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TOWARDS A FURTHER BIOCHEMISTRY OF SILICON

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Abstract

Silica plays an important role in the colloidal stabilisation of commercially produced beer, i.e in the treatment to reduce the tendency of a beer to form a visible haze on cooling or extended storage. The aims of this research wre to further the understanding of the mechanisms by which these hazes are formed and to clarify the role of silica in reduction of haze-forming potential of a beer.

Research was carried out to examine whether or not the raw materials used would themselves contribute silica to the brew, resulting in haze formation. Much time was devoted to developing a reliable method for measuring total silica content within fractions of plant cell wall material extracted from the raw materials provided for use during the course of this research. Graphite furnace atomic absorption spectroscopy and inductively coupled plasma atomic emission spectroscopy techniques were employed and after assessment it was shown that the latter is the more reliable technique for silicon analysis due to the problems encountered with the reactions occurring in the furnace tube during analysis using the former technique with these samples. *Equisetum telmatia*, a heavily silicified plant, was investigated similarly to ascertain whether or not any silica is bound within the pectic & hemicellulosic plant cell wall fractions. It was shown that there was indeed silica bound within these fractions and following specific application of chemical and enzymatic degradation techniques it was possible to release previously bound silica into a form detectable by the molybdenum blue assay. The work suggested that the mechanism of binding for the bound silica mirrored that during colloidal stabilisation of beer.

It became clear following this work that the raw materials of brewing would not contribute a significant amount of silica to the brew, and would be unlikely to contribute to haze formation as a result of contributing silica to the brew. Studies were then carried out to assess the proteinaceous material incorporated into the beer as a result of the brewing process and the interactions with a range of silicas.

Beer proteins can be broadly categorised into two main categories – haze active and foam positive. The foam positive proteins derive largely from globular proteins and their foam-stabilising action has been attributed to interactions between protein side chain amino acids and iso-alpha acids resulting in the formation of non-polar, surface active compounds which form stable foams. Haze active proteins are typically rich in proline and glutamine and are a major component of beer haze, which also comprises carbohydrate and polyphenolic compounds, largely dimers of catechin and epicatechin.

In order to investigate these materials, model extractions of proteins, carbohydrates and proanthocyanidin materials were carried out under conditions modelling those of the brewhouse. The extracted materials were further investigated by amino acid analysis and by addition to a tris-catecholato silicon (IV) model system to investigate the interaction of these materials with reactive silica. These studies showed that the extracted proteins interacted with the reactive silica, affecting the kinetics of deposition of silica within the system.

Electrophoresis of these extracted materials carried out showed that the molecular weight of protein materials incorporated into solution was broadly similar for a variety of malts and a commercial brew provided for investigation. A number of protein assays were also investigated for their usefulness in assessing protein levels of beer. The several common protein assays were shown to have a limited response to beer proteins and shown to under-represent haze active proteins.

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Chapter 1 - Introduction

At the outset of this project a number of aims were set out for the work. Initially, it was proposed to investigate the plant material used in the production of beer, including the development of chill hazes and to understand the role of silica released into the system from these materials. Further study revealed that the silicon contained within plants such as barley and hops was unlikely to play a significant role in the production of storage or chill hazes as the amount contributed is relatively small and the silica produced by solubilisation from the plant materials would lack the specificity of the adsorbents commercially employed to extract undesirable haze active protein material. The primary aim of this part of the proposed research then shifted toward understanding the interaction between silica as a colloidal stability agent and the protein mixture present in beer.

It was also hoped to develop a model system for beer to carry out trial experiments, though due to the nature of the complexity of beer it proved impossible to come up with a satisfactory composition for a model. Model studies of silica precipitation in the presence of selected amino acids and polypeptides and polyphenols extracted from beer at various stages of preparation were carried out in order to improve our understanding of interaction between these species and silicas in the form of reactive silica generated in the tris catecholato potassium model system and with industrial silica absorbents.

1.1. Introduction to Beer Haze

In order to understand the mechanisms involved in the formation of hazes in beer, it is necessary to possess an understanding of the brewing process and the ways in which the various components of beer are imparted to the brew (Bamforth, 2000). A haze can be simply defined as a cloudiness of beer made up of solid materials suspended in solution (Siebert; McMurrough). There are different classes of haze, depending on the causes of the perceived cloudiness and methods of quantifying the relative amount of haze in a brew also exist (Mundy, 1999). Haze is broadly classified as being either biological (i.e. microbial) or non-biological. Within the classification of non-biological haze there are several different types of haze according to their cause. Typical causes of haze include the combination and precipitation of protein-polyphenol complexes (chill haze), β-glucans from inadequately modified malt (which are known to cause 'invisible' or 'pseudo' hazes), starch, pentosans (from wheat-derived adjuncts), oxalate (this leads to precipitation of calcium oxalate as a solid, so called 'beer stones') and other things such as can-lid lubricants (Bamforth, 1988, 1999). By far the most common cause of haze is the precipitation of protein-polyphenol complexes (McMurrough, 1999). Other workers have carried out research on haze formed by other methods (Kaneda, 1988,1989,1990,1991,1997; Bamforth, 1988). The type of haze relevant to this research programme is this protein-polyphenol chill haze, the reversible haze formed on cooling of beer. Chill haze has been defined as the first manifestation of the loss of colloidal stability of beer (Chapon, 1994), and physically is best described as haziness of beer caused by a combination and precipitation of protein matter and tannin molecules during the secondary process of fermentation which becomes visible when beer is refrigerated too fast, too cold or for too long. It appears at around 0°C and disappears on warming to around 20°C

(Rabin & Forget, 1998). The tendency of a beer to form a haze is defined as its colloidal stability (Bamforth, 1998, 1999). This haze is formed when low molecular weight polyphenols cross-link with protein through weak interactions such as hydrogen bonds. Particle sizes for this type of haze are typically 0.1 to 1.0 μm but polyphenols are prone to polymerisation, and when they interact with proteins after this process they form a 'permanent haze'. Chill haze is important, as it is its formation that leads to the eventual formation of permanent haze (Bamforth, 1999).

1.2. The Brewing Process

Beer can be simply defined as containing four major components – water, alcohol, proteins and polyphenols. These can be further subdivided, in the case of beer proteins into haze active (foam negative) and foam active (haze negative) types (Siebert, 1998). Similarly, polyphenols can be divided into flavour molecules and easily oxidised haze active polyphenols (Huige, 1993). This makes reference to the tendency of a protein to either form haze or stabilise foam and also to the tendency of a polyphenol to form haze. The most important polyphenols in beer can be classified as simple flavanols, polymeric flavanols and complex flavanols. Simple flavanols include (+)-catechin, (-)-epicatechin, dimeric flavanols and trimeric flavanols, polymeric flavanols are the 'oxidation' products of simple flavanols and complex flavanols are water-soluble associations between polypeptides and tannins.

Although beer can be simply defined according to the terms used previously it should be remembered that in fact beer is a highly complex mixture, containing over 400 different compounds that have been characterised to date. These include macromolecules such as proteins, nucleic acids, carbohydrates and lipids. Some of the constituents of beer are derived from the raw materials and survive the brewing process unchanged whereas others are the result of chemical and biochemical transformation during the malting, mashing, boiling, fermentation and conditioning processes. It is the combination of all these constituents that make up the character of beer but in general, different beers and lagers contain different proportions of the same compounds rather than novel constituents.

The simplest possible definition of the brewing process is to state that beer is produced as a result of alcoholic fermentation by yeast of extracts of malted barley. In order to understand the mechanisms behind, and reasons for, the stabilisation of beer, it is

necessary to understand the chemical processes involved in the production of beer. The processes involved are complex, particularly the chemistry of barley germination and the essential oils and resins supplied to the brew from hops. The following is an explanation of the major processes in the brewing operation. The information given here has been drawn from a number of sources (Bamforth, 1998; Rabin & Forget, 1998; Hough, 1985; Hough & Briggs, 1981 & 1982; Varman & Sutherland, 1994).

Brewing can be summarised into the following operations:

1. Malting the barley.
2. Milling the malted barley to obtain the grist.
3. Adding warm water to the grist to form the mash. Malt enzymes are encouraged to solubilise the degraded endosperm of the ground malt.
4. Separating the wort from the spent grain and sparging the mash.
5. Boiling the wort with hops to stop enzyme action, sterilising the wort, coagulating certain proteins and imparting distinctive flavours and aromas to the wort from hops.
6. Clarification, cooling and aeration of the wort to provide an ideal medium for yeast growth and fermentation.
7. Fermenting the wort with yeast to convert much of the carbohydrate into alcohol and carbon dioxide. Other yeast metabolites contribute to flavour and aroma.
8. Maturing and clarifying the beer.
9. Packaging the beer, usually after it has been sterile-filtered or pasteurised.

1.2.1. Malting and Milling

Barley starch supplies most of the fermentable sugar from which the alcohol in beer is derived. The starch is enclosed in the form of granules in a protein matrix surrounded by relatively thin cell walls. Malting degrades the cell walls by hydrolysis and breaks down much of the protein within the grain by limited germination. The germination, or modification as it is termed, is carried out for long enough that the cell walls and protein are degraded, and the starch-degrading enzymes required later in the process are synthesised, but not long enough for excessive growth of the embryo to occur as this would use up parts of the starchy reserve that the brewer requires. This process also softens the grain for milling. The malting commences with steeping of barley in water at 14-18°C for up to 48 hours, until the grain reaches a moisture content of 42-46%. Most steep tanks are shallow sided, flat-bottomed vertical cylinders. Air is introduced to the steep water via either perforated pipes or is pulled through the water by suction. The steep tank permits aerobic conditions to be present in the steep water.

When the barley grains are first immersed into the steep water the moisture content of the grain rises sharply but the rate of increase slows down progressively with time. The steeping is interrupted after 12-24 hours by draining the steep water. Each barley grain remains covered with a thin film of liquid through which air can permeate, a condition known as air-rest. The drained steep water is disposed of, although as it contains a large amount of dissolved and suspended solid organic material (e.g. grain dust etc.) it must be treated as effluent prior to discharge.

Following a few hours air-rest the barley is re-immersed in a second steep water. Raising the moisture content of the barley enables the grain to germinate, a process that usually takes 3-5 days at 16-20°C. The aim of germination is to produce enzymes capable

of hydrolysing the cell walls, protein and starch and to ensure that these soften the steely endosperm by removing the cell walls and about half the protein, whilst leaving the bulk of the starch behind. The cell walls of barley contain two major polysaccharides, β-glucans and pentosans. β-glucan, which accounts for some 75% of the wall, is a straight chain polymer of glucose, similar to amylose. The difference between β-glucans and amylose lies in the way the glucose units are joined together. This means that the β-glucans require a different set of enzymes to be produced than those responsible for starch breakdown, and also that their physicochemical properties are markedly different. Pentosan is a hemicellulose material present in cereals and other types of plant material. These yield pentose (sugars with 5 carbon atoms). Traditionally, the grain was spread on a malting floor at a depth of approximately 10cm and maintained at a temperature of 13-16°C. If the temperature of the grain is required to be increased the piles would be manually made larger, i.e. deeper, and vice versa for a required reduction in temperature. Modern maltings use mechanical methods, which do not require the labour intensity of the malting floor and allow a far greater volume of malt to be produced. Following malting, germination is halted by kilning and progressively raising the temperature from 50°C to around 110°C. The malt is dried to 4% moisture content and the malt stabilised, germination stops and the enzymatic digestion processes halted. It is important not to destroy the enzymes, although certain of the enzymes are heat-sensitive, as they are required to generate fermentable sugars in the mashing stage. The initial stage of drying is termed 'free drying' where heated air flows through the grain bed at flow rates up to 6,000 $m^3min^{-1}t^{-1}$(where t=tonnes). At this stage, the temperature of the air entering the grain bed (the 'air-on' temperature) is approximately 50-60°C. This lowers the moisture content of the grains to approximately 23% by evaporation. The remaining water is more resistant to

being driven off from the bed and is largely associated with grain components. Because the water is not being easily volatilised the temperature of the air leaving the grain bed (the 'air-off' temperature) rises. This is due to the loss of the cooling effect caused by the evaporation of water from the grain bed. At this stage, the air-on temperature is increased, leading to a further reduction in the moisture content of the grain from 23% to approximately 12%. The remaining water is firmly bound within the grain and the air-on temperature is further raised until the water content is approximately 6%. Following this, the final stage, the airing phase, is used to lower the moisture content level to the final specification required, typically less than 4%. During the airing phase the air-on temperature may be anywhere between 75°C and 110°C, a figure dependent on the type of malt required. The use of lower temperatures produces malt light in colour, typically for use in lager style beers. Higher temperatures produce a darker malt colouration and create a markedly different flavour spectrum than the low temperature malts. Speciality malts, such as Crystal, Black and Chocolate, are produced for use in small quantities by the brewer to produce colour and a distinct type of flavour. Examples of the conditions used to produce such malts are given in the table below:

Table 1.1: Production Regimes for Some Coloured Malts

Type	Colour (°EBC)	Production Regime
Crystal	75-300	The surface moisture is dried off at 50°C before stewing over 40 minutes with the temperature increased to 100°C, followed by curing it at 135°C for less than two hours.
Chocolate	500-1200	Lager malt is roasted by taking temperature from 75°C-150°C over 1 hour, before allowing temp. to rise to 220°C.
Black	1200-1400	Similar to chocolate malt, but roasting is even more intense.

Reproduced from C.W. Bamforth, *Beer: Tap Into the Art and Science of Brewing*, Plenum Press, NY, 1998.

In the brewery the malted grain must first be milled to produce relatively fine particles (grist). These obtained particles, which are for the most part starch, are mixed with hot water in a process called mashing. It is important that milling keeps the barley husk as intact as possible whilst the endosperm is ground to fine particles which readily release their extract. If the husks of the grains are badly damaged they are less effective in forming a permeable filter bed during recovery of wort from the solution of sugars and proteins produced during the mash. The broken husk also releases more undesirable materials to the brew than the intact husk.

1.2.2. Mashing and Wort Production

Mashing is the enzymatic stage of the brewery operation and following milling the grain is mixed with water, which enables the enzymes to start acting. It is important to ensure the particles are efficiently hydrated, and this is aided by mixing provided by 'rousers' within the mash vessel. When mixing it is important to minimise the physical damage to the particles, particularly the barley husks, and also to be aware of the intake of oxygen into the mash. It is known that intake of oxygen at this stage can promote staling of the beer, and so is to be avoided with careful mixing. The water itself must possess the right chemical characteristics, i.e. mineral salt composition, which varies according to the brewers' requirements. Apart from obvious requirements, such as absence of taints and hazardous materials, the calcium content in water is of great importance. Top fermented ales require a comparatively high level of calcium, for example the water used by traditional brewers in Burton-on-Trent contains approximately 350ppm compared with the bottom fermenting lagers brewed in Pilsen which have a calcium level of less than 10ppm. Adjustment of the mineral concentrations in water to a similar composition to that used in Burton is a process known as 'Burtonisation' and in order to match the composition used in Pilsen de-ionisation of water is required. The calcium in the water (sometimes referred to by brewers as 'liquor') plays several roles. Firstly, it promotes the action of the enzyme α-amylase, a diastatic enzyme produced by malting barley. This enzyme converts soluble malt starch to dextrins and maltotriose during mashing, and work best at a pH of 5.6-5.8 units. Dextrin itself is an unfermentable carbohydrate, which contributes to the final gravity and body of the beer. Secondly, the calcium in the brewing water reacts with phosphate from the malt to lower the pH to the appropriate level for brewing, in the pH 5.6 region. The pH is lowered later in the process by the action of the yeast to around 4.2 pH

units. Thirdly, the calcium ions precipitate oxalic acid, naturally occurring in malt, preventing the later formation of calcium oxalate, also known as 'beer stones' and causing problems such as blockages in dispense pipes. Finally, the calcium promotes flocculation of yeast. Flocculation is the phenomenon by which yeast cells aggregate into masses and sink to the bottom of the brew towards the end of the fermentation process, contributing to clarification of the brew.

Typically, a mash will have a thickness of three parts water to one part malt. Mashing begins at a relatively low temperature, approximately 50°C, to enable the heat-sensitive enzymes to work, which includes the enzymes responsible for degrading any cell wall polysaccharides that survive the malting process. After about twenty minutes the temperature is raised to approximately 65°C. It is at this temperature that starch is gelatinised, a process that can be likened to melting. It involves the conversion of starch from a crystalline, difficult to digest structure into a disorganised state readily accessible to the amylase enzymes responsible for converting it into fermentable sugars. These include α-amylase, mentioned earlier and β-amylase, which is responsible for the conversion of dextrins and soluble starches into maltose, maltotriose, glucose and α-limit dextrins. The enzyme functions optimally in the temperature range 57-66°C and is destroyed at 75°C. The mash is held ~65°C for an hour before it is raised to 76°C, which stops most enzymatic activity and reduces viscosity, improving the fluidity of the mash.

The sugar solution produced by this process is known as 'wort', or more specifically, sweet wort (the term bitter wort applies to the bittersweet sugar solution produced after wort boiling and the addition of hops to the brew). The wort is separated from the spent grains in a vessel called the *lauter tun*. The aim of lautering is to recover as clear a wort as possible (bright wort) containing as much as possible of the soluble

products of mashing (the sum total of which is called the extract) in a relatively concentrated form – so-called high gravity wort. To assist in the washing of the breakdown products (carbohydrates and protein) from the mash, hot water is used to sparge the grains, i.e. wash the sugar solution from the grains.

1.2.3. Wort Boiling and Hopping

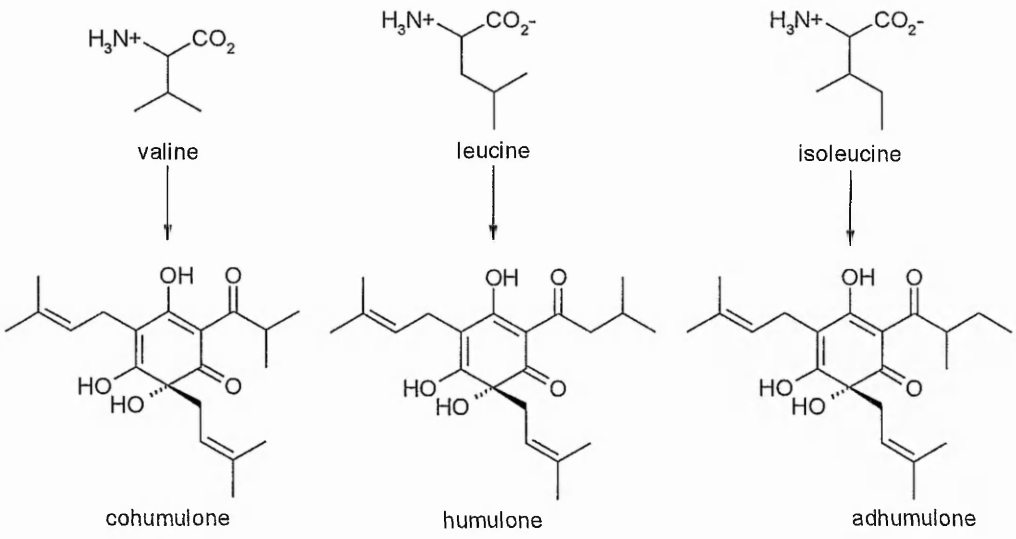
The wort flows directly from the lauter tun to the kettle where it is boiled, usually for one hour. There are several reasons for this process. The major reason is the extraction of bittering materials and aroma components from hops. Traditionally, whole hop cones are added although it is common for hops to have been previously processed and to be used in a pre-milled and pelletised form. If this is the case no vegetative matter survives. If whole cones are used the hop residue remains after the boil and forms the so-called 'hop back' to form the filter medium through which the bittered wort is separated, similar to the barley husks in sweet wort filtration. If pelletised hops are being used the next stage is the whirlpool which makes use of centripetal forces to clarify the wort. Boiling also stabilises the wort and causes the precipitation of certain undesirable proteins. It is at this stage that some brewers add adjunct sugars, an extra source of fermentable sugars for the fermentation process.

Hops contain resins and essential oils that are extracted in the boil. The resin fraction of hops contains several components of complex chemistry but brewers only consider the α-acids as significant as it is these molecules which impart the bitterness required by the brewer to the brew. These molecules, also known as humulones, can make up as little as 2% of the weight of the dry hop or as much as 15%. High α-acid content hops, such as the *Northern Brewer* variety, require only a small amount to be added to the brew to impart bitterness, but also make a proportionally lower contribution of essential oils, i.e. give a lower aroma potential. Use of a lower α-acid content hop, such as Fuggles, requires larger proportions, relative to the high α-acids species, to achieve a desired bitterness, which in turn leads to greater aroma potential. Unfortunately, use of these hops leads to a higher proportion of undesirable materials such as tannins in the brew, giving

the use of high α-acid varieties the advantage of imparting less undesirable material linked to the formation of haze from the hops into the brew.

The α-acids are synthesised from the amino acids valine, leucine and isoleucine in the growing hop.

Figure 1.1: The α-Acids and Their Precursors

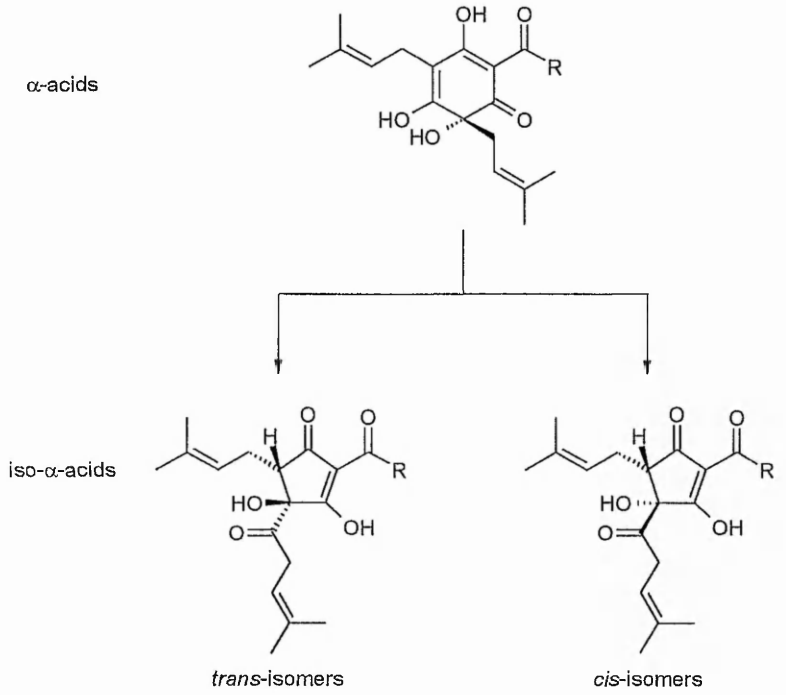


When wort is boiled in the kettle the α-acids are rearranged (isomerised) to form iso-α-acids. This rearrangement typically takes between thirty and ninety minutes, though this may vary as the solubility of the α-acids varies according the wort gravity. These products are much more soluble than the α-acids, and are more bitter. At the end of the boiling any unmodified α-acids are lost with the spent hop material, leaving only iso-α-acids. This is not a particularly efficient process with no more than 50% of the α-acids being isomerised and less than 25% of the overall bittering potential of the hops actually being incorporated into the beer. Modification of the α-acids leads to the formation of two

isomers (cis and trans) of each of the α-acids. The six α-acids differ in the quality and intensity of their bitterness.

For commercial use, iso-humulones are sometimes added in the form of isomerised extracts after fermentation, which is discussed later in this chapter.

Figure 1.2: The Isomerisation of α-Acids

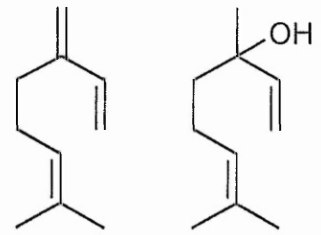


Also incorporated into the beer at this stage are the 'essential oils'. The oil component of the hop ranges from 0.03% to 3% of the weight of the hop, with seedless varieties containing a higher percentage of oil. The oils are produced in the hop late in ripening after the majority of the resin has been formed. These oils are responsible for the characteristic 'hoppy' aroma associated with beer. The oils are very volatile, and if all the hops are added to the brew at the start of the boil most of the oils will be lost by volatilization from the boiling wort. In traditional lager brewing a handful of hops are

held back and added late in the boil, a practice known as late hopping, to ensure that some of these essential oils remain in the brew. In traditional ale brewing a handful of hops is added to the cask at the end of the brewing process, enabling a complete mixture of oils to give the beer produced its distinctive character.

The oils are a complex mixture of over 300 compounds with no clear relationship yet having been established between the chemical composition of the oils and the aroma characteristics imparted to the beer. In general, it is thought that myrcene, the major hydrocarbon in the oil, is an undesirable feature of the oil, whilst components such as linalool and geraniol offer attractive aroma notes, figure 3.

Figure 1.3: Structure of Some of the Essential Oils from Hops



The use of whole hop cones in modern brewing is relatively rare, and, as mentioned earlier the most common procedure for hopping is to add hammer-milled and pelletised hops.

As a result of the inefficient utilization of the α-acids in the wort boiling, a great deal of research has been carried out into extracting the essential oils and resins from hops and the modification of the α-acids. Liquid carbon dioxide is used to extract the oils and resins, and the α-acids can be isomerised in the CO_2 by either chemical means or the use

of light. These pre-isomerised extracts can be used to add bitterness directly to the finished beer, which is a far more efficient use of the bittering compounds as a result of the greater extent of isomerisation of the α-acids by this route.

Apart from incorporating the hop components into the brew, wort boiling concentrates the wort (depending on the rate of evaporation allowed, typically varied between 4% and 12%), drives off unwanted flavour molecules (due to their volatility), deactivates any surviving enzymes from the mashing and wort separation stages and sterilises the wort. Boiling also coagulates much of the protein from the malt, a process promoted by tannins extracted from malt and hops. This precipitate, known as 'trub' or 'hot break', removes a large amount of material with the potential to form haze in the finished brew.

1.2.4. Wort Clarification

Following wort boiling, most breweries employ a whirlpool stage, particularly if the brewer has employed pre-processed hops or hop extracts. The wort is passed tangentially from the kettle into a large vessel and allowed to swirl for approximately an hour or so. The centrifuge is typically a cylindrical tank 5m in diameter and the trub collects into a conical pile at the centre of the vessel by centripetal force, leaving 'bright' wort above it. The wort is drawn off through pipes at the base of the vessel to avoid disturbing the trub. Following clarification of the wort, the material must cooled before the yeast can be added. For mainland European lager brewing the wort is cooled to approximately 6°C, and for English ale brewing to perhaps 15-20°C. This is typically done using plate heat exchangers. Once the wort is cooled, more proteins may precipitate out of solution, the so-called 'cold break'. These solids also include a certain amount of lipids. The final stage before fermentation is the introduction of oxygen into the wort, which the yeast requires for growth. Although the fermentation process leading to the production of alcohol is anaerobic, yeast requires a certain amount of oxygen to produce certain parts of its cell membrane and allow it to grow.

1.2.5. Fermentation

There are two general types of yeast used in the brewing process, top fermenters and bottom fermenters. These are named because of their respective tendencies to either float to the top or sink to the bottom during fermentation. Top fermenters are traditionally used for ale brewing in the UK, and bottom fermenters are associated with the production of lager-style beers. Although the traditional fermenting systems are still in use the most common system is the cylindroconical tank, within which the distinction between flotation characteristics of the yeast become blurred.

Fermentation is primarily concerned with the conversion of sugars into alcohol and the rate at which this occurs is in direct proportion to the temperature and to how much yeast is 'pitched' into the fermenter. Ale fermentations at 20°C are usually complete within a couple of days whereas lager fermentations at 6°C can take several weeks.

Fermentation is about more than just the production of alcohol, otherwise the temperature could be ramped and the process accelerated. This process is also about producing a subtle mix of flavour compounds and the balance of these compounds will depend upon the yeast strain(s) (*sacchromyces cerevisiae*) used. Brewers tend not to divulge much information on the yeast strains that they use, as it is these that are largely responsible for the particular taste characteristics of their product.

Most substances of the wort diffuse freely through the yeast cell wall although some (such as hop resins, proteins and polyphenols) tend to adsorb onto the outer surface of the cell wall. The yeast uses the sugar in wort by an anaerobic fermentative process to convert it into carbon dioxide and ethanol, releasing energy:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy}$$

The situation in brewing is that the yeast is not being grown but rather used purely to produce alcohol. This is diametrically opposite to the approach taken to produce yeast commercially for baking, where a small amount of sugar present in the presence of oxygen encourages respiration:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_20 + \text{energy}$$

Respiration produces approximately 14 times as much energy as fermentation per molecule of glucose and the yield of yeast from this process is high. The brewer wishes very little yeast to be grown, as this uses up sugar from the brew, which would otherwise be fermented to produce alcohol. As there is always a certain amount of oxygen available (as explained earlier, the yeast requires a certain amount to be present) there will be a small amount of yeast produced but the high sugar-low oxygen environment of the fermenter will lead to the production of alcohol rather than further yeast growth. The brewery fermentation could be described more accurately in the form of the following equation:

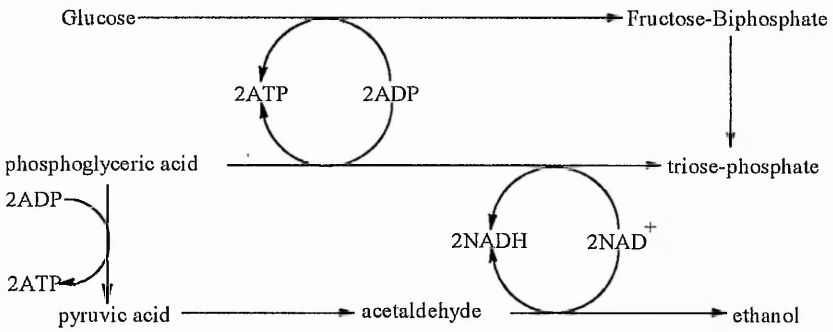
Maltose (100g) + amino acid (0.5g) $\rightarrow$ yeast (5g) + EtOH (48.8g) + CO_2 (46.8g) + 209 kJ

This equation relates more directly to the brewhouse operation than the previous expression as this fails to consider the question of yeast growth and the production of other metabolites such as lactic acid, glycerol, and succinic acid, albeit in relatively small quantities.

Yeast is capable of fermenting a wide range of sugars including sucrose, glucose, fructose, galactose, mannose and maltotriose. Production of ethanol, the major fermentation end product, and the most important to the brewer, occurs by the aerobic

formation of pyruvate via the Embden-Meyerhof pathway and the subsequent anaerobic decarboxylation of pyruvate to acetaldehyde. Finally, acetaldehyde is reduced to ethanol with concomitant reoxidation of NADH (the reduced form of the cofactor nicotinamide dinucleotide). This can be simply shown by the following diagram:

Figure 1.4: The production of ethanol



Following fermentation the brewer is left with a 'green' beer with alcoholic strength in direct proportion to the concentration and fermentability of the sugars in the wort. At this stage, two products can be obtained from an all-malt wort, either beer or scotch. Scotch is produced from distillation of unhopped beer.

1.2.6. Maturation

Maturation is considered to include all the transformations between the end of primary fermentation and the final filtration of the beer. These include carbonation by fermentation of residual sugars, removal of excess yeast, adsorption of various non-volatiles onto the surface of the yeast and their subsequent removal, precipitation of haze-forming complexes and progressive changes in aroma and flavour. The precipitation of haze-forming complexes is discussed in detail later in this chapter.

Ale-type beer is traditionally conditioned for 1-2 weeks at 12-20°C either in bulk tanks or in individual casks with controlled venting. At this stage finings are added to the

cask to give a clear beer. The method of action and required characteristics of a fining material has been researched (Leather, 1998; Freeman & Baron, 2002). Traditionally, it is at this stage that hops with a good 'nose' or aroma were added to the beer – a process known as 'late hopping'.

1.3. Haze Formation

The exact mechanism of haze formation remains uncertain but is known to be a result of the combination of proline-containing polypeptides and polyphenols. The proline rich polypeptide fragments originate from the hordein fraction of barley and the polyphenols are dimeric forms of flavanol species found in beer. Free amino acids do not appear to bind to polyphenols (Siebert, 1996) and this work will later discuss interactions between free amino acids and reactive silica.

Initial formation haze normally occurs as a result of chilling the brew (around 0°C) which dissolves upon warming. This haze is believed to be formed through non-covalent bonding which explains the solubility of the complex formed on warming. The particle sizes formed at this stage vary between approximately 0.1 and 1 μm in diameter and can be removed by filtration prior to packaging as long as the brew remains chilled.

Permanent haze forms if chill haze is allowed to remain for an extended period. The covalent bonds will be replaced with covalent bonds which lead to permanent complex formation. The resulting particle size is greater than that of chill haze (~1-10μm). These reactions are catalysed by oxygen and metal ions and the amount of haze increase over time (O'Rourke, 1994). The rate of formation has been shown to be related to the amounts of haze-active polypepetide and polyphenol present.

An equilibrium state has been proposed for the early stages of haze formation (Chapon, 1994) whereby during initial chill haze formation haze precursors an move freely between the free state and the loosely associated chill haze. Once the chill haze converts to the permanent haze no return to the soluble state is possible.

As previously mentioned, the exact mechanism of protein-polyphenol interaction remains unconfirmed but it has been proposed that the two stage process for permanent

haze formation arises due to the requirement for some kind of reaction needing to take place before permanent haze can form (Siebert, 1999). Two mechanisms for this have been proposed, firstly the polymerisation of simple flavanoids to form complex polyphenol structures which will then combine with protein and form haze. Secondly a mechanism proposing oxidation of existing complex polyphenols which then combine with protein to form haze. The second mechanism has been supported by work which found labelled oxygen from beer headspace incorporated into beer haze (Siebert, 1997c)

In order to further consider the mechanisms of haze formation, it is necessary to further understand the composition of the components which lead to its formation.

1.3.1. Haze-Forming Proteins

As little as $2mgl^{-1}$ of protein is sufficient to induce a haze of 1 EBC (European Brewery Convention) unit (Chapon, 1994). Most beers contain rather more than $2mgl^{-1}$ of protein so it is apparent that there is more protein available in beer than is needed to form haze. True proteins are considered to be over 17kD beer protein is technically a mixture of proteins and polypeptides but is normally referred to as protein. The proteins that cause haze in beer have been studied in detail (Asano, 1982) and are thought to originate primarily from the hordein fraction of barley malt although recent work suggests that the reasoning of haze active proteins originating only from the hordein fraction of barley malt and foam active proteins originating only from albumins may be flawed as the foaming proteins will eventually come out of solution as haze but only after precipitation of the hordein derived proteins. Amino acid analyses of the hordein derived haze active proteins showed relatively large amounts of the amino acid proline (Asano 1982). Studies of similar haze inducing proteins from apple juice have shown proline levels to be about 5-16% of the amino acids in the protein (Siebert, 1999a) and approximately 20% for beer.

Initially the protein-polyphenol haze complexes are held together by weak interactions and this 'chill haze' can be dispelled by warming. The mechanism seems to be a non-covalent interaction in which protein molecules are held together by polyphenolic compounds acting as bridges. A model for the formation of haze has been postulated (Siebert, 1996a).

It has been shown that the mole percentage of proline in a protein is linearly related to its' haze-forming ability in a model system with 400mgl^{-1} catechin as the polyphenol (Asano, 1982). Initial research postulated hydrogen and/or hydrophobic bonding as being important (Asano, 1982) with later work showing that interactions were largely hydrophobic in nature. Proline is required for haze-forming activity, in that all polypeptides investigated without proline failed to form a haze and those with proline did form haze to some degree (Siebert, 1996a, 1996b). More recent studies have proposed that as well as the presence of proline providing binding sites for proline-polyphenol interaction, the physical conformation of these proteins as a result of containing high levels of proline residue affects haze activity (Yang, 2001). Cereal storage proteins such as gliadin (relevant to wheat beers) and hordein as well as the proline-rich salivary proteins, contain a proline-rich sequence (typically primarily proline and glutamine residues) of approximately 5-8 residue length. These repeat in tandem and comprise much of the protein.

These proline residues cause the protein to maintain a generally extended conformation, maximising the available binding area. These proteins typically have an open and rod-like structure. Barley hordein proteins exhibit this rod-like structure and are rich in β-turns with a loose spiral conformation. This maximises both hydrophobic interactions and hydrogen bonding. The open structure facilitates access to the potential

binding sites of interior of the protein by the large polyphenols (such as the proanthocyanidins, haze-active polyphenols). Other work has lent support to the theory of the proline-governed physical conformation of these proteins being a critical factor in their haze activity (Hagerman, 1981). This research found that binding of polyphenols to globular proteins (such as the barley albumins) is generally weaker than binding to the loose, random conformation proteins such as the proline-containing proteins of beer.

1.3.2. Haze-Active Polyphenols

Polyphenols in beer originate from both barley and hops and are ubiquitous in higher plants (Doner, 1993). For this work, the term polyphenol is applied to cover all molecules with two or more phenol rings. Beer polyphenols come largely from malt and partly from hops. Polyphenols are responsible for flavour formation and stability in beers as well as being active in the formation of haze.

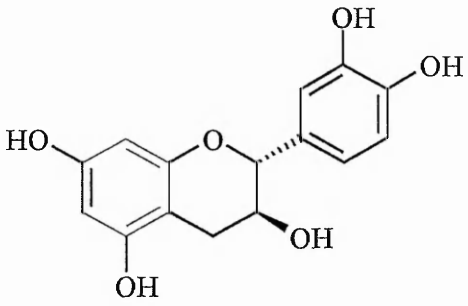
Beers are generally accepted to contain in the region of 100-300mgl^{-1} polyphenol which can be subdivided into two distinct types. The first subdivision comprises derivatives of hydroxybenzoic and hydroxycinnamic acids and the second flavanols and their derivatives. Flavanols comprise ~10% of the total polyphenol content of beer and include those polyphenols related to colloidal instability. Flavanoid polyphenols (i.e. polymeric forms flavanols) all have the same structure of two six-carbon rings linked by a three-carbon unit. These molecules are often hydroxylated to varying degrees and sometimes glycosylated or methylated. (Doner, 1993). Flavanols in beer include catechin, epicatechin, gallocatechin and epigallocatechin which can exist as monomers, but are commonly joined to form flavanoids as dimers, trimers and larger polymers.

The beer polyphenols deemed most responsible for haze formation are proanthocyanidin B3 and prodelphinidin B3 (dimers of catechin and epicatechin) (Arts, 2000; Delcour, 1985; Friedrich, 2000; Lazarus, 1999; Montanari, 1999 & Whittle, 1999) and the levels of these polyphenols in beer has been shown to be directly related to the rate of haze formation (Asano, 1984; Eastmond, 1974; McMurrough, 1992, 1994 & Van Gheluwe, 1979). These species are present at relatively low levels (~3.3% of total beer polyphenols) but account for about 32% of beer flavanoids (McMurrough, 1994). It has also been shown that using proanthocyanidin-free malt produces beer that without any stabilisation is highly resistant to haze formation (Delcour, 1984, 1985 & Erdal, 1986).

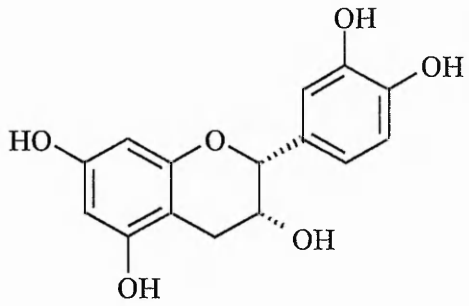
The absence of proanthocyanidins in the seeds of corn, rice and wheat shows that they are not necessary for the development of cereal grains. Varieties of barley without proanthocyanidins have been bred (the variety Galant was the first registered type) and unstabilised beers produced from them have been shown to possess a colloidal stability markedly better than that obtained with traditional methods and unmodified malt (Erdal, 1986). Use of these malts has not been found to be detrimental to the flavour of fresh beer, and it has been shown that proanthocyanidins from hops do not contribute to the flavour of fresh beers (Delcour, 1985) and also do not contribute greatly to the bitterness of beer. The benefits gained from using proanthocyanidin-free malt have to be preserved by using a hopping product that does not contribute undesirable polyphenols to the brew.

The prominent proanthocyanidin species in beer are predominantly procyanidin B3 and prodelphinidin B3, which are dimers of the monomeric proanthocyanidins typically found in beer.

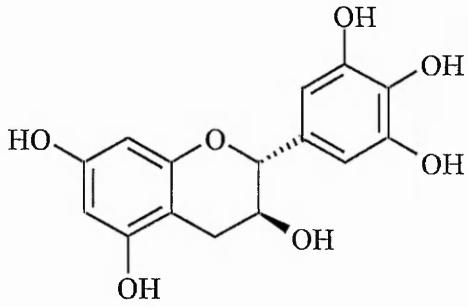
Figure 1.5: Proanthocyanidin monomers typically found in beer



(+)-Catechin

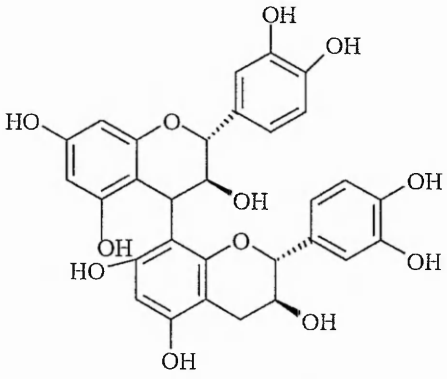


(-)-Epicatechin

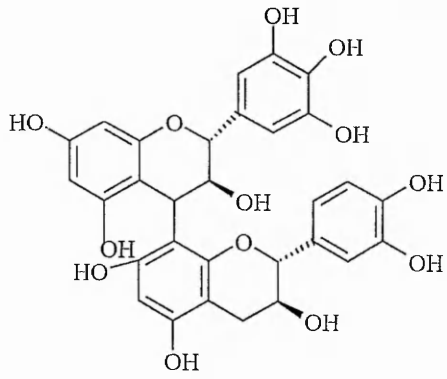


gallocatechin

Figure 1.6: Structures of the prominent proanthocyanidin dimers in beer



Procyanidin B3



Prodelphinidin B3

The term proanthocyanidin derives from the fact that the interflavanoid bonds are readily cleaved by acid and the resulting flavan-3,4 diol-carbenium ions yields anthocyanidins in the presence of traces of oxygen, i.e. prodelphinidin, procyanidin or propelargonin yield delphinidin, cyanidin or pelargonin respectively (McMurrough, 1994).

This class of compound is very susceptible to free-radical reactions, either initiated by oxygen or by plant oxidase/peroxidase enzymes. This leads to cross-linking and re-arrangement of the monomer units to form a more random structural configuration. Barley grains and malt contain (+)-catechin, two dimeric flavanols and four trimeric flavanols based on combinations of (+)-catechin and (+)-gallocatechin (McMurrough, 1983). Contents of catechin between 25mgkg^{-1} and 75mgkg^{-1} in barley have been reported. Levels of the dimers prodelphinidin B3 and procyanidin B3 cover the range 186-362mgkg^{-1} and 130-276mgkg^{-1} respectively. Collectively, levels of the four trimeric species have been found to vary between 336mgkg^{-1} and 671mgkg^{-1} (McMurrough, 1983). Whether or not the trimers survive the brewing process intact is an area of some disagreement. Trimers and other oligomers have been found in some beers (McMurrough, 1983) and not in others (McMurrough, 1992). Prodelphinidin B3, procyanidin B3, (+)-catechin and (-)-epicatechin are measurable in most beers (McMurrough, 1983; McMurrough, 1992; Delcour, 1984; Eastmond, 1974). There is no doubt that the levels of measurable flavanols in beer are far lower than can be extracted from barley or malt. Catechin is more rapidly and completely extracted from ground malt than dimeric flavanols. During mashing and boiling large losses in dimeric proanthocyanidins occur. Heat induced protein precipitation at this stage is accompanied by removal of dissolved polyphenols. In the 'hot break' malt and hop polyphenols are precipitated, but their role in the precipitation is more passive than active, although their role in cold break formation is pronounced. Workers have shown that hop polyphenols exert little influence during hop boiling, do not participate greatly in the reactions with the proteins of the wort and remain mainly in solution (Srogl, 1997). These workers produced several points:

1. Hop polyphenols are easily released to water solution whilst barley polyphenols are not.
2. Barley polyphenols are not released to the wort solution even under boiling or mashing with ground malt
3. Hop polyphenols are released to the wort in significant quantity
4. Malt polyphenols are not released to the solution during mashing and are only released after the addition of some non-malt enzymes
5. Barley polyphenols are apparently transformed into soluble forms during malting

1.3.3. Haze Formation

The actual mechanism of haze formation has been proposed to occur in several ways. Siebert (1999a) proposes that each polyphenol has two binding sites and each polypeptide three binding sites. The greatest amount of haze will occur when the number of polyphenol binding sites is equal to the number of polypeptide binding sites. In the case of beer, where there are more polypeptide binding sites than polyphenol, two polypeptides could be joined but further binding would be unlikely due to the relative shortage of polyphenol binding sites. For other systems, such as apple juice, where more polyphenol binding sites are available than polypeptide, all the polypeptide binding sites would be filled by polyhenols but no further binding would be likely due to the relative shortage of polypeptides in the system.

This system clearly gives importance to the ratios of haze-active polyphenol and haze-active polypeptides and suggests that the closer to an equality in binding sites a system is, the larger the complex molecules formed are likely to be. (Siebert 1996a&1996b).

1.4. Beer Stabilisation

The most effective way to reduce the tendency of a beer to form haze, i.e. improve the colloidal stability of the beer, is to reduce the levels of the components which lead to haze formation (Mussche 1999; Narziss, 1992 & O'Rourke 2002). Typically in modern brewing, the levels of sensitive protein are reduced by adsorption with silica hydrogels and the levels of haze-active polyphenols are reduced with the application of polyvinypolypyrrolidone (PVPP).

The following properties are required of a beer stabiliser (Basarova, 1990):

1. Hygenically safe and acceptable
2. Insolubility in beer, alkaline and acid solutions and in currently used solvents
3. A chemical structure that enables sorption of haze-causing substances
4. Textural (morphological) properties of the sorbent that govern the effectiveness and sorbtion specificity (particle dimensions, surface area, diameter and volume of the pores)
5. Minimal undesirable non-selective adsorptive activity towards bitter hop substances, coloured beer compounds, foaming substances and other extract components ensuring the full taste of beer
6. Technological properties warranting minimum beer loss at charge dosing and at dosing during filtration not influencing the process smoothness (including the flow rate, wet volume, bulk weight and filtration resistance)

1.4.1. Silica Gel Protein Adsorbents

Silica hydrogels are used to treat beer against chill and permanent haze. They are added as a slurry (i) in the beer line prior to the conditioning or aging tank, (ii) to the tank directly or (iii) prior to powder filtration (Hough, 1976). Silica gels are produced from sodium silicate. An aqueous solution is added to sulphuric acid and the pH level is maintained below four. The liqour sets to a solid, coherent mass called a hydrogel. Sodium sulphate formed during the reaction is washed out of the gel. If the pH of the hydrogel is increased either at the making stage or by steeping in hot water, the pores contract but there is little change in surface area. This material is called a xerogel.

Both hydrogels and xerogels can be milled to give fine, free flowing powders. For a given pore diameter, hydrogels tend to have larger surface areas than xerogels. Hydrogels typically have water content in the 45-65% range and pore diameters from 25 Ångstroms upwards. Surface areas are high, typically several hundred m^2g^{-1} and can be as high as a $1000m^2g^{-1}$. Average particle size is typically a few microns.

Silica gels absorb proteins over a range of 4,600 to 40,000Da (Hough, 1976). Silica xerogels have been traditionally most commonly used though recent innovations in hydrogel technologies are changing this. Silica sols are also sometimes used, though rarely.

The microstructure and technological properties of silica gels are dependent on the polymerising and condensing processes employed during production and sorbents with a wide range of properties can be produced by varying the rate of adding acid, the reaction temperature, the pH and the washing and milling procedures (Hough, 1976). The sorption capacity of silica gels depends on the particle size, surface area, porosity and the number of free hydroxy groups in the silicic acid aggregate. A compromise must be sought

between the stabilising effect and the particle size effect on filtration conditions. The sorption specificity depends on the distribution of the pores for a given pore volume. With nitrogenous compounds of over 10,000 Da being thought to be responsible for haze formation a pore size in the region 2.5nm is dictated. Peptide sorption proceeds quickly and a contact time of 2-5 minutes at a dosage of up to $100ghl^{-1}$ in beer proves adequate.

The use of silica gel as a specific protein adsorbent in beer has been popularly used for the last thirty-five years. Research into the production of more effective stabilisers has continued (Fernyhough, 1990; Smith, 1990; Matsuzawa, 1991; Ito, 1993; O'Rourke, 1994; Fernyhough, 1994; Guzman, 1996; Nock, 1997; Wannerberger, 1996 & Kondo, 1997).

Silicas hydrogels (SHG's) can be produced via the previously mentioned acid gel route (Basarova, 1990) or by other routes. One such route is the 'precipitate' or alkaline sol route (Fernyhough, 1990). This route involves destabilisation of the complex polysilicic acid anions present in the silicate solution by partial neutralisation of the Na_2O present in the solution. As a result of the high pH and high electrolyte content more complex structuring occurs. This means that absolute control of the mixing regime is essential during the precipitation stage.

Effective stabilisation of beer can be summed up by two basic principles:

1. Selective adsorption and removal of haze-forming proteins due to interactions with active adsorption sites, i.e. isolated surface silanol (SiOH) groups on the amorphous silica particle
2. Selective removal of such components by permeation based on the pore structure and pore diameter distribution.

The binding of proteins to silica gel is considered to be analogous to the binding of haze-forming proteins to polymerised polyphenols (Fernyhough, 1990; Fernyhough, 1994;

Siebert, 1997a; Siebert, 1997b; Siebert, 1996a; Siebert, 1996b; McMurrough, 1995a), i.e. via bonding of protein carbonyl groups to hydroxyl groups on either the polyphenol catechol ring or the silica gel surface. The conditions for both reactions are similar and favour the type of protein in each case. Initially, at least, the adsorption stability is due to the number of bonds between the silica gel surface and the haze-forming protein. In order to fulfil the stabilisation requirements listed earlier it is important that only the 'undesirable' compounds, i.e. the haze-forming components, are removed and the proteins responsible for foam and mouthfeel (body) remain unchanged. If the pores are large then foam retention may be reduced so it follows that the suitability of a silica gel for use as a stabiliser depends on its ability to remove haze-forming proteins and its inability to absorb foam positive proteins.

The exact pore size is the most important physical factor of the silica gel. A minimum pore size is required for the access of haze forming proteins to the inner surface of the gel, whilst a maximum pore size is required to prevent the access of larger foam positive proteins. This restricted pore size means that larger haze particles, perhaps from an intermediate stage in haze formation or a haze particle that has been degraded by enzyme action, will be too large to fit into the gel. These larger components can only be removed by adsorption onto the surface of the gel or by physical entrapment during the filtration. This is the thinking behind the tendency of American breweries to use exceedingly tight filtration beds in order to enhance the colloidal stability of their beers when using silica gel as a chillproofing agent (McKeown, 1999).

Recent work has investigated beers treated with silicas and has shown that treating a beer with silica has little effect on the total protein levels, the level of protein Z (from

albumin) and the level of LTP1 (from hordein); however, the levels of other hordein derived proteins were greatly reduced (Sheehan, 2000).

Different beers vary in their stabilisation requirements for a number of reasons. Some of the main reasons are:

1. Differing malt qualities
2. Product diversification, e.g. the use of specialist malts
3. Varying processing conditions
4. New processing strategies
5. Certain beers have a tendency to form a 'post pasteurisation' haze

Silicas offer a number of different options for difficult to stabilise products:

1. Customised hydrogels and xerogels

   The silica gel may be aged under pre-determined conditions which allows the smaller silica particles to rearrange and produce some larger diameter pores. These will shrink on drying but can be large enough to admit specific haze components from a particular beer.

2. Calcined xerogels

   Calcination of certain xerogels can produce a high proportion of single silanol groups on the silica surface. An overall reduction in the number of silanol groups on the silica occurs by condensation of adjacent silanols and the loss of bound water on heating. Protein adsorption to the surface of the gel is enhanced as single surface silanol groups are responsible for the specific adsorption of a variety of colloidal molecules (Fernyhough, 1994).

3. Co-gels/Stabiliser blends

Specific blends of amorphous silicas or sequential treatments by amorphous silicas differing in pore volume, pore size, surface area and particle size have been found to improve stability for specific beers. Supplementation of amorphous silicas with one or more different solid sorbents which are effective in removing haze precursors from beer is an option for treating certain hard to stabilise beers. Examples include polyvinylpolypyrrolidone (commonly referred to as PVPP), synthetic magnesium, calcium or zinc silicates, or natural clays (e.g. bentonite). As with using silicas, these different materials may be applied as a physical admixture or as sequential treatments. Recent work (Leiper, 2002) has investigated the use of a combination of PVPP and isinglass to stabilise beer. This work, in common with most other research in this area, concluded that the combination of stabilising agents had a beneficial effect on the long-term stability of the resulting beer. The use of mixtures of silica and PVPP gives optimisation of permeability (and therefore permeability of the filter bed) when using a 60% silica/40% PVPP treatment. PVPP and its mode of action have been the subject of a great deal of research (McMurrough 1992, 1994, 1995a, 1996a, 1996b, 1997a, 1997b, 1999).

The use of calcined xerogels or SHG/PVPP mixtures is a considerably more expensive method of colloidal stabilisation than the use of simple silica hydrogels, but is an efficient way of stabilising difficult to stabilise products. The use of silica hydrogels is also thought to improve beer quality through the ability of SHG to remove fatty acids. Fatty acids are progenitors of stale flavours and aldehydes and have a negative effect on foam quality (Fernyhough, 1990). There are also indications that the use of SHG reduces the dissolved oxygen content in the beer. No studies have indicated any detrimental effects of using SHG on beer (Fernyhough, 1994).

Xerogels typically have a high adsorption capacity for haze-forming proteins but tend to be poor in filtration and dusty to handle. SHG's are less dusty and easier to filter. Recent work (Nock, 1997) has concentrated on producing SHG's with the stabilisation capability of a xerogel and the filtration properties of a hydrogel, without the use of additive materials for surface activation, i.e. through the use of pure amorphous silicas. This has been successfully carried out by optimisation of permeability, average particle size and pore diameter. The latest silicas, known as XL (Nock, 1997), have achieved this aim and have a typical average particle size of 10μm, a permeability of 0.15 Darcys and a pore diameter of approximately 80 Angstroms.

1.4.2. PVPP Polyphenol Adsorbents

PVPP has been used commercially as adsorbent for beer phenolics since 1961 (McMurrough, 1995). This additive selectively removes simple flavanoid polyphenols and the products of their oxidation and this product is commonly employed with silica gel to remove haze-active polyphenols from the brew. Although PVPP is expensive, it can be recovered from the brew and prepared for re-use, reducing the overall economic impact of the material to the brewer. Research has shown that the use of PVPP can diminish the levels of simple flavanoid oligomers and tannoids to tolerable levels (McMurrough 1992; 1995a; 1995b; 1997a; 1997b; 1999).

1.5. Equisetum Telmateia Studies

This research was also intended to investigate the silica incorporated in *Equisetum telmatia*, horsetail, as previous studies within the research group had suggested that silica was actually incorporated into certain extractable plant cell wall materials (pectins and

hemicelluloses) in a bound form, rather than being associated with these fractions in an unbound manner. To this end, extraction of these materials, analysis for silicon content and selective digestion by chemical and enzymatic methods was proposed to attempt to identify the location of any bound silica present within these fractions. Earlier work had been carried out (Harrison, 1995) which had identified macromolecular assemblages intimately associated with biogenic silica in plants that was released on solubilisation by treatment with HF following treatment to remove cytoplasmic cell contents and the highly polysaccharidic cell wall. This amino acid composition of the obtained material was rich in Pro-Glu, Pro-Lys and Ser-Asp-Gly sequences. The high proline level, and similarity with HA proteins as identified by Siebert, suggests a similar mechanism of interaction for these associated silicas with the plant cell wall and the mechanism by which proline and silicas associate in chillproofing for beers. As a result of this, studies were carried out to investigate the location of these bound silicas within the plant cell wall and to begin to understand the mechanism of association to compare it to the associations observed in beer. The experiments previously carried out had effectively stripped much of the organic material from the silica with left the sample difficult to test further. A different approach was proposed, to fractionate the organic material and further analyse these fractionated pectic and hemi-cellulosic materials for the presence of silica. Following confirmation of the presence of bound silica it was proposed that chemical and enzymatic digestion methods would be applied to the samples to attempt to achieve release of the bound silica into solution, identifying the location of the silica and allowing examination of the binding sites and likely method of binding.

Chapter 2 – Experimental Methods

Unless indicated otherwise all experiments were carried out in grade 'A' glassware. Solutions were prepared using distilled deionised water obtained from a StillPlus apparatus fitted with Purite D330 cartridges. Any dispensed volumes were measured using Gilson pipettes.

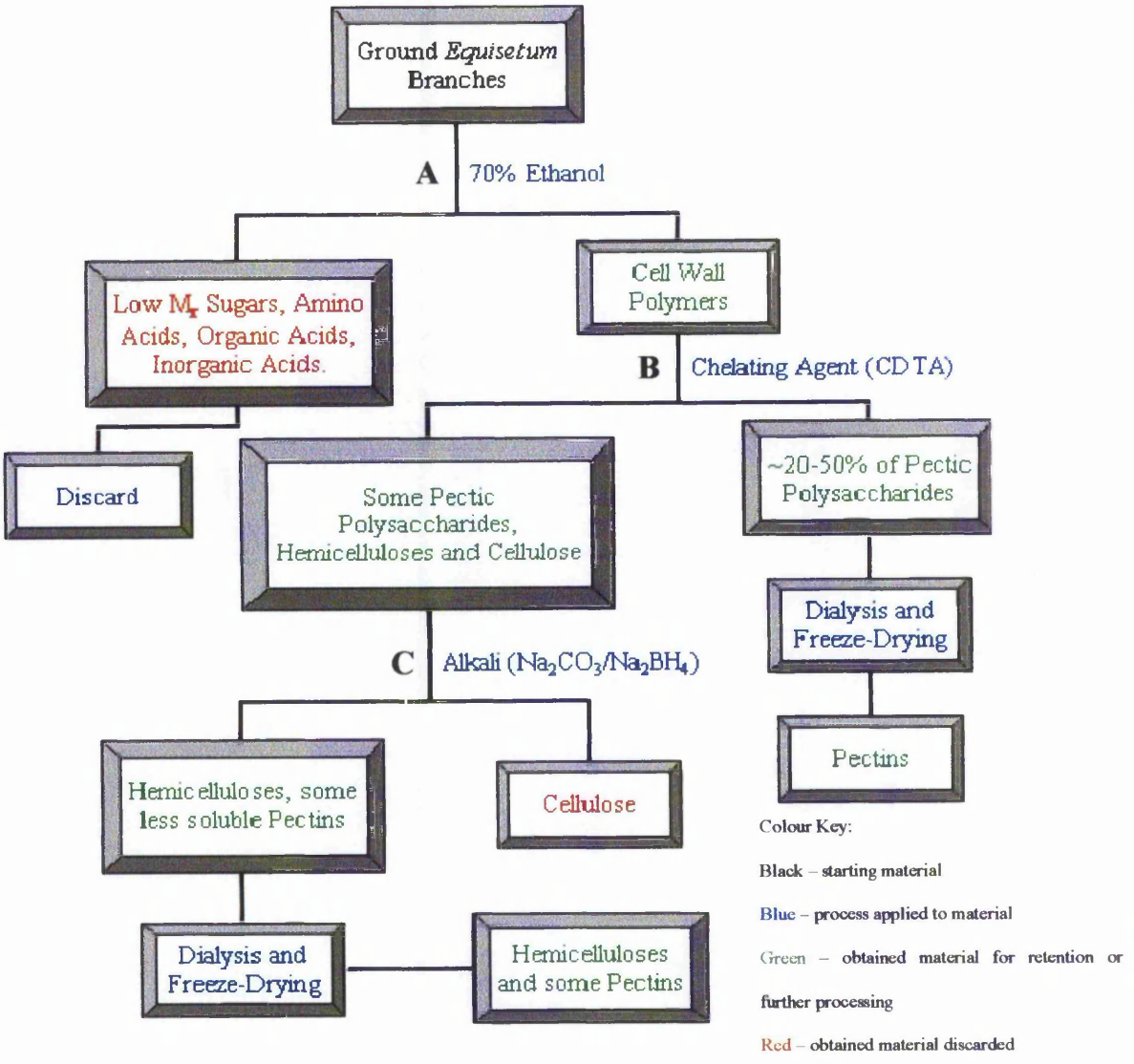
2.1. Chemical Methods

These methods include those employed to extract fractions of plant cell wall materials, and in the case of malted barley and hops, to obtain protein and polyphenol samples to investigate further their composition and their behaviour relative to silica. These also include methods used to digest extracted plant cell wall components into smaller constituents to assess release of bound silica. Methods used for the estimation of protein concentrations in solution are also described here, along with methods used to precipitate proteins from solution to enable extraction and further study. The protocol employed for investigating the interaction of industrially produced silica absorbents with beer samples is also described here.

2.1.1. Scheme of Pectic and Hemi-Cellulosic Material Extractions

These methods described in the scheme below were applied to *Equisetum* material collected from Wytham Woods near Oxford. These extractions enable the plant cell wall to be fractionated by chemical methods in order to obtain the pectic and hemi-cellulosic materials from the plant cell wall. This was carried out to facilitate further investigation into the possibility of silicon being incorporated into the pectic and hemi-cellulosic

fractions of *Equisetum*. Methods used to further investigate these samples are described later in this chapter.



2.1.1.1. Preparation of an Alcohol Insoluble Residue (AIR)

This is represented in the scheme above as stage A. The AIR was prepared by adding cold ethanol (70%) 200ml to approximately 20g of ground plant material. The tissue was vortexed periodically and cooled to 0°C between vortexings. The mixture was stirred at about 0°C for several hours before being filtered. The solid was washed with

further 70% ethanol aliquots and freeze-dried. The dried material was stored in a tightly sealed container to prevent water uptake.

2.1.1.2. CDTA Pectin Extraction

This procedure is represented in the above scheme, labelled B. Use of aqueous chelating agents, such as CDTA (trans-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetate), gradually solubilises pectic polysaccharides. The actual proportion of these materials solubilised varies depending on the plant but is assumed to be approximately 50% of the total pectic material. The efficiency of this extraction can be raised by the application of heat, which results in the solubilisation of more of the pectin but causes additional degradation even at pH values 3-5. For the purposes of these extractions efficiency was improved by repeating the extraction.

Approximately 10g of AIR material was added to 50mmol solution of CDTA. The mixture was left stirring for 12 hours, and then the mixture was centrifuged. The supernatant was stored and the process repeated with the plant material and CDTA solution. The supernatant was retained. The supernatant was then dialysed with distilled de-ionised water in SpectraPor molecularporous membrane at 4°C, with three changes of water per day for approximately two days. The solid material remaining was extracted for a second time as described above.

2.1.1.3. $Na_2CO_3/NaBH_4$ Pectin/Hemicellulose Extraction

This extraction is applied to the remaining solid material following the CDTA extraction above, and is represented on the extraction scheme as route C. Alkalis are usually used following application of a chelating agent to solubilise hemi-celluloses. These are solubilised very effectively by this treatment, which also extracts some of the

pectic material not extracted by the chelating agent. Alkalis cause 'peeling', the stepwise removal of terminal polysaccharides by β-elimination, which can be suppressed using $NaBH_4$.

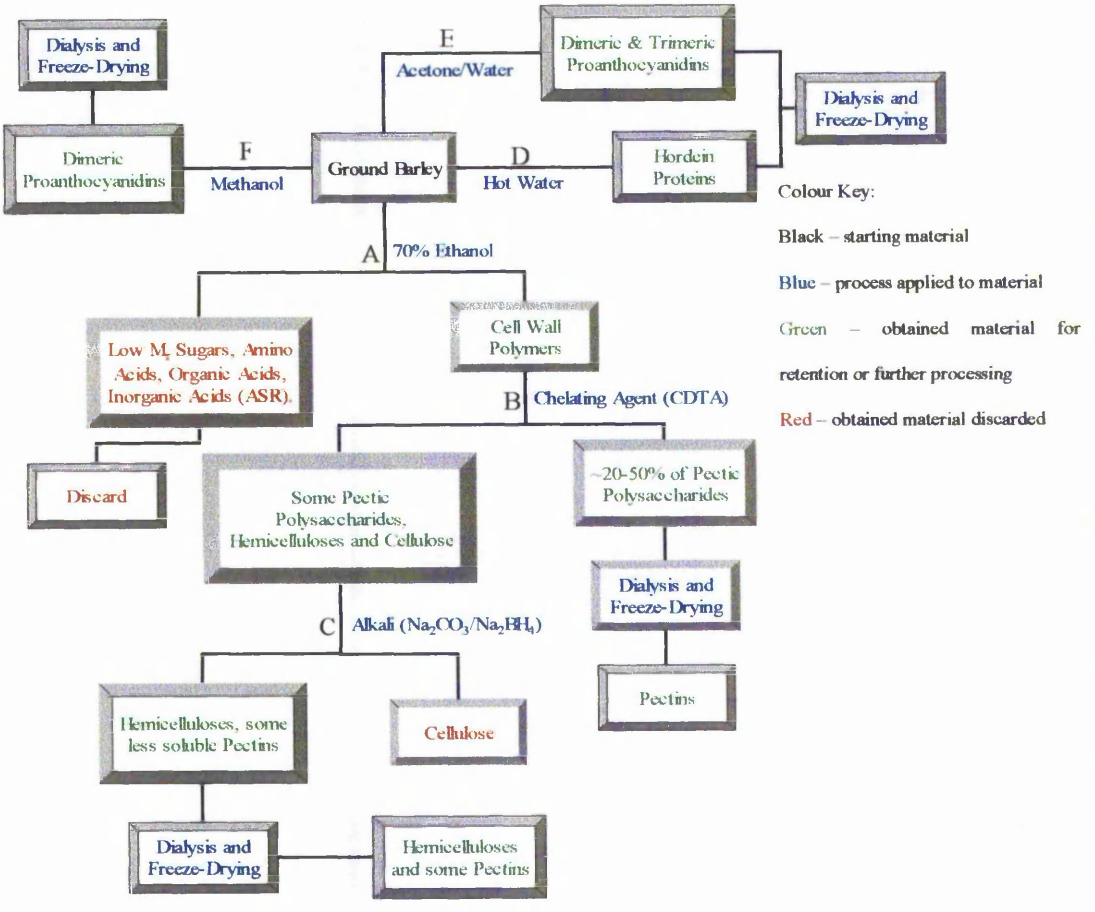
The solid material obtained from the previous extraction was added to a solution of Na_2CO_3 (50 mmol) and $NaBH_4$ (20mmol). The solution was placed in the cold room at 4°C and stirred for 12 hours. The centrifuging and washing process described above was repeated, the supernatant was stored and the extraction process repeated for a second time.

This process extracts some pectic polysaccharides as well as the hemicellulosic materials.

2.1.2. Protein and Polyphenol Extractions

2.1.2.1. Scheme of Malt Extractions

This scheme below represents the extractions carried out on malt material supplied by breweries for this research. This enabled extraction of the pectic and hemicellulosic components of the plant cell walls, as previously described for investiagtion. Also, methods modeling brew-house operations were employed to extract protein from the material in order to try to further understand the mechanism by which haze is formed and beer can be stabilised. Methods developed by other workers were applied to extract polyphenolic materials from the material, specifically proanthocyanidin materials, to further investigate their role.



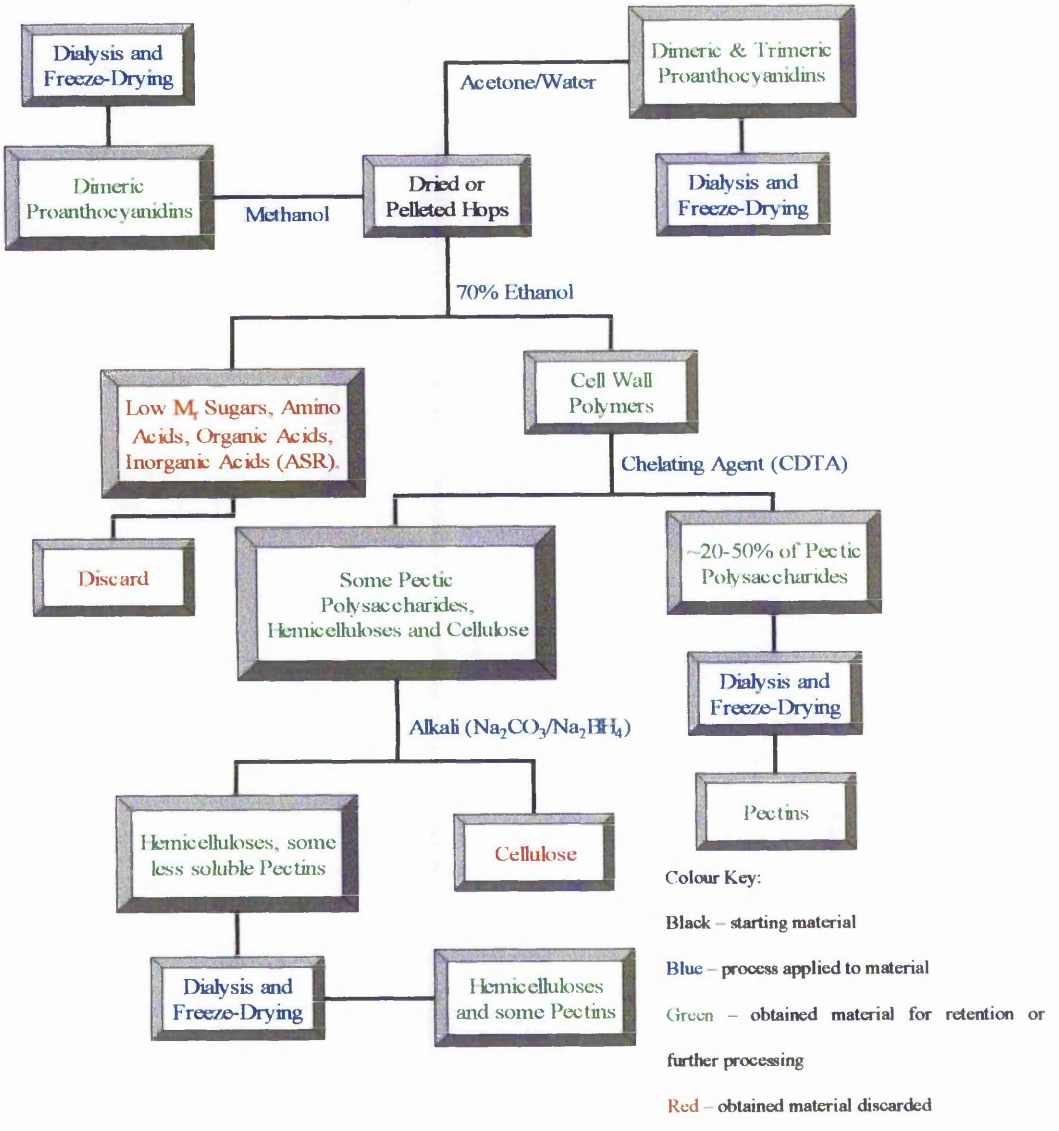
2.1.2.1.1. Protein Extraction – Hot Water Extractions

The protein extraction carried out is shown on the above scheme as D. 50g of milled barley malt (brewery supplied) was added to 200ml of water. The temperature was raised to 70°C and held for an hour. Following heating, the material was cooled and centrifuged to remove solid material from the liquor. This obtained liquor was rich in protein and carbohydrate and is analogous to the wort produced industrially in the brewing process. Freeze-drying using a Christ-α-1-4 sublimation freeze-drier produces a solid material for further experimental use.

Extractions A, B & C were carried out as previously described. The proanthocyanidin extractions are described in the section following this.

2.1.2.2. Scheme of Extractions From Hops

The methods previously described for extractions carried out on *Equisetum* and malted barley were similarly applied to hop material supplied by breweries, as illustrated in the scheme below.



2.1.3. Polyphenol Extraction

2.1.3.1. McMurrough's Method

Dimeric species only:

A 50g sample of malted barley, mill ground for two minutes, or as supplied milled for brewery use, was extracted in 150mL of ethanol in a CO_2 atmosphere for 1 hour. Following extraction the mixture was filtered on sintered glass. The liquid volume of the filtrate was reduced to ~10mL by rotary evaporation at 35°C. The resulting solution was filtered through a 0.4μm filter, freeze-dried and retained.

Dimeric and Trimeric Species:

A 50g sample of malted barley mill ground for two minutes, or as supplied milled for brewery use, was extracted in 150mL of 3:1 acetone: water in a CO_2 atmosphere for 1 hour. Following extraction the mixture was filtered on sintered glass. The obtained filtrate was salted out by the addition of 5g NaCl and shaking for 10 minutes. After standing for 1~2 hours an upper acetone phase was formed which was rotary evaporated to ~7ml at 35°C. The resulting solution was filtered through a 0.4μm filter, freeze-dried and retained.

2.1.3.2. Whittle's Method

This is a modification of the McMurrough method for the extraction of a mixture of dimeric and trimeric polyphenolic species.

Following rotary evaporation, the remainder of the acetone layer was extracted with 3 successive aliquots of diethyl ether. For each extraction, the aqueous layer was retained and the ether layer discarded. The use of ether was found to remove large amounts of catechin (a monomeric polyphenol species) and material which causes yellow colouration of the product, whilst at the same time having little effect on the yield of polyphenols obtained from the extraction.

2.1.4. Digestion Experiments

Chemical and enzymatic agents were applied to samples obtained form plant cell wall material as earlier described. Analyses were carried out at all stages to assess the levels of bound (undetectable by the molybdenum blue assay) and unbound (detectable by the molybdenum blue assay) silica within the solutions under investigation.

2.1.4.1. Acid Digestion

Plant cell wall fractions extracted as previously described were solubilised in 1M and 6M HCl. These samples were then stored overnight at room temperature (1M and 6M) and incubated at 38°C overnight. Following exposure to the acid, the silica content of the sample was measured and compared with the value obtained for a solution of untreated material.

2.1.4.2. Alkali Digestion

Prepared as above but with 1M and 6M NaOH rather than HCl

2.1.4.3. Enzyme Digestion

Samples as described above were treated with solubilised driselase at 38°C overnight and analysed for free silica in the same way as the previous samples.

2.1.5. Protein Precipitation

Several methods were applied to extract protein material from either model prepared solutions or commercial beer materials for further analysis.

2.1.5.1. Ammonium Sulphate Precipitation

To a solution containing protein material to be precipitated ammonium sulphate was added to produce a saturated solution, approximately 4M or 528 gl^{-1}. Gentle stirring was applied to the sample at 4°C for an hour, taking care not to stir too vigorously.

Presence of foam on the sample, if observed, indicated the ammonium sulphate may be denaturing the protein samples (Copeland, 1994).

Following stirring for an hour the sample was centrifuged at 4°C at 15000g. The supernatant was decanted off and discarded. The solid residue was re-suspended in water prior to being dialysed to remove the large amounts of salt remaining within the sample. The protein material was obtained following dialysis by lyophilisation.

2.1.5.2. PEG Precipitation

This method was also employed to precipitate protein from samples under investigation. To 50ml of a sample under investigation, 75ml of 50%w/v of PEG-6000 was added (Ingham, 1984). The beaker was incubated at room temperature and mixed gently for one hour using a shaking water bath. Following this, centrifugation was employed to remove the precipitated material. This material was then suspended in distilled deionised water and dialysed to remove associated unwanted impurities. There was no requirement to remove excess PEG from the samples as it is optically transparent and helps prevent loss of protein by adsorption on glass.

2.1.6. Wort Preparation

The previously described ammonium sulphate precipitation method was selected to precipitate protein material from model wort, prepared in-house by mixing milled grist (as supplied by Castle Rock, Shepherd Neame, Greene King, Smiles and the ICBD) with hot water (liqour in brewing parlance) in a sealed vessel to minimise oxygen uptake of the brew. This process started at 50°C and after 20 minutes the temperature was increased to

65°C and held for an hour. 'Bright' wort was recovered from the mash by filtration. The resulting wort was unboiled and unhopped.

Whilst this is not necessarily an accurate model of the conditions employed by a brewer during production, it provides an approximation and a standard condition to test differing types of malt.

2.1.7. Silica Stabilisation Trials

Silicas supplied by the Crosfield company were added at $100ghl^{-1}$ concentration to all-malt wort supplied by the International Centre for Brewing and Distilling at Heriot-Watt University and produced in their pilot scale brewery.

50ml aliquots of the wort solution were treated with the supplied silicas and placed in a thermostatted water bath at 4°C overnight with shaking. Following treatment the silica was removed by centrifugation (at 4°C) and retained. The supernatant was also retained and assessed for protein content using the Bradford Assay, the BioRad DC Assay and the Pierce BCA Assay. The silica, with adsorbed proteins, was also retained for further treatment. The silica was solubilised by the addition of 1%HF. Following solubilisation, the HF was neutralised and the sample dialysed to remove the salts added as a result of the previous procedure. After dialysis the protein material was obtained from the sample by lyophilisation.

Samples of adsorbed protein as well as samples of the un-adsorbed protein were sent for amino acid analysis.

2.2. Analytical Methods

The methods described in this chapter were used to analyse samples for silicon, analyse protein materials for amino acid composition and molecular weight and to analyse the kinetics of the tris-catecholato silicon model system in the presence of additives to further understand the interaction between reactive silicon and beer constituents.

2.2.1. Methods for Silicon Content Determination

2.2.1.1. The Molybdenum Blue Colorimetric Assay

The sample under investigation was added to 1.5mL of a molybdate solution ($20gL^{-1}$ ammonium molybdate tetrahydrate and $60mL.L^{-1}$ conc. HCl). After ten minutes 7.5mL of a reducing solution ($20gL^{-1}$ oxalic acid, $6.67gL^{-1}$ 4-methylaminophenol sulphate (metol), $4gL^{-1}$ anhydrous sodium sulfite and $100mL^{-1}$ concentrated sulphuric acid) was added to the assay solution and the colour allowed to develop at room temperature for a minimum of two hours before being read at 810nm on a Unicam UV2 UV/Vis Spectrometer. The assay was calibrated against stock solution of silica, prepared by dilution from a 1000ppm standard solution (BDH).

This gives a measure of 'free', i.e. unbound, silica within a sample. Polymers of silica larger than a dimer and any form of bound silica are not detected by this assay.

2.2.1.2. Graphite Furnace Atomic Absorption Spectroscopy (GFAAS)

GFAAS was used to analyse aqueous samples for silicon content. A Perkin Elmer 1100B atomic absorption spectrometer fitted with an AS-70 autosampler and an HGA 700 graphite furnace attachment was used.

Calibration was carried out in the range 0-300μgl^{-1} with respect to Si (diluted from 1000ppm BDH silicate standard solution) and Ar was used as the purge gas.

$Mg(NO_3)_2$ (Aldrich) was employed as a matrix modifier at 100mgl^{-1} strength (Aldrich).

The graphite tubes were of the pyrolitically coated variety (Lightpath Optical).

Each analysis was repeated five times to give an average result

A sample volume of 15µl was dispensed from the Perkin-Elmer autosampler and the following programmed steps used:

Table 2.1: Analysis parameters employed for GFAAS

Furnace Program Step	Temperature(°C)	Ramp	Hold	Gas Flow	Read
Step1	80	5	5	300	
Step2	120	10	30	300	
Step3	500	10	30	300	
Step4	1350	10	15	300	
Step5	2650	0	5	0	*
Step6	2700	1	3	300	

2.2.1.3. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

Dr. Rhodri Thomas at Edinburgh University's Department of Chemistry carried out ICP-AES analysis of aqueous samples for silicon content using a Thermo Jarrell Ash IRIS ICP-AES. This method, along with GFAAS, is discussed in greater detail in the following chapter.

2.2.2. Methods for Protein Analysis

This section includes assays for protein content (Coomassie blue, BioRad DC Lowry variant and BCA), the method employed for molecular weight determination (SDS-PAGE) and the method employed to analyse amino acid composition of protein samples.

2.2.2.1 Coomassie Blue Assay – The Bradford Method

0.10mL of protein solution containing 10-200μg protein added to 5.0mL Coomassie blue dye reagent (100mg Coomassie Brilliant Blue G-250 (Sigma) dissolved in 50mL of 95% ethanol to which 100mL of 85% phosphoric acid (Avocado) had been added and the whole diluted to 1L with water)

After 5 minutes, the absorbance was measured at 595 nm. Standard curves were prepared using Bovine Serum Albumin (BSA).

Based on the Bradford method (Bradford, 1976) this method uses a dye, Coomassie Brilliant Blue G-250, which has a negative charge and binds with positive charges on the protein. The dye exists in a red form (A_{max} = 465nm) and a blue form (A_{max} = 595nm). The red form is the predominant form in solution, and when its negative charge binds to the positive charges on the protein, it is converted into the blue form. Because many proteins have nearly identical response curves, the method can be applied widely using a single set of standards (in this case, BSA); furthermore, it is much less susceptible to interfering substances. The reaction is highly reproducible and rapid and is essentially complete after two minutes with colour stability for about an hour. Detergents such as sodium dodecyl sulfate (SDS) and Triton X-100 do interfere.

Whilst this method does give responses for microgram quantities of protein, it cannot be considered to give absolute concentrations as protein-dye binding is dependent

on the relative abundance of basic (particularly arginine) and aromatic amino acid residues in the protein.

2.2.2.2. The Lowry Protein Assay

Protein solution was mixed with alkaline copper tartrate before addition of Folin-Ciocalteau phenol reagent, which gave a blue-green coloured product. After fifteen minutes the absorbance of this product was measured at 750nm. Standard curves were prepared using BSA. The reagents for this assay were purchased from Bio-Rad™ as a Detergent Compatible Lowry Assay pack.

This two step assay, also known as the Folin-Lowry Method, is a modification of the Biuret Method. Two colour reactions are used – the biuret reaction with alkaline copper (II) and the reaction of a complex salt of phosphomolybdotungstate, called the Folin-Ciocalteau phenol reagent, which gives an intense blue-green colour with the biuret complexes of tyrosine, tryptophan, cysteine and histidine.

This method is 100 times more sensitive than the Biuret method, but is susceptible to interference by several substances, such as K^+, Mg^{2+}, NH^{4+}, EDTA, Tris, carbohydrates and reducing agents (2-mercaptoethanol, dithiothreitol etc.).

2.2.2.3. The BCA (Bicinchoninic Acid) Assay

Protein solution was added to a solution containing sodium carbonate, sodium bicarbonate, BCA sodium tartrate and copper sulfate. Initial colour development occurs quickly, and was accelerated to completion by heating at 37°C. Absorbance was measured at 562nm.

This assay was standardised with BSA and is prone to interference with ammonium sulphate, EDTA, sucrose, glucose, glycerol, tris and glycine among other materials.

2.2.2.4. Sodium Dodecyl Sulphide – PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Extracted protein samples were loaded onto each lane in the gels at 10μg concentration. The gels comprised a 4% acrylamide stacking gel and a 10% acrylamide separating gel. The gels were electrophoresed with electrode buffer (see below) at 50mA using a BioRad minigel apparatus and a Powerpac 300 for approximately one hour, until the tracking dye ran to within 1cm of the bottom of the gel.

Molecular weight markers (Sigma, cat no. SDS-7) were loaded onto the first lane of each gel in 10μg concentration for standardisation and analysis purposes. The markers were prepared as per instructions provided and stored at -20°C until needed. The marker mix consisted of (approximate molecular weight in brackets):

Bovine albumin (66Da)

Egg albumin (45 Da)

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 Da)

Bovine carbonic anhydrase (29 Da)

Bovine pancreas trypsinogen (24 Da)

Soybean trypsin inhibitor (20.1 Da)

Bovine milk α-lactalbumin (14.2 Da)

The gels were prepared according to Hancock & Poxton (1988).

2.2.2.4.1. Preparation of the Acrylamide Gels

2.2.2.4.1.1. 10% Separating/Lower Gel

To 4.2ml distilled water, 8.75 ml diluted separating buffer (see below) and 5.8ml of a 30% acrylamide stock solution were added and mixed in a flask. Following this the catalysing mixture was added, comprising 25µl TEMED and 875µl APS (ammonium persulphate, 15mgml^{-1}). The gel mix was used immediately as once the catalyst was added the acrylamide gelled quite quickly so needed to be quickly pipetted into the glass plates to the line below the wells. Water was placed on top of the gel layer to exclude oxygen and facilitate gelation. The gel takes approximately 40 minutes to set fully. Once the gel was set the water was poured off and the upper gel poured.

2.2.2.4.1.2. 4% Stacking/Upper Gel

To 3.5ml of distilled water, 5ml of stacking buffer (see below) and 1.5ml of a 30% acrylamide stock solution was added and mixed in a flask. Following this the catalysing mixture was added, comprising 20µl TEMED and 500µl APS (ammonium persulphate, 15mgml^{-1}). Once the gel was poured to the level of the top of the lower plate the comb was inserted into the top of the assembly to form the wells. This gel takes a similar time to set as the lower gel.

2.2.2.4.2 Preparation of Buffer Solutions

2.2.2.4.2.1 Double Strength Separating Buffer

0.75M Tris, pH8.8

0.2% SDS

To 700ml distilled water, 90.86g Tris (hydroxymethyl) aminoethane was added and the pH was adjusted to 8.8 with 1M HCl. Following the addition of 2g SDS the buffer was made up to 1 litre with distilled water.

2.2.2.4.2.1. Preparation of Double-Strength Stacking Buffer

0.25M Tris, pH 6.8

0.02% SDS

To 300ml distilled water, 15.14g Tris was added. The pH was adjusted to 6.8 before the addition of 1g SDS. The buffer was made up to 1 litre with distilled water.

2.2.2.4.2.2. Preparation of Electrode Buffer, pH 8.3

0.025M Tris

0.192M glycine

0.1% SDS

To 1.5L distilled water, 6.06g of Tris and 28.3g of glycine were added. The pH was adjusted to 8.3 before the addition of 2g SDS. The buffer was made up to 2 litres with distilled water.

2.2.2.4.3. Sample Loading

The central chamber of the bracket was filled with electrode buffer to the level of the top of the large plate. The samples were injected into the wells using a Hamilton syringe at a volume of 10~20μl. The cell was connected to the power pack and the power output set to 50mA for approximately 30 minutes. Bubbles were visible from the electrode wire when the power was flowing. The gel was observed until the blue line of

the samples was just below the grey 'v' at the bottom of the bracket. At this time the power supply was switched off.

2.2.2.4.4. Fixing and Staining using Coomassie Blue Staining Procedure

The gel was immersed in fixer (see below) and shaken gently (to avoid tears in the fragile gel) for at a minimum of two hours. Following fixing, the gel is immersed in the staining solution (see below) and gently shaken for 90 minutes. After this time has elapsed the stain is poured off and retained as required (stain can be re-used 2 or three times, though after the risk of protein material diffusing into the solution and causing spurious bands on the gel outweighs the economic benefit of re-use) and the gel is immersed in the de-staining solution. The gel should be shaken in this solution until the background is relatively clear. This typically takes around four hours, with at least two changes of the de-staining solution.

2.2.2.4.4.1. Preparation of fixing solution for Coomassie Blue stain

To 325ml distilled water, 125ml ethanol and 50ml acetic acid was added (10% acetic acid, 25% ethanol).

2.2.2.4.4.2. Preparation of staining solution for Coomassie Blue stain

Prepared as fixing solution with 0.02% Coomassie Blue added.

2.2.2.4.4.2. Preparation of de-staining solution for Coomassie Blue stain

The de-staining solution is prepared as 10% methanol and 10% acetic acid in water.

2.2.5. Amino Acid Analysis

Samples were sent away for total amino acid analysis (i.e. samples completely hydrolysed) at the MRC Department, Department of Biochemistry, Oxford University and were performed by Tony Willis using an Applied Biosystems 420a Amino Acid Analyser with nor-leucine as an internal standard.

Further details on how these analyses were carried out and the limitations of the technique can be found in section 4.4.2 .

2.3. Methods for Model System Studies

This section details the synthesis of the model system complex and the method employed to investigate the silica oligomerisation reaction of the model system.

2.3.1. Synthesis of the Potassium Tris-Catecholato Silicon (IV) Complex

Chemicals: The catechol, if found to be discoloured, was purified by sublimation under vacuum. The ethanol was dried by distillation and stored over molecular sieves [Aldrich, 5A, 8-12 mesh]. Mineral oil was removed from the potassium [Aldrich] surface with adsorbent tissue just prior to use. All other chemicals were used without further treatment or purification.

Method: To catechol (5g, 45.41mMole), stirred under nitrogen in a three necked round bottom flask, absolute ethanol (20ml), was injected via a septum. Once the catechol had dissolved an ethoxide solution, prepared by adding potassium (1.21g, 31.1mMole) to absolute ethanol (20ml), was injected into the system with the immediate formation of a white precipitate. This was heated to approximately. 50°C using a water bath. Following

this tetraethylorthosilicate, TEOS, (3.38ml, 16.01 mMole) [Avocado or Aldrich, 98+%] in absolute ethanol (5ml) was added via the septum. The reaction was then incubated at 50°C for one hour followed by cooling at ~0°C in an ice bath for a further hour. The obtained white precipitate was vacuum filtered and washed once with cold absolute ethanol before being stirred in diethyl ether for thirty minutes to remove excess unreacted catechol. The precipitate was again filtered and the latter procedure repeated once more. The final filter cake was dried in the vacuum oven at 70°C overnight.

The complex identity was confirmed by ^{1}H NMR spectroscopy with a singlet at 6.63ppm being shown with no residual catechol.

2.3.2. Model System Studies

The potassium complex prepared earlier prepared as a 50mmol solution and the pH of a 5ml aliquot was altered from ~8 to 7.00 ± 0.01 within one minute of addition of around 20µl of concentrated HCl. The specific amount of HCl to be added was determined by experiment, with the amount required to bring the pH down to the required level being the appropriate amount to use. The pH was monitored for a minute to ensure the pH at t=1 minute was 7.00 ± 0.01. 20µl aliquots were removed at defined time intervals and added to plastic containers containing 16ml of distilled deionised water and 1.5ml of the molybdate solution. Ten minutes after the addition of the complex solution 7.5ml of the reducing solution was dispensed into the mixture to produce the blue colouration. After two hours the absorbance at 810 nm was measured and a plot of absorbance against time after addition of HCl to the complex solution obtained.

Chapter 3 – Comparison of the Techniques Employed for Bound Silicon Analysis

The analytical techniques employed during the course of this study to investigate levels of bound silicon within samples, GFAAS and ICP-MS, have been briefly discussed in the previous chapter. Here, the techniques will be discussed more fully and the results obtained compared.

3.1. Graphite Furnace Atomic Absorbtion Spectrocopy (GFAAS)

Graphite furnaces are primarily employed to atomise solids, slurries and solutions for atomic absorption, a method also known as electrothermal atomisation. The sample is, in the case of a slurry or solution, dried, converted into a heat-stable elemental form (if required) and atomised by rapidly increasing the furnace temperature to the atomisation point. An inert atmosphere (in this case Ar) is provided to minimise unwanted chemical reactions with the analytes and to protect the furnace itself, as discussed later in this chapter. This method allows longer residence times of the analyte in the light path than that obtained through the use of flames AAS, though the background tends to be larger, sample material can be lost and interaction between the analyte and the furnace is also possible.

The following sections discuss the steps of this analysis type in more detail as well as detailing the action taken to overcome the problems associated with this technique.

3.1.1. Analysis Overview

The sample to be analysed is dispensed into a small graphite tube, which can be electrically heated. The temperature of the tube is then increased stepwise to separate the stages of drying, thermal pretreatment of the material under investigation and dissociation of the sample into free atoms (atomization). During the drying and thermal pretreatment stages an inert gas (Argon) is passed through the tube to remove solvent and matrix vapours. Some matrix components are removed prior to atomization, which takes place in an inert atmosphere. During the atomization the gas flow is stopped to allow a longer residence time in the tube for the ions under detection – of the order of tenths of a second, approximately 100 times longer than for flame AAS. This gives a larger number of atoms available for radiation adsorption allowing the use of small sample aliquots dispensed straight into a tube.

As there is no nebuliser in furnace AAS, physical properties of the sample such as viscosity and surface tension have little effect on the sensitivity of the technique. Also, no sample is lost in the spray chamber, resulting in far higher atomization efficiency than for flame AAS techniques. Furnace AAS can also be used for analysis of solid samples.

3.1.2. Graphite Tubes

There are two types of graphite tube available for use in GFAAS:

1. Spectrally pure high density graphite
2. Spectrally pure high density graphite with a thin layer of pyrolytic graphite

Both types of tube have several grooves in the inner surface close to the ends. This provides favourable temperature profiles for quantitative sample decomposition and also prevents samples from 'creeping' to the cooler tube ends. These grooves allow the use of

sample volumes of up to about 125µl for aqueous solutions. For organic solvents lower volumes (around 50µl) can be used.

Graphite is not a chemically inert material at the temperatures used in GFAAS. It can react in many ways with sample constituents, solvents and acids and can influence the partial pressure of species in the vapour phase. The surface and porosity are of great importance for the reactivity of the graphite. The atmosphere inside the graphite tube may also play an important role in reacting with the sample constituents if it is not inert, so Ar is used for this purpose.

Normal graphite has a relatively coarse, layered surface. This allows elements to penetrate the lattice, where, at elevated temperature, they can react with the graphite. Strongly oxidising elements rapidly destroy a tube in this way. Stronger interferences may be observed because the interfering species may be retained in the graphite tube and released only at higher temperatures.

Pyrolitically coated tubes have a much more even and less dense surface that does not allow sample or solvent to penetrate, which offers a number of advantages over uncoated tubes. For elements that form refractory carbides (e.g. B, Mo, Ti, U, V, lanthanides etc.) pyrolitically coated tubes offer greater sensitivities and reduced 'memory effect'. Pyrocoated tubes show little advantage when determining easily volatilised elements (e.g. Cd, Pb) in sample matrices. Reduced interferences are found in pyrocoated tubes with certain matrices, e.g. chloride compounds, because they are not retained in the lattice. Pyrocoated tubes provide a longer lifetime because of their impermeable surface, particularly in the presence of oxygen or other oxidising agents.

The tubes typically have a useful lifetime of about 50-300 firings depending on atomisation time, temperature, gas flow rate and the sample under analysis. As the tubes

age they show decreased sensitivity, and should typically be replaced when the sensitivity drops 20-25% below the original value. Precision may also decrease with increased tube aging. For maximum tube lifetime of both coated and uncoated graphite tubes the atomisation temperature should be kept as low and the atomisation time as short as possible.

Atomisation temperatures above 2650°C are rarely justified, similarly, atomisation times of greater than 5 seconds are rarely required. Instead of higher temperatures, max-power heating at a lower temperature should be employed. Interrupted gas flow should only be used until a maximum signal is registered. Once the maximum is registered gas flow can be restored until the baseline signal is reached. This is only applicable to measurements where peak height is being used for determination – measurements using peak area should be carried out under the same gas flow conditions throughout the whole integration time until the signal has reached the baseline again.

New graphite tubes should be conditioned prior to use by 'heating out' to remove any impurities on the tube and in the tube material. Initially, the temperature should be ramped from ambient to 2650°C within 60 seconds. Tubes should be repeatedly heated at 2650°C for five-second periods until the conditioning is complete. This can be confirmed by observing the signal detected during each heating of the tube until a constant level is obtained.

3.1.3. Cooling Water

Water is used to allow the graphite tubes to cool quickly between readings and therefore enable a high sampling frequency. At optimum water temperature (20-40°C) and flow rate (~2.5lmin^{-1}) the furnace should be ready to accept the next sample about twenty seconds from the end of the program. If the water is too cool, or flowing too fast,

condensation may occur causing attenuation of the radiation beam. The water (usually supplied from a mains source) does not come into contact with the tube, but passes through a cooling jacket arrangement around the tube. This means that the composition of the cooling water is not an issue with regards to the analysis being carried out.

3.1.4. Inert Gas

This is prerequisite for GFAAS. It serves two primary functions – firstly, this gas prevents the hot graphite parts of the furnace from burning down as a result of contact with the ambient air at high temperature, and secondly the gas flow purges the tube of any accompanying sample material volatilised during the drying and thermal pre-treatment steps.

The gas flow is split into two streams; an external protective gas stream around the graphite tube and an internal purge stream that enters the tube at both ends and leaves via the central bore (the site of sample injection). The external gas flow is not critical, although it protects the furnace from the air, lengthening tube life and allowing more repetitions to be carried out in the same tube. The internal purge gas flow must be exactly controlled as it must remove volatilised material from the tube and prevent these components from re-condensing on cooler parts of the furnace. An asymmetric gas stream during the thermal pre-treatment step can cause much higher non-specific attenuation during atomisation than would be expected, due to re-condensed matrix constituents.

During atomisation it is desirable to keep the analyte atoms in the beam for as long as possible to give the highest possible absorbance. As a result of this the gas purge stream can be stopped (the GAS STOP step). The sensitivity increases obtained may be minimal but it is preferable to use these conditions because the gas stream also has a

cooling effect. Thermal equilibrium is less likely to be achieved in the graphite tube during atomisation under gas flow conditions whereas gas stop conditions favour equilibrium conditions.

Argon is generally recommended as both protective and purge gas as it provides the best sensitivity and will not react with either graphite or the analyte atoms formed. Other gases may have disadvantages – lighter noble gases (such as He) cause severe sensitivity loss for many elements. Use of N_2 as a purge gas also causes sensitivity loss for some elements (for example Al) due to the formation of monocyanides.

3.1.5. Optimisation of Parameters

In flame analysis, detection of the sample, dissociation from the matrix and the generation of the analyte ground state occur almost instantaneously. In the furnace the same processes occur during the drying, thermal pre-treatment (char) and atomisation steps. This means that analyses carried out by GFAAS take longer than those carried out by flame AAS and the various step temperatures require careful selection for the process to be carried out effectively.

When a sample under analysis contains unknown compositions and concentrations of other associated elements as well as the element under investigation it is essential to assess whether standard conditions can be used directly or whether alterations are necessary to improve performance. Alterations may include both time and temperature as well as the use of extra pre-treatment steps prior to atomisation; however, the pre-treatment and atomisation should not be changed too much from recommended conditions or loss of sensitivity may occur due to incomplete char or atomisation.

The thermal pre-treatment programs are in place to decompose and/or remove all accompanying materials thoroughly so that atomisation can be carried out with the minimum of interference from other associated material within the sample under investigation. The success of this step is largely determined by the physical properties of the materials present in the sample, so the thermal stability of the compound is therefore of great importance. The more volatile the accompanying materials and the less volatile the element, the easier the separation is. As a result of this it is often advisable to use a matrix modifier to convert the analyte element into a lower volatility compound. Careful selection of program parameters can have great influence on the interference levels experienced during the determination of the analyte element.

3.1.6. Drying Time and Temperature

The purpose of the drying step is to remove the low-boiling (lower boiling point) liquids from the sample. The drying temperature should be selected on the basis of the boiling point of the solvent or of any liquid component of a solid sample. Typically, a temperature slightly higher than that of the solvent boiling point should be chosen. For dilute aqueous solutions (such as those under investigation in this study) a drying temperature of between ~100-150°C should be employed. Solutions containing large amounts of dissolved solids may have a boiling point higher than that of the pure solvent as a result of intermolecular forces arising due to interaction between solvent and sample constituent components. Drying times are largely dependent on the sample volume – a sample volume of 100µl will take approximately 60 seconds to dry. This time is dependent on the temperature employed and the relative volatility of the solvent to be removed.

Care must be taken when selecting the drying temperature to ensure that only rapid evaporation occurs and not boiling, which occurs when too high a temperature is employed for drying. Boiling of the sample is obvious due to an hissing sound emanating from the furnace. When a sample contains a mixture of solvents ramp drying, i.e. gradually increasing the drying temperature over a period of time, may need to be employed. Boiling can also be detected by using a mirror to look into the tube itself; however, care should be taken when doing this, particularly if the drying phase is followed immediately by an atomisation step as the radiation generated is potentially damaging to the eyes. Sample boiling may lead to spattering of the sample which can lead to poor analytical precision and sample loss.

3.1.7. Thermal Pre-Treatment Time and Temperatures

Thermal pre-treatment (charring) is used to remove those components of the sample matrix which are more volatile than the compounds of the element under analysis. This reduces the likelihood of background attenuation as well as of chemical interference. For dilute aqueous samples a pre-treatment time of ten seconds at 300-500°C may be sufficient. The parameters may have to be optimised for certain samples and temperature ramping can also prove useful. The pretreatment stage exists to achieve two aims. Firstly, a sufficiently long pretreatment time and high enough temperature should be employed to volatilise as completely as possible any interfering or smoke producing sample matrix constituents. Secondly, the pretreatment time must be short enough and the temperature high enough to ensure no loss of the analyte element during the thermal pretreatment step.

A matrix modifier, for example a salt or an acid, is added in large excess to ensure that the element under investigation is converted to a compound of high thermal stability to reduce the potential for sample loss during this stage.

The maximum char temperature is determined by the thermal stability of the element under analysis, or the compound in which it is present. The time used for pretreatment as well as the temperature program (ramp rate and hold time) is determined by the matrix components. Pretreatment times should be long enough to allow the background signal to the return to the baseline prior to atomisation.

3.1.8. Optimisation of Thermal Pre-Treatment Temperature

The drying and atomisation parameters are set to the optimum determined previously or taken from the recommended conditions, and the thermal pretreatment time is set to thirty seconds or longer, depending on the sample volume. A sample aliquot is chosen that is expected to give an absorbance signal of about 0.2-0.5 and the thermal pretreatment temperature to about 200°C.

Using the recommended conditions for analysis, the sample is analysed using a series of increasing pretreatment temperatures- usually in 100°C increments. A plot of pre-treatment temperature vs. atomisation signal (absorbance or absorbance x seconds) is obtained. At the lower pretreatment temperatures the absorbance will normally remain constant until the maximum pretreatment temperature is reached – in this case 600°C. It is not normally necessary to establish the entire pre-treatment curve from 200°C up to the point where the element of interest is almost completely volatilised in the thermal pre-treatment step. Usually, the recommended pre-treatment temperature is taken first and a temperature of 100°C higher and 100°C lower than that are also taken. If the signal obtained for the lower temperature is the same as that for the recommended temperature,

and the signal for the lower temperature is reduced relative to the signal obtained at the recommended temperature, then that recommended temperature can be used for the sample pre-treatment. If all three results show a decline in sensitivity with respect to increasing temperature, the pre-treatment curve should be extended to lower temperatures, and the maximum pre-treatment temperature for the material under investigation determined.

If the element seems to have a much higher volatility than expected – indicated by much lower pre-treatment temperature that that suggested in the recommended conditions – then a matrix modifier should be added to establish recommended conditions by decreasing the volatility of the element. Temperatures more than 200°C lower than the recommended conditions should not be employed as there may be a decrease in the effectiveness of the separation of the materials accompanying the element as well as too large a temperature step between the pre-treatment and atomisation steps.

The thermal pre-treatment step is used to remove any constituents of the sample which produce background adsorption, so for 'smoke' producing samples the optimisation procedure will be more complex than that for a pure solution.

3.1.9. Temperature Ramping

Temperature ramping enables the temperature to be increased at a continuous constant rate, if required, to ensure better handling of complex samples within the furnace. In the analysis of complex samples (especially those containing organic constituents such as those under investigation here) stepwise temperature increase gives poor analytical precision. This is caused by the pre-treatment step not being complete. This is usually a result of the presence of a number of different components with different boiling points

within the sample under analysis. Using ramping allows each of the components in a multi-component system to be subjected to a thermal treatment more closely related to the range of boiling points present within the sample. Also, for a multi-component system, the range of boiling points between the least and most volatile components may be large. Too high a pretreatment temperature for this mixture could cause spattering of the most volatile components, leading to both sample loss and poor analytical precision.

When developing a method for analysis, the following guidelines will govern the thermal pretreatment temperature:

1. The thermal pretreatment temperature will be related to the amount of matrix to be removed.
2. The initial thermal pretreatment temperature (ramp start point) should be slightly lower than the point at which the most volatile constituent will starts to boil or decompose. A stepwise increase to this temperature will reduce the overall analysis time.
3. The final ramp temperature should be about 50°C higher than the boiling point of the least volatile component to be removed, but lower than the temperature at which the analyte is lost. The ramp should always be followed by an isothermal period (hold time) that allows the matrix to fully decompose or be fully removed.
4. The ramp rate selected should be selected so that any chemical reactions in the furnace tube do not become too violent.
5. For a sample containing several components of widely differing thermal properties, a sequence of temperature steps and/or ramps, followed by

isothermal periods, may give the most effective thermal pre-treatment in the shortest times.

6. The maximum thermal pretreatment temperature will be governed by the thermal stability of the analyte element, which should be determined as previously described.

3.1.10. Matrix Modification

Matrix modification has been proposed as a technique to enable better separation of an analyte element from its component mixture. The matrix modifier either makes the component matrix more volatile, or stabilises the analyte element. A reagent, for example an acid or a salt, is added to the sample in high concentrations to convert the analyte element to a well-defined compound with known properties. This also has the effect of enabling easily reproducible conditions for thermal pretreatment for a variety of matrices.

These modifiers can form complexes with the analyte in order to raise its vaporisation temperature. This means the atomisation of the analyte occurs with a greater delay after the matrix vaporisation during the thermal pretreatment step, reducing the background. Use of matrix modifiers also helps to lessen the difference in matrix effects between individual samples and between samples and standards.

3.1.11. Discussion of Silicon Atomisation and Reactions Occurring During GFAAS

When considering the analysis of silicon content by GFAA it is necessary to consider the atomisation mechanism of silicon within the furnace. It has been shown that small changes in the partial pressure of O_2 within the tube result in dramatic changes in the type of silicon compounds formed in the tube, i.e. $Si0_{2(s)}$, $SiO_{(g)}$, $SiCl_{2(g)}$, $SiS_{(g)}$, $SiN_{(g)}$ and $SiC_{2(g)}$ (Frech & Cerdegren, 1980). These workers concluded that a low partial pressure of

oxygen is necessary for the formation of silicon carbides. Other workers (Sturgeon & Burman, 1985) found that the time at which maximum atom concentration is achieved is influenced by the O_2 content of the purge gas. The purge gas is not the only source of O_2 in the graphite furnace but research into the addition of O_2 to the purge gas (Rademeyer & Vermaak, 1992) showed that the peak maxima could be shifted by 50°C by the addition of oxygen to the purge gas. The shift of the absorbance signal is attributed to the suppression of the rate of dissociation as a result of the increased O_2 partial pressure. These conditions are also not favourable to the formation of carbides. Si atoms are produced by the formation of two intermediate species, SiO and SiC_2. During the initial stages of the atomisation step the Si atoms are produced by decomposition of SiO_2 thus:

$$SiO_2 \longrightarrow Si_{(g)} + O_{2(g)}$$

This is a proposal supported by the fact the increased partial pressure of O_2 suppressed the Si signal. Following this, and dependent on conditions in the tube, one of two further reactions may occur, thus:

$$\text{(i)}\ Si_{(g)} \xrightarrow{2C_{(s}} SiC_{2(g)} \xrightarrow{SiO_2 \text{ or } SiO} Si_{(g)} + CO_{(g)}$$

This reaction occurs when O_2 partial pressure is below a certain value, possibly 101.325×10^{-10} Pa (Cedergren, 1989). Once the partial pressure of O_2 exceeds this critical value the second reaction will occur:

$$\text{(ii)}\ Si_{(g)} \xrightarrow{O_{2(g)}} SiO_{2(g)} \xrightarrow{2C_{(s)}} Si_{(g)} + 2CO_{(g)}$$

This is supported by the theory that increased partial pressures of oxygen lead to reduced formation of carbides due to their blocking of active sites on the surface of the carbon by chemisorption.

This goes some way to explaining some of the problems with GFAAS analysis of silicon content. The formation of Si atoms by two distinct routes could lead to 'spikes' of

Si being detected during the early stages of the atomisation step formed as result of the decomposition of SiO_2.

Whilst the addition of O_2 to purge gas has been shown to have marked results, purge gas is not the only source of oxygen in the furnace. Whilst the purpose of the purge gas is to exclude air from the furnace, and to protect the hot graphite parts from burning down in the atmosphere at high temperature, the gas flow is stopped during atomisation to increase the residency time of the atoms in the detection beam. This could lead to a change in the partial pressure of oxygen within the tube. The purge gas itself, as well as the purge gas flow rate may also have a role to play in the introduction of oxygen or other contaminant gases into the system. Also, as the silicon present in the samples under investigation is likely to be in the form SiO_2, decomposition of the sample to give $Si_{(g)}$ atoms results in a release of oxygen into the furnace atmosphere. This in turn leads to an increased partial pressure of oxygen within the tube and may, in turn, cause the reaction shown above in equation (ii). This could lead to the production of Si atoms, followed by the production of further SiO_2 before the production of Si atoms and CO at a later stage in the tube, precluding the likelihood of obtaining an accurate reading of the silicon content of the sample. It can be seen that the partial pressure of oxygen within the furnace tube may fluctuate according to the reactions occurring within the tube. The initial thermal decomposition of SiO_2 causes an increase in the partial pressure of O_2 within the system. This causes the formation of free, unbound Si atoms and carbon monoxide (equation (ii)) which in turn results in a lowering of the partial pressure of oxygen within the system. This then provides favourable conditions for the formation of silicon carbides. As the overall level of carbides within the system increases, and the level of SiO_2 decreases, conditions for the formation of carbides become more favourable with the likely result that

some or all of the silica may end bound in a solid form with carbon from the graphite tube and therefore not be detected during the atomisation step.

As discussed above, when the partial pressure of oxygen is low in the furnace, the reaction outlined in equation (i) is more likely to occur. This leads to the formation of silicon carbides, which require a very high temperature to dissociate the compound into its constituent atoms. As the furnace is already, in the case of the equipment used at NTU, operating at close to its maximum temperature, the result may be that the carbides are not degraded as a result of the heating action in the furnace. This in turn leads to the possibility that a build-up of silicon carbides occurs within the tube, as the temperature at the end of the analysis (step 6) may not prove sufficient to remove these species as silicon carbide sublimates at approximately 2700°C. It is not possible to run the furnace at this temperature for sustained periods of time before the safety cutout activates, and firing for prolonged periods at this high temperature dramatically shortens tube life.

The effects discussed here are dependent on a number of external variables – primarily the partial pressure of O_2 within the furnace, the level of SiO_2 within the sample under investigation and the reactive sites available on the surface of the carbon tube to react with either oxygen or silicon atoms. These are difficult to quantify, and indeed very difficult to standardise. The addition of oxygen to the purge gas is likely to provide an environment more conducive to the production of free silicon atoms rather than silicon carbides; however, this may lead to problems with 'burning down' of the hot graphite furnace parts during the atomisation and tube cleaning stages of the analysis program (i.e. the high temperature stages of the analysis).

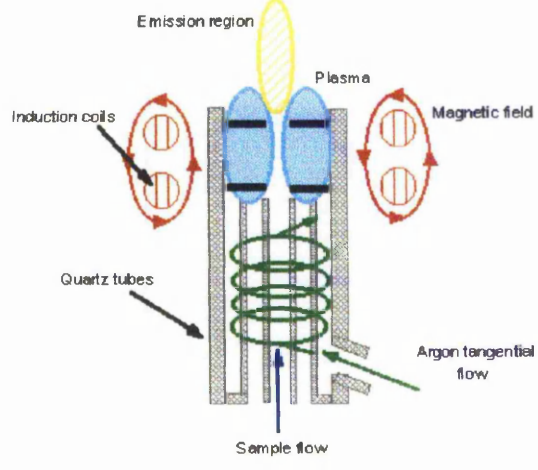
Another problem that must be considered is the difficulty of using a suitable calibration standard for a multi-component system of variable constitution and comprising

of largely uncharacterised materials. This technique was being applied to assess materials comprising of primarily pectins with some hemicelluloses; primarily hemicelluloses with some pectins or a combination of the many materials, characterised and otherwise, that comprise beer. Ideally, each sample type requires a specific calibration but it is impossible to prepare certified reference standards of these materials. In the case of the plant cell wall extracted materials, these are particular for the plant from which they were extracted, which in this case is a plant known to contain large amounts of silica. Beer, by its very nature, is variable in composition according to the materials and conditions employed by the brewer during the production process. To date, a useful model to reflect beer has not been agreed upon and as the silica under investigation is believed to be imparted into the brew primarily by the plant materials used for brewing it is difficult to see how an appropriate standard could be produced.

This problem is not unique to this technique, as it is also an issue in the use of ICP-MS. ICP-MS is the most widely accepted technique for measuring silicon content in a sample. Whilst this technique avoids the problem of carbide formation found in GFAAS, the calibration issue remains a problem. Inter-laboratory comparison trials of results obtained using ICP-MS to analyse samples for silica content have shown that results obtained by individual researchers show large standard deviation values and are frequently not directly comparable to those obtained by other researchers under the same conditions (Van Dyck, 2000).

3.2. Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES)

ICP-AES is a multi-element analysis technique that uses an inductively coupled plasma source to dissociate a sample into its constituent atoms and ions, and cause them to emit a characteristic wavelength by exciting them to a higher energy level. This is accomplished through the use of an inductively coupled plasma source, usually argon, a monochromator to separate wavelengths of interest and a detector to measure the intensity of the emitted light. The plasma is used as a sample cell that will excite atoms. These atoms emit energy as they return to the ground state and it is this energy of a specific wavelength that is directed to the detector by the monochromator. This technique uses similar instrumentation to AAS techniques (flame and furnace) except that no external radiation source (e.g. hollow cathode lamp as used in GFAAS). The atomisation temperature is typically in the range 6000-8000°C, which will efficiently atomise most elements. The equipment is capable of analysing a sample for multiple elements. The argon plasma torch provides a consistently high and uniform temperature and the elements have a relatively long residence time which gives a linear response over a large concentration range. A typical plasma source is shown below:



Reproduced from Dr Judy Ratliff's ICP-AES page on the Murray State University website

The argon flow is represented by the green arrow in the diagram, and the sample flow by the blue arrow. The ICP, so named because the plasma is not directly connected to the loop but where the energy is added inductively, is a continuous (in time) plasma induced in a flowing stream of argon. The energy to ionize the argon is added through a radio frequency magnetic field, supplied by induction coils around the tube with an ac current with a 30MHz frequency and 2KW-power level. The argon that passes the coil is seeded with free electrons from a tesla discharge coil. These electrons are accelerated by the surrounding field and hit the surrounding atoms causing heating and further ionization, which sustains the plasma state. The ions present from the initial tesla spark accelerate in a circular pattern perpendicular to the stream exiting the top of the quartz tube. The alternating current reverses the current direction in the induction coils and as a result changes the direction of the ions by reversing the magnetic field orientation, which results in further collisions with argon atoms. This, in turn, results in further ionisation of these argon atoms and intense thermal energy. As a result of this a flame shaped plasma forms on top of the torch. The plasma is suspended away from the glass through a combination of the helical path of the cooling gas flow and the shape of the radio frequency electromagnetic field, which helps prevent the silica tube from melting. If the gas flow and radio-frequency power are regulated correctly a doughnut shaped region with a high, constant temperature is formed at the base of the plume. As there is no electrode contact in the plasma source spatially separated excitation and emission zones are obtained which results in a simple background spectrum and a high signal to noise ratio.

The sample under investigation is introduced as an aerosol by use of a nebuliser or atomiser, normally a cross-flow or Babington type nebuliser to overcome the problem of blockage. Electrothermal vaporisers and high solid nebulisers are also employed.

ICP-AES is less prone to chemical interferences (interferences as a result of chemical changes in the analyte) than GFAAS, though spectral interferences are possible and there are some element limitations. Spectral interferences can be caused by light scattering from particles and droplets, molecular absorption and emission, radiation from the matrix atoms, the light emitted by all heated objects and as a result of self- reversal of spectral lines due to the absorbance of emitted radiation by cooler parts of the source. Particles originate from incomplete atomization while droplets are a result of poor nebulisation. Particles are unlikely to be found in an ICP torch purely as a result of the very high atomisation temperature reducing the likelihood of incomplete atomisation. The problems as a result of matrix radiation and molecular absorption commission can be overcome with background compensation techniques. All heated objects from red hot to white hot emit blackbody radiation. In the case of ICP, the emitter can be the plasma as well as any unatomised particulate material passing through. Spectra line reversal, along with blackbody radiation, can be compensated for but the plasma conditions must be highly stable in order to do so with high precision.

3.3. Results

The plant cell wall material extraction techniques discussed in chapter 2 were applied to samples obtained from hops, malted barley, the commercial brewing process and from *Equisetum telmatia* samples. The primary purpose of these studies was to develop a method for detection of overall levels of silica (whether bound or unbound) within a sample for comparison with results obtained from the molybdenum blue assay for free (i.e. unbound silica). This information, in turn, allows an estimate to be obtained of silica that

may be either in a bound oligomerised form, or incorporated in some form within the material under analysis.

Table 3.1.: Silicon Content by GFAAS and ICP-AES & the Molybdenum Blue assay for Pectic & Hemicellulosic Plant Cell Wall Fractions Extracted From *Equisetum telmatia*

	GFAAS	ICP-AES	Moly Blue
Sample Name	Si/μgmg^{-1}	Si/μgmg^{-1}	Si/μgmg^{-1}
Extraction 1 C1	0.52	0.70	0.16
Extraction 1 C2	0.73	1.24	0.29
Extraction 1 N1	0.91	16.31	0.15
Extraction 1 N2	0.74	8.25	0.15
Extraction 2 C1	0.78	0.78	0.18
Extraction 2 C2	0.41	1.50	0.34
Extraction 2 N1	0.62	19.61	0.16
Extraction 2 N2	10.84	2.79	0.05
Extraction 3 C1	0.59	0.78	0.04
Extraction 3 C2	0.26	1.82	0.14
Extraction 3 N1	0.46	19.09	0.20
Extraction 3 N2	10.83	27.89	0.19
Extraction 4 C1	0.49	0.57	0.04
Extraction 4 C2	2.98	1.09	0.11
Extraction 4 N1	15.99	18.76	0.19
Extraction 4 N2	9.03	29.17	0.18

Legend
C1 - First pectin extraction
C2 – Second pectin extraction
N1 - First hemicellulose extraction
N2 – Second hemicellulose extraction

It can clearly be seen from the above table that the results obtained by these separate techniques for the same samples show a large degree of variance. The values obtained from the separate extraction experiments show approximately the same trend, i.e. there is more silicon within the hemicellulosic than pectic fractions as detected by these methods. There is disparity between the levels of silicon detected for the first hemicellulosic fractions, with experiments 1 & 2 showing markedly less silicon in the second extraction as opposed to experiments 3 & 4, which show considerably more.

In order to consider the relevance of these results it is necessary to compare with the readings obtained from the molybdenum blue assay for the same solutions. This assay is used to obtain a measurement of the level of free, unbound silica and can be used to discriminate between the bound and unbound levels of the sample.

The results contained in the table above indicate that there is a significant amount of silica contained within the plant cell wall components of *Equisetum* which is either bound within the fractions or present in some form undetectable by the use of the Molybdenum Blue assay; i.e. in the form of a polymer larger than a dimer. Using the methods applied here it was not possible to discriminate between silica bound or polymerised silica; however, experiments were carried out to degrade the pectins and hemicelluloses using both chemical and enzymatic methods and to measure any detectable increase as a result of these treatments.

The method described in chapter two for analysis of these samples by GFAAS was the result of a great deal of 'tweaking' the variables to try to obtain a stable and reliable method for analysis of samples to ascertain the levels of silicon contained within. By preference, ICP-MS would have been the analytical method of choice for this sort of analysis, but at the time of this research this technique was not available within the department.

Analysis by GFAAS is relatively simple, i.e., the stages of treatment the sample undergoes are uncomplicated and contain variables that it is possible to easily alter using the controlling software for the equipment. Each element likely to be analysed has a standard 'recipe' as set out in the accompanying Perkin-Elmer literature, and it is this that was taken as a starting point for method development. The aim of this part of the research was, as previously stated, to develop a reliable and accurate silicon analysis technique

which would suffice for all the different types of sample being worked with. A couple of factors meant that a general silica standard had to be employed. Firstly, certified reference standards modelling different plant species are either not available or very difficult to come by. Secondly, the variety of sample types under analysis (including hops, malted barley, *Equisetum*, beer, model wort) did not lend themselves to anything other than a general standard in order for one method to be useful, and comparable, across the range.

Certified silica standards, long used by the group for calibration purposes, were obtained from BDH – a large amount was purchased from the same batch to ensure conformity across the stock – and it this standard, and dilutions thereof, that were used to calibrate the equipment. Pyrocoated graphite tubes were obtained from Lightpath Optical, in sufficient number that the tube material could also be considered reference.

Initial studies focussed on varying the parameters of the equipment to provide a reliable calibration technique. The equipment used an auto-calibration system, so calibration was primarily a matter of preparing the appropriate range of calibration solutions and loading them onto the auto-dispenser. The solutions were placed in acid washed plastic vessels, in order to minimise external interferences, having been prepared in similarly acid-washed plasticware, with all accurate measurements being carried out using Gilson pipettes with disposable plastic tips. In turn, the calibration of these pipettes was checked prior to use to reduce the risk of dilution and systematic error in the standard production.

The first variable to be explored was the drying temperature. This is obviously quite an important step, as discussed previously in the chapter. The purpose here is to remove the low boiling solvent components from the sample in such a way that sample loss is kept to an absolute minimum and interferences from such things as 'smokey'

solvents are reduced or avoided. Obviously, the drying temperature is limited by the boiling point of the solvent , in the case of this study water. Employing temperatures too high results in the sample boiling in the tube, rather than drying gradually, and causes spattering and sample loss. Experimentation revealed a good temperature for this sample injection to be 80°C, lower than the solvent boiling point, which was held for five seconds and then ramped upwards at a rate of 5°C per second to 120°C. By this time the sample will have lost all the water (~25μl) injected. This temperature was held for ten seconds to be certain of the solvent removal before proceeding to the next step. Varying these parameters involves the use of a mirror, not dissimilar to that of a dentist, to observe the sample during the drying stage within the tube. With the use of the mirror it is possible to observe whether the sample boils – though this is usually also evinced by a hissing and sputtering sound emanating from within the furnace. Temperatures in the range 50 - 250°C were employed to assess their potential usefulness. At 50°C, the sample did not dry fully, causing sample boiling at the next stage, and temperatures above 120°C led to similar problems at this stage of the analysis.

Following this, development of an appropriate thermal pretreatment or 'char' stage was required. Using temperatures in the typical range of 300-500°C did not result in a baseline signal, i.e. the signal did not return to the baseline despite varying the time the sample was maintained at this temperature. Consideration of the complex sample matrix under analysis in the case of the samples extracted form the plant cell wall material suggested that there were likely to be large amounts of non-volatile material to be removed prior to the silicon analysis stage of the program. It was decided to employ an additional step, at a higher temperature, to further remove these associated non-volatile components. Temperatures up to 1350°C were tested and it was decided to employ this

temperature. Although this did not always give a return to baseline signal, it was more effective than any of the lower temperatures employed. It was decided not to employ a higher temperature than this as studies with a simple calibration solution showed higher temperatures affected the amount of silicon detected in the atomisation phase – possibly as a result of the early atomisation of silica whilst the inert gas flow was still active. During this stage it is necessary to maintain the gas flow to remove the volatilised sample components not under investigation which would otherwise affect the reading. This was unfortunately not a systematic error and therefore could not be allowed for, but a random error.

The atomisation temperature employed in this analysis remained largely fixed. This was a result of the high temperature required to adequately atomise silicon within the furnace. Temperatures lower than this for a standard sample gave lower responses than would otherwise be expected, though this is of less importance when considering the calibration procedure is carried out under these conditions so a standard response should be obtained. It made sense to atomise as much as possible of the available silicon , both in terms of obtaining an accurate result and of removing as much as possible of any possible contaminating material from within the tube itself. A higher temperature may have given better and more consistent results, but employment of such was limited by the maximum operation parameters of the machine and the requirement to have a final higher 'burn-off' temperature available to remove any remaining sample from the tube. The temperature employed for the burn-off stage of this analysis, 2700°C is the maximum operating temperature for the furnace equipment. Prolonged periods of time at this temperature cause the safety cut-out to activate, resetting the machine and causing the loss of any data sets stored on it at the time.

The effect of matrix modifiers ($Ca(NO_3)_2$ and $Mg(NO_3)_2$) on the analyses in question were investigated. Unusually, these had a detrimental effect on the quality of the results obtained. Calibration proved difficult with wildly variable results obtained in the presence of either of these commonly employed modifiers. Matrix modifiers are intended to facilitate the removal from the sample under investigation of undesirable materials during the char stages of the analysis by complexing with them to enable their easier removal. It is clear that modifiers would not be required in the calibration standard (solutions of the certified BDH silica standard) but also that they must be included as part of the calibration to allow their behaviour during the analysis to be accounted for. Further studies using matrix modifiers with samples under investigation revealed no benefit in the results obtained. These results, coupled with the calibration problems, led to the decision not to employ a matrix modifier. The action of these modifiers is poorly understood and difficult to accurately quantify, and the actual effect on the results obtained of the addition of the modifiers was overall detrimental to the quality and precision of the analyses.

Once the method described in chapter 2 had been developed, it was applied to analyse samples extracted from the plant species under investigation as part of this research. This process was not simple as further problems were encountered during the course of the analyses. These included problems with the autosampler, which dispensed variable amounts of sample on occasion, rendering the obtained results useless. As previously discussed, the graphite furnace parts have a life measured in the number of firings they are able to stand before the material starts to degrade. The large furnace parts are less prone to damage from repeated firing, but did have to be replaced during the course of the research. The furnace tubes themselves, typically expected to last in the region of 300 firings, performed worse than expected – probably as a result of the high

temperatures and relatively long hold times employed in the analysis. Unfortunately, it is difficult to see when a tube is nearing the end of its useful life unless the tube is seriously physically degraded – by which time the results obtained in the course of the analysis would be at best of questionable value. As the tube ages, the sensitivity decreases and it is generally only detectable by regular analysis of a known standard and comparing the obtained results over a period of firings. It is difficult to say for certain that the furnace conditions were at all times consistent as a result of changing parts of it as required. Whilst the variables that could be controlled were (inert gas flow and cooling water rates), as much as possible, gas pressure regulator failure, water pressure variability and sample volume variability as a result of the autosampler were all experienced during the course of the studies.

3.4. Chemical and Enzymatic Digestion of Extracted Plant Cell Wall Components

In order to understand the mode of action of the chemicals being applied to these materials it is necessary to consider the samples themselves. The first extractions from the alcohol insoluble residue of the plant cell wall material (C1 & C1) primarily extract pectic materials by the use of a chelating agent, whilst the secondary extractions (N1 & N2) primarily extract hemicelluloses. This has been explained more clearly in section 2.1.1 of the experimental chapter.

Hemicelluloses and pectins are found within plant cell walls bound to microfibrils of cellulose to from a network of cross linked fibres. Hemicelluloses themselves are polymers of simple sugars or uronic acids and can simply be classed as non-cellulosic wall polysaccharides other than pectins. Although this is a wide definition these types of material share common chemical properties in that they are extractable by NaOH but not by cold chelating agents and can hydrogen bond to cellulose. They consist of several polysaccharides including xyloglucan, callose, β-glucan and xylans. Pectins can be described as 'block' polymers containing 'smooth' blocks (homogalacturonan) comprising contiguous unbranched α-D-galacturonic acid (GalA) residues, and 'hairy' blocks containing numerous other sugars, including Rha, Gal & Ara. These 'hairy' blocks are resistant to pectinase. The smooth blocks contain occasional rhamnose residues which may be spaced at regular intervals (approximately every 25 GalA units). These Rha residues can be hydrolysed with acid to break up the GalA backbone. Acid hydrolysis of the 'hairy' blocks (a class which includes the rhamnogalacturons) results in the release of the side chains from the core due to the highly acid labile glycosidic linkages by which these side chains are attached to the core. There has been suggestion that silanolate groups (C-O-Si) are covalently linked to pectins (Fry, 1988).

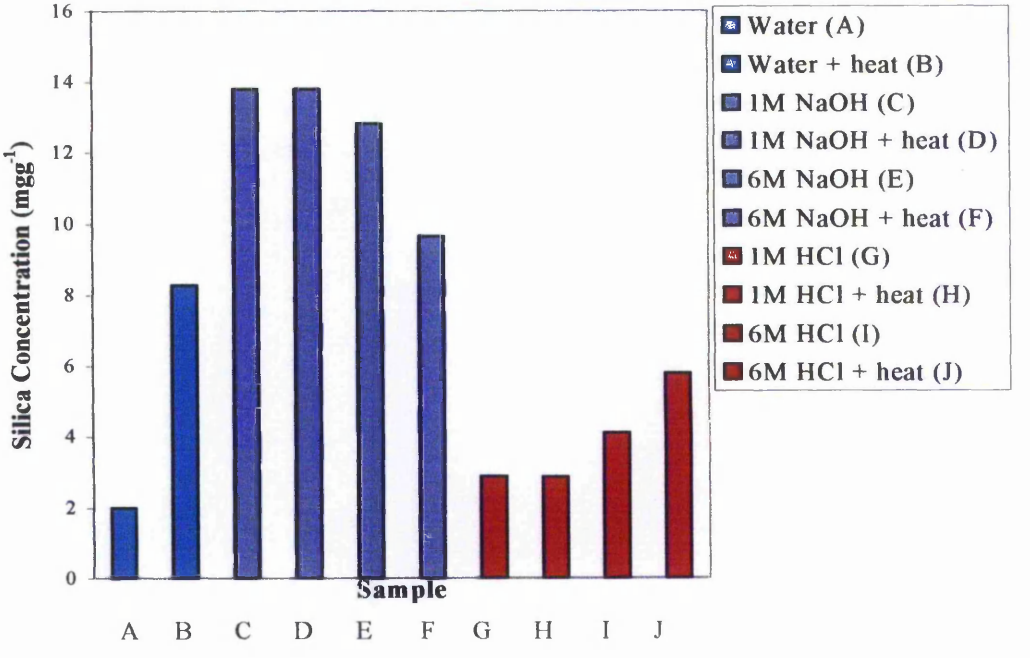
Following this, the digestion experiments were designed to make use of chemical and enzymatic methods to selectively hydrolyse the extracted plant cell wall components to investigate whether or not any bound silica, previously detected by GFAAS or ICP-AES, was released into the solution – i.e. an increase in the levels of silica detectable within the sample by the molybdenum blue assay.

As discussed above, it is relatively easy to hydrolyse the materials extracted from the *Equisetum* material through the use of strong acids, strong alkais and enzymatic agents. Addition of strong alkali to the extracted materials results in 'peeling', the stepwise removal of sugar units from the reducing terminus. Alkali will also degrade these materials by internal cleavage as a result of elimination degradation reaction of certain glycopeptide bonds (for example, sugar-serine and sugar-threonine). Acids degrade these materials by hydrolysis of the backbone of the molecule (at rhamnose residues) and also of the highly acid-labile glycosidically-linked side chains. Following treatment the samples were analysed for both unbound and unpolymerised silica (by the molybdenum blue assay) and for all detectable silica (by the use of GFAAS). In addition to the addition of acids and alkalis, the materials were hydrolysed by the addition of an enzyme, driselase. The use of pure enzymes to degrade polysaccharides represents an ideal method due to the predictable specificity of the action of a pure enzyme. Unfortunately, in practice pure enzymes are difficult to obtain and impure mixtures are used. This makes it harder to predict exactly the mechanism of action of the enzyme upon the polysaccharide under hydrolysis. In general, driselase can be classed as a mixture of exo- and endo- enzymes. Exo-enzymes attack the non-reducing ends, cleaving off one or two sugars at a time. Endo-enzymes attack mid-chain in the polysaccharide, cleaving sugar-sugar bonds. Driselase is obtained from the fungus *Irpex lacteus* and is known to be

highly active on plant cell walls (Fry, 1988). The mixture comprises of a number of exo- and endo-hydrolases, including (among others) cellulose-cellobiohydrase, D-glucopyranoosidase, cellulase and pectinase. Driselase often completely dissolves non-lignified walls and exhaustive driselase digestion can be a useful alternative to acid hydrolysis.

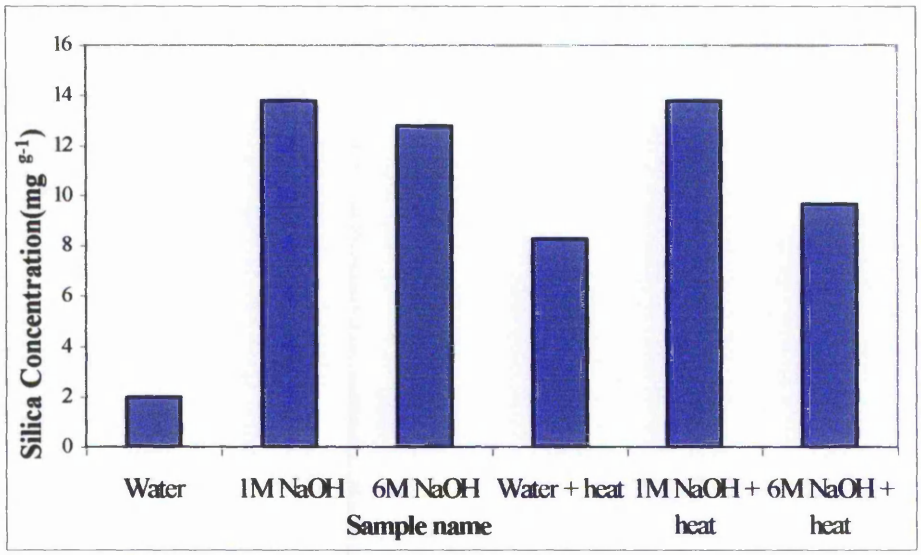
It had quickly become apparent that the silica contained within the raw materials of beer, eg barley husks, rice adjuncts etc., played little part in the formation of long-term beer haze. It is more likely that any silica imparted to the brew from the raw materials is likely to have a beneficial effect by removing proline-containing haze-active proteins from the brew during early treatment stages in the mash. As a result of this, the digestion work was not extended to cover materials extracted from *Humulus lupulus* or *Hordeum vulgare*. This work was carried out to further investigate the belief that *Equisetum* incorporates, in some form, bound silica with the pectic and hemicellulosic fractions of the plant cell wall.

Graph 3.1: Silica Detectable by Molybdenum Blue Assay (mg Si per g pectin)



Graph3.2: Silica Detected by Molybdenum Blue Assay for Alkali-Treated Samples

(mg Si per g pectin)



Graph 3.3: Silica Detected by Molybdenum Blue Assay for Acid-Treated Samples

(mg Si per g pectin)

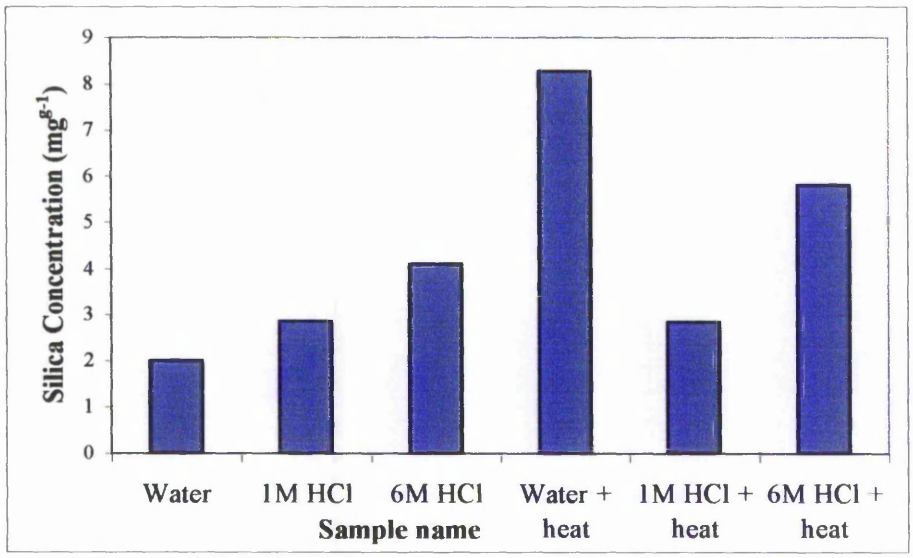
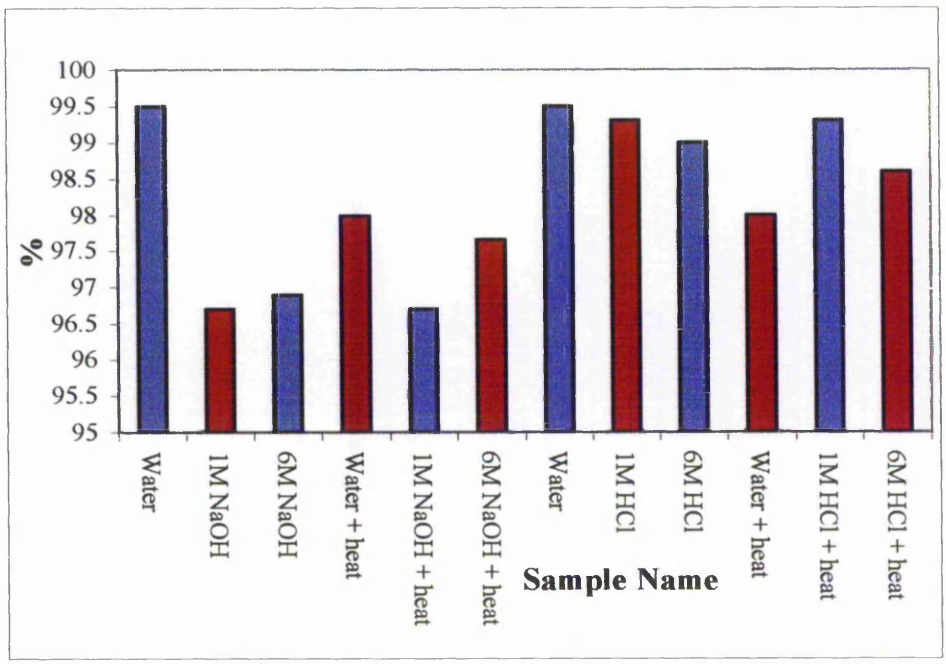


Table 3.2: Silica Content analysed by Molybdenum Blue Assay & GFAAS of Extracted Plant Cell Wall Material from *Equisetum*

Sample	SiO_2 (mgg^{-1}) Moly Blue	SiO_2 (mgg^{-1}) GFAAS	%SiO_2 Bound
Water	2	413	99.5
1M NaOH	13.8	413	96.7
6M NaOH	12.8	413	96.9
Water + heat	8.27	413	97.99
1M NaOH + heat	13.8	413	96.7
6M NaOH + heat	9.64	413	97.66
Water	2	413	99.5
1M HCl	2.87	413	99.3
6M HCl	4.11	413	99
Water + heat	8.27	413	98
1M HCl + heat	2.85	413	99.3
6M HCl + heat	5.8	413	98.6

Graph 3.4: Percentage of Total Silica Remaining Bound Following Treatment With Chemical Additives



From graphs 3.1, 3.2 & 3.3, it is clear that dissolving the samples in water and placing in a water bath caused an increase in detectable levels of free silica. The reasons for this are

unclear but may be related simply to the solubility of silica itself being improved as a result of incubating the solution.

The addition of alkali to the samples caused an increase in detectable silica levels (relative to aqueous solution) at both 1M and 6M strength. This is due to alkaline degradation by 'peeling' and by internal cleavage of the polysaccharide, releasing previously bound silica into the solution. This supports the hypothesis of the presence of silica in a bound form in the main 'backbone' of the molecules. Heat appeared to play little part in the amount of silica released into solution, in that the results obtained correlated with those obtained without sample incubation with no apparent increase in the final silica levels. It may be that the addition of incubation to the treatment regime results in a quicker reaction with the polysaccharide, i.e. heat catalysed. As the reactions were allowed to run for a 24-hour period and measurements were only taken at the end of this period it is not possible to state whether or nor this is the case.

Dissolving the samples in 6M NaOH resulted in increased levels of detectable silica relative to the aqueous solution, but decreased relative to the levels obtained from treatment with 1M NaOH. This was more pronounced when the solution was incubated. The reason for this is most likely to be polymerisation of the 'free' silica in solution as a result of the pH change in the system, combined with the addition of heat to the system which accelerates the rate of polymerisation. The molybdenum blue assay can only detect single or dimeric orthosilicic acid polymers, so any polymers larger than this (e.g. trimers and larger oligomers) will go undetected by the assay, resulting in a different value being obtained for the reactive silica level of the solution.

Addition of acid to the samples had little effect on the levels of detectable silica released into aqueous solution. There was some increase in the level of the detectable

silica at 1M concentration which becomes more pronounced at 6M concentration. The reason for this is likely to be that the acid hydrolysis of the side-chain linkages does not release much in the way of bound silica into solution. It is possible the hydrolysis of the rhamnose residues in the backbone of the molecule, breaking up the polysaccharide, does not release silica into the solution but this is difficult to confirm without further investigation.

The results obtained using driselase were unfortunately not useful. Addition of driselase into the solution caused problems with the molybdenum blue assay, meaning it was not possible to obtain any useful information on whether or not any silica was being released into solution; however, the use of enzymes for digestion still has validity – a new method for their use needs to be formulated to enable any useful information to be developed from this work.

Chapter 4: Silica & Beer Proteins

Work was carried out using a model wort, supplied by the ICBD, and samples taken from different stages of the production of a commercial beer, provided by the Smiles brewery, Colstons Yard, Bristol. The model wort was prepared at the ICBD 2hL pilot brewery. An all-malt wort was produced from lager malt obtained from Baird's malts, of the Chariot variety. The wort was prepared as described in Leiper's thesis (Leiper, 2002). Several different protein assays were applied to estimate the amount of detectable protein contained within the model wort under investigation before and after treatment with silica material supplied by Crosfield-ICI (SD2489, SD2490, SD2513, SD2514) to remove protein material capable of associating in some with the silica additives. The samples provided by the Smiles brewery and the model wort samples were subjected to amino acid analysis of the protein material present in solution before and after treatment (in the case of the model wort) and at different stages in the production of the beer (in the case of the Smiles samples) to enable comparison of the levels of different amino acid types as a result of the addition of the silica. Full details of the amino acid analysis results can be found in an appendix at the end of this work.

4.1. Protein Extraction Experiments

As discussed in chapter two, proteinaceous materials were extracted from beer raw materials as well as from commercial and model brews. The extractions from raw materials were designed to simply model the process of wort production in the brewhouse by 'mashing' the milled malted barley samples provided by the breweries in hot water. This is a simple procedure and results in soluble sugars and proteins being extracted from the malt into the hot liquor. The maximum mashing temperature was set at 65°C. The mash was raised from approximately 50°C to reach 65°C as this was felt to be a good representation of the brewhouse operation. The solutions were agitated constantly through the use of a magnetic stirrer bar and after an hour at 65°C the bright wort was recovered from the mash by centrifugation and filtration of the mixture. The wort, comprising a solution of sugars and proteins, was then freeze-dried to obtain solid samples for further investigation. The wort was not boiled and consequently there may have been some residual enzyme activity. One of the purposes of boiling the wort is to denature the enzymes in solution and stop any further action. The actual amount of material obtained depended largely on the malt under investigation. The pale malts tended to give a relatively low yield in comparison with the darker malts under the same conditions. This is most likely a result of the relative amounts of modification these malts have undergone during the malting process. A black or chocolate malt is heated at a greater temperature than a light malt such as a crystal and consequently will have undergone more modification as a result of heating than the light malt. Consequently, more material may be easily released from these malts under the mash conditions.

When discussing the relative amounts of material obtained from these varying malts it is perhaps useful to consider their use within the brewery. Typically these malts, which

have undergone a greater degree of modification, are employed as flavouring and colouring malts. In the case of lighter Pils and crystal malts, to introduce sweet flavours without greatly affecting the colour of the brew, and in the case of the darker malts, such as the brown and black malts studied here, to add smoky and burnt tones to the palate of the brew and to considerably darken the colour of the resulting beer.

In the case of the samples under analysis here, 10g of milled barley malt was extracted in 100ml of distilled/deionised water over the temperature range described above and yields of solid material in the range 0.05 – 0.2g were obtained. The physical appearance of the obtained material varied also according to the malt, with the black malts producing 'fluffy' residues after freeze-drying and the un-modified Maris Otter malt producing a comparitively finely powdered residue. This extraction is not particularly scientific, but gives a good starting point to enable examination of the components of the barley malt most likely to be incorporated into the beer.

Protein materials for further investigation were also obtained from commercially produced beers provided by the Smiles brewery and by ICBD. The proteins in this case were precipitated from the brews by the use of ammonium sulphate precipitation techniques. This is a well established biological technique for the extraction of proteins from solution which is often employed as proteins which have been precipitated from solution by this route can often be re-suspended and retain their biological activity; i.e., the precipitation mechanism has little effect on the proteins themselves except to remove them from solution. The only disadvantage of this technique is that relatively large amounts of the salt remain with the precipitate and have to be removed by dialysis from the sample prior to any further experimental steps being carried out.

The efficacy of this technique in recovering solubilised proteins from solution was assessed using a standard BSA (Bovine Serum Albumin) solution. This is not a particularly useful standard for comparison with beer protein, but it is a widely used general standard for calibration of protein assays. Approximately 40-50% of the standard was recovered from solution by this method.

Applied to the samples provided by the Smiles brewery, the results were less impressive still. The amount of protein removed from solution by this method (as detected by the Bradford assay) varied from 18-35%. Assessment of the PEG (Poly Ethylene Glycol) precipitation method also described earlier gave even worse results than those obtained from the ammonium sulphate method. It became apparent that despite repeated attempts to get this method working very small amounts of protein, if any, were being extracted and were not producing yields suitable to allow further research. Research into this method was discontinued and though methods such as ultrafiltration were investigated the ammonium sulphate method was adopted as the cheapest and easiest way of obtaining protein from solution for further investigation.

4.2. Model Wort Experiments

Table 4.1: Protein content in ($mgml^{-1}$) of model wort samples used in the silica stabilisation trials

Sample	Coomassie Blue Assay	BioRad DC Assay	BCA Assay	AA Analysis
Model Wort	12.7	5.9	4.06	n/k
SD2489	3.3	5.39	2.08	79
SD2490	3.9	5.15	2.07	38
SD 2513	3.3	5.74	2.06	35
SD 2514	7.6	5.78	2.08	162

These materials were obtained by precipitation with ammonium sulphate from the wort solution under investigation, followed by de-salting and lyophilisation

4.2.1. Untreated Wort

Table 4.2: Relative % Abundance of Specific Amino Acid Types for Untreated Wort

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	9.13	Pro	18.64
Glu	16.23	Tyr	0.24
Ser	5.41	Val	6.98
Gly	9.80	Thr	0.40
His	2.37	Cys	0.45
Arg	3.01	Iso-Leu	3.52
Thr	4.61	Leu	6.18
Ala	9.82	Phe	3.21

(48.35% non-polar, 20.91% polar, 25.36% acidic, 5.3% basic, 3.45% aromatic)

This beer wort was prepared at the International Centre for Brewing and Distilling at Heriott-Watt University, Edinburgh. The reason for the use of this particular brew was that the brewing conditions were known, and could be reproduced, thus giving a standard beer for this experiment. This does not resolve the problems encountered with attempting to provide a standard model for beer as this brew is only truly representative of its constituent materials. It did, however, provide a start point from which to work when assessing the impact of the silica stabilisation aids supplied by Crosfield for investigation as part of this research. The following results were obtained as described in chapter two and are the result of employing model stabilisation conditions with the silica additive as part of the system. These analyses were carried out on the amino acids remaining in solution, i.e. those remaining after treatment with the silicas. Other workers have carried out experiments to analyse the proteinaceous material retained by the silicas during treatment (McKeown, 1998).

4.2.2. SD2489 Treated Wort

Table 4.3: Relative % Abundance of specific amino acid types for wort treated with silica SD2489

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	6.16	Tyr	0.09
Glu	8.18	Val	4.43
Ser	37.02	Met	0.23
Gly	7.48	Cys	0.08
His	1.33	Iso-Leu	2.43
Arg	1.47	Leu	4.37
Thr	2.90	Phe	2.12
Ala	6.78	Lys	2.21
Pro	12.73		

(33.09% non-polar, 47.57% polar, 14.34% acidic, 5.01% basic, 2.21% aromatic)

4.2.3. SD2490 Treated Wort

Table 4.4: Relative % Abundance of specific amino acid types for wort treated with silica SD2490

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	8.56	Tyr	0.08
Glu	12.71	Val	6.68
Ser	5.56	Met	0.25
Gly	10.73	Cys	0.07
His	2.19	Iso-Leu	3.60
Arg	1.76	Leu	7.06
Thr	5.06	Phe	3.46
Ala	10.34	Lys	2.55
Pro	19.17		

(50.56% non-polar, 21.5% polar, 21.27% acidic, 6.5% basic, 3.54% aromatic)

4.2.4. SD2513 Treated Wort

Table 4.5: Relative % Abundance of specific amino acid types for wort treated with silica SD2513

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	7.54	Tyr	0.13
Glu	12.99	Val	6.65
Ser	4.76	Met	0.64
Gly	11.28	Cys	0.13
His	1.94	Iso-Leu	3.50
Arg	1.86	Leu	6.57
Thr	4.07	Phe	3.22
Ala	11.48	Lys	2.39
Pro	20.85		

(52.91% non-polar, 20.37% polar, 20.53% acidic, 6.19% basic, 3.35% aromatic)

4.2.5. SD2514 Treated Wort

Table 4.6: Relative % Abundance of specific amino acid types for wort treated with silica SD2514

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	8.70	Tyr	0.65
Glu	12.16	Val	6.10
Ser	5.72	Met	1.41
Gly	11.65	Cys	1.28
His	1.86	Iso-Leu	3.36
Arg	3.22	Leu	5.79
Thr	4.44	Phe	2.94
Pro	27.05	Lys	3.66

(46.65% non-polar, 23.74% polar, 20.86% acidic, 8.74% basic, 4.31% aromatic)

4.3. Smiles Samples Experiments

Table 4.7: Protein content (in mgl^{-1}) of samples provided by Smiles Brewery

Sample	Coomassie Blue Assay	BioRad DC Assay	Amino Acid Analyis
Wort	13.9	5.9	132.19
Green Beer	3.5	5.2	73.69
Fined Beer	2.4	4.9	68.79

These samples were obtained during the brewing process for a batch of commercial beer being prepared by this brewery. These samples were analysed in order to facilitate an understanding of the changes in amino acid composition occurring within a beer during the brewing process. These samples were also treated with the Crosfield-supplied silicas to investigate their effect on the composition of amino acids and further understand the proteins removed from solution by the actions of these adsorbents.

Considering the results from table 4.7, it is clear that different assays when applied to these beer samples give different results. The results obtained for both Coomassie Blue and the BioRad DC assays on the Smiles samples are roughly as expected, showing an overall decrease in the level of detectable proteins during the brewing process. This shows that both assays are of use when considering these types of samples, although it is not possible to rely on either for a definitive answer. The methods of action of the assays used during the course of this work are discussed in greater depth later in this chapter. As a result of the amino acid analysis carried out on these samples, it is possible to give a definitive answer as to the amount of amino acid contained within the sample, which is also shown in the table 4.7. As can be seen, these results suggest that the assays typically employed to estimate protein levels massively underestimate the levels in beer. Other workers have also found this to be the case (Hii, 1982).

4.3.1. Amino Acid Analysis of Samples Provided by Smiles Brewery

These samples are of interest as they have come from a commercial brewing process. These samples present an opportunity to study a commercial beer and to observe the changes in protein content and relevant amino acid levels during the brewing process. Samples were obtained at three key stages – wort, green beer and fined beer. These brewers employ the traditional finings method for stabilisation, in keeping with the brewery and the product as well as the commercial sensibilities of the market share they are seeking for their products.

4.3.1.1. Smiles Wort

Table 4.8: Relative % Abundance of specific total amino acid types for Smiles Wort

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	9.34	Tyr	0.71
Glu	16.33	Val	5.74
Ser	6.79	Met	0.95
Gly	11.69	Cys	1.96
His	1.64	Ile	3.42
Arg	2.37	Leu	6.21
Thr	5.22	Phe	2.96
Ala	6.98	Lys	3.05
Pro	14.63		

(40.89% non-polar, 26.37% polar, 25.67% acidic, 7.06% basic, 39.65% aromatic)

This sample is taken at a very early stage of the brewing process and is the result of mashing the malt in hot water – the protein and sugar-rich 'wort' discussed in chapter one. This wort was found to contain $132mgl^{-1}$ amino acid material by amino acid analysis and consideration of the amino acid distribution makes it seem likely that haze would form in this fraction. This is partly due to the very high concentration of amino acid material

within the brew, the large amounts of proline and glutamine (though the figure for glutamine level will also include any glutamic acid present in the sample as this will converted to glutamine during the 6M HCl sample preparation for this assay) which indicate a likelihood of haze-forming ability and the overall relative levels of hydrophobic and hydrophilic amino acids within the sample.

4.3.1.2. Smiles Green Beer

Table 4.9: Relative % Abundance of specific total amino acid types for Smiles green beer

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	9.03	Tyr	1.40
Glu	15.71	Val	5.03
Ser	6.57	Met	0.98
Gly	13.12	Cys	2.53
His	1.54	Ile	3.24
Arg	2.10	Leu	5.55
Thr	5.01	Phe	2.81
Ala	5.86	Lys	3.22
Pro	16.48		

(39.95% non-polar, 28.63% polar, 24.74% acidic, 6.86% basic, 7.82% aromatic)

This sample is obtained from a later step in the brewing process. This is the last stage prior to fermentation of the beer. At this stage the wort has undergone further mechanical procedures and has been boiled and cooled to precipitate the hot & cold breaks which reduce the level of amino acids within the sample. This is illustrated by the decrease in the level of amino acid in this sample – from $132mgl^{-1}$ for the wort to $74mgl^{-1}$ to the green beer.

4.3.1.3. Smiles Fined Beer

Table 4.10: Relative % Abundance of specific total amino acid types for Smiles fined beer

Amino Acid	% Abundance	Amino Acid	% Abundance
Asp	9.26	Tyr	0.43
Glu	16.24	Val	5.60
Ser	7.41	Met	0.79
Gly	12.33	Cys	1.79
His	1.58	Ile	3.32
Arg	.88	Leu	5.99
Thr	5.49	Phe	3.14
Ala	6.39	Lys	3.10
Pro	16.25		

(41.48% non-polar, 27.45% polar, 25.5% acidic, 5.56% basic, 3.57% aromatic)

This sample is obtained from the latter stages of the brewing process. This beer has undergone fermentation and has been treated with finings, a traditional stabilising agent described in chapter one. Also by this time the beer has undergone a period of cold storage to promote the removal of as much of the remaining sensitive protein as possible. Isinglass and gelatin, commonly found in finings, are primarily polyphenol absorbents, whilst bentonite, an aluminium silicate, acts as a protein adsorbent. The protein level by this time has dropped further, with an amino acid content less than the green beer. Once the beer has reached this stage of the brewing process it is clear that a lot of material has been removed, but it is also clear that this has not been a particularly specific treatment. This is evident from the level of proline in the fined product. Whilst the protein level has been reduced considerably, it has been shown that a protein level of as little as $2mgl^{-1}$ is sufficient to cause haze (Bamforth, 1999) and considering the amino acid composition of this fined beer, it is reasonable to say that this beer would possess a tendency to form haze. As a result of the fermentation process, large amounts of amino acids are adsorbed by the

yeast cells added to ferment the beer. This accounts for much of the free amino acid in beer, though yeast does not absorb the free proline available in beer. Consequently, it can be said that fermenting the beer, whilst causing an overall reduction in the level of protein material, refines the protein to leave behind the haze active rpoline material.

4.4 Protein Assay & Amino Acid Analysis

Consideration of the amino acid analysis of the untreated wort sample used in the stabilisation trials clearly shows the reasons for the poor response of the protein assays employed during the course of this study. Each of these assays has either a slightly different mode of action (Williams, 1995), i.e. respond to different functional amino acids, or a different susceptibility to interference by external contaminants.

4.4.1. Protein Precipitation & Assay Techniques

The experimental procedures for the use of these techniques has been previously discussed in chapter 2. This section considers the mechanisms of response of the various methods employed and their usefulness when considering beer protein material.

4.4.1.1. Coomassie Blue Assay

The dye employed in the Coomassie Blue assay (Bradford, 1976) reacts primarily with arginine functional groups. Other basic residues such as hystidine and lysine, as well as the aromatic residues (tryptophan, tyrosine and phenylalanine) give slight responses. Considering the amino acid composition of the proteinaceous material obtained from the wort it can be seen that the level of arginine, the primary reacting residue, as well as of the other reacting amino acids is relatively low compared to the level of other amino acids such as proline and glutamine. This assay is considered as detecting proteins with a

molecular weight of over 5,000Da (Compton, 1985 & Hii, 1982). As has already been discussed, beer proteins can arbitrarily divided into haze forming (undesirable) and foam positive (desirable) proteins (Dale, 1992; Evans, 1995; Kano,1993; Kaufman, 1994; Lusk, 1995; Onishi, 1994 & Sorensen, 1993). Haze forming proteins, which can comprise up to one third of the total protein in beer, are rich in proline (a cyclic amino acid which can make up as much as 20% of the protein) and glutamine (which can make up to 30% of the total protein). Haze-forming proteins are typically between 1,000 and 40,000Da in weight. Based on this information it is reasonable to conclude that this assay under-represents the haze-forming proteins in beer from the hordein fraction of barley.

In comparison, the foam-positive proteins, which largely originate from the albumin and globulin fractions of the malt, are relatively rich in basic amino acids such as the previously mentioned histidine, lysine, arginine and the aromatic phenylalanine, tyrosine and tryptophan. Studies have shown that these proteins typically have a molecular weight of over 10,000Da (Brierly, 1996; Clark, 1988, 1991, 1991a, 1991b, 1994; Dale, 1993; Sarker, 1996 & Sharpe, 1997).

It can be concluded that the dye-binding would under represent the proteins originating from the hordein fraction of the malt but would emphasise proteins of the type shown to be important in beer foam production and retention (Asano, 1982).

The overall low protein level indication of this assay relative to the true protein content of the beer is due to the fact that far more of the protein in beer (~60%) originates from the hordein fraction, compared with the approximate 10-15% originating from the globulin and albumin fractions of the barley. This assay primarily has use in indicating the levels of high molecular weight, foam-positive proteins.

Research by other workers (Hii, 1982) has also shown that the Coomassie Blue dye gives variable results with different proteins. Some proteins produce a linear plot of concentration vs. absorbance at 562nm whilst others do not. The typical calibration standard, and the one employed for the purposes of this study, is Bovine Serum Albumin (BSA). BSA is relatively rich in arginine (~5%) which accounts for its strong response to the dye; however, this standard does not directly relate to beer proteins due to the compositional differences between the two types of protein. Beer protein produces a response about one seventh as strong as that of BSA.

In order to obtain a useful measure of the protein in beer a standard more closely resembling the proteins found in beer is required; however, this is not an easy proposition due to the wide variety of materials and methods employed in beer production and the difficulty on producing such a standard. It is nonetheless apparent that for a more accurate quantitative response the protein employed as a standard should contain similar levels of arginine as the protein found in beer.

4.1.1.2. The BioRad DC Assay

The BioRad DC Assay is a detergent-compatible modification of the Lowry assay (Benadoun, 1996) and is a combination of two distinct reactions. Initially, there is a copper independent reduction of the acid-complex Folin reagent, principally by the side-chains of tyrosine and tryptophan and to a lesser extent of cystine and histidine. The second reaction is a reduction of the acid complex mediated by copper complexation to the peptide chain, principally in the histidine and arginine residues (Aslam, 1998).

The principal chromogenic amino acids are tyrosine and tryptophan. Whilst the colour value of mixtures of free amino acids is additive, the colour yield for peptides can be more or less than the sum of the component free amino acids. It is dependent on

peptide sequences, chain length and exposure of the relevant functional groups. The peptide linkages can also be chromogenic as reduction of the mixed acid can also occur from dipeptides consisting of otherwise non-chromogenic amino acids.

Due to the large number of chromogenic sequences in protein it is not possible to predict the chromogenicity of different proteins based on their structure. As for the Coomassie Blue assay, it is difficult to find an appropriate standard without having further information on the sequences and conformations of proteins found in beer. Even then, it is difficult to predict the average response of the different protein types to the assay and therefore to find a suitable 'catch-all' standard for beer protein determinations. It should also be noted that whilst this assay is detergent compatible, it is prone to interference from carbohydrates and metal ions, both of which can be found in beer in small concentrations. Unless the concentrations and exact identities of these materials were determined from the sample under investigation, and the calibration doped to include similar levels, it is difficult to estimate the accuracy of this assay without further study.

4.4.1.3. The Pierce BCA Assay

The Pierce BCA assay makes use of the reaction between bicinchonicic acid (BCA) and proteins (Brown, 1989; Smith, 1985; Walker, 1994 & Wiechelman, 1988). It is similar to the Lowry assay in that both rely on a biuret reaction between peptide bonds and copper atoms. Whereas the Lowry assay uses Folin reagent to enhance the colour response of the reaction, the BCA assay makes use of the sodium salt of BCA. This is a highly specific chromophore for Cu^{1-} and gives a simplified, one-step protein determination with good tolerance to non-ionic detergents and simple buffer salts; however, some compounds commonly used in protein preparation can affect the colour development of the BCA chromophore. This includes ammonium sulphate, used in this

study to purify protein from a variety of samples. Ammonium sulphate suppresses the absorbance at 562nm induced by the presence of protein material. There are two possible methods for this – either Cu^{1-} may be become oxidised back to Cu^{2+}, resulting in fewer BCA-Cu^{1-} ordered complexes or the interfering compound may directly block the formation of the complex. In order to overcome this, an established protein precipitation method making use of sodium deoxycholate and trichloroacetic acid (DOC & TCA) is recommended to avoid contamination of the sample with interfering substances (Brown *et. al.*, 1989). Again, in common with the other assays discussed in this chapter, the results from this assay cannot be considered absolute due to the method of action of this assay.

4.4.1.4. Ammonium Sulphate & PEG Precipitations

The ammonium sulphate precipitation employed to extract the protein material relies on the hydrophobic character of the protein. Water solvates the added salt ions, decreasing the solvation of the protein molecules. This decrease exposes hydrophobic areas of the protein structure, which interact with each other to give aggregates that precipitate out of solution; however, this 'salting-out' effect is not fully understood (Pryzbyain & Bailey, 1991). It is assumed that this method will cause the precipitation of the majority of the protein material within a sample. As it is not possible (for reasons previously discussed) to absolutely measure the protein content of beer, coupled with the fact that much of the material in beer is not true protein, it is not possible to predict the efficiency of this method with regards to removal of proteinaceous material from beer. One disadvantage of this technique is that relatively large concentrations of the salt remain associated with the final sample which must be removed prior to further work. For this study the salt was removed by dialysis with distilled de-ionised water in SpectraPor

molecular tubing. Employment of this technique should reduce the likelihood of problems with the BCA assay as a result of interference from ammonium sulphate.

As well as ammonium sulphate precipitation, precipitation by the use of polyethylene glycol (PEG) was also employed to precipitate proteins from the samples under investigation. This works in a similar way to the ammonium sulphate precipitation previously described. Despite the fact that most proteins will precipitate at 30%w/v PEG, very small yields of material were recovered and use of this technique was discontinued.

4.4.2. Amino Acid Analysis

4.4.2.1. Discussion of the Analysis Technique

The amino acid analyses reported in this these were carried out at the MRC Immunochemistry Unit at the University of Oxford, using an Applied Biosystems Model 420A PTC derivatiser with an on-line Applied Biosystems Model 130A PTC Amino Acid Analyser.

In order to consider the results obtained from the use of the technique, it is necessary to understand the method of analysis and also the limitations thereof. Amino acid analysis is a means of quantifying the amounts of each amino acid present in a protein or peptide, and can be separated into four distinct steps:

1. Hydrolysis
2. Derivitisation
3. Separation of derivatised amino acids
4. Data interpretation and calculations

4.4.2.1.1. Hydrolysis

A known amount (2nmols) of internal standard (norleucine) is added to the sample under analysisy.. As norleucine is not a naturally occuring amino acid in proteins, is stable to

acid hydrolysis and can be readily chromatographically separated from other protein amino acids, it makes an excellent internal standard. The sample, containing at least 1nmols of amino acid, is then transferred to a specially cleaned and furnaced hydrolysis tube and dried under vacuum. The tube is placed in a vial containing 5.7 N Hcl and a small amount of phenol, and the protein is hydrolysed by the HCL vapours under vacuum for 22-24 hours at 110°C. Following hydrolysis, the sample is dissolved in distiled water containing EDTA (to chelate metal ions) and 20-40% of each sample is placed on a glass amino acid analyzer sample slide. Hydrolysis can have varying effects on different amino acids, summarised in the table below:

Table 4.1: The Effect of Aacid Hydrolysis on Various Amino Acids

Amino Acid(s)	Effect
Valine, isoleucine	Bonds are not easily broken - timecourse hydrolysis needed for really accurate result
Threonine, serine	Slowly destroyed by acid hydrolysis – timecourse hydrolysis needed for really accurate result. Serine is a common contaminant
Methionine	Partially oxidised during acid hydrolysis
Asparagine, glutamine	Converted to aspartic Acid and Glutamic Acid respectively
Tryptophan	Completely destroyed by acid hydrolysis
Cystine	Destroyed by acid hydrolysis. Needs to be derivatised by oxidation or reduction and alkylation

4.4.2.1.2. Derivatisation

The hydrolysed/free amino acids cannot be detected by HPLC unless they have been derivatised. This process is performed automatically on the Amino Acid analyzer by reacting the free amino acids, under basic conditions, with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acid derivatives. This process takes

approximately 30 minutes per sample. A standard solution containing a known amount (100pmol) of 17 common amino acids is loaded on 3 or 6 separate amino acid analyzer sample spots and derivatised. This provides the calibration file that can be used to determine amino acid content of the sample. Following dervatisation, a methanol solution containing the PTC-amino acids is transferred to a narrow bore HPLC sytem for separation. The effect of common contaminants on derivatisation is summarised in the following tables:

Table 4.2: Effects of Common Contaminants on Derivatisation Yield

Effects of common buffer salts and detergents on amino acid derivatisation.

All solutions were added to 100pmols of hydrolysate amino acid standard in a 20µl volume.

Concentration of salt solutions 50mM; concentration of detergent solutions 0.1% (v/v).

Data from Applied Biosystems user manuals.

Additive	**Effect**
Ammonium Acetate	No negative effect on results
Sodium Acetate	His low, Tyr, Val, Ile, Leu, Phe and Lys slightly low
Triethylammonium acetate	His and Thr slightly low
Ammonium Bicarbonate	Thr slightly low
Sodium Bicarbonate	His and Tyr low, Ile, Leu and Phe also low
Sodium Borate	No negative effect on results
Sodium Chloride	No negative effect on results
Sodium Phosphate	Low and variable yields of most amino acids
Triethylammonium phosphate	No negative effect on results
3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)	Very large late eluting peak obscures Phe and Lys
N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES)	Gives an artifact peak which co-elutes with Met
[2-hydroxyethyl]iminotris[hydroxymethyl] methane (TRIS)	His slightly low, artifact peak co-elutes with Tyr
Sodium dodecylsulphate (SDS)	His and Thr slightly low, Cys and Lys yields are good
Triton X100	His and Thr slightly low, Cys and Lys yields are good

Table 4.3: Effects of Trace Contaminants on Amino Acid Derivatisation

All solutions added to 100 pmols of hydrolysate amino acid standard in a 20µl volume.

Concentration of metal solutions was 20ppm.

Data from Applied Biosystems user manuals.

Metal	Effect
Aluminium	Asp and Glu very low, all other amino acids low except Pro
Boron	No significantly adverse effects, His and Thr may be slightly low
Copper	Cys and Lys almost gone, His low, Asp and Glu slightly low
Iron	Glu, Ser, His, Thr, Cys, Lys all low
Lead	Asp , Ser, Thr, Lys slightly low
Nickel	Almost no recovery of any derivatives exceptt Thr and Pro
Zinc	Acidic and basic amino acids and Cys very low, Ser slightly low

4.4.2.1.3. HPLC separation

The PTC amino acids are separated on a reverse phase C18 silica column and the PTC chromophore is detected at 254nm. All of the amino acids will elute in approximately 25 minutes. The buffer system used for separation is 120mM sodium acetate pH 5.50 as buffer A, and 70% acetonitrile as buffer B. The program is run using a gradient of buffer A and buffer B with an initial 7% buffer B concentration and ending with a 60% buffer B concentration at the end of the gradient.

4.4.2.1.3. Data Interpretation and Calculations

Chromatographic peak areas are identified and quantitated using a data analysis system attached to the amino acid analyzer system. A calibration file is prepared from the average retention times (in minutes) and areas in (in (Au)) of the amino acids in 3 to 6

standard runs. Since a known amount of each amino acid sample is loaded onto the analyzer, a response factor ((Au)/pmol) can be calculated. This is then used to calculate the amount of amino acid (in pmols) in the sample. The amount of each amino acid is calculated by dividing the peak area of each (corrected for the differing molar absorptivities of the amino acids) by the internal standard (norleucine) in the chromatogram, and multiplying this by the amount of the internal standard added to the original sample. After the picomole by height of each amino acid has been calculated, the data can be manipulated to yield more useful information. The chromatography software will extrapolate back to 2nmoles of internal standard (norleucine) and give a result for the total amount pipetted into the hydrolysis tube originally.

Mole percent represents the amount of each amino acid present as a percentage of the total amino acids recovered in the sample. Mole percent can be useful for samples in which there is no known composition or molecular weight, non-specific molecular weights or where the sample contains mixtures of proteins, free amino acids and other components, as in the samples under investigation in this case. Amino acid analysis can also give useful information on composition by molecular weight where the weight of the ample is known, composition by residue where the number of times a residue occurs in the sample is known, and the minimum molecular weight for samples where neither the molecular weight nor the composition of the sample are known.

4.4.2.2. Results & Discussion

It is clear that for the system under investigation, interference from contaminating substances listed in tables 4.2 & 4.3 is unlikely. Traces of detergent are not likely to be acceptable in a commercial brew, and the detergents listed were not part of the protein purification techniques applied to the model system. Whilst trace amounts of metal ions may be expected in a beer, these are not likely to be in concentrations sufficient to cause interference to the assay due to links between metal ions and problems with beer, for example 'gushing'. It is therefore reasonable to conclude that the assay should work effectively for this system, based on the information supplied by the MRC Immunochemistry unit, which carried out the assay work.

Considering the amino acid analyses of the pre- and post-treatment wort samples (included in full in appendix A), it can be clearly seen that the addition of the silica adsorbents supplied by Crosfield alters the protein composition remaining in the beer solution (table 4.11). For the purposes of this study, the amino acids of most interest were proline and glutamine as these amino acids are identified as occurring in sequences together in haze-forming proteins and proline is implicated as the site of interaction between these proteins and the haze-active polyphenols. It has been demonstrated that the tendency of a protein to form haze is directly related to the proline content of the protein.

Table 4.11: Proline and glutamine content as a percentage of total amino acid composition of untreated wort and samples treated with the Ineos supplied silica adsorbents

Sample	Proline %	Glutamine %
Untreated Wort	18.64	16.23
SD2489	12.73	8.18
SD2490	19.17	12.71
SD2513	20.85	12.99
SD2514	27.05	12.16

Initial consideration of these results shows that only wort treated with silica SD2489 shows a drop in the relative levels of the amino acids associated with haze-forming proteins. It is difficult to ascertain what the effect of the silica additive is in the overall protein level of the wort sample.

It should be remembered that silica hydrogel acts as a protein adsorbent by both adsorption onto the surface of the silica and admittance of proteins into the pores of the gel. In order to consider the effect of the silicas on the proteins of the wort, it is necessary to consider the physical characteristics of the silicas themselves.

Table 4.12: Physical characteristics of the supplied silica adsorbents

	SD2489	**SD2490**	**SD2513**	**SD2514**
Permeability (darcy)	0.14	0.17	0.18	0.07
APS (micron)	13.0	19.2	11.4	9.4
PH (5% slurry)	5.7	7.0	7.0	4.3
H2O loss at 105C	5.4	10.3	6.3	24.6
%TVM	10.3	13.7	9.4	29.5
SA (m2/g)	816	435	322	492
PV (ml/g)	1.55	1.04	1.74	0.28
Mean PD (Ångstrom)	76	95	216	23

As discussed in the earlier introduction, a mean pore diameter of 2.5nm (1 Ångstrom is equivalent to 0.1nm) is considered to be optimal for the removal of haze-forming proteins. The binding of proteins to silica gel is considered to be analogous to the mechanism of haze formation between the proline-containing proteins and haze-active polyphenols. It would appear that whilst these adsorbents have the ability to remove undesirable protein material, they lack specificity required to discriminate effectively to prevent the undesired removal of useful protein material from the brew. Research into the use of silica adsorbents has shown that large pores denigrate the foam-retention capacity and mouthfeel of a beer by allowing the admittance of relatively large proteins into the pores of the hydrogel.

Based on the provided information for these samples, the silicas can be classed as hydrogels given the large surface area values. Hydrogels are the most commonly applied silica stabilising agent in the modern brewhouse

SD2489 was the only silica treatment which post-addition showed an overall reduction in the relative levels of proline and glutamine, indicating a better specificity for these haze-forming proteins than the other trialled materials. It therefore follows that for this particular all-malt wort, the physical properties of this particular material were most effective in removing protein material likely to contribute to haze formation. The wort material treated with the other silicas showed increased relative levels of proline and glutamine post-treatment. This does not necessarily lead to the conclusion that these materials are ineffective in removing haze-active protein, but do indicate that where these materials may adsorb haze active protein, their physical characteristics also lead them to remove the larger foam-positive proteins from the brew. Without in-depth investigation of the material retained on the silica surface as well as trapped within the pores of the

materials, it is difficult to conclude anything further about the net effect of these adsorbents. It could however be surmised that treatment of this particular brew with these materials could be deleterious to the quality of the finished product produced.

It is possible to engineer the physical characteristics of these adsorbents to improve their specificity for a particular brew by making use of techniques such as aging or calcination to vary the pore size of the silica produced in order to provide the most effective protein removal profile for a particular brew.

Protein assay and amino acid analysis of the process samples provided by the Smiles Brewery (Colstons Yard, Bristol) shows the changes in the protein concentration and composition of a typical beer during the brewing process.

Considering the detectable protein concentration, it can be seen that the overall level of protein within the brew diminishes during the brewing process. This is expected, as within the brewing process there are several processes that contribute to removal of protein from the brew and, as a result, increase the colloidal stability of the resulting product.

Looking at the brewing process directly, the highest level of protein would be expected in the wort. Wort is formed by the addition of warm water to the malt grist to form the mash. This causes enzymes such as α-amylase to convert soluble malt starch into dextrins and maltotriose and also incorporates soluble proteins into the brew. Green beer, i.e. newly fermented beer, has been through several stages that contribute to protein loss from the brew. The first stage following production of wort is wort boiling and hopping. During this stage the boiling causes precipitation of certain proteins (the 'hot break') which are removed either through the use of a 'hop back' in traditional brewing or by the centripetal forces generated by a whirlpool in modern brewing techniques. The hop back

is formed where whole hop cones are used as an additive to the brew. Essentially, the solid residue from the hops forms a filter bed, which removes the solid material from the brew. Most modern brewing techniques involve the use of hammer-milled or pelletised hops, and the residue from these is removed by the use of a whirlpool, along with precipitated proteins and other insoluble materials. Following the wort boiling stage the wort is cooled prior to the addition of the yeast. Cooling the brew causes more protein material to precipitate from the solution (i.e. 'chill haze' forms) which is removed by filtration. Additionally, the yeast itself may play a part in removing haze-active protein as a result of the tendency of these materials to adhere to the surface of the yeast cells. The yeast is of course recovered from the brew for further use, removing whatever materials may be physically associated with it.

Fined beer, in addition to the procedures already mentioned, has been through maturation and treatment aimed at improving the clarity of the finished product. Traditional treatment at this stage involves the addition of 'finings' which is something of a catchall term that can include a mixture of organic or mineral substances used to cause impurities, yeast and other suspended matter to coagulate and precipitate from the brew for easy removal. Isinglass, gelatin, bentonite (aluminium silicate clay), egg albumin, Irish moss (a type of seaweed), charcoal, wood chips and casein are all examples of colloidal agents used for this purpose. Isinglass has been shown to have very little effect on the long-term stability of beer, though improvements in foam stability as well as an increase in the rate of fermentation as well as a decreased level of tannoids in the beer have been associated with its use (Leiper, 2002). Some of these traditional methods are not commonly used, though Anheuser Busch advertises the use of beechwood chips as an indication of quality in its beer. The beechwood strips employed in this process are

typically a couple of centimeters wide and can be up to a foot long and work by providing a large surface area for deposition of undesirable materials from within the brew. Beechwood aging has been shown to remove polyphenol material from the brew (Stewart, 2003). Isinglass is primarily gelatin in composition and works by coagulating with haze-active polyphenols in the same way as the haze-active proteins. Bentonite is essentially a simple silica additive, which acts as a protein adsorbent. The drawback of this material is the lack of specificity of its action – too long a contact time with the beer will cause damage to the foaming characteristics sought by the brewer for the finished article.

The protein assays, due to the limitations earlier discussed, can be assumed to refer primarily to the high molecular weight, long chain proteins contained within the brew. With this in mind, the results obtained from these assays show that there is a clear reduction in the levels of those proteins assumed to be of beneficial character as far as customer expectation of the finished brew is concerned. It may be that, purely by virtue of their large size, these proteins themselves contribute to the overall tendency of a beer to produce a visible haze by agglomeration and precipitation, although not necessarily by forming the protein-polyphenol complexes shown by the proline-containing haze-active protein. It is possible that the removal of some species that could be considered beneficial to the brew may result in a decrease in the haze-forming tendency of the finished product.

The amino acid analysis data obtained for the material extracted from the wort and green beer samples by precipitation shows that the overall composition of the protein within the brew does not vary greatly as a result of the process stages between these two samples. The relative level of proline remains fairly constant at 16.48 and 16.25 mol% respectively. A decrease in the level of proline containing proteins would be expected due to the precipitation of this class of material experienced as part of the 'cold break'

These results are in agreement with results obtained by other workers (Asano, 1982) which show the level of detectable protein decreasing through the brewing process. Asano also demonstrated that the level of proteins considered to be foam-positive (those of a relatively high molecular weight and low level of proline) were reduced during the brewing process. The decrease in foam positive proteins as a result of the wort boiling stage comes about as a result of interactions between the polyphenols, humulones and iso-humulones with the foam-positive proteins leading to precipitation from the brew. Analysis of the various fractions of proteins obtained by beer (separated by molecular weight) showed that the foam-positive proteins occur in the molecular weight range 10,000 to 100,000 Da and the following data was obtained:

Table 4.13: Amino acid composition of three distinct molecular weight bands of beer proteins

Amino Acid composition of protein (mol%)	**RelativeMolecular Weight**		
	Higher	**Medium**	**Lower**
Gly	6.1	7.5	9.2
Ala	6.7	8.6	7.4
Val	4.1	6.4	5.5
Leu	5.7	8.3	5.3
Ile	2.4	3.4	2.8
Ser	6.9	7.5	5.9
Thr	3.6	3.9	4.1
Cys	0.0	0.0	1.9
Met	0.0	0.0	1.1
Phe	2.2	4.1	1.5
Tyr	0.9	1.1	1.7
Pro	3.9	8.7	10.3
Asp	5.6	6.6	8.5
Glu	9.0	14.2	14.3
Lys	3.6	3.3	2.5
Arg	5.7	3.4	3.6
His	3.4	3.4	2.5

This data supports the assertion that the larger weight proteins contain less proline than the low molecular weight proteins originating from the hordein fraction of the barley grain. It can be seen that proline content of protein material in beers correlates approximately with the molecular weight of the protein.

Similar research by another group (Dale, 1989) found that protein material with a molecular weight of greater than 60,000Da originates from yeast mannan-protein or, in the case of beer prepared with torrified wheat, from wheat proteins. The fraction of molecular weight 40,000-60,000Da is consistent with having been derived from cereal albumins and globulins. Analysis of a variety of commercially prepared beers showed the final amino acid composition of the brew was relatively consistent and that the major amino acids in beer prepared from 100% malt are glutamic acid/glutamine, proline, glycine and aspartic acid/aspargine (Sorenson, 1978).

The low molecular weight proteins have been shown through the use of ELISA techniques (Sheehan, 1997) to originate from the hordein fraction of barley (molecular weight of less than 8,600Da, comprising approximately 64% of total wort polypeptides).

Investigation of the amino acid composition of haze-forming proteins extracted from beer (Asano, 1982) and from the malt fractions hordein, albumin and globulin gave the following results:

Table 4.14: Amino acid composition of various proteins extracted from beer and brewing raw materials (Asano, 1982)

Protein content (%)		Haze Forming Proteins			Malt	
Amino Acid	I (1,000-10,000Da)	II (19,000Da)	III (16,000Da)	IV (40,000Da)	Hordein	Albumin & Globulin
Gly	9.3	4.7	9.2	7.5	1.8	8.7
Ala	6.1	3.1	7.4	8.6	2.1	8.6
Val	3.6	2.2	5.5	6.4	3.1	6.4
Leu	3.3	2.3	5.3	8.3	4.7	6.5
Ile	2.2	2.3	2.8	3.4	2.8	3.2
Ser	4.6	3.4	5.9	7.5	3.3	6.0
Thr	0.4	2.0	4.1	3.9	1.4	3.5
Cys	1.2	0.9	1.9	0.0	0.6	1.5
Met	0.7	0.5	1.1	0.0	0.6	1.7
Phe	1.2	2.6	1.5	4.1	4.4	2.7
Tyr	1.6	2.0	1.7	1.1	1.6	2.7
Pro	5.5	19.9	10.3	8.7	18.2	7.3
Asp	6.6	3.0	8.5	6.6	1.2	9.3
Glu	12.1	20.9	14.3	14.2	29.1	9.6
Lys	3.9	1.3	2.5	3.3	0.3	3.6
Arg	4.2	1.4	3.6	3.4	1.3	4.3
His	2.3	0.6	2.7	3.4	0.9	1.7

These results clearly support the origination of haze-forming proteins from the hordein fraction of barley by comparison of the analyses of the fractions with the analysis of the material from the barley fractions. Studies of the development of chill haze over 70 days showed beer haze to consist of 31-50% protein, 13-17% polyphenol and 39-57% carbohydrate. These studies also demonstrated that whilst most amino acid levels in chill haze remained approximately the same during the formation of the haze, proline levels were highest initially and then decreased during the course of the storage period. This indicates that initially the proline containing proteins contribute primarily to the formation of the haze. As time elapses, the protein content of the haze increases, whilst the relative

level of proline in that protein decreases. This indicates that after the initial haze formation (0-3 days) the proline containing, haze-active proteins play the primary role in haze formation. As time passes, other, larger proteins with lower proline levels (i.e. those thought of as foam positive and non haze-active) are incorporated into the haze, lowering the overall percentage of proline in the composition of the protein.

This supports the assertion that initial chill haze formation is not necessarily detrimental to the quality of a beer as a result of its primarily comprising of undesirable haze active proteins and polyphenols, and also due to the fact that on warming short term chill haze will re-dissolve. Over time, other desirable proteins are incorporated into the haze and extended cold storage will undoubtedly lead to removal of proteins associated with foam formation and retention, and mouth feel, being removed from the beer. Haze allowed to form and remain over an extended period of time becomes permanent, i.e. will not re-dissolve on warming.

The most likely reason for the haze activity of the proline containing proteins is the structure of proline itself. The structure of the proline rich proteins is unfolded due to the pyrrolidine ring of the proline. This unfolded conformation facilitates entry of the haze active polyphenols into the interior of the protein. As well as this, the pyrrolidine ring cannot form intra- and inter-molecular hydrogen bonds with the oxygen atoms of the peptide bonds and so these free oxygen atoms readily form bonds with the hydroxyl groups of polyphenols contained within the brew. The hydrophobic nature of proline also means that it would also participate in hydrophobic bonding between the haze-forming proteins and polyphenols.

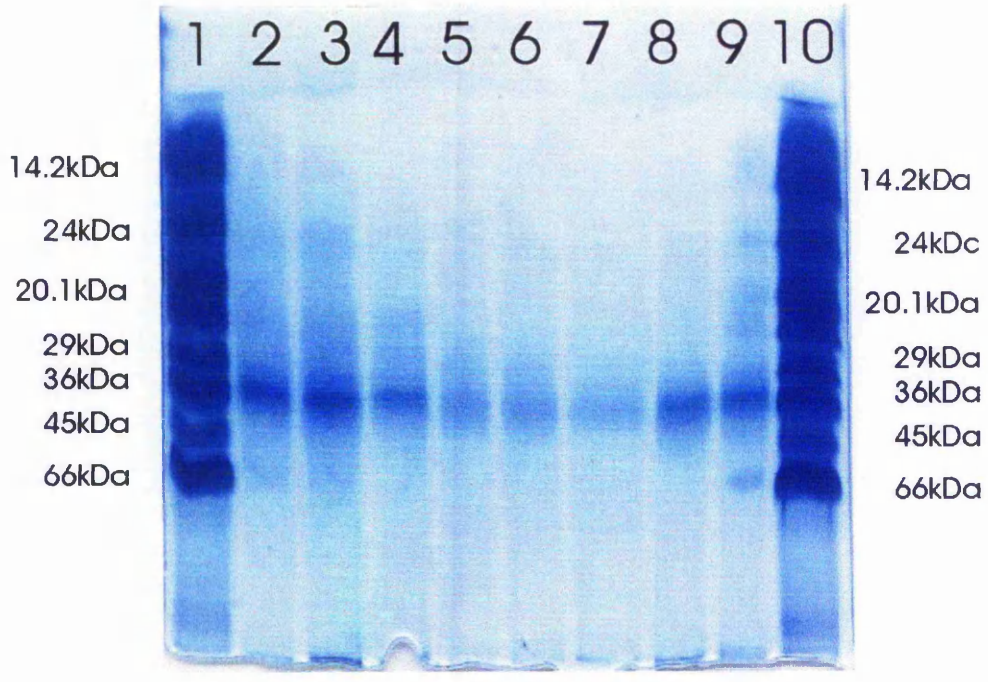
With this in mind, the results obtained from the addition of additives to the catecholate model system described earlier appear to support the assertion that proline is

the amino acid which gives these proteins their haze forming properties. This hypothesis is further supported by unrelated research which demonstrated a an interaction between salivary proline-rich proteins and dietary tannins (Baxter, 1997 & Mehansho, 1987). Precipitation runs of the model system were carried out in the presence of polyproline, glutamic acid and aspartic acid. The results clearly showed that the presence of the polyproline in the model system as an additive caused a decrease in the levels of silica detectable by the molybdenum blue colorimetric assay. As has been discussed, this assay detects free, unbound silica and does not show a response for bound silica particles of trimer size or over. The other species tested as additives into the model system showed no effect on the levels of detectable silica from the solution.

4.5. Protein Electrophoresis by SDS-PAGE

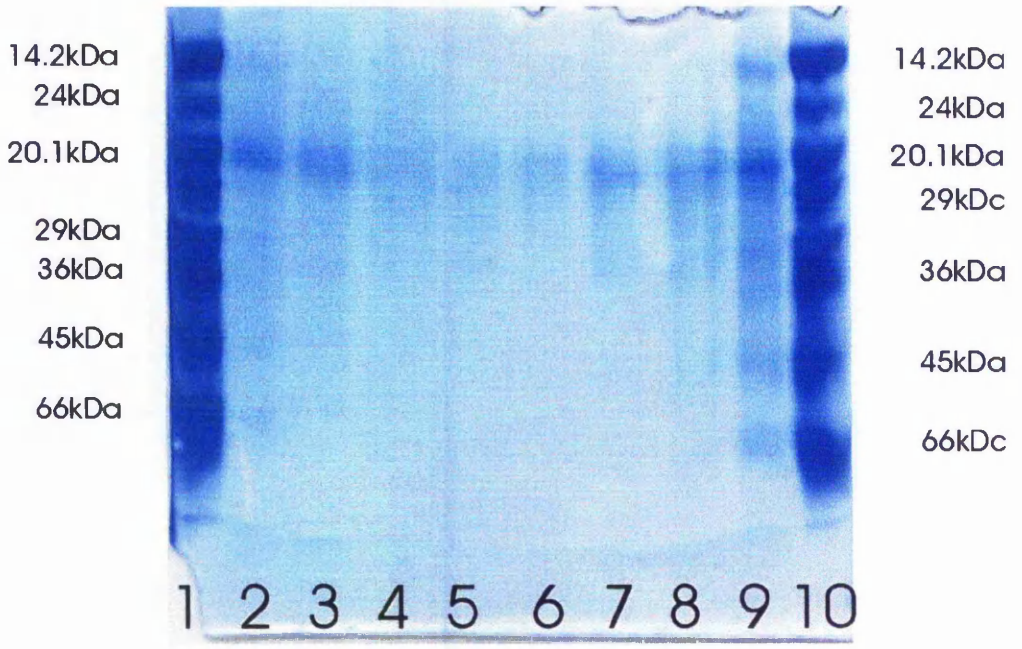
Gels were prepared according to the method detailed in chapter 2. Samples of protein extracted both raw materials (the brewery supplied malted barleys) and from in-process samples of beer (supplied by the Smiles Brewery) were analysed against a marker (Dalton mark VII-L for SDS Gel Electrophoresis, Sigma SDS-7) containing a mixture of proteins of approximate molecular weights 14,200 – 66,000 Daltons. Following electrophoresis the gels were stained according to the Coomassie Blue staining protocol, also detailed in chapter two. Following are examples of the gels obtained:

Figure 4.1: SDS-Page of Precipitated Beer Proteins



Lane 1: Marker SDS-7
Lane 2: Preicpitated Protein Fined Beer1
Lane 3: Preicpitated Protein Fined Beer2
Lane 4: Preicpitated Protein Fined Beer3
Lane 5: Preicpitated Protein Green Beer1
Lane 6: Preicpitated Protein Green Beer2
Lane 7: Preicpitated Protein Green Beer3
Lane 8: Preicpitated Protein Wort1
Lane 9: Preicpitated Protein Wort2
Lane 10: Marker SDS-7

Figure 4.2: SDS-PAGE of Heat Extracted (Mash) Proteins from Malted Barley



Lane 1: Marker SDS-7
Lane 2: Mash Protein Hook Norton Black
Lane 3: Mash Protein Hook Norton Brown
Lane 4: Mash Protein Hook Norton Brown B
Lane 5: Mash Protein Greene King Black
Lane 6: Mash Protein Greene King Black B
Lane 7: Mash Protein Castle Rock Black
Lane 8: Mash Protein Castle Rock Maris Otter
Lane 9: Mash Protein Castle Rock Crystal
Lane 10: Marker SDS-7

Considering these two gels generally, it can be seen that the resolution obtained was quite poor and as such the results can only be used to give a general idea of results. This was true for all the gels run in this program of research; however, time constraints limited developing this technique any further. It is also apparent that there is some overrun of the marker into the next lane, partially obscuring any bands obtained there. This most likely to be a result of operator error – i.e. poor injecting technique or damaged or deformed wells on the gel itself.

It is also important to consider the earlier discussion of dye binding assays in this chapter. Following further research, it is clear that the Coomassie Blue assay was not the best staining method to employ for this analysis.

Considering the results obtained for proteins precipitated from the process brewing samples (those provided by Smiles) it can be seen that there is an apparent band in the region of 36-45Da detectable by the Coomassie staining. In the case of the proteins extracted from malt barley under mashing conditions, the visible band is in the region of 20-29Da. These gels show that the protein molecular weight does not vary markedly for the proteins extracted from the various malt samples. This is important as the malts used for this study came from a variety of sources, and most likely growers, and had all undergone differing proprietary malting techniques, but would most likely be treated in a similar way to produce beer. This is important from the point of trying to conceive of beer as a standard system, or indeed designing a suitable standard to model beer. The Coomassie detectable protein in the process brewing samples from Smiles again does not appear to show any marked variance in molecular weight through the stages of the brewing process.

This technique would have more use if a different stain was employed, rather than Coomassie blue. As previously discussed, this dye is useful only for proteins of the type that are unlikely to contribute in any meaningful way to the production of haze. In order to gain much information on the molecular weight distributions of proteins within these samples a technique with more sensitivity to proteins of the amino acid compositions found in haze-forming proteins needs to be employed.

5. The Molybdenum Blue Assay

The rationale behind the use and the mechanism and mode of action of both the molybdenum blue colorimetric assay and the model precipitation experiments has been well established (Perry, 1992; Harrison, 1995; Evans, 1989; Loton, 1994; Keeling-Tucker, 1999; Govett, 1961; Alexander 1953 & 1954; Mullin, 1955; Iler, 1976, Jolles , 1898; Strickland, 1952).

The Molybdenum Blue assay is a photometric method of determining silica concentrations. The assay makes use of the ability of orthosilicic acid to form a yellow coloured heteropoly acid (Alexander 1953, 1954), silico-12-molybdic acid, with molybdate ions in active solution.

Silicic Acid + Acidified Molybdate → Heteroplymolybdic Acid

$$7Si(OH)_4 + 12H_6Mo_7O_{24}.4H_2O \rightarrow 7H_8[Si(Mo_2O_7)_6].28H_2O$$

This yellow silicomolybdic acid species exists in two forms, an α- and a β- (*cis*- and *trans*- geometrical isomers) (Ferrari, 1951; Strickland, 1952). These forms have the same empirical formula but differ in their stability and the conditions under which they are formed. Silicomolybdic acid can be readily reduced in an acidic medium to the blue α-silicomolybdous acid, hence the molybdenum blue assay (Mullin, 1955). This is more sensitive than the yellow method as a stronger absorbance is obtained with smaller concentrations of orthosilicic acid for the blue form in comparison to the yellow form. In order for this reaction to be successfully employed the reduction step must take place within fifteen minutes of the formation of the initial silicomolybdate species.

In aqueous solution the silicomolybdic acid anion exhibits Keggin type structure (Cotton, 1988) with full tetrahedral symmetry. There is a cavity formed by the MoO_6 octahedron in which the single Si atom can sit, bonded to a tetrahedron of oxygen atoms

(Govett, 1961). As a result of this, the formation of silicomolybdic acid from the monomer is fast, with the reaction typically being complete within 75 seconds at 20°C (Alexander, 1953). This is the reason the assay only gives a response to free (i.e. unpolymerised) silica, as only the orthosilicic acid species is able to fit into the binding cavity.

Kinetic studies of the reactions between the molybdic acid reagent and polysilicic acids (i.e. dimers and higher weight polymers) have shown that the reaction obeys the first order rate law, as is the case for orthosilicic acid. As the size of the polysilicic acid molecules increased the rate of reaction decreased. Studies of the reaction of disilicic acid with molybdic acid showed that the reaction takes approximately ten minutes to proceed to completion at 20°C. As a result of this, it was hypothesised that in order for the reaction to occur to completion the disilicic acid must fully de-polymerise to orthosilicic acid in order to react with molybdic acid. This is supported in turn by consideration of the previously mentioned Keggin type structure of the silicomolybdic acid anion only allowing the depolymerised orthosilicic acid species to bind within the limited physical space of the cavity contained within.

The Molybdenum Blue assay has undergone revisions and the method as modified by Perry (1997) and Keeling-Tucker (1999) has been employed during the course of this study.

5.1. Polymerisation of Orthosilicic Acid as a Result of Catecholate Complex Dissociation

The polymerisation process that occurs as a result of the dissociation of the potassium *tris*-catecholato silicon (IV) complex involves the production of trimers prior to the formation of structures such as cyclic tetramers and hexamers. The catecholato complex dissociates following the addition of acid to the system to give orthosilicic acid. This octahedral silicon catecholato complex has an overall double negative charge which is spread over the six surrounding oxygen atoms (from the three catechol groups) to result in an overall slightly positive (δ+) charge on the central silicon atom and a small negative charge on the surrounding oxygen atoms as a result of the associated inductive effect. As a result of these negative charges on the oxygen atoms the complex is destabilised by the presence of H^+ ions. This in turn leaves the central silicon atom vulnerable to attack by water molecules in solution, which bond to silicon and are stabilised by the release of H^+, leading to further attack on other bound oxygen atoms of the catechol group. This leads to the release of catechol from the complex and to the presence of orthosilicic acid in solution. At a concentration of ~2mmol of orthosilicic acid this solution is stable; however, the addition of the HCl to the 50mmol complex solution causes the pH to level out to 7 (±0.1) and subsequently the release of approximately 25mmol of orthosilicic acid to the solution – the extent of the complex breakdown is a function of pH – resulting in a supersaturated solution. Consequently, the solution then undergoes oligomerisation in order to relieve the supersaturation.

At the very early stages of the reaction the rate of change in concentration of orthosilicic acid may not be consistent with those required for third order kinetics with respect to the rate of disappearance of orthosilicic acid. During this early period, the

primary reaction occurring is dimerisation; i.e. the condensation of orthosilicic acid species to form disilicic acid. This reaction is typically completed within approximately 2 minutes of the addition of the acid to the complex solution and the subsequent pH reduction.

Once this stage of the reaction is complete, there is a short period of time when apparent third order kinetics are obeyed. This suggests that the principal reaction occurring is the formation of trimers; i.e. disilicic acid to trisilicic acid. This stage of the reaction occurs for approximately 3-15 minutes following the pH adjustment.

After 15 minutes, and when a significant number of trimers have formed, the principal reaction is the interaction of a monomer with an oligomer which is as large as or larger than a trimer.

As the system reaches dynamic equilibrium with respect to the change of concentration of the orthosilicic acid in the solution, the particles continue to grow. This process occurs by smaller particles dissolving and precipitating into larger particles – a process known as Ostwald ripening.

This suggests that third order kinetics may not be observed in the time period up to 2 minutes, but are expected to be observed for the time period 3~15 minutes. For the time after this reversible first-order kinetics are expected to be observed until the concentration of orthosilicic acid drops to approximately the equilibrium level where the dominant reaction occurring will be Ostwald ripenening.

The period 0~2 minutes is referred to by some authors as an induction period (Makrides, 1980; Rothbaum, 1990) but it is more likely that this time period covers the formation of dimers, giving no measured change in the concentration of orthosilicic acid

as the molybdate method will decompose these disilicic acid polymers to two equivalent of orthosilicic acid as a result of the conditions of the assay.

Data obtained from this assay can be used to directly estimate levels of silica in an unbound within a sample (as previously discussed) and also to provide kinetic information relating to the polymerisation reaction occurring during Ostwald ripening. This was employed during the course of these studies to investigate interactions between free silica and components relating to beer.

The measured rate of a reaction is often found to be proportional to the concentrations raised to some power. The expression:

$$\text{Rate} = -d[A]/dt = k[A][B] \text{ where k is the rate coefficient} \quad \textbf{(5.1)}$$

is an example of a second order relationship, in this case a bimolecular process with respect to species A + B. For the oligomerisation reaction it is possible to calculate at which stages the reaction obeys 1st order, reversible order, 2nd order or 3rd order kinetics. The data obtained from these experiments was analysed according to the methods developed by other workers within the group (Loton, 1994; Keeling-Tucker, 1999) using third order and reversible first order rate expressions. A third order reaction can be generally represented by the expression: A + B + C → Products. In this case, A, B & C are the same, i.e. orthosilicic acid. At times in the reaction where third order kinetics is observed a plot of $1/[Si(OH)_4]^2$ will give a straight line. The gradients of these straight line plots give values for the rate constants of the various stages of the reaction. In the case of the third-order plot, the gradient is twice the rate constant. This can be derived by considering the change in concentration, x, of the species, A, B & C, with respect to time, t. Using a, b & c as expressions for the initial concentration of these species gives the following expression:

$$dx/dt = k(a-x)(b-x)(c-x) \quad \textbf{(5.2)}$$

In the case of this system, all the starting concentrations will be the same so 'a' can be substituted for all three species to give:

$$dx/dt = k(a-x)^3 \quad \textbf{(5.3)}$$

This expression can be rearranged to give:

$$dx/(a-x)^3 = kdt \quad \textbf{(5.4)}$$

which can subsequently be integrated to give:

$$1/2(a-x)^2 = kt + \text{constant} \quad \textbf{(5.5)}$$

Initially, t=0 and x=0 and the constant becomes $1/2a^2$ giving:

$$Kt = (1/2(a-x)^2) - 1/2a^2 \quad \textbf{(5.6)}$$

Rearranging to give an expression in the form y = mx + c:

$$½(a-x)^2 = kt + 1/2a^2 \quad \textbf{(5.7)}$$

In this study (a-x) represents the concentration of orthosilicic acid. A plot of $1/[Si(OH)_4]^2$ will therefore give a line of gradient 2k.

At times in the process when reversible first order kinetics are followed, a plot of $(\ln\{[Si(OH)_4] - [Si(OH)_4]_\infty\})$ results in a straight line. This can be derived by considering the following:

$$nSi(OH)_4 \underset{-k}{\overset{+k}{\rightleftharpoons}} \text{Silica} \quad \textbf{(5.8)}$$

Or in general terms, reagent A converting reversibly to product P. This gives the rate equation:

$$-d[A]/dt = d[P]/dt = k_+[A] - k_-[P] \quad \textbf{(5.9)}$$

At equilibrium t tends to infinity giving:

$$k_+[A]_\infty - k_-[P]_\infty = 0 \quad \textbf{(5.10)}$$

The equilibrium constant, K, can be expressed as:

$$K = [P]_\infty/[A]_\infty = k_+ / k_- \quad \textbf{(5.11)}$$

Expressing the concentration of product in terms of reactant gives:

$$[P]_\infty = [A]_0-[A]_\infty \quad \textbf{(5.12)}$$

Taking the relationship between product and reactant concentrations and the forward and back rate constants given in equation **5.11** and substituting **5.10** into **5.11** gives:

$$[A]_\infty = [A]_0(k_-/k_++k_-) \quad \textbf{(5.13)}$$

Similarly, rearrangement of **5.12** gives:

$$[A]_\infty = [A]_0 - [P]_\infty \quad \textbf{(5.14)}$$

Substituting this expression into **5.11** gives:

$$[P]_\infty = [A]_0(k_+/k_++k_-) \quad \textbf{(5.15)}$$

Substituting [P] in **5.9** with the expression in **5.12** gives:

$$D[A]/dt = -(k_++k_-)\{[A]-[A]_\infty\} \quad \textbf{(5.16)}$$

The final term in **5.16** can be simplified by **5.13**:

$$d[A]/dt = -(k_++k_-)([A]-[A]_\infty) \quad \textbf{(5.17)}$$

Which is integrated to:

$$\ln([A]-[A]_\infty/[A]_0-[A]_\infty) = -(k_++k_-)t \quad \textbf{(5.18)}$$

And finally rearranged to give:

$$[A] = [A]_\infty + ([A]_0-[A]_\infty) \exp[-(k_++k_-)t] \quad \textbf{(5.19)}$$

thus proving a semi-logarithmic plot of $\ln([A]_t - [A]_\infty)$ (or, in this case, $\ln([Si(OH)_4] - [Si(OH)_4]_\infty)$) will give a straight line of gradient $-(k_++k_-)$.

During times in the oligomerisation process when second order kinetics are observed, no loss of orthosilicic acid with respect to time will be observed.

$[Si(OH)_4]$ is the concentration of free orthosilicic acid at time t and $[Si(OH)_4]_\infty$ is the concentration of free silica after four hours. This is considered to be representative of the equilibrium concentration.

The catecholate complex used was synthesised specifically for the purposes of these studies, and was a relatively reliable synthesis giving yields of typically 70% of the predicted maximum. The purity of the produced material was confirmed by the use of ^1H NMR spectroscopy, and an example of an obtained spectra can be seen in the appendices following. The complex can now be sourced ready synthesised from chemical suppliers.

5.2. Conversion Rates

As discussed, under the conditions of the molybdenum blue assay the catecholate complex decomposes to release orthosilicic acid into solution. The total quantity of orthosilicic acid measured by this method arises from two sources – the breakdown of the complex due to the pH reduction of the original solution, $[Si(OH)_4]_{free}$, and breakdown of the complex under the conditions of the assay, $[Si(OH)_4]_{complex}$. In order to calculate the true levels of orthosilicic acid from the dissociation of the complex under neutral pH conditions it is necessary to calculate a conversion rate for the dissociation of the complex to orthosilicic acid under the conditions of the molybdate test. This work has previously been carried out by other workers (Perry, 1992; Keeling-Tucker, 1999) and the results obtained were applied to the data obtained here to enable kinetic analyis.

5.3. Results

Experiments were carried out to investigate changes to the kinetics of silica deposition within the model system in the presence of both model additives and additives obtained from beer raw materials and in-process samples. The polyphenolic species

extracted from hop material, as described earlier, was also employed but the results proved difficult to replicate. These samples really required further purification by chromatography to determine more exactly their composition. The obtained materials, smelling characteristically of hop oils, were very difficult to handle. Following their extraction and washing in diethyl ether to remove the yellow colouration observed in the samples, they were never effectively solubilised for further works. It became apparent that in order to dissolve a substantial amount of these materials for addition to the model system would require the use of an organic solvent which would potentially cause problems within the system.

5.3.1. Kinetic Changes In The Model System In The Presence Of Model Additives

It was decided to investigate the kinetics of the catecholate model system in the presence of differing concentrations of proline and glutamine/glutamic acid. These amino acids had been shown by other workers (Asano, 1982; Siebert, 1996a&b) to occur in significant concentrations in beer proteins shown to exhibit haze activity. Siebert later proposed that in order for a protein to exhibit haze-forming activity it must contain proline (1999) but proposed no explanation for the occurrence of glutamine residues with these haze-active proline residues. Proline and glutamine have been shown to occur in regular ordered clusters within those proteins known to form haze, typically in the sequence Pro-Glu-Glu-Pro, and it had not been previously demonstrated whether or not glutamine had a significant role itself to play in the binding of haze active proteins to haze active polyphenols. The behaviour of proline under the conditions of this assay will not necessarily represent that behaviour exhibited by proline containing proteins in beer. This is due to the fact that proline acts as a zwitterion at pH 7, roughly the pH of the polymerisation (i.e., proline will form an ion that has both a positive and a negative charge

under the conditions of the polymerisation. Glycine and serine are also capable of forming a zwitterion under these conditions). Research by other workers (Loton, 1994) had previously investigated the effect of organic additives on the polymerisation of silica, including the effect of proline. This research demonstrated a reduction in the detectable levels of free orthosilicic acid at any time of the polymerisation in a system containing proline, relative to a polymerisation containing no additive. This suggests that either proline acts as a catalyst for the early stages of the polymerisation or that certain amounts of silica are bound into a form not detectable by the molybdenum blue assay by the proline. These workers also showed serine to affect the levels of free orthosilicic acid during the polymerisation, which was attributed to the ability of both amino acids to act as zwitterions at the experimental pH and a mechanism of binding between these and orthosilicic acids was also proposed between two of the hydroxyl groups on the silicic acid molecule and the amino acid.

The additives used in the course of these experiments were obtained from Sigma and underwent no modification other than solubilisation in distilled deionised water to facilitate addition to the model system. It is worth noting that these additives caused differences to be noted from the blank system. A pinkish tinge to the solution was observed, as opposed to the brown solution normally observed, and there was a visually obvious difference between the physical appearance of the precipitated silicas obtained from the additive runs compared with the silica obtained from the blank system. Additionally, precipitated solid matter is observed at an early stage of the reaction. The actual time of appearance of this precipitate appeared to be affected by the concentration of the additive. For example, for the system containing 200ppm poly-L-proline the precipitate was observed at approximately 25 minutes into the run. At a concentration of

800ppm the precipitate is first visible at ~ 5 minutes into the run. It is theorised that this precipitate is either poly-L-proline coming out of solution as a result of the changing chemistry of the solution as the polymerisation proceeds; or, the formation of silica-amino acid aggregates which are subsequently precipitated out of solution. This effectively would be mirroring the formation of haze in beers as well as the mechanism for silica stabilisation of beer.

It was hoped to carry out further investigation both into the precipitated silicas normally obtained as a result of this polymerisation reaction, following on from work carried out as an undergraduate by this researcher, and also of the precipitated material. Unfortunately, time constraints meant that it was not possible to progress with these studies.

Additive (dissolution)	Third-order rate constant ($mol^{-2}dm^6s^{-1} \times 10^6$)	First-order rate constant (precipitation) ($s^{-1} \times 10^{-4}$)	First-order rate constant ($s^{-1} \times 10^{-4}$)
Blank	1.0 ±0.23	5.2±	1.08±
500ppm L-Proline	1.33±0.035	3.8± 0.11	5.38±0.05
800ppm Poly-L-Proline	5.27±0.09	4.9±0.14	2.5±0.37
600ppm Poly-L-Proline	8.58±0.82	6.8±0.23	9.39±0.62
200ppm Poly-L-Proline	9.33±0.007	1.5±0.05	2.59±0.11
500ppm L-Glutamic Acid	5.35±0.18	3.85±0.03	1.61±0.35
500ppm Poly-L-Glutamic Acid	1.085±0.034	3.0±0.054	4.73±0.035
Maris Otter Protein 500ppm	8.33±0.007	4.9±0.036	2.04±0.02
Crystal Malt Protein 500ppm	5.1±0.47	6.1±0.074	5.095±0.09
Black Malt Protein 500ppm	1.83±0.24	3.9±0.07	8.04±0.32

The results obtained from these experiments (consisting of five repeats, running three reactions per repeat experiment) indicated that the addition of proline to the complex

dissociation resulted in an overall reduction in the levels of free silica detectable by the molybdenum blue assay. This clearly shows that there is in fact likely to be some interaction between proline (and, by extension, proline-containing proteins) and reactive, or unbound, silica. The experiments containing glutamine did not show any reduction in the overall level of detectable silica, as expected. This confirms that the role of glutamine in haze formation is not one of direct involvement in the physical binding of the haze forming components. It may be that glutamine has a conformational role to play, in that these Pro-Glu-Glu-Pro sequences may be the most conformationally open to facilitate access by the large proanthocyanidin species to the reactive proline residue within the protein. Since all proteins in beer that contain proline have been shown to have some degree of haze activity, but those most haze active contain these previously discussed Pro-Glu-Glu-Pro sequences it is reasonable to conclude that the presence of glutamine residues in proximity to proline residues increase the haze activity for a given protein. In order to test this hypothesis it would be necessary to a great deal more information about the amino acid sequence of the haze-positive proteins in beer to enable modelling of the protein and further experimentation.

5.3.2. Kinetic Changes In The Model System In The Presence Of Additives Extracted From Beer or Beer Raw Materials

Following on from the model additives to the system, additives extracted from both beer itself and from brewing raw materials were added to the system to investigate their effects. As expected, these heat-extracted protein materials (from the model mash extractions described in chapter two) showed similar results to those obtained with the proline additives. The degree of reduction in the amount of free silica detected varied

depending on the additive, as was expected. Comparing proteins extracted from three different varieties of malt illustrates this point.

Protein extracted from three varieties of malt – Maris Otter, Crystal and Black - was added to the model system to assess the result. The protein material obtained from the Maris Otter variety malt did not appear to interact with the silica of the model system. This malt is pale variety which has not undergone a great degree of modification by heating. This malt generally imparts less protein into the brew during the course of mashing than other, more modified types and this malt also imparts little colour to the brew. The Crystal malt is treated at a higher temperature than the Maris Otter type and also imparts much more colour to the brew (75-300°EBC as opposed to 15-30°EBC). This malt showed some interaction with the free silicic acid but a lesser interaction than that observed with the material extracted from the Black malt. Black molt has undergone substantial modification by heat and readily imparts protein material and strong colour (1200-1400°EBC) to the brew under mashing conditions. Considering the amino acid analysis of the materials extracted from these materials explains further the reason for the differences in the interaction between the protein and the silica:

Malt Variety	Proline Content (mol %)
Maris Otter	19.05
Crystal	21.94
Black	30.32

These results show clearly the relative difference in the levels of proline in the extracted proteins, and give some hint as to the likely haze-forming tendency for brews prepared from these varieties.

6. Conclusions

6.1. Protein Assays of Beer Proteins

The results obtained from these analyses yielded a several conclusions. Firstly, these works revealed the lack of a suitable 'catch-all' method for analysing beer protein content. Consideration of the readily accepted colorimetric methods used to measure protein content (the Bradford assay, the BCA assay and the BioRad DC assay) has shown that these assays do not respond to all the varied protein material types found to occur in beer. Recent work (Siebert, 2000) has led to the development of an assay specifically for haze-active (HA) proteins, which presumably works as a result of the similarity of the dye molecule to the HA polyphenols from beer. It is conceivable that combination of these two assays might enable a better estimation of the overall level of protein within a particular beer sample. Attempting to monitor changes in protein level using the assays employed in this work did not prove useful as these assays specifically respond to the protein material least likely to be removed from the brew by conventional stabilisation techniques. Secondly, these experiments provided a stern warning against regarding the result of a particular protein assay as being absolute – comparison of the varying results obtained by the different assays clearly illustrate this point. It is worth noting that the response for the Bradford assay showed a much larger level of dye-responsive protein in the beer at the wort stage than at either of the two later stages of production for the Smiles-produced brew. This indicates that there is a lot of protein within the wort which is removed prior to the later stages of the process, as a result of stages such as wort boiling (the 'hot break') and cooling (the 'cold break'). This indicates that these precipitations are quite effective at reducing the overall level of protein in the brew prior to the use of any engineered or traditional stabilising agent.

6.2 Amino Acid Analyses

6.2.1. Silica Stabilisation Trials

These trials, carried out using engineered silicas (provided by Ineos Silicas) aimed to study the different effects on a sample all-malt beer brewed at the two-hectolitre pilot brewing plant operated at the International Centre for Brewing and Distilling. The reasons for employing this particular brew were discussed in chapter 4.

Protein material precipitated from the wort showed a markedly different composition before and after treatment with the silica. Whilst it was difficult to work out the amount of protein removed from the brew relative to the total amount of protein present, it did allow consideration of the protein types being removed from the system. In the case of SD2489, the relative abundance of most amino acids was reduced but the most prominent change was in the levels of both proline and glutamine. This strongly indicates that the adsorbent acts on those proteins known to be haze-active, i.e. those containing proline. As previously mentioned, proline is a requisite for a beer protein to be haze-active so removal of any of this type of material will result a decrease in sensitivity to haze formation of the eventual brew. The other treated samples showed more complex results. It seems apparent from the results that only silica with specifically engineered physical characteristics has the specificity to be a useful adsorbent for HA proteins. This is almost certainly due to the surface functionality of the different silicas and, most importantly, of the pore size and distribution across the silica surface.

6.2.2. Amino Acid Analysis of Samples Provided by Smiles Brewery

These samples were of a commercial beer, produced for sale primarily in the area local to the brewery and the South-West of England generally. This beer is prepared according to traditional methods and, unlike the pilot plant beer provided by the ICBD, includes hops in the brewing process. Analysis of these samples allowed examination of the levels of different amino acids during the brewing process, and to relate these levels and changes in levels to the processes undergone during the production of the sample. These samples confirmed that, initially the wort contains high levels of proline-containing proteins – the levels of proline in this sample were far higher than the levels of any other amino acid type. Once the sample has been through the wort boiling stage it is clear that a substantial amount of the haze active proteins are removed. This green (so-called due to high levels of acid aldehydes in the brew at this stage, which can make the beer smell of apples) beer is then further treated with 'finings'. Finings are primarily applied to reduce particulate matter from within the brew, which it does in two ways, coagulation and flocculation. Coagulation between the isinglass (normally collagen of some kind) comes about as a result of electrostatic attraction. At beer pH (normally between 3.8 and 4.2), the collagen particles have a net positive charge whilst most of the particulate matter (including live yeast cells) are net negatively charged. Flocculation occurs also as a result of the same electrostatic charges, but in this case bridging of two or more collagen molecules occurs to produce a linked particle. In each case, the action is relatively non-specific and is not enough to fully stabilise a brew against haze formation. In order to carry out further stabilisation one of the previously discussed and more specific stabilisers must be employed. Considering the total amino acid analysis of the fined beer, it is clear there is

some removal of proteinaceous material but this is best considered a general treatment with no special relevance to stabilisation against haze formation.

6.3. Protein Electrophoresis by SDS-PAGE

Whilst the actual results from these experiments yielded little in the way of results useful to this project, the recent development of a method of a dye specific for haze-active proteins may make this technique more useful. More work is required in order to improve the resolution of this technique and also to develop better standards for use with beer proteins.

6.4. Digestion Experiments

The results obtained as a result of the digestion experiments support the theory that there is in fact silica present in the pectic and hemicellulosic fractions of *Equisetum*. This follows earlier work carried out by the research group which suggested this was the case. *Equisetum* is a highly silicified plant, which makes use of silica for structural purposes and although the silica found in this plant occurs associated with polysaccharides, it has not been previously proven that there is silica incorporated within these plant cell wall fractions.

Although the enzyme digestion did not yield results as part of this project, it is clear that driselase is potentially a useful source of pure enzymes. The use of pure enzymes would allow the operator to predict with a much higher degree of certainty the mode of attack of the enzyme under investigation. The use of driselase itself could be likened to something of a 'scattergun' approach, but could theoretically give an indication of how much bound – or certainly how much releasable unpolymerised silica – it is possible to release from the molecule. Use of individual enzymes, or even reduced

mixtures containing perhaps two or three different enzymes might allow the operator to begin to predict where the silica is being released from and to begin to surmise how it is in fact bound.

6.5. Molybdenum Blue Assay/Catecholate Model System Results

These studies showed that silica and protein do have a tendency to bind to each other with fairly strong binding (i.e. the silica remains bound under the conditions of the molybdenum blue assay, indicating a stronger bond than that occurring in the silicic acid dimer formed during the course of the oligomerisation reaction). Glutamine showed no affinity for silica under these conditions, indicating the possibility of a physical role for glutamine in haze formation rather than a direct interaction role.

Consideration of the results obtained using the proteins extracted by the model wort protein extraction further support the interaction of proline and silica. The degree of effect of the protein appeared to be related to the amount of proline contained within the sample.

The efficacy of silica as an adsorbent for haze active protein is directly as a result of the ability of silica to bind to proline. Other proline-containing proteins, which may be linked to other properties of beer than the ability to form haze, may not contain the Pro-Glu-Glu-Pro sequences found in haze positive proteins and consequently have a poor ability to form haze. It is clear that silica will have a tendency to bind to proline but it is also clear that silica has the capability to remove other proteins not related to the tendency of a beer to form haze.

Samples of finings and isinglass supplied by one the collaborating breweries for this project were analysed for their amino acid composition and the results showed that

finings contains around 16.7% proline and similarly about 16.9% glutamine. Isinglass was shown to contain 30% proline and 32.4% glutamine. These amino acid compositions roughly correspond to those obtained from the analysis of proteins extracted from malted barley. Both these materials will have a tendency to bind to haze-active polyphenols when added to the brew, subsequently reducing the overall likelihood of haze formation in the resulting brew. This is a less specific approach than applying engineered absorbents such as silica and PVPP but clearly one would expect the materials to be of some limited use in beer stabilisation.

7. References

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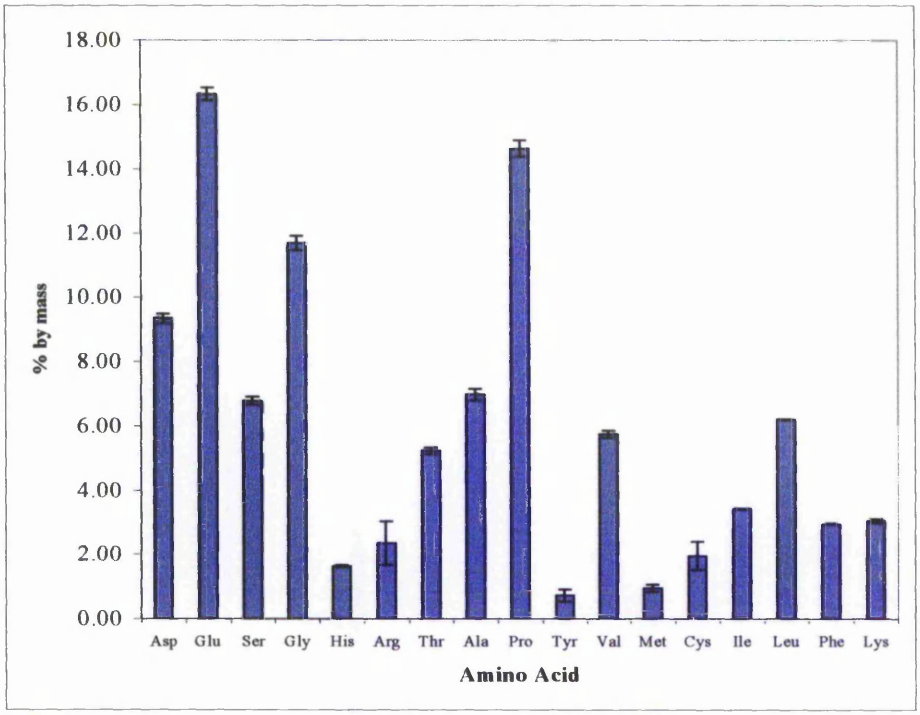
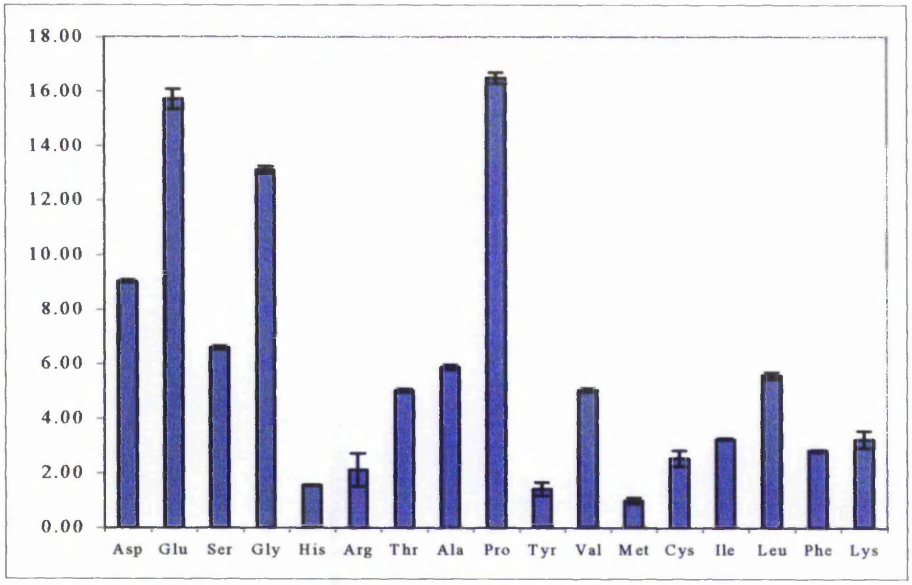
Appendix 1: Amino Acid Analysis Data

Graph A1: Amino Acid by % Mass for Smiles Wort Samples



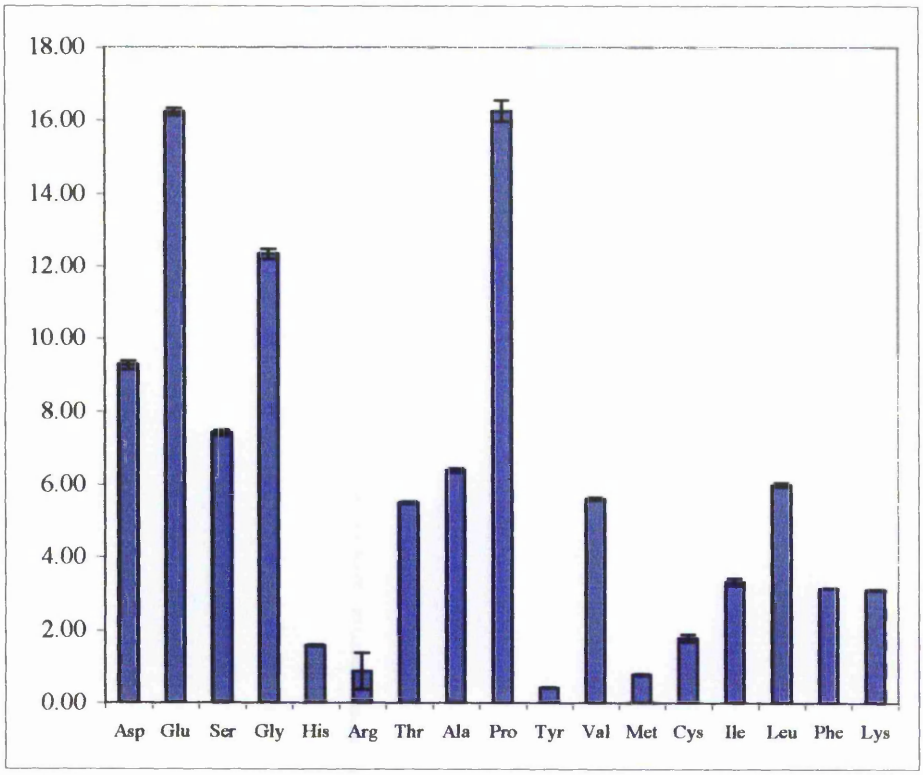
graph includes error bars showing standard error for these samples

Graph A2: Amino Acid by % Mass for Smiles Green Beer Samples



graph includes error bars showing standard error for these samples

Graph A3: Amino Acid by % Mass for Smiles Fined Beer Samples



graph includes error bars showing standard error for these samples