling compounds in fresh and forced beers), metal ion content and beer oxidative stability as measured using an ESR forcing test.

The formation of off-flavours in beer is a natural reaction happening when beer is stored for ageing (Vanderhaegen et al., 2006). The first experiment involved different fining strategies and the quantification of beer staling compounds through a force ageing trial. No major significant changes in concentrations have been observed between isinglass, the two hop extracts, or, an unfining control. One compound (3-methylbutanal) was found to be significantly impacted by the fining protocol, with significantly higher concentrations observed when using hop extracts. This compound is however, a natural compound present into beer, and also used as a flavouring agent (Saison et al., 2009). 3-methylbutanal is an intermediate in the catabolism of leucine (Smit et al., 2009). The increase of this compound from the addition of the hop extract could be related to high levels of leucine present into the hop extract. This experiment has only been performed using accelerated ageing

techniques (Bamforth et al., 2009; Saison et al., 2010) and should be repeated using natural (ambient) ageing to confirm the results observed.

As well as their noted antioxidant capacity, proanthocyanidins have been identified as a metal ion chelators, with a special affinity for iron (Santos-Buelga et al., 2000). Iron is a problematic metal ion in beer as it negatively impacts on the flavour stability as well as sensory properties (Jenkins et al., 2018). Two major experiments, one including a laboratory beer, and a beer produced at the research brewery have involved the application of the hop extract and the further metal ion quantification of the beer after clarification. For the laboratory beer trial, the hop extract significantly decreased the iron concentration of the beer after clarification, more so than isinglass finings. A non-significant difference has been observed with the research brewery beer. The difference was shown more obvious in the laboratory case as the research brewery beer metal ion concentrations are much lower due to the water treatment facilities upstream and process control to favour a minimal content of metal ions. These two experiments confirmed however the action of proanthocyanidins in forming iron complexes (Guo et al., 2007; Wu et al., 2010), which are potentially removed as a part of the clarification process. The commercial hop extract also contained various amount of metals ions, which also increased the global concentration of the fined beer. However, it is noteworthy that the addition of these polyphenols really assisted to the decrease of iron content in beer. Other reductions have also been found but with less significance than the iron decrease. It is also important to notice that the hop extract itself is also composed of metals (copper) and could potentially increase the global concentration of the finished beer. Consequently, a noticeable improvement was observed in beer oxidative stability (T150) when using hop extract finings in beers. The addition of the hop extract

significantly reduced the propensity to form free radicals in ESR experiments. It is reasonable to think that the decrease in iron content is implying a beneficial impact in oxidative stability as this transition metal acts as a catalyst for the Fenton reaction, producing free radicals. Polyphenols have shown to enhance the flavour properties and beer flavour stability (Aron et al., 2010). Spent hop extracts from CO₂ extraction also offered great potential for innovative flavouring (Jaskula-Goiris et al., 2014). Since proanthocyanidins are able to prevent oxidation of other compounds present, this addition can represent a potential improvement in beer freshness, and also a longer shelf-life of the product.

Although some benefits have been clearly demonstrated using hop extract finings, further experiments, including sensory studies and analysis of beer flavour volatiles, would be required to demonstrate the ability of the hop extract to improve finished beer flavour stability. This product may offer the brewer, in addition to reducing filtration costs, a novel advantage in terms of the flavour stability of the finished beer.

Chapter 5: Conclusions and future work

5.1 Conclusions

Preliminary data and research performed by Linforth et al., (2015) had pioneered the development and the application of the novel hop extract in green beer. Although first results and literature pointed to the efficiency of acetone/water extractions (Kallithraka et al., 1995; Anioł et al., 2008), robust experiments had to be performed in order to validate and optimise the extraction solvent mixture most selective for the active material (Section 2.3.1). The overall aim of this study was the development of a novel clarification aid for green beer. Beer clarification can be performed using this product, with desirable quality performance relative to isinglass finings. Fining performance has been evaluated through measurements of colloidal stability, sediment evaluation haze analysis post-fining and beer filterability. The hop extract addition produced lower sediments compared to isinglass post-fining (Section 2.3.6.1 and 2.3.6.2). These sediments are both composed of yeast and proteins. Sedimentation is achieved with these two fining agents after 24 h on a laboratory scale and was evaluated through visual evaluation. After removal of residual yeast, isinglass fined beers obtained lower haze than hop extract fined beers, for the 4 different blond beers evaluated in Section 2.3.6.1. The hop extract has the potential to fine chill-haze proteins but is slightly less effective to bind to all the haze responsible proteins that isinglass does, resulting in a slightly higher haze post-clarification (Table 2.5). This residual fine haze observed for hop extracted treated beers is however not sedimenting after time resulting in effective colloidal stability. Filterability trials have given similar results as the addition of hop extract on its own

resulted in slightly lower performance than isinglass fined-beers (Section 2.3.7). Isinglass has been able to remove more haze material during clarification compared to the hop extract alone.

Fining using the hop extract on its own into dark beers (e.g. stouts) has been problematic as although the hop finings generated sediment, indicating an action, they tended to leave behind increased fine haze material (Section 2.3.6.3). Only a very high dose of auxiliary finings in combination with the hop extract allowed results similar to isinglass finings post-fining and this does not represent a viable option for the development of this extract. However, this product on its own added into dark beers can assist global clarification as these suspended small aggregates formed could be further filtered through other processes such as filtration (Section 5.2) or the addition of PVPP during the clarification (Section 2.3.6.4). These processes would achieve improved clarity when bottled.

An important beneficial action of using the hop extract in combination with silicate-based auxiliary fining has been found. Haze removal using this combination of finings was able to match the performance of isinglass (Section 2.3.6.1), and also better filterability results have been observed compared to isinglass (Section 2.3.7). The two fining agents worked synergistically for haze removal. The application of combinations of these two fining agents was tested in-depth in two blond model beers. The addition of auxiliary finings was unnecessary for simple model beers (home-brew kit) where haze removal was mainly driven by the addition of the hop extract, but necessary for more complex beers (all-grain), as in such beers haze was significantly reduced further with the addition of auxiliary finings (Section 2.3.5). The hop extract has also been proven suitable for pilot brewery scale use, with a

beneficial impact on haze reduction as well as filterability compared to an unfined control (Section 2.3.6.5, Section 2.3.7). It is very important to optimise the introduction of this extract into brewery fermentation vessels as high shear rates due to pumps or mixers could induce poor sedimentation and fining efficiency (Section 2.3.2, Zhang et al., 2019).

Many other properties in usage were discovered through the characterisation section of the thesis (Chapter 3). Varietal differences in proanthocyanidin concentration and fining performance have been observed using hop extracts from different varieties, with the best results obtained for products with the highest analytical PA concentration (Section 3.2.2). However, the option to select a specific hop variety for product manufacturing from spent hops is difficult logistically as the hop varieties which are processed are dictated by their brewing usage and are typically high alpha hops. Prototype products used in this research were manufactured from mixed varieties of spent hops, indicating that the waste is not currently separated according to variety.

The characterisation of hop extracts from different varieties was first conducted using the phloroglucinolysis technique (Section 3.2.3.1) based on recent literature (Li et al., 2006; Bosso et al., 2016; Neilson et al., 2016). Results indicated that polyphenolic hop extracts clearly contain polymeric molecules, and also gave the first indication that better fining performance was obtained with the use of hop extracts with the highest degree of polymerisation. This principle agrees with the theories proposed by (Siebert et al., 1996, 1999, 2009) as binding sites, stoichiometry and size of the aggregates formed are the fundamental principles of efficient flocculation and clarification.

The development of a novel method to characterise the molecular size distribution in plant polyphenolic extracts using the analytical ultracentrifuge (described from Section 3.1.3.2) enabled in-depth profiling of the molecular size distribution of hop extracts from different varieties. Although this equipment has a general use for the characterisation of pure proteins and polysaccharides, (Cole et al., 2008; Harding, Abdelhameed and Morris, 2010; Schuck et al., 2014) the adaptation of this technique to characterise complex mixtures of polyphenols was achieved for the first time, with reproducible results and success for hop extracts (Section 3.2.3.2). All polyphenolic hop extracts had a heterogeneous size distribution, with the highest sedimentation coefficient peaks being observed for hop samples with the best fining performance. These results confirmed and validated the phloroglucinolysis results, as better fining action was found for hop extracts containing the highest concentrations of large polymers. Sedimentation equilibrium studies were applied to calibrate the molecular scale, and polymers up to 100 kDa were detected for Saaz hop (Section 3.2.3.3). Samples of Saaz hop repeatedly showed a greater content of high molecular weight proanthocyanidins than other hop varieties investigated. Not surprisingly therefore in all studies, it was found to be the most active in terms of fining performance as well as having the highest concentration of PA as determined by the acid-butanol assay. Saaz has been proven to be the most stable hop variety (variation in content and composition of hop secondary metabolites during long term storage) (Mikyška et al., 2012), with also highest antioxidant activity (Jaskula-Goiris et al., 2014). The DPPH (1,1-diphenyl-2-picrylhydrazyl) antiradical potential method was found to be best for the determination of antioxidant properties of hops and was found also maximum for Saaz hop (Krofta et al., 2008).

The analytical method using the ultracentrifuge represents the first application to the AUC to complex polyphenol solutions. Other botanical sources known to be rich in polyphenolics were also tested for polyphenol content and fining efficiency at first. These botanical extracts presented various proanthocyanidin concentration, and also acted as efficient beer fining agents. Extracts with a high concentration of PA from the acid butanol hydrolysis assay (Figure 3.10) gave better haze removal in beer post-fining (Section 3.2.6.1, Figure 3.11).

Using the AUC method, these botanical extracts also have shown size heterogeneity and the presence of high molecular weight polymers (Section 3.2.6.2). The presence of high molecular weight polymers conferred an efficient fining action. Even if most of the materials studied would be cost-effective to produce, it is novel to notice that proanthocyanidins from numerous sources and especially high molecular weight polymers are responsible for beer clarification. This novel methodology was pioneered throughout this thesis and should find extensive application in polyphenol research for the study of this diverse and bioactive class of compounds. As proanthocyanidins are used and studied with high interest in the medical field (Howell, 2002; Gu et al., 2004; Pedan et al., 2016), this may well assist a better understanding of reactions involving these compounds.

Whilst exploring the potential of the hop extract to clarify haze, other benefits have been found during this research study. The action of the hop extract to reduce the iron content of beers (Section 4.2.2) is another important point to consider as this ion is a pro-oxidant transition metal ion which can catalyze oxidative reaction leading to flavour changes and this can be a real problem for brewers (Zufall et al., 2008; Jenkins et al., 2018). The reduced T150 signal found in ESR experiments

(Section 4.2.3) using hop extract finings could also represent another benefit as it potentially implies an improved beer shelf life. Both of these positive impacts have also been found for isinglass treated beers compared to an unfined reference. However, beers treated with the hop extract showed better results with statistical significance than isinglass. This represents an additional point favoring the usage of the spent hop extract in beer fining.

One of the main challenges of this commercialisation is that all beers should be pre-tested before introducing the extract into large amounts of beers. Because of the complexity of beer fining and the large variety of products and their composition, dosage trials always have to be performed prior to application on a large scale. However, this principle also applies to the usage of isinglass and most other commercial fining materials.

This product could represent a novel, more sustainable, way to clarify green beer as it is firstly sourced from food waste, from hops, and represents also a vegetarian option for the brewer.

5.2 Further work

- The use of a combination of hop extract and silica auxiliary finings is beneficial for fining activity. However, it is possible that the leftover concentration of polyphenol can be important after fining. The use of PVPP in combination with preliminary finings in lager beer would be of interest to investigate and might induce better beer clarity. This product is widely used by brewers to lower polyphenol content in their filtration processes and could represent an efficient combination of removing both haze proteins and polyphenols from beer.
- The use of PVPP in combination with the hop extract in dark beers was detrimental towards haze removal (Section 2.3.6.4). Because of the potential for the hop extract to create additional haze, it is also possible that this haze material could be further removed by filtration. Filterability trials using this procedure would be a useful piece of data to determine if the hop extract could be applied successfully into the dark beer market.
- Spray drying the hop extract can represent a more economical way to sell this product to the global market. The feasibility of drying the hop extract into a powder has been studied and it is possible to transform the active material into a powder without any extra carrier. However, no work has been performed on the stability of powders with the time of storage, to observe if the solid material would deteriorate over time. If this was selected as an appropriate physical form in which to market the fining agent then future

shelf-life studies would be required to determine product physical and microbial stability and its fining performance versus storage time.

- After the carbon dioxide extraction, the spent hop material is still composed of proteins (almost 20%). Many protein extract from other vegetables are developed as a fining agent. If these would perhaps have a fining mechanism more closely to isinglass, then they might represent a main interest in fining and it would be interesting to study their extraction and fining activity.
- In addition to AUC, other techniques can be applied to validate the presence of the heterogeneous distribution of hop proanthocyanidins. Other techniques such as Dynamic Light Scattering may bring also a new perspective into the characterisation of the proanthocyanidin fraction of hop extracts. This technique has proven very powerful to define protein sizes (Jachimska et al., 2008; Lorber et al., 2012), and has also been used to study the aggregation of proanthocyanidins with gelatin proteins (Su et al., 2015).
- The separation of the high molecular weight fraction of hop proanthocyanidins from the lower molecular weight polyphenolics has still not been achieved satisfactorily. As the proanthocyanidin polymers display a different polarity (Watson et al., 2013) than the small monomers, investigations using analytical electrochemistry could assist the separation of these polymers from the initial matrix. Future recommendations would include the use of capillary electrophoresis. This technique can perform a

clear separation of heterogeneous samples by their affinity and their charge. This technique has been proven useful for the separation of small flavan-3-ols and dimeric proanthocyanidins (Kreimeyer et al., 1998). Enzymes such as laccase could also be used to produce smaller subunits, that could be sequenced further using the phloroglucinolysis reaction.

- If the separation of the high molecular weight fraction would be successful, extensive research could be applied for the shape analysis of each fraction through AUC. This technique has a high potential in molecule conformation (Graceffa et al., 1988; Cole et al., 2008). This technique would possibly explain the differences found between sedimentation coefficient and molecular weight, as peaks with regular spacing as been found on SV experiments, and large spacing as been found on the molecular scale with SE experiments.
- As efficient fining activity has been conferred to the presence of large polymers, research in organic chemistry could involve the synthesis of large polymers to produce more efficient products.
- The trial involving the analysis of the staling volatiles profile of beer used an accelerated forced ageing procedure (Section 4.2.1). The replication of this experiment with normal (room temperature) conditions of storage would add value and context to the data produced and determine if the hop extract could change the staling chemistry of beer.

- The effect of the hop extract on beer sensory properties has so far not been profiled in detail. Literature research has shown that beers enriched with hop polyphenols are considered more pleasant than beer without any addition (Forster, 2007; Aron et al., 2010). Descriptive analysis (Stone et al., 2008) will generate quantitative and qualitative data to describe the similarities but also the difference among a set of beers, with and without the hop extract. This experiment would help define the main sensory attributes generated by the addition of the hop extract. This experiment combined with an acceptance and preference test to a larger consumer panel (Lawless et al., 1999) would also validate the fact that beer treated with hop polyphenols would be preferred by consumers and assist the commercialisation of the product for brewing companies.

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Appendixes

SPECIFICATIONS SHEET:

PRODUCT NAME:
Finings Adjunct

PRODUCT CODE:
FADJ

COMMODITY CODE:
13012000

PACKAGING:
12.5, 25, 200, 600 and
1,000 kg

STORAGE:
Store 10-20°C

SHELF LIFE:
Six months

ALLERGENS:
Contains Sulphur
Dioxide

Specifications

Product	Finings Adjunct	Product Code	FADJ
Composition	A stabilised acidic solution of an inorganic silicate sol		
Appearance	A colourless, slightly opaque liquid		
Odour	Sulphur Dioxide (SO ₂)		
Analysis			
Flavour		Does not adversely affect beer flavour	
Specific Gravity @20°C		1.015 ± 0.001	
Sulphur Dioxide (ppm)		>150 ± 25 *	
Haze (EBC)		<2.5	
pH		2.05 ± 0.15	
Microbiology			
Total Viable Count (cfu/ml)		<1,000	
Maximum limits of impurities (ppm)			
Arsenic		3	
Lead		10	
Copper		50	
Zinc		25	
Copper and Zinc		50	
Issue No.	4	Date	09/04/2018
Written by	E Wray	Authorised by	RJ Haywood

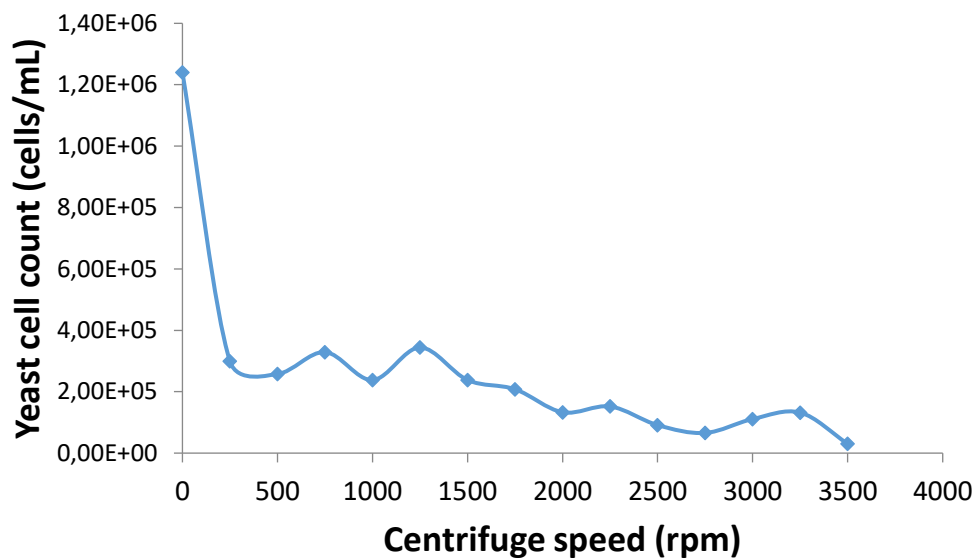
Appendix 1 Silicates auxiliary finings data sheet

Residual Yeast removal

In many experiments it is necessary to first remove yeast before proceeding to measure indexes of beer haze or colloidal stability. A trial was designed to determine the minimum centrifugation required to spin out the majority of yeast cells, such that any influence on other suspended particles was minimised.

The impact of velocity varies depending on rotor dimensions, bucket size and centrifugation angle. The beer used was a typical homebrew kit, each sample contained around 300 mL and the beer was spun at speeds ranging from 250-3500 rpm at 12°C for 15 min.

It was clearly shown (Appendix 1) that most of the yeast was removed by a low spinning velocity. In order to remove only yeast and not the others particles contained in the beer, hence, in further experiments the beer will be spun at 500 rpm for 15 min before haze analysis.



Appendix 2 Suspended yeast cell count (cells/mL) of a green kit beer centrifuged at different velocities for 15 min.

Charnwood brewery beer fining activity data

Appendix 3 Haze recordings and sediment depth of Charnwood Brewery beer treated with a range of different fining strategies at 4°C and 12°C. Haze measurements have been performed after 24h of clarification.

Fining at 12°C	Sediment depth (mL)			Haze 12°C (EBC)	
	3 h	6 h	24 h	H90	H25
<i>HE 2 mL/L</i>	5.5	5.25	4.2	1.74	1.74
<i>Aux 2 mL/L</i>	5.75	6.5	5.2	1.68	2.3
<i>Control</i>	3	3.5	3	4.89	7.82
<i>IG 10mL/L</i>	11	12	13.5	1.56	4.81
<i>IG 20mL/L</i>	15	17	15	2.59	5.66
<i>Aux 2mL/L + IG 2.5mL/L after 15"</i>	10	10.5	8.5	1.23	2.54
<i>Aux 2mL/L + IG 10mL/L after 15"</i>	18	20	14	1.47	3.01
<i>Aux 2mL/L + HE 2mL/L after 15"</i>	7	12	6	1.34	1.33
<i>HE 2mL/L + Aux 2mL /L after 15"</i>	7.5	10	6.5	1.12	1.6
<i>HE + Aux 2mL/L same time</i>	6	11.5	6	1.2	1.49

Fining at 4°C	Sediment depth (mL)			Haze 4°C (EBC)	
	3h	6h	24h	H90	H25
<i>HE 2 mL/L</i>	7	6.5	5.25	1.68	1.8
<i>Aux 2 mL/L</i>	8.5	7	5.25	1.14	3.13
<i>Control</i>	3	3.2	4	5.14	10.49
<i>IG 10mL/L</i>	18	16	13	1.03	1.32
<i>IG 20mL/L</i>	24	20	18	1.14	2.61
<i>Aux 2mL/L + IG 2.5mL/L after 15"</i>	16	14	9	0.92	1.38
<i>Aux 2mL/L + IG 10mL/L after 15"</i>	27	23	17	1.53	2.68
<i>Aux 2mL/L + HE 2mL/L after 15"</i>	11	10.5	9	1.48	1.35
<i>HE 2mL/L + Aux 2mL /L after 15"</i>	12.5	10.5	8	0.71	0.65
<i>HE + Aux 2mL/L same time</i>	11	10.25	9	0.89	0.94