Effects of Amino Acid Profile, Endoprotease Activities and Wort Quality on Fermentability under Different Malting and Brewing Conditions

by

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To all the beings of light

(A todos los seres de luz)

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LIST OF ABREVIATIONS

AA: amino acids

AAL: Apparent attenuation limit

Alpha: α-amylase

AMQ: 6-aminoquinoline

AQC: 6-aminoquinolyl-N-hydroxysuccinimyl carbamate

Beta: β-amylase

CV: Coefficient of variation

DP: Diastatic power

EPs: Endoproteases

FAN: Free amino nitrogen

Fine: Fine grind extract

Fri: Friability

GA_3: Gibberellic acid

HMS: High maltose syrup

KI: Kolbach index

LD: limit dextrinazyme

Moi: Moisture

MP: Malt protein

MPs: Metalloproteases

SN: Soluble nitrogen

SOMoi: Steep out moisture

SP: Soluble protein

Std: Standard deviation

TCA: Trichloroacetic

VDK: Vicinal diketones

Visc: Viscosity

W: Congress wort

B-glucan: β-(1-3)-(1-4)-D-glucans

ABSTRACT

The quantity of alcohol produced through wort fermentation is fundamental to a malt's quality. Good fermentability is dependent on many malt quality parameters but requirement for proteases to provide amino acids and peptides for yeast is poorly understood. The thesis investigated relationships between amino acid profiles, endoprotease activities and fermentability under different malting and brewing conditions. Methods for measuring individual wort amino acids, endoprotease activity and fermentability were modified or developed to better understand the relationships. Levels of lysine and glycine were affected the most by malting and the variability was not always well predicted by the standard FAN analysis. Cysteine endoprotease activity developed similarly to total amino acids levels but associations were not significant Amino acids were limiting to fermentability at low yeast pitching rates and with the use of high maltose syrups but malt modification was the key determinant of fermentability. Studies on non fermentable sugar content were recommended.

Chapter I

General Introduction

1.1 General Introduction

Barley is one of the cereals of wider diffusion in the world, occupying the fourth place after wheat, maize and rice. It is used for a wide range of purpose: animal feed (cattle, pigs, and poultry), human food, and the most significant high value use is for producing malt, a raw material for brewing and distilling. As processes become more automated in breweries, it is necessary to know the major behaviour expected from a given variety of barley. Today it is evident that new varieties of malting barley must fulfill the quality specifications of the regional and international markets.

Fermentability determines the quantity of alcohol that can be produced from a certain quantity of malt, which determines the value of the malt in the brewery. Fermentation is a biological process that is significantly affected by: strain and age of the yeast used; wort composition; as well as the conditions of fermentation (temperature, oxygenation of wort and length of fermentation). Each brewery tends to use its own strain of yeast and, therefore, results from tests with certain strains are not always of consequence (Kunze, 2004).

A function of malt is to provide a source of assimilable nitrogen for yeast. During malting and mashing, proteases partially degrade barley storage proteins into amino acids and peptides that are critical for brewing quality beer (Jones and Marinac, 2002). Some of the nitrogenous substances that are produced during malting and mashing process will be assimilated by yeast and these are mainly amino acids, ammonium ion and some di and tri peptides. To produce malting varieties with known potential for producing these fractions, we need to ascertain which proteolytic enzymes are involved and how they operate during malting and brewing process (Jones and Budde, 2005).

Jones et al. (2005a) studied endoproteases from barley, malt and wort, and found four classes of endoproteolytic enzymes in germinated barley seeds: cysteine, aspartic, serine and metalloproteases. These enzymes are responsible for hydrolysis of barley

storage proteins during malting and mashing. Most studies on endoproteases were carried out on cysteine and serine proteases. The cysteine class plays an important role in the hydrolysis of barley proteins during malting and mashing, while none of the aspartic proteases that have been purified and characterized seem to be involved in hydrolysing barley storage proteins. Fontanini and Jones (2001) suggested that serine endoproteases play a little role in the solubilisation of storage proteins. On the other hand, the role of metalloproteases has been almost overlooked (Jones, 2005a).

Free amino nitrogen (FAN) of wort generally varied in concert with soluble proteins (SP) levels and endoprotease activities. Jones and Budde (2005) reported that FAN and SP values did not change proportionately, probably because the wort SPs are released from insoluble storage proteins by endoproteases while FAN is produced by exoproteases operating predominantly on the SP fraction. They found that all of the endoproteases classes affected the formation of FAN, although the main contribution to FAN release was by the non serine endoproteases, probably due to their promotion of increased SP levels, and thus, increasing substrate levels for FAN formation (Jones and Budde, 2005).

Several studies were conducted on amino acids in barley, malt wort and beer during 1963 and 1987 (Jones and Pierce, 1963; Pierce, 1987). Jones and Pierce (1967), classified the amino acids to four groups according to their speed of absorption from wort under brewery conditions, or, using a second classification, according to the “essential” nature of their keto analogues in yeast metabolism. In addition, they found that proline was the major amino acid found in malt worts, but only under aerobic conditions is it completely removed from fermented wort (Jones and Pierce, 1963; Pierce, 1982; O’Connor-Cox and Ingledew, 1989). Other studies on wort amino acids have shown that lysine supplemented fermentations were faster than: the control or methionine–supplemented fermentations. Accordingly, they concluded that lysine is a

“key” wort amino acid in the fermentation process (Lekkas et al., 2005). Furthermore, Edney et al. (2005) found that individual amino acids (serine in combination with cysteine) explained some of the variability in fermentation, despite masking by other parameters such as level of enzymes and β-glucan.

The purpose of this thesis was to understand the relationship between free amino acid levels, endoproteases activity, wort quality and fermentability levels. The relationship was investigated with, three main objectives:

1. Measure the effects of different malting programs on amino acid profile and endoprotease activities
2. Follow changes in amino acid profile and endoprotease activities during malting and brewing production under a commercial program
3. Study the impact of different amino acid profiles and malt quality parameters on fermentability

Chapter II

Literature Review

2.1 Overview of the malting and brewing processes

2.1.1 Malting process

In barley grain, the most abundant component is starch, which comprises approximately two thirds of the dry weight of the grain. For brewing, barley grains must be modified by the malting process, which results in the synthesis or release of starch degrading enzymes.

Malting is essentially a biological process in which water is imbibed by barley for one or two days (steeping), the barley allowed to germinate for four to five days, and dried above 80ºC (kilning) to stabilize the malt product and to produce the flavours and colours characteristic of malt. Although malted barley is a key ingredient in brewing and distilling, malt is also used extensively in the manufacture of non alcoholic malt beverages, and as an ingredient in baked goods (Agu, 2005a).

The malting process begins with the steeping of barley in water to achieve a moisture level sufficient to activate metabolism in the embryo and aleurone tissues, primarily the development of hydrolytic enzymes (Fig. 2.1.1). Moisture uptake by the starchy endosperm is also crucial for the mobilization, through the action of the enzymes, of the food reserves found in the endosperm. The enzymes migrate through the starchy endosperm, progressing from the embryo end to the distal end (Bamforth and Barclay, 1993; Agu, 2005b).

During the germination phase, proteases and starch degrading enzymes are developed and accumulated. Moreover, the cell walls and protein matrix of the starchy endosperm are degraded, exposing the starch granules and rendering the grain friable and readily milled (Fig. 2.1.2). Without adequate modification, starch granules remain unavailable for hydrolysis during brewing and the concentration of cell wall components, mainly high molecular weight β-glucan remain high, causing elevated wort viscosities and processing problems (poor lautering and long filtration times).

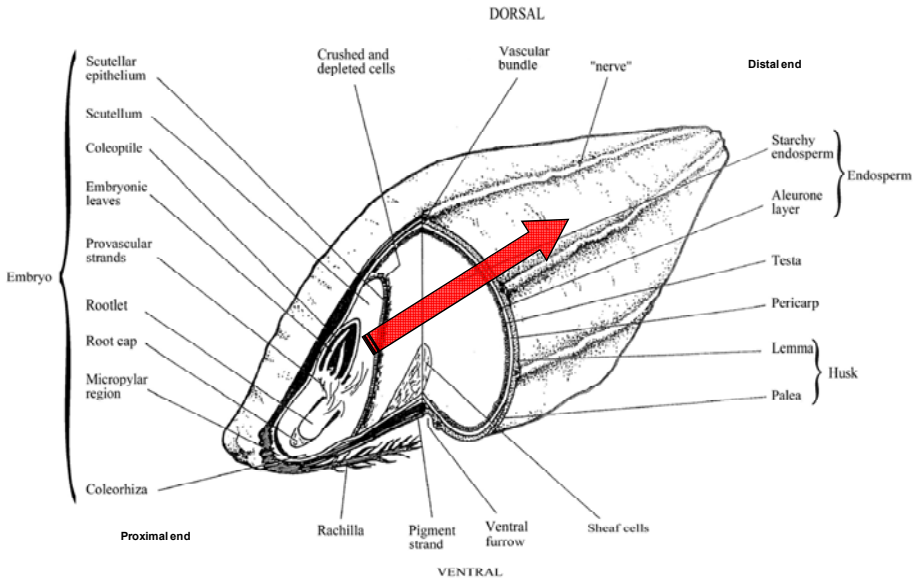


Fig. 2.1.1 Schematic view of a barley grain. Red arrow indicates enzymes movement (adapted from Briggs, 2002).

After a period of germination sufficient to achieve even modification, the "green" malt is kilned to arrest germination, and stabilize the malt by lowering moisture levels. During the kilning process, where Maillard reaction products are mainly produced; undesirable raw flavours are removed and pleasant aroma, taste and colour characters of the malt are introduced. Kilning, however, must be carefully regulated to ensure survival of enzymes that will be essential in the brewery to hydrolyze the malt starch into fermentable sugars (Briggs, 1998; Edney and Mather, 2004; Evans et al., 2005).

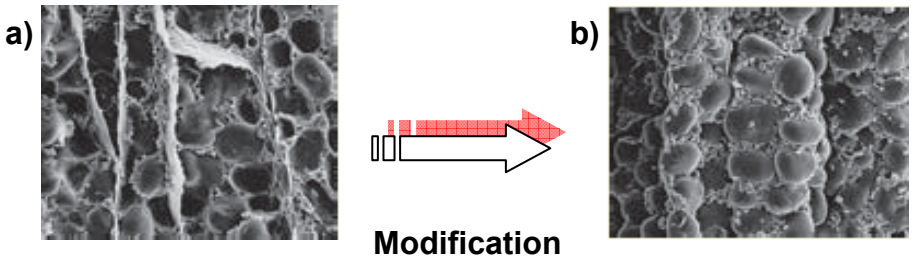


Fig. 2.1.2 a) Barley endosperm, starch granules imbedded in protein matrix; b) malted barley endosperm, starch granules after cell wall and protein matrix degradation

2.1.2 Brewing process

Beer is made by yeast-mediated conversion of fermentable sugars (glucose, fructose, sucrose, maltose and maltrotriose) into alcohol. The characteristic properties of many beers (colour, foam, and flavour) are a direct consequence of the malted barley used in their production (Bamforth and Barclay, 1993; Evans et al., 2005).

In the brewing process, malt is milled allowing water access to grain particles during the mashing phase. Milled malt is mixed thoroughly with two to four times the volume of hot water. The aim of the mashing process is controlled hydrolysis of starch to produce the required simple sugars. The majority of the starch is hydrolyzed by the amylolytic enzymes: α-amylase, β-amylase, limit dextrinase and α-glucosidases, during this stage.

Mashing is typically performed isothermally at 65ºC or by using a ramped temperature profile from approximately 45 to 70ºC. Although, alterations in mashing conditions can be used to change wort composition to a significant extent, temperatures above 60ºC are required for starch gelatinization, which in turn is necessary for the rapid and complete enzymatic starch degradation. However, the enzymes that hydrolyze proteins (endoproteases) and residual cell walls (β-glucanases) are relatively heat–labile and are usually destroyed prior to starch gelatinization (Briggs et al., 1981).

The liquid fraction of the mash contains a range of solubilized substances such as, simple sugars, dextrins, more complex polysaccharides, amino acids, peptides, proteins, other nitrogenous materials, vitamins, organic and inorganic phosphates, mineral salt, polyphenols, small quantities of lipids as well as other minor components. Some of the solubilized substances were produced during the malting process but others, mainly the fermentable sugars and dextrins were only released during mashing (Briggs et al., 1981; Evans et al., 2005).

Various separation methods exist to separate the sweet wort from spent grains. The resulting sweet wort must now be stabilized with boiling during which hops are also

added. Polyphenols extracted from the hops help precipitate proteinaceous substances in wort that might otherwise drop from solution in the finished beer, giving unacceptable hazes. Hops also provide the typical bitter (iso α-acids) and aromatic (essential oils) characteristics to beer. Boiling of wort inactivates all enzymes, precipitate some proteins and lipids, and kills microorganisms (Bamforth and Barclay, 1993; Wainwright, 1998).

During the last stages of brewing, wort is usually clarified with a whirlpool or centrifuged. Clarified wort is cooled, subsequently aerated or oxygenated, and pitched with yeast to begin fermentation. Through fermentation, brewing yeast utilize a wide variety of nutrients to support growth and generate energy. Carbohydrates and amino acids are the most significant nutrients, allowing yeast to convert fermentable sugars to alcohol and produce various flavour components. After fermentation, a series of maturation and stabilization stages are generally performed, before the finished beer is packaged (Bamforth and Barclay, 1993; Coghe et al., 2005).

In summary, barley needs to be malted before brewing to allow the synthesis of enzymes that: soften the grain, hydrolyse polymeric substances that obstruct wort separation, convert starch to fermentable sugars during mashing, release from protein the amino acids and peptides that are part of the nutrients required by yeast for efficient fermentation and eliminate proteins that endanger beer quality (haze formation) (Bamforth and Barclay, 1993).

2.2 Malt quality

The quality of malt has a decisive influence on the beer production process and ultimately on beer quality. Malt analyses provide an estimate of the brewing value of the malt and a basis for malt evaluation in relative terms that will serve to guide commercial transactions (Briggs et al., 1981; Pitz, 1990).

2.2.1 Malt extract

Malt extract is the most important quality parameter for malt. It gives an indication of the potential volume of beer that can be expected from a malt. Malt extract refers to the soluble material that can be extracted from the malt during the mashing process. It includes the simple sugars and amino acids as well as larger proteins and carbohydrates that will remain in the fermented wort adding flavour, aroma and mouth feel of the final beer. Malt extract is dependent on the barley, germination capacity and catabolic activities mediated by amylase, protease, and glucanase activities (Ullrich et al., 1997; MacGregor et al., 1999; Collins et al., 2003).

Congress mashing is a standard laboratory method (EBC, 1998) for estimating malt extract. The Congress program begins with stirred mash being held at 45ºC for 30 min to promote proteolysis by proteases, the lower the mash temperature, the greater the proportion of total soluble nitrogen arising as free amino nitrogen. The temperature is then raised at one degree per minute for 25 min to a final temperature of 70ºC. This step favours rapid starch conversion and high extract formation due to starch gelatinization and amylolytic attack. The mash temperature is held at 70ºC for an hour during which enzymes are inactivated, but residual starch is fully gelatinized and cellular structures are broken down. The traditional Congress mash protocol is sometimes criticized for not predicting brewing performance. As a result alternative mash protocols have been examined with some resulting in better prediction of commercial results (Schmitt et al., 2006).

2.2.2 Cytolysis

Malt extract is very dependent on the degree of cytolysis, the degradation of endosperm cell walls. The degree of cytolysis occurring during mashing is minor compared with the cytolysis that occurred during malting where cytolytic enzymes act to

convert the hard (steely) endosperm into a friable (mealy) endosperm of malt (Kühbeck et al., 2005).

Malt friability measures the degree to which a barley has modified during malting. It is performed in a friabilimeter, which mechanically separates husks and soft textured friable endosperm from ungerminated kernels and undermodified portions of the endosperm. Poor friability levels are associated with high protein level, reduced germination, poor modification and large kernel size (Wentz et al., 2004).

The major components of the starchy cell walls endosperm are β-(1-3)(1-4)-D-glucans (β-glucans) (Fincher and Stone, 1993). These polymers are broken down to various degrees during malting and mashing. When high molecular weight β-glucans are solubilised from the cell walls, these polymers increase the viscosity of wort and the resulting fermented beer. High viscosities retard the rate of filtration and β-glucan molecules can easily clog the fine pores of mash filters. Malting barley cultivars should have low endogenous β-glucan levels or the ability, through enzymatic hydrolysis, to reduce β-glucan levels to insignificant amounts in a germination time that does not result in excess protein modification. It can represent as much as 4% of the dry weight of malting barley, thus signifying considerable lost potential in fermentable sugars (Edney et al.,1998; Fox et al., 2003; Kanauchi and Bamforth, 2008).

Jin et al. (2004) studied the influence of β-glucan molecular weight on wort viscosity and they established that while various components in wort and beer contributed to their viscosities, β-glucans (between 31–443 kDa) increased solution viscosities. They found that in the range of β-glucan concentrations studied (0–1000 mg/L), viscosity increased linearly with molecular weight and concentration of β-glucans. Viscosity of wort and beer influence the brewing process and beer quality in several aspects. For instance, beer viscosity positively contributes to its body. As well, a high beer viscosity can retard the drainage of liquid from foam bubble walls and lead to better head retention of beer

foams. However, as mentioned earlier, high viscosities of wort and beer can lower the efficiency of many unit operations including stirring of mashes, pumping, wort separation from spent grains, wort boiling, cooling of wort, beer clarification, as well as beer filtration (Jin et al., 2004).

2.2.3 Wort clarity and colour

Wort turbidity is a well known indicator associated with the quality of the resulting wort and therefore is of outstanding importance. Clear worts are desired after lautering as they result in fewer problems later in the process, although hazy wort do not necessarily lead to hazy beer. Well-modified malts give clear worts, while less well-modified malts give slightly opalescent or hazy worts. Many authors have reported a correlation of wort turbidity to an increase of lipid substances in wort, particularly of long-chain fatty acids (Stewart and Martin, 2004; Kühbeck et al., 2006).

Wort colour is another quality parameter measured from mashed wort. Colour variation in wort is due to non-enzymatic browning reactions, the Maillard reaction, that take place during kilning in the malting process, and wort boiling in the brewing process. A non-enzymatic browning reaction is a chemical process that produces a brown color in foods without the activity of enzymes. Browning is caused by the formation of unsaturated, coloured polymers with different composition. In this case, the sugars interact with the amino acids, producing a variety of odours and flavours. This reaction is called “Maillard reaction” and is the basis of the flavouring industry with the type of amino acid involved determining the resulting flavour (Hodge, 1953).

2.2.4 Proteolysis

Another important factor related to colour development is the degree of malt modification, in particular protein modification. The degree of protein modification directly

affects the overall modification of the malt and, as a result, can lead to less degradation of cell walls and greater unavailability of starch. On the other hand, a high degree of protein modification releases many degradation products, for example amino acids, into the malt. The numerous amino acids released can create Maillard products during the kilning with sugars that have also already formed.

The yield of soluble nitrogen is influenced by mashing conditions. Thick brewery mashes produce more soluble nitrogen than thin laboratory mashes. The proteins are partially precipitated in the copper boil and those (or at least the very large polypeptides) that survive into beer contribute to head (foam) stability, the mouth feel of the beer (body) and, sometimes, to the formation of undesirable hazes. The low molecular weight materials, in particular ammonium ions and most of the amino acids serve as the source of nitrogen that support yeast growth. The proportion of the different nitrogen fractions differ among worts and is dependent on the original barley as well as processing conditions (Briggs, 1998; Qi et al., 2004).

Kolbach index is an indicator of the proteolytic modification of the malt and reflects protein solubilisation during malting and mashing. It is calculated from the ratio of soluble wort protein to the total malt protein. Kolbach index does not however provide information on the size of the protein breakdown products (Wentz et al., 2004).

Protein modification also involves the production of wort amino acids and small peptides (dipeptides and tripeptides), collectively known as free amino nitrogen (FAN). This quality parameter is considered to be a good index for potential yeast growth and fermentation efficiency. Adequate levels of FAN in wort ensure efficient yeast cell growth and, hence, a desirable fermentation performance. Generally the specifications for a normal fermentation require FAN levels between 140-160 mg/L. The FAN level of the wort is largely dependent on the malt or the grist used, whilst the mashing programme has only a limited influence. The increase in free amino acids in mashing is not more

than 50% and is not really significantly influenced by changing the mashing programme (Edney and Langrell, 2005; Lekkas et al., 2005). Enari in 1975, concluded that barley variety, nitrogen content and the malting technique all influence the FAN level of the wort. As FAN level affects the fermentation these factors also has the influence on the fermentability of the wort.

2.2.5 Fermentability

Fermentability is often discussed in terms of apparent attenuation limit (AAL). AAL is the decline in the specific gravity of worts that occurs as worts are fermented by yeast, and the lower limit is reached when fermentation ceases. The reduction in gravity results from the removal of the fermentable sugar and the accumulation of ethanol, which has a density below that of water. AAL is calculated from the difference between the original specific gravity and the specific gravity after fermentation. Under best practice brewing conditions, the yeast will normally consume all available fermentable sugars (glucose, fructose, sucrose and maltose), producing alcohol as a by-product of this activity. Some yeast can also ferment maltotriose, but slowly. A few will ferment the minor sugars isomaltose, panose and isopanose. Therefore, wort fermentability can differ when different yeasts are applied (Enari, 1975; Briggs, 1998; Evans et al., 2005).

2.2.6 Starch-degrading enzymes

The previous discussion has centred on the determination of malt quality through analysis of a laboratory-produced wort. Analysis of the starch-degrading enzymes is also an important component of malt quality.

DP (diastatic power) is a key parameter of malting quality since it is an estimate of the capacity of a malt to degrade starch into fermentable sugars. This hydrolysis is accomplished primarily by four starch degrading enzymes: α-amylase, β-amylase, limit

dextrinase and α-glucosidase. This analysis is performed on a different method of extract compared to standard Congress mashes (Olson et al., 1948; Briggs, 1998).

α-Amylase is an endo-acting enzyme that attacks the starch chain internally to produce oligosaccharides and/or limit dextrins containing α-1,4, α-1,6-linked glucose residues. After the first day of germination, the aleurone begins to produce α-amylase. The levels of α-amylase formation are highly dependent upon gibberellic acid and in its presence, the enzyme continues to be secreted by the aleurone (MacGregor et al., 2009).

β-Amylase is an exo-acting enzyme that cleaves starch from non-reducing ends of each starch chain to produce the disaccharide, maltose. It is synthesized during the development of the barley grain and responds positively to nitrogen fertilization in parallel with hordein proteins. β-amylase has free, latent and bound forms. In malt, typically 78-99% of β-amylase is in the free form (Evans et al., 2008). This enzyme is relatively heat labile and rapidly loses activity when mashing temperature rise over 62.5ºC (MacGregor et al., 2002; Bamforth, 2003; Fox et al., 2003).

Limit dextrinase is an endo-acting enzyme that hydrolyzes α-(1-6) linkages, and thus removes the branch points in amylopectin or α-limit dextrins allowing further hydrolysis with β-amylase. This enzyme is also present in a free, latent and bound form. In malt, only 8-20% is in the free form (Evans et al., 2008). Limit dextrinase appears to have only limited action during mashing likely due to inhibition by low molecular weight malt inhibitors (MacGregor et al., 1994). Recent research found limit dextrinase levels to be limiting to fermentability and it has been suggested limit dextrinase has an important role during mashing (Evans, 2008).

α-Glucosidase is an exo-acting enzyme that primarily cleaves α-(1-4) linkages to produce glucose. This enzyme may play a role during mashing by hydrolysing small

maltodextrins to glucose, but the importance of its action remains unclear (MacGregor et al., 2002; Bamforth, 2003).

2.3 Processing conditions

The objective of any commercial malthouse is to produce the best quality malt in the shortest production time. This requires programs that are tuned for each individual barley variety and to the malt specifications requested by the brewer. The first consideration in producing the type of malt required is selection of an appropriate malting barley variety and then consideration of the quality of the barley grain sample. Malt process can then be altered accordingly including alterations to the hydration process (steeping/spray), the use of additives, and changes to the germination process including grain moisture content, temperature profile, duration of germination period as well as the frequency and vigour of turning. Finally, variations on drying and cooling process (kilning or roasting) can be performed.

2.3.1 Barley varieties

Barley varieties are classified as malting varieties only after demonstrating a potential to produce a high yield of hot water extract after a reasonable period of malting and if the quality of the wort is acceptable to produce beer without problems. In short, it must lead to the production of a beer which conforms to market requirements (Moll, 1979). It is important to point out that environmental conditions under which the variety grows and matures greatly influence its quality. Some of these effects are related to: disease problems, barley nitrogen content and β-glucan levels (Pitz, 1990; Fox et al., 2003).

Non-malting, feed barley, varieties are cheaper to purchase than malting varieties and various efforts have been made to malt them. Adequate malts may be made from

some poor feed quality barleys only if changes in the malting process are employed, e.g.: extended period of germination, addition of gibberellic acid, low-temperature kilning schedules. Often considerable extra effort and expense is involved in preparing adequate malt from poor quality barley (Briggs, 1998).

Hulless barley (naked) malt offers a tremendous opportunity for the brewing industry due to higher extract levels (3–5% higher than covered malting barley), and the absence of undesirable hull compounds such as tannins and polyphenols. However, malting of hulless barley presents a number of challenges owing to differences in chemical and physical characteristics (Edney and Rossnagel, 2000). Like all naked grains, hulless barley is easily damage during malting. The lack of protection results in excessive acrospire damage during turning and handling that stop growth. In general hulless grains take up water rapidly and give high extracts as expected since the husk limits grains swelling (Li et al., 2006). From the brewing point of view, hulless malts are not attractive because the absence of husk gives wort separation problems; furthermore, they present high viscosities and higher malting losses. However, McCaig et al., 2006, did brew beer from three Canadian hulless barley varieties and found the beer sensory results and overall beer quality to be satisfactory with no defects noticed.

2.3.2 Steeping

Malting grains at different steepout moistures substantially alters the progress and pattern of modification. The aim of steeping is to: clean the grain, hydrate it to the correct extent, prepare it to grow steadily and modify uniformly during germination. Rapid water uptake and homogeneous water distribution must be balanced against malting losses and malt performance (Nischwitz et al., 1999). Increasingly higher steep out moistures are used (45-46% moisture), although poor quality (steely barleys) may required 47-48% moisture, or they may be sprayed in the germination compartment to increase their

moisture content to within this range to ensure the same degree of modification as malt produced from barley of better quality. Over-steeping can cause grain death, but usually irregular germination is the consequence (Briggs, 1998).

Windisch (cited by Briggs, 1986) was the first one to demonstrate the use of air rests in which the grain rested in air between immersions, removing carbon dioxide and ethanol, which may inhibit germination. These accelerate germination and reduced the risks of over steeping. During air rests the grain became warm and reimmersion served to cool it, as well as providing more moisture (Briggs, 1986). The correct balance of wet and dry periods during steeping is critical to hydrate the endosperm fully without over saturating and thus inactivating the embryo (Nischwitz et al., 1999).

2.3.3 Germination

Steepout moisture levels will significantly affect germination. Grain with high moisture content grows vigorously, with strong respiration and rootlet growth compared to grain germinating at a lower moisture content. Malting times tend to be shortened at high moisture levels which can be helpful in processing difficult barleys that are slow to modify. The more vigorous growth of high moisture barley results in elevated levels of soluble nitrogen, diastatic power and α-amylase while final levels of total malt nitrogen are little altered. β-glucan contents and wort viscosity are reduced. Malt friability, colour and fermentability are increased. On the other hand hot water extract values usually are lower than those germinated at low moisture content, and malting losses are higher, owing to elevated respiration and extra rootlet growth. It is important to consider that different barley varieties respond differently to changes in steepout moisture (Sebree, 1997; Briggs, 1998).

Late applications of water to germinating grain can rehydrate the embryo fully and cause excessive embryo growth without appreciably wetting the endosperm or

accelerating modification. The dryer regions of the endosperm may not modify (Briggs, 1986). Dudley and Briggs (1977) found that water spray applied on day 3 of germination slightly enhanced: a) malting losses, and b) soluble nitrogen (SN) on germination days 6 and 7; without significantly altering the extract. However, once germinated, grain with a high-moisture content grows vigorously with strong respiration and rootlet growth, relative to grain that germinated with lower moisture content. Levels of soluble nitrogen, diastatic power and α-amylase are elevated by wet malting (Briggs, 1998).

The influence of germination temperature has been thoroughly investigated. Since, within limits, grains grow faster at elevated temperatures it seems attractive to malt at the highest possible temperature that the grain will tolerate and so finish the process in the shortest possible time. However, as the temperature increases, there is an increasing predisposition for grain to germinate unevenly; heat output increases, respiration and grain growth are accelerated (Dudley and Briggs, 1977; Bamforth and Barclay, 1993).

2.3.4 Additives

A range of additives and special techniques have been used during malting to: a) reduce dormancy, b) alter the microbial population, c) accelerate germination and modification, d) reduce the levels of soluble polyphenols, e) increase the extract, f) reduce malting losses and g) alter the composition of the malt (Briggs et al., 1981). Studies have shown that the major gibberellin in malting barley is GA_1 with smaller amounts of the closely related compound gibberellic acid, GA_3. GA_3 is available commercially, prepared from cultures of the fungus *Gibberella fujikuroi* and it is probably the most widely used additive when legally or contractually permitted. It may be used in malting to overcome residual dormancy and to accelerate modification process allowing malting to finish two days sooner than in untreated malt (MacLeod, 1963).

The process begins as gibberellins diffuse from the embryo and they progressively trigger responses in the aleurone layer, increasing its respiration rate. The major changes in the aleurone are dependent on respiration and are enhanced by improving the supply of oxygen. The central action of the aleurone layer in response to gibberellin is the syntheses and release of a large sequence of hydrolytic enzymes, including α-amylase, β-glucanase, proteases, phosphatases and enzymes that degrade nucleic acids. These are formed at the expense of the reserves of the tissues itself. Unlike the embryo, aleurone tissue is not dependent on a supply of external nutrients for making the hydrolytic enzymes, and subsequently releases. However, the aleurone layer may also take up simple molecules, generated in the adjacent modifying starchy endosperm tissue, for conversion to enzymes and other substances (Fig. 2.3.1). Some hydrolytic enzymes are formed when the aleurone layer is hydrated, but their release into the endosperm is strongly accelerated by, or is wholly dependent upon, the arrival of gibberellins (Fincher and Stone, 1993).

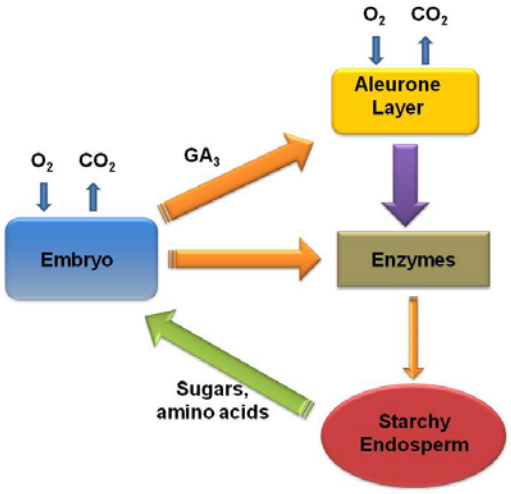


Fig. 2.3.1 Interactions between the embryo, the aleurone layer and the starchy endosperm (adapted from Briggs, 2002).

By the time that hydrolytic enzymes are accumulated in the starchy endosperm, modification of the endosperm structure starts resulting in increased hydrolytic products including: sugars, oligosaccharides, amino acids and peptides as well as inorganic ions. This process is enhanced by additions of GA_3, due to an acceleration of the rate of modification (Briggs, 2002). The marked stimulatory effect of gibberellic acid on the

accumulation of free amino acid that occurs in malting, and the fact that levels of endopeptidases and some carboxypeptidases are also greatly enhanced by addition of this hormone, while some of the other enzymes are not, emphasizes the importance of these enzymes in degrading the major protein reserves of the starchy endosperm and enhancing the soluble nitrogen fraction (Fig. 2.3.2) (Briggs, 1998). Responses to added GA_3 are highly dose dependent and vary between barley varieties and different samples of one variety (Fig. 2.3.3). The manner in which GA_3 is applied to the grain is important.

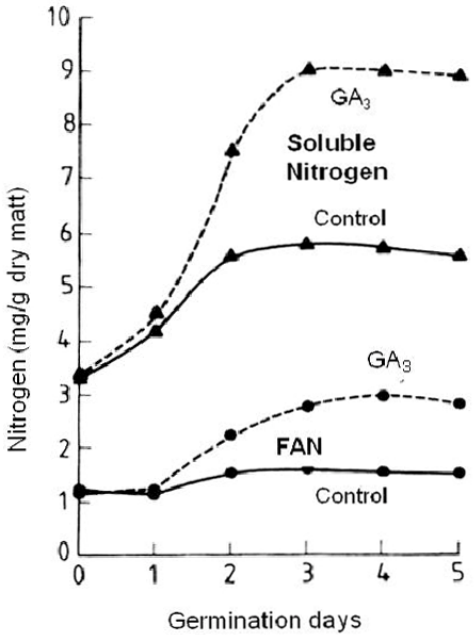


Fig. 2.3.2 Changes in the levels of SN and FAN malted with (dashes lines) or without addition of GA_3 (solids lines) (adapted from Briggs, 1998).

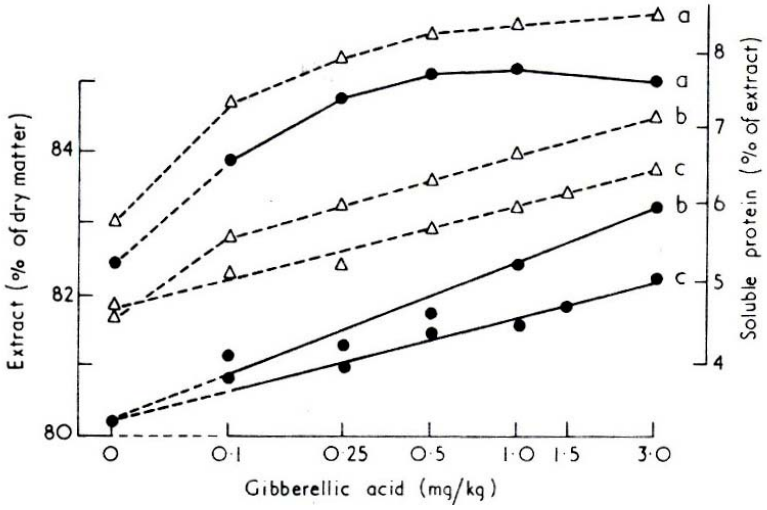


Fig. 2.3.3 Increased values of extract (●) and soluble nitrogen (∆) found after malting three barley varieties (a, b and c) for a fixed period after dosing with various amounts of GA_3 (from Briggs, 1981).

The most efficient application is to spray the grain with the solution of GA_3 at the beginning of germination, when the grain is already chitted. When a dose of the hormone usually in the range 0.005-0.25 mg GA_3/Kg barley is chosen, all the advantages mentioned will be obtained, with the production of a malt that is at least equal, and in many ways may be superior, to traditional malts (Briggs et al., 1981; Bamforth and Barclay, 1993). Evans et al. (2009) used two different GA_3 doses: 0.5 and 1.0 ppm to show the level of GA_3 does not have as dramatic an effect on the levels of α-amylase, limit dextrinase and KI as was expected. They found that the extent of stimulation appears to depend on the barley source, perhaps indicating that barley produced or stored under different conditions may have varying levels on endogenous GA_3 or responsiveness to GA_3.

In summary, additions of GA_3 tend to overcome dormancy, accelerate the malting process (higher degree of modification is achieved in a shorter time), enhance the respiration rate, stimulate the production of some enzymes, and improve in general terms malt quality. It is important to note that enzymes do not all increase in the same manner in response to the gibberellic acid nor do other malt parameters alter “in parallel” (Dudley and Briggs, 1977; Briggs, 2002).

2.3.5 Kilning

Many of the analytical values of finished malts are strongly influenced by the last stage of the malting process, kilning. Malt is kilned to produce a dry, stable product (facilitating storage and transporting), of fixed composition, from which the roots can be readily removed. The dry product is friable, which means that it can be easily crushed (Bamforth and Barclay, 1993).

In the initial stages of kilning, development of some enzymes can continue and malt modification continues to progress. In the later stages of kilning, where higher

temperatures are encountered, some enzyme destruction occurs. The malts also develop character (colour, malty flavour and aroma). The development of these latter characteristics is largely dependent upon Maillard reaction, which occurs more easily at higher temperatures and at higher moisture contents (Coghe et al., 2004; Coghe et al., 2006). On the other hand, enzyme survival occurs best when malt is initially kilned at the lowest possible temperature until moisture levels are reduced and then temperatures are allowed to rise. These facts dictate the nature of the kilning schedule needed to make each specific type of malt (Briggs et al., 1981; Sebree, 1997). As stated earlier, Maillard is the key browning reaction that occurs during kilning. Since the reaction is based on the interaction between amino acids and reducing sugars, colour development occurs more readily in well modified malts, which are richer in reducing sugars and soluble nitrogenous substances. In general terms, reducing sugars interact with amino compounds to produce initially Schiff's bases, but in later stages aldosamines and ketosamines are formed via Armadori rearrangements. The latter products may condense with another sugar to give diketosamines. Diketosamines are unstable and break down to give a range of products including reductones (Fig. 2.3.4). Consequently, some of these products interact and polymerize producing melanoidins, high molecular weight substances that are coloured, and low molecular weight substances that contribute to aroma and flavour (Hodge, 1953; Briggs, 1998).

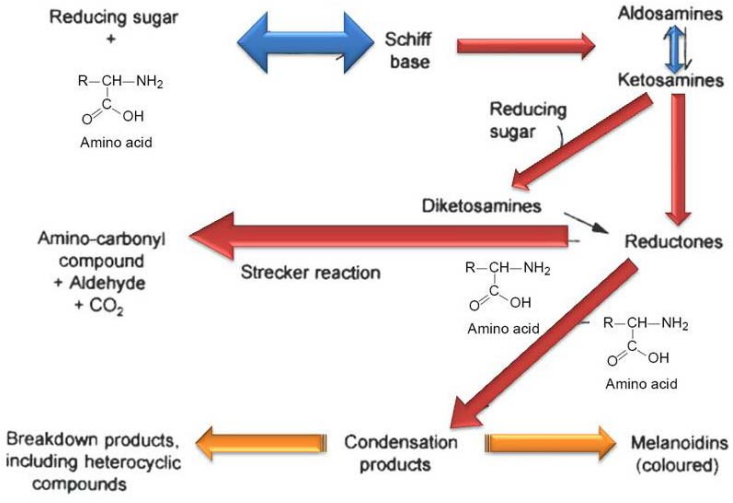


Fig. 2.3.4 Chemical steps involved in the formation of melanoidins and flavour and aroma substances (adapted from Briggs, 1998).

Changes going on in the grain during kilning are dependent on both the temperature and moisture content. Generally, with a moisture content greater than 25% and at temperatures below 40ºC, the drying malt is still respiring, acrospires continue to grow, some enzymes continue to increase in amount (α-amylase, β-amylase and endo-β-glucanase), modification continues, β-glucan levels continue to decline and the levels of soluble nitrogen increase. The drier the grain the higher the temperature must be for amylolysis and proteolysis to occur, increasing the levels of reducing sugars, peptides and amino acids (Briggs et al., 1981; Lloyd, 1988). In contrast, at curing temperature 70-80ºC activities of enzymes such us β-amylase and β-glucanase are reduced. At the end of kilning process (85-90ºC) the inactivation is even greater, for example β-amylase activity can be reduced to between 21 and 41% (dry weight) of levels in green malt (Evans et al., 1997). Nevertheless, appreciable levels of amylolytic enzymes survive kilning, although many other enzymes, like peptidases and β-glucanase can be almost totally inactivated (Karababa et al. 1993; Briggs, 1998).

Kilning also has a profound role to play in the regulation of levels of heat labile S-methylmethionine (SMM) formed during germination of malt, which discomposes to DMS, part of which is oxidized to kilned malt precursor dimethyl sulfoxide (DMSO), and part is lost in the kiln fumes. During kilning, unwanted raw grain flavours (DMS precursors) are removed or destroyed. Some European types of lager required malt that gives rise to appreciable quantities of DMS (Lloyd, 1988).

Furthermore, during kilning, possibly carcinogenic nitrosamines, including N-nitrosodimethylamine (NMDA) can be produced. Studies showed that when kiln gases containing oxides of nitrogen (NOx) mix with malt, interactions occur with hordenine, present in the root and smeared on the malt from crushed roots, and giving rise to

NMDA. Using palliative procedures, such us indirect firing, burning sulphur in the kilns, and maintenance of an atmosphere low in oxides of nitrogen maltsters now routinely control NMDA to levels well below the legal limits (Bamforth and Barclay, 1993; Briggs, 1998).

2.4 Free amino acids in barley, malt and wort

In the early twentieth century brewers believed a low nitrogen content in malt was beneficial. Wort nitrogen level was thought to be the single most influential factor governing beer infection or contamination, and brewers therefore avoided barleys with high nitrogen content. Studies in 1950's demonstrated that yeast growth was governed to a large extent by the source and level of assimilable nitrogen in wort. As a result, brewers gradually reversed their thinking on the role of assimilable nitrogen in brewing fermentation (Pierce, 1987; O'Connor-Cox and Ingledew, 1989).

The brewing industry customary classifies nitrogenous constituents of wort into "assimilable" and "nonassimilable" fractions. The major assimilable constituents are the amino acids and small peptides, which are the usable wort nitrogen sources for yeast nutrition (Pierce, 1982). The study of these assimilable nitrogenous substances entered a new era around 1958 with the development of new, sensitive, and rapid methods for their analysis. Prior to this era, research was not possible into the production and fate of amino acids through the malting and brewing process. Since, total soluble nitrogen content of malt is not a good guide to assimilable nitrogen of malt, techniques that could differentiate between yeast assimilable nitrogen and unusable nitrogen containing compounds were necessary to identify the optimum nitrogen levels for fermentation (Pierce, 1982; O'Connor-Cox and Ingledew, 1989).

Table 2.4.1 lists the common amino acids that occur in barley proteins, and are thus detectable as free amino acids in wort. Barley also contains significant quantities of

other free amino acids, such as β-alanine, α-aminoadipic acid, γ-aminobutyric acid (GABA), and L(-)-pipecolinic acid. In addition to the free amino acids, peptides and proteins, wort also contains quantitatively minor nitrogen components, such as vitamins, purines, pyrimidines and their derivatives (Briggs, 1981; Patterson and Ingledew, 1999).

Levels of nitrogenous constituents in wort can vary by four means: 1) quality of barley used for malting, 2) malting schedule, 3) grist composition, and 4) mashing procedure (Pierce, 1982).

In this section, some of the most important sources (barley, malt and wort) and effects of free amino acids on brewing fermentation will be discussed. Factors such us barley characteristics, malting conditions, mashing procedure, wort composition and yeast assimilation are included. A description of the methodology applied during the years will then be covered.

Table 2.4.1 Common amino acids (from Tyler, 2001)

Name	Symbol 3 letter	1 letter
Acidic amino acids		
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Neutral amino acids		
Alanine	Ala	A
Asparagine	Asn	N
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Basic amino acids		
Arginine	Arg	R
Histidine	His	H
Lysine	Lys	K
Imino acid		
Proline	Pro	P

2.4.1 Effects of barley variety and barley quality on levels of free amino acids

Jones and Pierce (1963) found that barley varieties containing similar total nitrogen content had limited differences in free amino acid content in their malts. The one known exception to this observation was when varieties had been bred specifically for increased levels of specific amino acids. For example high lysine barley was found to have higher free lysine levels, although this was found to have no particular advantage in terms of malting quality (Burger and Porter, 1978). In normal varieties nitrogen content

of a barley had a more significant effect on levels of free amino acids found in the malt than did variety. The relationship between total nitrogen content and free amino nitrogen content was found to be linear but only when total nitrogen content was below 1.5%.

It is important to note that as total amino acids levels increased, relative proportions among individual amino acids remained constant with the exception of proline. In the case of proline, its concentration decreased relative to the total malt amino nitrogen content (Jones and Pierce, 1963; Pierce, 1982).

Generally the free amino acid content of malt increases during germination. Studies show that the increase becomes rapid between two and four days germination, when a maximum concentration may be reached. This is consistent with the development and liberation of proteolytic enzymes early in the germination process, which act on proteins to produce amino acids. As barley begins to grow and produce rootlets, some of these amino acids are required for synthetic process and are therefore, removed from the grain. In addition, it is possible that the production of amino acids may be restricted as the germination continues, due to the lack of suitable substrate for proteases to attack (Jones and Pierce, 1963; Jones and Pierce, 1967c).

Jones and Pierce investigated effect of adding a combination of potassium bromate and gibberellic acid during germination on free amino acid levels. They showed that the remarkable depression of amino acids production, due to the use of bromate, was not uniform and they concluded that any method which attempts to reduce malting loss by inhibition of embryonic growth is likely to produce malt with significant differences in free amino acid levels (Jones and Pierce, 1963; Jones and Pierce, 1967a).

Jones and Pierce (1963) classified the amino acids produced during germination into three groups, based on the time of release during germination. Concentrations of amino acids in the first group (typified by amides) increased to a maximum after approximately four days of germination followed by a sharp decline (Fig. 2.4.1). The

members of this group are: alanine, leucine, isoleucine, lysine, valine, arginine, glutamine and asparagine. The addition of GA_3 accelerates the production of these amino acids and also the protease activity responsible for their release.

The second group of amino acids (typified by glycine) reaches a maximum after approximately four days of germination, but thereafter remains constant (Fig. 2.4.2). This group included: aspartic and glutamic acid, glycine, threonine, tyrosine, histidine, tryptophane, and methionine. GA_3 also stimulates the production of these amino acids, but reducing the germination period does not have as marked an effect as in the first group (Pierce, 1982).

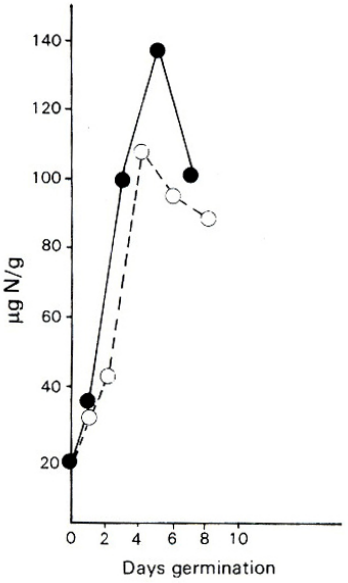


Fig. 2.4.1 Development of glutamine and asparagine during germination. (●) with GA_3 (○) without GA_3 (from Jones and Pierce, 1963)

The third group contains only proline, and its pattern of release is different from the rest. Its concentration increases uniformly during the germination period with no maximum reached even after eight days (Fig. 2.4.3). This development is not appreciable affected by the addition of GA_3 and thus the shorter germination periods used in conjunction with GA_3 supplementation will result in less free proline in the final product (Pierce, 1982).

2.4.2 Movement of amino acids within the kernel during germination

The amino acid composition of the storage proteins differs appreciably from the cytoplasmic proteins. Whereas the proportion of amides, glutamic acid and proline are extraordinarily high in hordein, the major storage protein in the endosperm, the cytoplasmic proteins are characterized by their relatively higher proportions of aspartic acid, alanine, glycine, lysine and arginine.

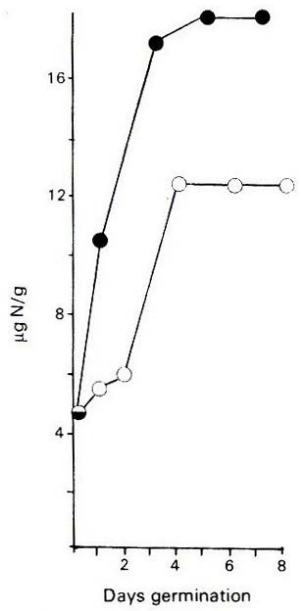


Fig. 2.4.2 Development of glycine during germination (●) with GA_3 (○) without GA_3 (from Jones and Pierce, 1963).

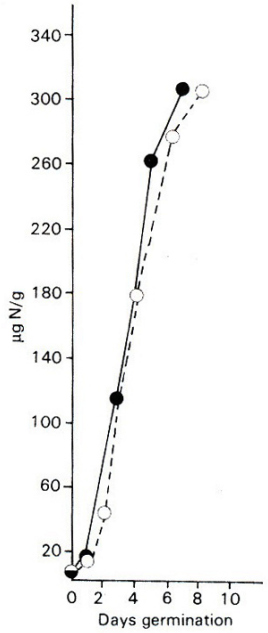


Fig. 2.4.3 Development of proline during germination (●) with GA_3 (○) without GA_3 (from Jones and Pierce, 1963).

Thus, conversion of storage proteins from the endosperm into cytoplasmic proteins in the embryo should require synthesis of aspartic acid, alanine, glycine, lysine and arginine at the expense of the amides, glutamic acid and proline (Jones and Pierce, 1967a).

To study the changes in amino acid composition of the whole grain throughout the germination period, Jones and Pierce (1967a) dissected endosperms and embryos from barley kernels. The loss of individual amino acids (free and combined) from endosperm was compared with the gain of these amino acids in the embryo.

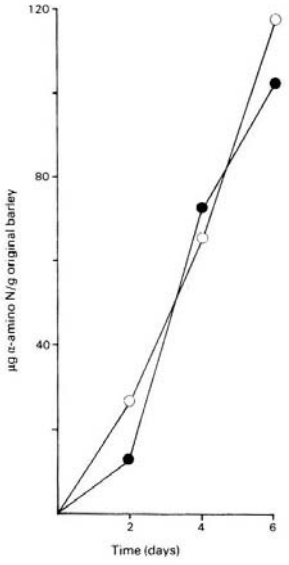


Fig. 2.4.4 Changes in alanine of endosperm and embryo (●) loss from endosperm (○) gain in the embryo (from Pierce, 1982).

It was found that for most amino acids the loss from the endosperm was equal to the gain in the amino acids by the embryo (Fig. 2.4.4). Some of the exceptions were glutamic acid and glycine which showed a considerably greater loss from the endosperm than was gained by the embryo (Fig. 2.4.5). Proline, aspartic acid and lysine were also unusual with larger amounts of these amino acids arising in the embryo than were lost from the endosperm (Fig. 2.4.6).

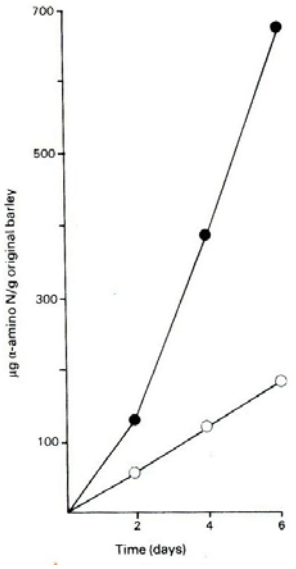


Fig. 2.4.5 Changes in glutamic acid of endosperm and embryo (●) loss from endosperm (○) gain in the embryo (from Pierce, 1982).

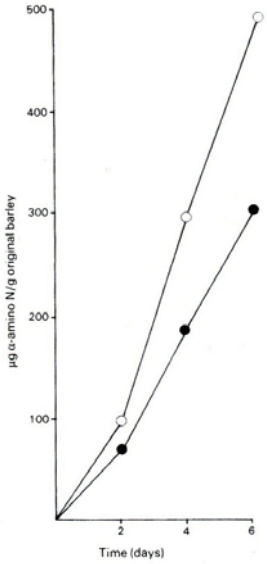


Fig. 2.4.6 Changes in proline of endosperm and embryo (●) loss from endosperm (○) gain in the embryo (from Pierce, 1982).

The release and production of proline cannot be conveniently classified into groups like the other free amino acids. This could be of considerable importance since proline is the major free amino acid found free in the grain during germination and also in the final beer. It has always been assumed that proline is produced by protease action on hordein because as mentioned previously, hordein is the major protein attacked by proteases during germination. However, studies indicate a large portion of the proline found in germinating barley is synthesised during germination from other amino acids or nitrogenous sources (Jones and Pierce, 1967a).

The major portion of the proline content of the embryo is found in the free state, and after 6 days of germination approximately one-third of this free proline has been synthesised from other nitrogenous sources, likely from glutamic acid or glutamine. It is possible that at the end of germination, when the proteins of the endosperm have been exhausted, proline may be used as a source of nitrogen, or for other synthetic processes (Jones and Pierce, 1967a).

2.4.3 Effect of kilning on free amino acids

Malt kilning conditions markedly influence the wort amino acid content, as would be expected. This is particularly true in the final stages of kilning, when higher temperatures used during the curing phase (82 to 104ºC) cause marked decrease in amino acids (Jones and Pierce, 1963). At the early stages of kilning reducing sugars and amino acids actually increase as metabolic process continue to function as the barley kernel germinates. However, as kilning temperatures rise interactions between sugars and amino acids, reducing their individual levels and increasing the amounts of melanoidins precursors and melanoidins. The formation of melanoidins via Maillard reaction is one of the causes of amino acid decrease (Briggs, 1998).

Coghe et al. (2005) studied the impact of dark specialty malts on free amino acid levels in Congress wort. In their study, worts were prepared with mixtures of different malt types such as pale, caramel and dark. In all of their prepared Congress worts, proline, leucine, arginine, phenylalanine, and valine were the five most abundant amino acids. Samples were poor in glycine and methionine and contained insignificant levels of cysteine. In general, they found that the levels of individual wort amino acids decreased with increasing wort colour. Furthermore, in the darkest wort samples, practically all amino acids were derived from the pale type malt. Their work showed similar trends

when wort colour was compared with FAN versus the sum of individual amino acids (Fig. 2.4.7).

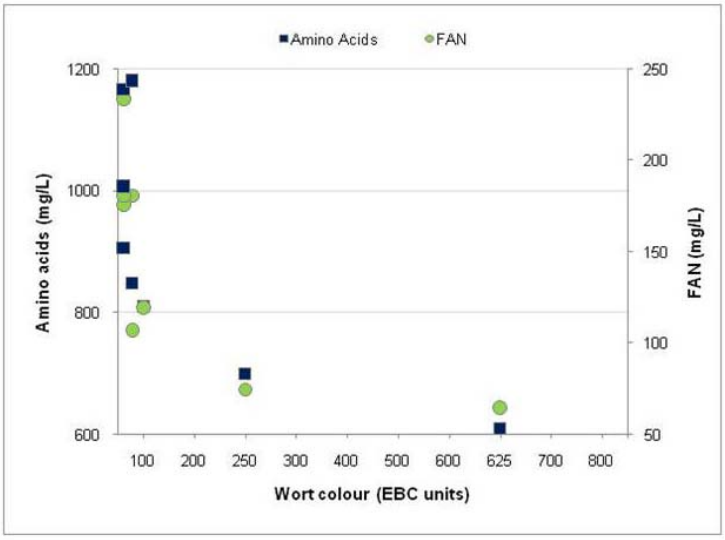


Fig. 2.4.7 Relation between total amino acid levels, FAN in Congress wort and wort colour (adapted from Coghe et al., 2005).

Dark caramel malts and roasted malts, therefore, appear to be extremely poor in free amino acids. Levels of amino acids significantly decreased with increasing wort colour due to the previous mentioned non enzymatic browning reactions during curing or roasting of malt. This may

Table 2.4.2 Free amino acid analysis of worts prepared from malt samples (milligrams per 100 g of malt)[a] (from Samaras et al., 2005).

amino acid	green malt	lager malt	pale malt[b]	cara malt	crystal malt	black malt	chocolate malt	roasted barley
aspartic acid	5.35	5.25	2.10	2.13	nd	nd	nd	nd
hydroxyproline	nd[c]	nd	nd	nd	nd	nd	nd	nd
threonine	2.68	4.20	2.10	1.60	0.52	0.51	0.51	0.51
serine	4.01	4.20	2.10	1.60	0.52	nd	nd	nd
glutamic acid	4.01	6.30	3.14	1.07	1.05	nd	nd	nd
asparagine	8.03	10.49	3.14	4.26	2.10	nd	nd	nd
glutamine	14.72	15.74	6.29	2.67	0.52	0.51	0.51	0.51
proline	16.05	30.43	9.43	9.06	3.67	0.51	nd	nd
glycine	1.34	2.10	1.05	0.53	0.52	0.51	0.51	0.51
alanine	4.01	5.25	3.14	1.60	1.57	0.51	nd	nd
valine	6.69	7.35	3.14	2.67	1.57	0.51	0.51	0.51
cystine	nd	nd	nd	nd	nd	nd	nd	nd
methionine	1.34	2.10	1.05	0.53	0.52	0.51	0.51	0.51
isoleucine	4.01	4.20	2.10	0.53	0.52	0.51	0.51	0.51
leucine	8.03	8.39	4.19	1.60	0.52	0.51	0.51	0.51
tyrosine	4.01	6.30	3.14	1.60	0.52	0.51	0.51	0.51
phenylalanine	6.69	8.39	4.19	2.13	1.05	nd	nd	1.03
γ-aminobutyric acid	5.35	4.20	2.10	1.07	nd	nd	nd	nd
ornithine	nd	nd	nd	nd	nd	nd	nd	nd
lysine	5.35	5.25	3.14	0.53	0.52	nd	nd	0.51
tryptophan	2.68	3.15	1.05	1.07	0.00	nd	nd	nd
histidine	2.68	4.20	1.05	0.53	0.52	nd	nd	nd
arginine	4.01	9.44	4.19	2.13	1.05	1.03	nd	1.03
total	111.04	146.90	61.84	38.91	17.29	6.18	4.12	6.68

[a] Values are single determinations of single extracts (dry basis). [b] Pale malt was provided by Muntons Malted Ingredients (Stowmarket, U.K.). [c] Not detected.

possibly be due to both protein denaturation and incorporation of proteins in the melanoidin structure. Consequently, low fermentability levels have been observed with dark malts (Coghe et al., 2005). Samaras et al. (2005) observed similar trends in amino acid levels in worts prepared from different malt and roasted malt samples (Table 2.4.2).

2.4.4 Effect of mashing on free amino acids

The mashing step results in solubilization of nitrogenous substances. The majority of the substances were released during the malting process and they are merely dissolved during mashing. However, some additional nitrogenous substances become solubilized during mashing as insoluble proteins undergo proteolysis (Briggs, 1998). The nitrogenous components of wort account for 4-5% of the total dissolved solids. The bulk (85-90%) of the total nitrogenous fractions is in the form of amino acids and small peptides (58%) followed by polypeptides (22%) and proteins (22%) (Boulton and Quain, 2001).

Jones and Pierce (1963) showed that free amino acids increase slightly during the initial period of mashing followed by a steady decline. The ratio of individual amino acids to total amino nitrogen present in the sweet wort generally remained unchanged throughout the process. Exceptions were the amides, whose proportions gradually decreased, presumably due to thermal decomposition at the higher temperatures which, prevail at the later stages of the extraction. The increase in basic amino acids during the early stages of mashing was delayed, presumably due to some process impeding their rapid extraction from the grist. The ratio of proline to the sum of all amino acids decreased throughout the extraction process with ratios reduced by approximately half during the entire extraction process.

The same publication also investigated the effect of different mashing temperatures on wort amino acid content. Results showed that alanine, valine, leucine and lysine are

produced in significant amounts compared to aspartic acid and proline which are not significantly affected by mashing temperature (Table 2.4.3). Furthermore, maximum yields of glycine, alanine, valine, leucine and lysine were achieved between 40ºC and 50ºC. It was apparent that lowering temperatures from this range had a smaller effect than a similar increase in temperature above this range.

Table 2.4.3 Effect of mashing temperature on amino acid composition of worts (from Pierce, 1982).

Amino acid	50°C Mash	70°C Mash
Aspartic acid	45	41
Glutamic acid	25	50
Alanine	108	60
Valine	142	94
Methionine	29	19
Leucine	138	96
Lysine	90	74
Arginine	97	77
Proline	450	422

Expressed as μg α-amino N/g.

The soluble nitrogen fraction of wort was shown as early as 1932 to be affected by pH. In particular any changes in mashing procedure that lowered the mash pH resulted in a considerable increase in amino acid content in the wort (Jones and Pierce, 1967c). The pH effect is likely due to the activity of carboxypeptidases. Mikola et al. (1971) found that carboxypeptidases were more active within the pH range used in mashing. They have an optimum pH of 5.2, and display a higher level of activity in a pH range of 4.0 to 6.2. Based on these results, the author's concluded that carboxypeptidases are the most essential enzymes for the production of free amino acids, and that they account for 70-80% of the reaction in mashing.

2.4.5 Effect of wort boiling on free amino acids

The boiling phase of the brewing process significantly reduces levels of free amino acids, particularly basic amino acids. As occurred during kilning, high temperatures during boiling enhance Maillard reactions, producing melanoidins. The addition of hops during this stage appears to have negligible contribution to wort amino acid composition (Jones and Pierce, 1967b; Boulton and Quain, 2001).

2.4.6 Free amino acids and their role in yeast fermentation

There is no doubt that the level of assimilable nitrogen present in wort profoundly affects nutrient uptake by yeast and subsequent fermentation performance. As mention previously there is an optimal level of assimilable nitrogen, 140-150 mg/L, for an adequate fermentation. Less assimilable nitrogen can lead to sluggish or "stuck" fermentations. Studies proposed nitrogen limitation as a major factor responsible for the decline in fermentation activity noted during the early stages of fermentation (O'Connor-Cox and Ingledew, 1989).

Amino acids can be classified according to their speed of absorption from the medium during fermentation. The pitching time required for each amino acid to reach half its initial concentration has been used in such a classification. However, this method of classification does not distinguish between those amino acids for which absorption begins immediately but then precedes at a slow rate, compared to those amino acids where removal only begins after a lag period but are the removed relatively rapidly. As a result, Jones and Pierce (1964) classified the amino acids into four groups according to the order at which initial removal from the medium commenced. Individual members of the four groups, as classified by this method, are given in Table 2.4.4.

Table 2.4.4 Classification of amino acids according to their speed of absorption from wort under brewery conditions (adapted from Pierce, 1964)

Group A	Group B	Group C	Group D
Glu	Val	Gly	Pro
Asp	Met	Phe	
Asn	Leu	Tyr	
Gln	Ile	Trp	
Ser	His	Ala	
Thr		Ammonia	
Lys			
Arg			

Group A are the amino acids immediately removed from wort after 20 hrs fermentation. Amino acids from group B are not removed from the medium rapidly, but are absorbed gradually during the fermentation. Group C present a considerable

lag in their absorption, their uptake appearing to coincide with the final removal of group A amino acids. Group D contains only proline, the major amino acid constituent of wort, which is only slightly absorbed by yeast (Fig. 2.4.8) (Jones and Pierce, 1964).

Yeast will only partially absorb, under normal brewing conditions, any excess amino nitrogen present in wort. When yeast is provided with a mixture of amino acids in excess of their requirements, they will preferentially absorb group A and B amino acids, leaving behind those from group C and D (Jones and Pierce, 1964). As a result final beer will contain none of the rapidly assimilable amino acids (group A & B) but will contain certain amino acids from groups C and D (glycine, alanine, tyrosine, phenylalanine, tryptophan and proline).

Under certain conditions changes in the rate of absorption of some amino acids have been demonstrated. For examples, absorption of glutamic acid and tryptophan has been showed to change under aerobic conditions, although the major difference between aerobic and anaerobic fermentation occurs with the uptake of proline. Proline was completely removed from wort under aerobic fermentation. The result is beer brewed under aerobic fermentation have a very different amino acid profile in the final beer (Jones and Pierce, 1964; Pierce, 1982).

Nevertheless, amino acid utilization patterns have always been presumed to be insensitive to changes in fermentation

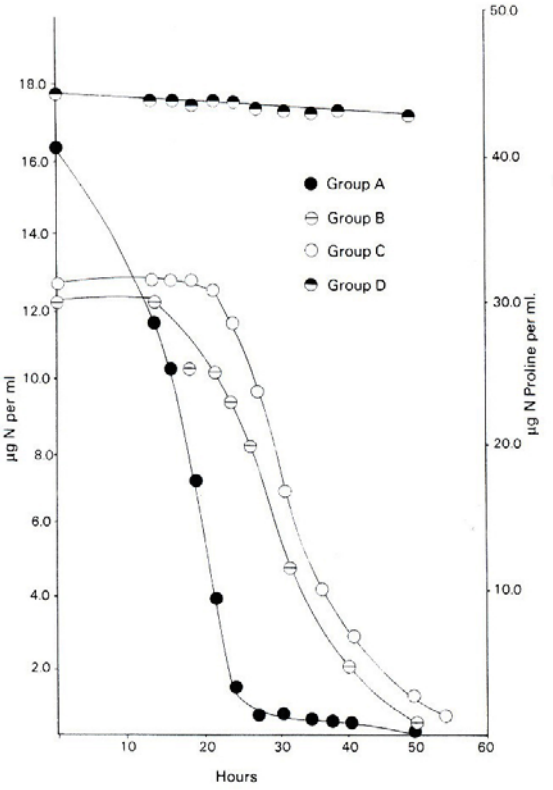


Fig. 2.4.8 Amino acid absorption by yeast (Jones and Pierce, 1964).

conditions. In fact, environmental conditions during fermentation such as: temperature, elevated CO_2 pressures, and oxygen can be important, especially for organoleptic parameters (O'Connor-Cox and Ingledew, 1989). Furthermore, the regular pattern of amino acids uptake is not generally affected by changes in the flocculence characteristics of yeast, except in the case of arginine, which was rapidly absorbed by the less flocculent type of yeast (Pierce, 1982).

Perpète et al. (2005) studied the sequence of absorption under different fermentation conditions and compared theirs results with those previously reported by Jones and Pierce (Table 2.4.5). They defined a "critical time" (Tc) for each yeast strain. Tc was defined as the time necessary for complete consumption of a first group of amino acids. They found that this class coincided with group A described by Jones, plus methionine but minus arginine. According to Ramos-Jeunehomme et al. (cited by Perpète et al., 2005), methionine should be effectively consumed after a few hours of fermentation. Tc also marked the beginning of consumption of a third group, which in accordance with the authors should only include glycine and alanine. They also reported that fermentation temperature did not significantly influence the uptake sequence.

Table 2.4.5 Comparison between the experimental conditions of fermentation used to study amino acid uptake (from Perpète et al., 2005).

	Jones and Pierce (9)	Palmqvist and Ayrapaa (14)	Ramos-Jeunehomme et al (15)	This Study
Strains	*Saccharomyces cerevisiae* Guinness 4200	*S. carlsbergensis* U15	*S. cerevisiae* 1278b and 2592, 59R	*S. cerevisiae* BRAS 291, BRAS 212 and BRAS 12 + industrial co-culture of 3 top fermentation yeasts
Stirring	Stirred (conical flask) and industrial vessel	Stirred and non stirred	Stirred	Stirred (conical flask), non stirred (EBC tube), and industrial vessel
Temperature	15.5 and 23°C	8-9°C	18°C	22 and 28°C
Medium	10-12°P	10°P	12°P	12°P
Group A	Glutamate, glutamine, aspartate, asparagine, serine, threonine, lysine, arginine	Asparagine, glutamine, serine, threonine	Glutamate, glutamine, aspartate, asparagine, serine, threonine, lysine, arginine, methionine	Aspartate, glutamate, serine, threonine, lysine, methionine (A'group)
Group B	Valine, methionine, leucine, isoleucine, histidine	Methionine, lysine, aspartate, leucine, glutamate, isoleucine, arginine	Valine, leucine, isoleucine, histidine	Valine, arginine, isoleucine, leucine, tyrosine, phenylalanine, tryptophane (B' group)
Group C	Glycine, phenylalanine, tyrosine, tryptophane, alanine	Valine, histidine, phenylalanine, alanine, tyrosine, tryptophane, glycine, proline	Glycine, phenylalanine, tyrosine, tryptophane, alanine	Glycine, alanine (C' group)
Group D	Proline		Proline	Proline

2.4.7 Absorption of amino acids by yeast

Jones and Pierce investigated yeast grown under brewery conditions in a semi defined medium, simulating wort, with labelled ^{14}C and ^{15}N-L-leucine. The results showed that there was no significant spread in ^{14}C labelling to other amino acids, but the appearance of ^{15}N randomly distributed among all the amino acids in the yeast showed that extensive transamination had occurred. This experiment proved conclusively that intact assimilation of amino acids into yeast protein did not take place (Pierce, 1982). Thus, total amino acid absorption is governed more by the total assimilable nitrogen level (FAN) in the medium than by the concentration of specific amino acids.

Another experiment was shown that there was a relatively high specificity in respect of the transfer of carbon skeletons of amino acids in the medium to carbon skeletons of yeast protein amino acids. Simple sugars in the medium also contribute significantly to the carbon skeletons of amino acids in yeasts. From these experiments it became clear that for certain amino acids it is most desirable that their relative proportions be maintained in wort at standard values, whilst for other amino acids the relative concentrations are not significant apart from their contribution to the total assimilable nitrogen present (Pierce, 1982). Based on that, Jones and Pierce (1967a) classified the amino acids according to the “essential” nature of their keto analogues in yeast metabolism (Table 2.4.6).

Table 2.4.6 Classification of amino acids on basis of their essential nature (from Pierce, 1987).

Class 1	Class 2	Class 3
Aspartic Acid	Isoleucine	Lysine
Asparagine	Valine	Histidine
Glutamic Acid	Phenylalanine	Arginine
Threonine	Glycine	Leucine
Serine	Tyrosine	
Methionine		
Proline		

The concentration of Class 1 amino acids was considered not important. During initial stages of fermentation, a large proportion of their carbon skeletons are obtained from amino acids in the medium. In the later stages, however, the keto acids analogues are synthesized from sugars. The initial concentration of these amino acids in wort is not

critical since the by product of their synthesis are being produced in significant quantities at the end of fermentation. Proline can be included in this group since its concentration in wort is not critical. Initially this amino acid is synthesized from glutamic acid but later in the fermentation intact assimilation of this amino acid occurs, but it should be remembered that the amount of proline in yeast protein is small (Pierce, 1982).

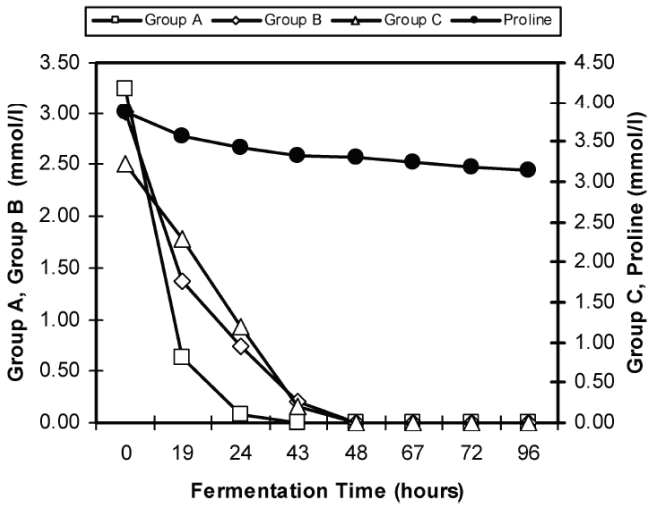
The initial concentration of Class 2 amino acids in wort is critical since in the later stages of fermentation, the major sources of theirs carbon skeletons came from the synthesis of the keto analogues from sugars in the medium. Thus, although initially by products are formed by synthesis of amino acids from sugars, these are further metabolized during the later stages of fermentation when no further by products are formed. Hence, changes in the concentration of this group of amino acids may considerably affect beer quality (Pierce, 1982). The carbon skeletons of the Class 3 amino acids are derived entirely from exogenous parent amino acid and there is little contribution from sugar synthesis. Consequently, deficiencies of these amino acids in wort may result in major changes in the nitrogen metabolism of yeast, affecting the final quality of beer (Pierce, 1982).

2.4.8 Fermentability of amino acid supplemented wort

Lekkas et al. (2007) investigated further the role of lysine and methionine in wort fermentation. They found that all the amino acids from group A (Table 2.4.4) were completely absorbed within the first 43 hours fermentation. Total consumption of both groups B and C amino acids was achieved at 48 hours fermentation, with methionine noted to be removed from wort rapidly by the yeast. As expected, proline uptake was limited (Fig. 2.4.9).

Free amino acids are known to affect both biomass accumulation and ethanol production in addition to yeast vitality. Based on that, Lekkas et al. carried out two supplementation experiments, with five times base levels of lysine and methionine respectively. Addition of L-lysine enhanced yeast metabolic activity and shortened the fermentation time from 96 h to 48 h. The majority of the amino acids were taken up during the first 43 h of fermentation, and the same effect was also observed for ammonia, which was taken up by 19 h. This confirms the classification of ammonia in group C by Jones and Pierce, although the reasons for incomplete removal are not apparent (Lekkas et al., 2007).

Fig. 2.4.9 Amino acid absorption pattern for lager fermentations conditions (from Lekkas et al., 2007).



Two vicinal diketones (VDKs), 2,3-butanodione and 2,3-pentanodione are fermentation by-products resulting from biosynthesis of valine, leucine and isoleucine. VDKs are considered undesirable in most beers, particularly in lagers because they impart buttery flavour, and cleaner organoleptic profiles are expected. Elevated VDK levels were observed in lysine supplementation (Cyr et al., 2007; Lekkas et al., 2007).

On the other hand, supplementation of worts with L-methionine had an inhibitory effect on yeast fermentation and extended the fermentation time from 96 h to 103 h. The presence of added methionine prevented absorption of group C amino acids. VDKs levels in the fermented wort were lower than the control. This could be because the elongation of the fermentation time observed lead to an increase in diacetyl removal by yeast (Lekkas et al., 2007).

2.4.9 Methodologies for amino acid quantification

In June 1958, Spackman et al. published a fully automated variant of their column chromatographic process (Fig. 2.4.10) which involved the continuous colorimetric analyses of the column effluents. This new process was, of course, much less labour intensive and a completed analysis was achieved in one day rather than one week. Starting around 1987, much smaller columns were used and an amino acid analysis could be completed in only thirty minutes or fifty times faster still (Pierce, 1987). Today, fully automated equipments allow analysis of all amino acids in only 9.5 minutes per sample.

High performance liquid chromatography (HPLC) represented an important contribution to the study a wide range of applications in brewing. In particular, the system found its major application in the analysis of non-volatile compounds such us amino acids, which are difficult to analyze effectively by other analytical systems. The same classical separation system utilizing ion exchange, applied by Spackman et al., has been adapted to the HPLC system (Garza-Ulloa et al., 1986).

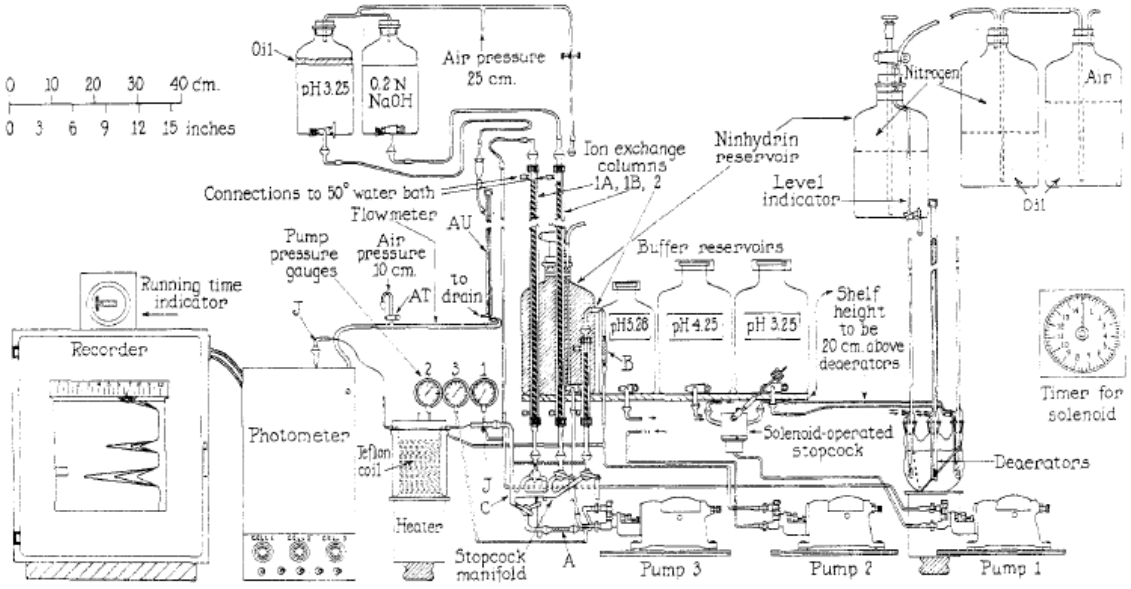


Fig. 2.4.10 Automatic recording apparatus used in chromatographic analysis of mixtures of amino acids (from Spackman et al., 1958).

Some HPLC methods use postcolumn derivatization in which the amino acids are separated on an ion-exchange column followed by derivatization with ninhydrin, fluorescamine, or o-phthalaldehyde (OPA). Another approach has been to derivatize amino acids prior separation on a reversed phase HPLC column. Examples of this technique are dansyl, phenylisothiocynate (PITC), 9-fluorenylmethyl chloroformate (Fmoc) and 6-aminoquinolyl-N-hydroxysuccinimyl carbamate (AQC) (Tyler, 2001).

The difference between precolumn or postcolumn derivatization refers to whether the amino acids are derivatized before or after the chromatographic separation. All of the other steps in the process, like sample preparation, hydrolysis, quantification (integration), and report preparation are common to both methods. Studies shown that precolumn derivatization offers significant advantages over postcolumn derivatization in the following areas: single wavelength detection, 10-fold greater sensitivity, and 45 min run times. However, the major disadvantage with precolumn derivatization is that there is much less flexibility with the composition of the sample matrix (Smith, 2003).

In 1994, Cohen and De Antonis, introduced 6-aminoquinolyl-N-Hydroxysuccinimidyl carbamate (AQC) for precolumn derivatization. The advantages of this new method included rapid and simple derivatization protocol, excellent response linearity over at least two orders of magnitude, formation of highly stable urea derivatives, and detection limits below one picomole (Cohen, 2001).

The derivatization reaction (Fig. 2.4.11) has several advantages that simplify the analysis: the formation of the amine or amino acid derivatives is extremely rapid, occurring within seconds; excess reagent is hydrolysed to 6-aminoquinoline (AMQ) in less than 2 minutes, hence preventing any unwanted side reactions; and fluorescence emission maxima of AMQ and AQC-derivatized amines are approximately 100nm apart, allowing for selective detection of the desired analytes without significant reagent interference (Cohen and De Antonis, 1994; Cohen, 2003).

This method could be applied on different sample types, like grains, hydrolyzed food or feed, collagen containing samples, glycoproteins and intravenous solutions. Derivative yields are essentially unaffected by the presence of salts and high concentrations of carbohydrates present in the sample. This is especially important in the analysis of wort amino acids, since wort is mainly composed of carbohydrates (Cohen and De Antonis, 1994).

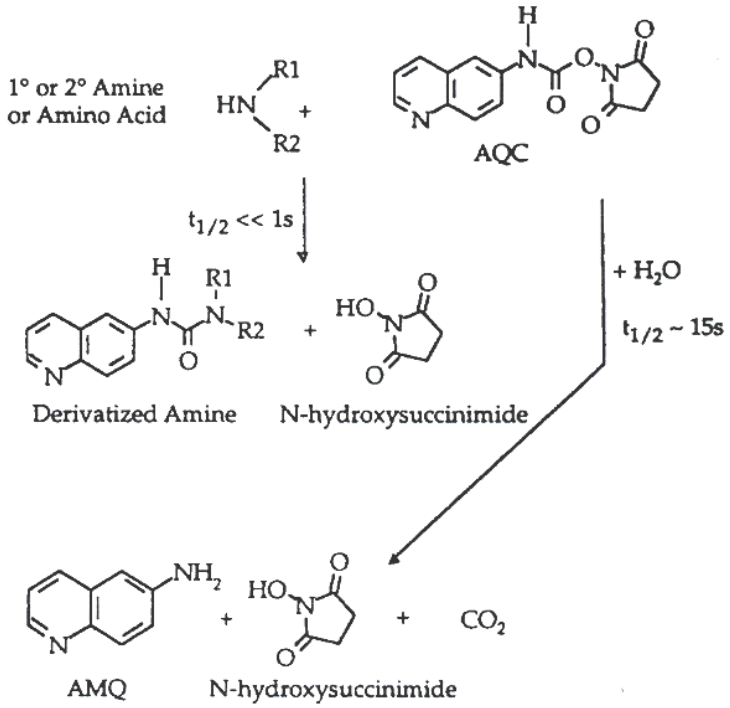


Fig. 2.4.11 Derivatization of primary (1º) and secondary (2º) amines with AQC. Also shown is the hydrolysis reaction of excess reagent (from Cohen and De Antonis, 1994).

2.5 Endoproteases

2.5.1 Proteases in barley, malt and wort

The hydrolysis of barley proteins into peptides and amino acids is one of the most important processes during and after barley germination. The degradation of endosperm stored proteins facilitates water and enzyme movements, enhances modification, liberates starch granules and increases soluble amino nitrogen (Briggs, 1998). Most of the proteins that are degraded are the storage proteins, mainly hordeins, but various albumins and globulins are also degraded during malting. The proteolytic enzymes responsible for this process are either synthesized and stored in the endosperm during grain development, or are synthesized and secreted during germination from the aleurone or scutellum.

Protease activity is the result of the activities of a very complex mixture of exo- and endoproteases. The barley proteins are initially solubilized by endoproteases and then further by exopeptidases. Most proteases act either on the interior of peptide chains (endoproteases, EPs) or on their termini (exopeptidases) (Palma et al., 2002; Jones, 2005a).

Exopeptidases have been differentiated according to their substrate specificity as, aminopeptidases, which are able to cleave peptides at the N-terminus, and carboxypeptidases, which degrade peptides at the C-terminus. Aminopeptidases are ubiquitous enzymes that have been identified in several tissues from a large number of plant species, although only a few of them have been purified and characterized so far. In barley, there is no evidence for the presence of aminopeptidases in the starchy endosperm, but these enzymes may be involved in storage protein mobilization within the aleurone and scutellar cells. Carboxypeptidases are one of the major classes of proteases found in seeds, although their presence has been also reported in mature green and senescent leaves. Seed carboxypeptidases are located in the protein bodies

of dicotyledonous plants and in the starchy endosperm of germinating cereals. As general features, seed carboxypeptidases have serine in their active site, a pH optimum within the 4–6 range, and low specificity (Palma et al., 2002). Five serine carboxypeptidases have been identified in germinating barley. Each enzyme has a distinct preference for particular amino acids at or near the COOH-termini of their substrate, but their specificities are complementary resulting, synergistically, in extensive degradation of storage proteins and peptides. Studies suggest the scutellum is the principal source of carboxypeptidases but that then they are detected in the starchy endosperm (Fincher and Stone, 1993).

Endoproteases are classified according to their catalytic mechanism, which implies specificity in the enzyme active sites. It is important to point out that the term endoprotease is equal to endopeptidases, which at the same time can be used synonymously with proteinase. In plants, four classes of endoproteases have been described: serine-proteinases (EC 3.4.21), cysteine-proteinases (EC 3.4.22), aspartic-proteinases (EC 3.4.23), and metallo-proteinases (EC 3.4.24) (Table 2.5.1) (Palma et al., 2002).

Table 2.5.1 Main endopeptidases (proteinases) reported in plants (from Palma et al., 2002).

Type of proteinase	Protease name
Serine-proteinases	ClpP
	Subtilisins
	Kexin-type proteins
Cysteine-proteinases	Caspase-like proteins
	Vacuolar-processing enzyme (VPE)
	Papain-like peptides
	Cathepsin-type proteases
	Asparaginyl endopeptidases
Aspartic-proteinases	Cardosins
	Cathepsin D-like proteins
Metallo-proteinases	FtSH
	Chloroplast-processing enzyme (CPE)
	Matrix-like enzymes

Zhang and Jones (1995a,b) characterized and classified 42 different endoprotease activities in germinated barley based on their pI values (isoelectric point), PAGE mobilities in a two dimensional (2-D) gel and biochemical characteristics. This method allowed separating the 42 endoproteases into individual activity spots, including 27

cysteine endoproteases, seven serine proteases, four metallo-proteases and four aspartic proteases (Fig. 2.5.1). This large number of proteases, which includes some isoforms, does not include exoproteases, which are not detected by this technique. An important finding from the 2-D studies was that within each protease class the individual members generally had similar pH optima but that between classes there were major differences. The cysteine and aspartic proteases were most active at pH values between 3.8 and 4.5, whereas the serine and metalloproteases were optimally active at pH levels from about 6.0 to 8.5 (Zhang and Jones, 1995a).

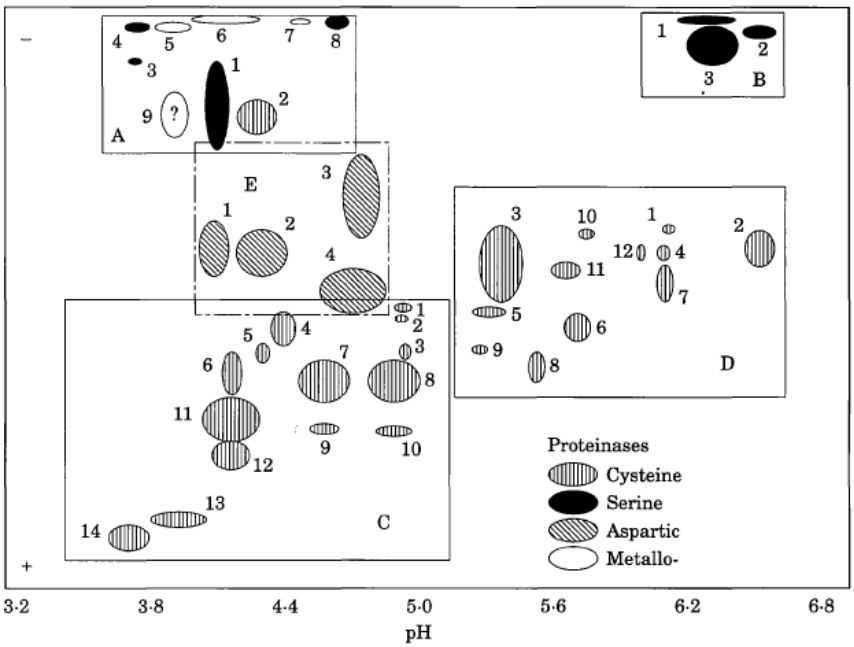


Fig. 2.5.1 Diagram of green malt endoproteolytic activities separated by 2-D method. The different endoproteases groups are boxed and labelled A-E. The activities within each group are indicated by numbers. The x-axis indicates the pI of each activity while the y-axis indicates the distance moved during PAGE (from Zhang and Jones, 1995a).

2.5.2 Endoprotease classes

Cysteine endoproteases (Cys-EPs)

Cys-EPs are the group of plant proteases that have been most thoroughly studied. The catalytic mechanism of these enzymes involves a cysteine group in the active site. In plants, many biological events have been reported where cys-EPs take part. In barley,

mobilization of proteins during germination is their main role. The best-known cys-EPs are listed in Table 2.5.1. The family of papain-like peptidases is the most thoroughly investigated of the cys-EPs. These enzymes are synthesized as zymogens, inactive enzyme precursors that require reducing agents for full activity (Koehler and Ho, 1990; Palma et al., 2002).

Serine endoproteases (Ser-EPs)

Ser-EPs are neutral proteases having an active Ser in the catalytic site, which interacts with the proteic substrate. Ser-EPs are involved in a variety of physiological processes, such as senescence, programmed cell death, xylogenesis, tissue differentiation, the infection of plant cells, pathogenesis in virus-infected plants, and germination (Palma et al., 2002).

In barley, the majority of Ser-EPs activity is found in the root and axis tissues, with some activity also found in the endosperm (Zhang and Jones 1995b). A serine protease of the subtilisin family (subtilase), hordolisin, has been isolated from barley malt, and identified by N-terminal amino acid sequencing and labelling with [^{14}C]-DFP (Simpson, 2001; Fontanini and Jones, 2002).

Aspartic endoproteases (Asp-EPs)

Asp-EPs have a preference for peptide bonds flanked by hydrophobic amino acid residues, and are active at acidic pH. Studies demonstrate that their hydrolysis was maximal when the substrate contained either aromatic or aliphatic amino acids. Although these enzymes have been described in a wide range of plant species, most studies have been conducted in barley grains, where asp-EPs are abundant. Less attention has been paid to aspartic proteases than to ser-EPs and cys-EPs, and, therefore, little is still known about their biological function (Jones, 2005a).

Metalloproteases (MPs)

Plant MPs are less known than the other groups of proteases. Most MPs contain Zn in their active site, although cobalt or manganese can also be present to activate the water molecule for the hydrolysis of peptide bonds. MPs were detected in all of the barley grain tissues, but the aleurone appeared to contain the greatest concentration of these enzymes (Zhang and Jones, 1995b).

2.5.3 Endoproteases assays

The protease activities of barley and malt have been measured mainly in two ways described below, but with a wide type of substrates, thus the results obtained from a given study can vary greatly, depending on which assay conditions (pH, reaction temperature, substrate) were used. Thus, appropriate analytical methods and conditions still need to be developed.

An 'in solution' quantitative assay

Jones et al. (1998) define a system to study quantitatively, quickly and efficiently individual or mixtures of endoproteases. The assay utilizes the coloured protein substrate, azogelatin, in an 'in solution' assay. The basic principle of the method mixes the protein substrate with the endoproteases, removing fractions at appropriate times by precipitating the unhydrolyzed substrate with trichloroacetic acid (TCA) and reading the absorbance of the supernatant at 440nm. The study demonstrated that several different endoproteases types were linear for 30 min or less, so that only reactions performed for 30 min or less would yield true initial reaction rates (Fig. 2.5.2)

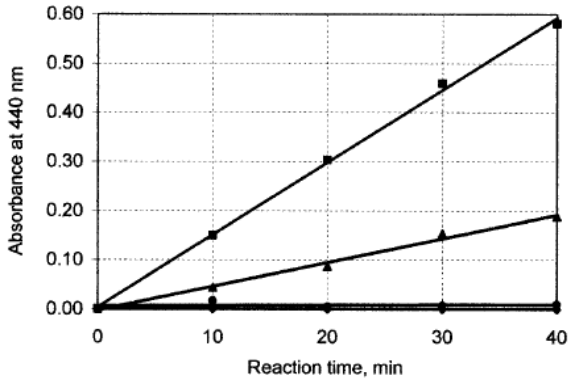


Fig. 2.5.2 Analysis of endoproteinase activity with azocasein substrate and TCA precipitation. (■) Green malt extract, no inhibitor; (▲) green malt extract with added proteinase inhibitor; (♦) no enzyme added; (●) no substrate added (From Jones et al., 1998).

Although azogelatin is readily hydrolysed by many enzymes comprising cysteine, metallo and serine proteases, the aspartic proteases hydrolyse azogelatin at a much slower rate (Fig 2.5.3).

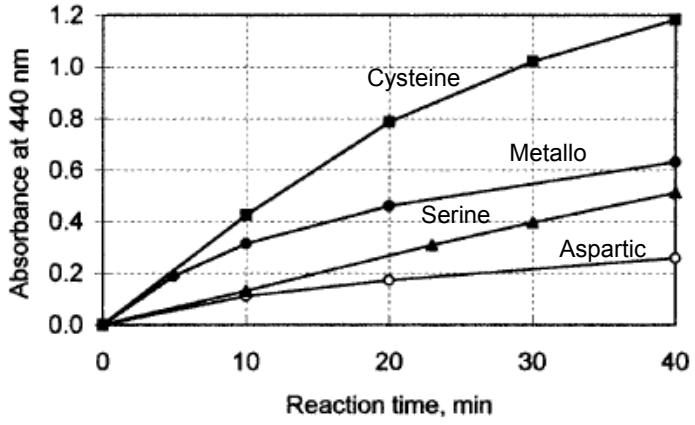


Fig. 2.5.3 Hydrolysis of azogelatin by commercial enzyme preparations. Hydrolysis was with (■) papain; (●)protease type XV; (▲) trypsin; (○) pepsine (adapted from Jones et al., 1998).

Another example of protein as substrate was published by Osman (2003a), who used a barley preparation known as 'highly degradable barley protein fraction' (HDBPF), as a 'natural' substrate for malt endoproteases assays. Osman (2003b) purified five barley-malt endoproteases and used the HDBPF as the substrate for activity detection and measurement Comparing HDBPF with gelatin properties as substrate, Jones concluded that HDBPF was not useful for proteases assays (Jones, 2005a).

Jones et al. (1998) established that the best substrate for measuring malt proteases is the gelatin coloured derivative, azogelatin. The azogelatin substrate has several

excellent attributes: (1) it is a protein so it should give a realistic view of how the enzymes act on a natural substrate; (2) it is hydrolysed by enzymes of all four of the common protease classes, although it is not as susceptible to attack by the aspartic proteases as it is by those of the other classes; (3) its hydrolysis products are coloured red and absorb light at 440 nm, so they can be monitored without derivatization. This also resolves problems associated with measuring the release of peptides at 280 nm, where contaminating amino acids, proteins, etc. also absorb; (4) the hydrolyses are also more reproducible, since only the hydrolysis of the azogelatin substrate is detected, whereas the commonly used 280 nm wavelength also detects the hydrolysis of any contaminating proteins, each of which might be hydrolysed at a different rate; (5) it is readily soluble between pH 3 and 10.5, the range of the proteolytic activity pH optima of the four malt proteinase classes; (6) it is readily precipitated with TCA; and (7) the azogelatin derivative is easily and reliably prepared from porcine skin type A gelatin of w300 bloom (Jones et al., 1998; Jones, 2005a)

In many studies, researchers have used the substrates azocasein, casein, and hemoglobin, which precipitate nicely in the presence of TCA and also are good substrates to identify endoproteases from malt. However, they are readily hydrolyzed by only a fraction of malt endoproteases and not at the same rate for all four endoprotease classes.

Flourogenic assay

Recently, Schmitt and Budde (2007a) developed a fluorogenic method to estimate malt endoproteases activities of larger sets of samples. The fluorogenic assay relies on an increase in fluorescence after endoprotease substrates are hydrolyzed into smaller fragments, effectively relieving the intramolecular fluorophore quenching in the intact molecule (Fig. 2.5.4). The assay included two substrates: one a labeled dipeptide (Z-

Phe-Arg-AMC) known to be hydrolyzed by cysteine proteinases, and the second a fluorescent gelatin derivative. The pH of the assay system was adjusted to favour the activity of the cysteine-class proteinases (pH 4.5, Z-Phe-Arg-AMC substrate) or the serine-class proteinases (pH 6.0, DQ-gelatin substrate) as appropriate. The use of fluorogenic substrates greatly simplifies the assay of proteinase activity, allowing activity surveys across much larger populations of malting barley. The low (<2%) CV for quadruplicate assays using DQ-Gelatin suggests that fewer replicates could be adequate for obtaining reliable estimates of activity.

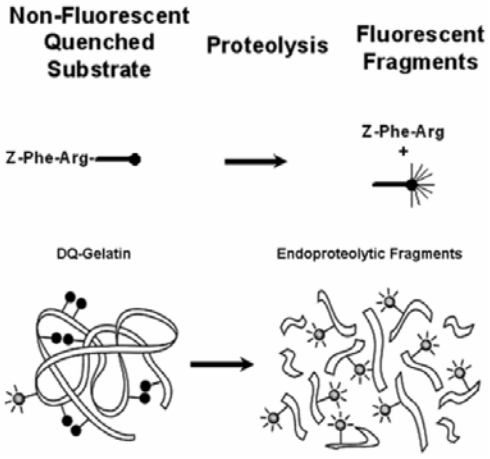


Fig. 2.5.4 Scheme describing two fluorogenic (fluorescence dequenching) protease assays (from Schmitt and Budde, 2007b).

2.5.4 Effect of processing conditions on endoproteases activities

Formation of endoproteases during malting and their stabilities to kilning

The overall endoproteolytic activity of barley grains is quite low with protease activity only becoming significant during the early phases of seed germination or malting (Jones, 2005a). Jones et al. (2000) found that under malting conditions the proteolytic activity at the end of steeping was very low, but began to rise at germination day 1 and was maximal by day 3. Sampling of green malt during the kilning phase of malting showed that there was no diminution of proteolytic activity during kilning even when the temperature was raised to 85 ºC.

Similar results were obtained by Osman et al. (2002) who assayed endoproteases with hemoglobin and glutelins as substrates, at pH 4.0 and pH 5.0 respectively. They found that more than 90% of the maximal endoprotease activity measured on day 4 of germination is retained in kilned malt.

In 2002, Kihara et al. investigated endoprotease activities in malt from various barley lines at pH 6.8 under reducing conditions, and found that malt had a wide genetic variation. Furthermore, cysteine activity presented the highest correlation to the content of soluble nitrogen and Kolbach index, suggesting that cysteine proteases play an important role in the solubilization of grain storage proteins during malting.

Effect of mashing on proteolytic activities

Proteases also partially degrade barley storage proteins, during mashing, into amino acids and peptides that are critical for brewing high quality beer. Previous studies showed that during mashing, the proteases responsible for releasing soluble nitrogen were inactivated at temperatures of 55-60 °C, indicating that it is unlikely that very much of the proteolytic activity would survive the final steps of the mashing process (Jones, 2005a; Jones, 2008).

Jones and Marinac (2002) studied the effect of mashing, under conditions that mimic the commercial process, on malt endoproteases. Results, from the 'in solution' assay, showed that the overall proteolytic activity was constant throughout a 50 min, 38 ºC 'protein rest' phase, but fell rapidly when the temperature was raised to 72 ºC for the 'conversion' phase

To define which classes of endoproteases effect changes in SP and FAN wort levels, Jones and Budde (2005) performed an experiment in which class-specific proteases inhibitors were added to barley malt mashes (Table 2.5.2). Previous studies showed that only the cysteine enzymes were involved in SP production. However, the

efficacies of the endoproteases in solubilizing malt proteins were in the order cysteine ≈ metallo > aspartic > serine ≈ 0, which roughly reflected how the inhibitors affected the mash endoproteolytic activities (Table 2.5.3).

Table 2.5.2 Endoprotease classes and their specific chemical inhibitors (adapted from Jones and Budde, 2005).

Protease class	Inhibited by:	Concentration, individual	Concentration, mixture study
Cysteine EC 3.4.22	E-64	1uM	4uM
Serine EC 3.4.21	PMSF	1mM	-
Aspartic EC 3.4.23	PepstatinA	5uM	25uM
Metallo EC 3.4.24	o-Phen EDTA	5mM 5mM	10uM -

The release of SP during mashing correlated well with the change in endoproteolytic activities of the mashes. Moreover, all four enzyme classes affected FAN levels but none altered any of the other measured wort characteristics. The FAN values of the wort generally varied in parallel with SP levels and endoproteases activities. The FAN and SP values did not change proportionately, probably because the wort SPs are released from insoluble storage proteins by endoproteinases while the FAN is produced by exopeptidases that operate predominantly on the SP fraction. It has been proposed that the FAN production is due mainly to the serine-class carboxypeptidases, which should have been inhibited by PMSF (serine class inhibitor). In contrast to its inhibiting of SP formation, PMSF caused the expected inhibition of FAN formation. The

Table 2.5.3 The effects of adding class-specific inhibitors to pH 6.0 Congress mashes (from Jones and Budde, 2005).

Inhibitor added	Soluble protein		Endoproteinase		FAN	
	Percent	%Inhibition	Activity[a]	%Inhibition	ppm	%Inhibition
Morex						
None	6.03	–	0.071	–	235	–
E-64	5.55	8	0.043	39	213	10
Pepstatin A	5.72	5	0.070	1	227	4
PMSF	5.99	1	0.061	14	224	5
o-Phen	5.30	12	0.047	34	227	4
EDTA	6.28	−4[b]	0.127	−79	278	−18
Harrington						
None	4.93	–	0.041	–	192	–
E-64	4.26	14	0.030	27	167	13
Pepstatin A	4.56	8	0.040	2	172	10
PMSF	4.67	5	0.030	27	168	12
o-Phen	3.93	20	0.033	20	150	22
EDTA	4.90	0	0.058	−42	184	4

[a] $\Delta OD_{440\ nm}$/60 min.
[b] Negative values indicate activation, not inhibition.

FAN-producing exopeptidases do, however, use the SP fraction as substrate, so any inhibitors that lowered the SP amounts would presumably also lower the level of FAN produced (Jones and Budde, 2003; Jones, 2005b).

Yano et al. (2008) found that cysteine and metallo-proteases significantly correlated to barley adjunct wort FAN levels. However, the methodology applied for wort preparation makes comparison with previous studies, where standard methodology for wort preparation was used, difficult.

Recently, Kihara et al. (2006) investigated levels of total proteinase activity and of each class (cysteine, aspartic, serine and metallo) of proteinase activity in malt from the cross of two standard malting barley varieties. The results from the study showed that the enzyme activity had a significant influence on the content of SP in malt, or KI, which indicates the degradation efficiency of storage protein in germinating barley. Cysteine protease activity had the highest correlation to KI among the classes of endoproteases.

2.6 Fermentability

2.6.1 Brewing fermentation

Fermentation is the process whereby wort is converted, as a result of the metabolic activity of yeast, to ethanol and a wide range of other fermentation by-products in order to produce beer (Stewart and Russell, 1993). The reactions, which support the growth of yeast during fermentation and the concomitant conversion of wort to beer, handle almost every aspect of cellular metabolism (Boulton and Quain, 2001).

The biochemistry of brewing fermentation is complex with many aspects still to be elucidated. Some aspects that affect brewing fermentation are: wort composition, the genotype of yeast strain, the phenotypic expression of the genotype, and the fermentation practice (Boulton and Quain, 2001).

Brewer's wort is a natural multifaceted nutritional environment, which provides a complete growth medium for yeast. The biochemical reactions which contribute to the assimilation and metabolism of its individual components are equally complicated. Wort components are: sucrose, fructose, glucose, maltose, maltotriose, dextrin material, as well as a mixture of amino acids, peptides, proteins, vitamins, ions and nucleic acids (Boulton and Quain, 2001; Cruz et al., 2003). Sulphur, phosphorous, zinc, copper, magnesium and a large number of other elements are also present in a wide range of concentrations (Ingledew, 2000).

Brewing yeast strains are heterotrophic organisms capable of utilizing a wide variety of nutrients to support growth and generate energy. A property of such organisms is possession of specific systems to accommodate the uptake of individual or related classes of nutrients. The most important processes in brewing fermentation are the assimilation of carbohydrates and nitrogenous compounds, which are highly regulated. When yeast is presented with a choice of nutrients, like what happens with wort, cells tend to first use those nutrients that are most easily assimilated. However, some nutrients are utilized in preference to others and the presence of certain nutrients can inhibit the uptake of others (Boulton and Quain, 2001). As a result, the uptake of carbohydrates and the various sources of nitrogen from wort are ordered processes which will be covered further in this section.

Changes that occur during typical lager fermentation are illustrated in (Fig. 2.6.1). During the initial lag phase, which lasts for 12-24 hours, there is limited or no observable change in specific gravity, yeast count and ethanol concentration. However, the oxygen concentration rapidly decreases, falling to undetectable levels within the first 24 hours (Boulton and Quain, 2001). The concentration of FAN begins to fall as soon as the fermentation starts which is accompanied by a rapid decline in pH. At the end of yeast growth, all these processes stop and the concentration of certain flavour compounds

begin to decrease. However, Inoue and Kashihara (1995) observed that the change in concentration of the fermentable sugars continues, whether the yeast is actively growing or not.

A crude mass balance for a typical fermentation may be written as follows:

Sugars (150g/L) + FAN (150mg/L) + Yeast (1g/L) + Oxygen (25mg/L)

Ethanol (45g/L) + CO2 (42g/L) + Yeast (5g/L)

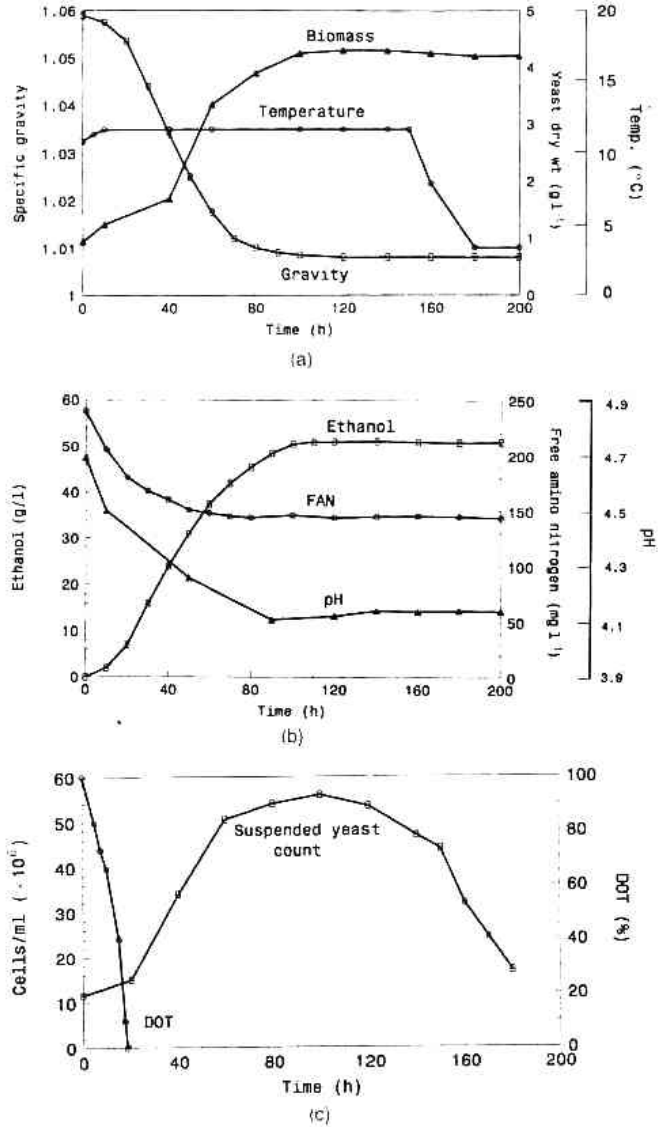


Fig. 2.6.1 Progress of a typical lager fermentation (from Boulton and Quain, 2001).

In general, it has been shown that it is the overall balance of key substances that is more important than any one nutrient in particular and that it is an imbalance of nutrients that is likely to cause fermentation problems (Van Zandycke and Fischborn, 2008).

2.6.2 Wort composition

Wort is a complex medium, so when yeast is inoculated (pitched) into wort it is introduced into an intricate environment (Stewart, 2006). All malt worts, prepared with an appropriate ionic composition, provide a medium with the potential to produce new yeast biomass, ethanol and flavour components in balanced and desired quantities. Although wort composition is somewhat under-characterized, it is possible to establish relationships between the assimilation by yeast of the major classes of wort nutrients and the formation of products of yeast metabolism. This equilibrium can be manipulated by modifying wort composition through changes to the ratio of carbon to nitrogen or by increasing the fermentable extract, for example, with sugar syrup adjuncts. Hence, manipulation in wort composition can be a deliberate strategy for producing desired changes in fermentation (Boulton and Quain, 2001).

Nitrogen metabolism

Nitrogen is one of the main elements found in many macromolecules of living organisms. It plays a central role in structure and function, and most organisms have sophisticated control mechanisms to provide a constant supply of nitrogen. As mentioned in previous sections, assimilation of amino acids in wort is ordered, and groups of amino acids have been identified on the basis of their rate of removal from the fermenting wort. Wort amino acids have also been classified according to the essential nature of the keto-acid analogues in yeast metabolism (Cruz et al., 2003). Yeast utilizes the major proportion of wort amino nitrogen to synthesize their structural and enzymic

proteins. Protein components of cell membranes play an important role in yeast ethanol tolerance levels and adaptation (O'Connor-Cox and Ingledew, 1989; Lekkas et al., 2005).

Yeast reproduction and biomass development is largely determined by the concentration of assimilable nitrogenous compounds. Changes in the concentration of individual amino acids in wort do not appear to alter the pattern of amino acid uptake significantly. Yeast will tolerate considerable variation in amino acid distribution as long as the concentration of free amino nitrogen is adequate for growth (Burger and Porter, 1978).

Genetic and biochemical differences among industrial yeast strains, including structural complexity, can have a strong effect on fermentation performance as affected by nitrogen source. Yeast performance is influenced by: 1) the ability of cells to transport the amino acids or small peptides across the plasma membrane and 2) enzymes required to generate the ammonia necessary for their metabolism. Once inside the cell ammonia can react with α-ketoglutarate, provided by the metabolism of the carbon source of the growth medium, to produce glutamate, which in turn can react with ammonia to produce glutamine. The amino nitrogen of glutamate serves as the source of 85% of the total cellular nitrogen and the amide group of glutamine is the source of the remaining 15% (Magasanik and Kaiser, 2002).

Nitrogen regulation (nitrogen catabolite repression) is the mechanism designed to prevent or reduce the synthesis of enzymes and permeases for the utilization of compounds that are non-preferred sources of glutamate and glutamine when a preferred nitrogen source is available. All of the pathways for the utilization of non-preferred sources of nitrogen feed into a common set of reactions for the production of glutamate and glutamine (Fig. 2.6.2).

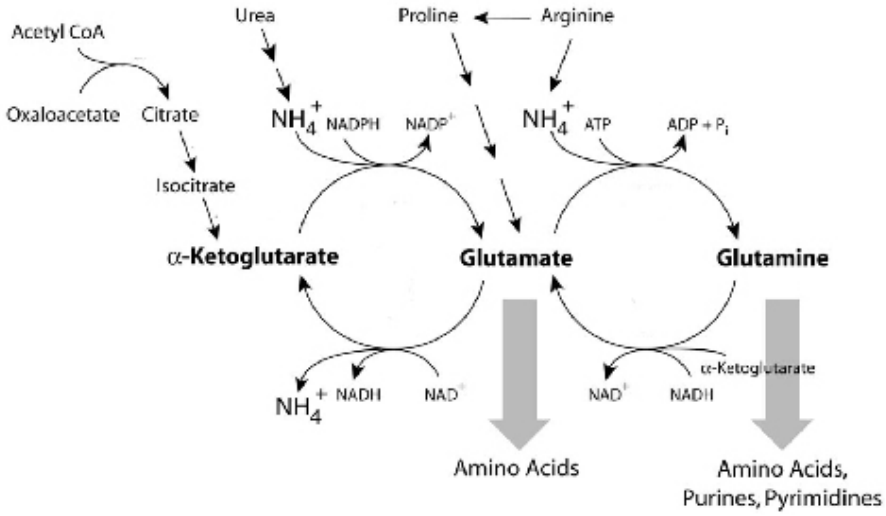


Fig. 2.6.2 Central pathways for nitrogen metabolism. The nitrogenous compounds in the cell are synthesized from either glutamate or glutamine. The major pathway for glutamate synthesis is through combination of ammonia with a-ketoglutarate, which is synthesized from acetyl CoA and oxaloacetate through the early steps of the citric acid cycle. Glutamine is synthesized by the combination of ammonia with glutamate. The pathways for utilization of a variety of nitrogen sources, including urea, proline and arginine, are shown (adapted from Magasanik and Kaiser, 2002).

It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast, and the non-preferred sources are proline, ornithine, γ-aminobutyrate, allantoin, and urea. There are two different criteria that can be used to judge the quality of a particular nitrogen source. One criterion is based on the growth rate that can be supported by the source of nitrogen. However, the differences in growth rate supported by very different nitrogen sources are often surprisingly small and it is therefore difficult, in practice, to make clear distinctions between the qualities of different nitrogen sources. A second criteria, is based on the level at which systems that use alternative nitrogen sources are derepressed during yeast growth. Thus, nitrogen sources that: 1) do not derepress the pathways for utilization of alternative nitrogen sources are generally considered to be preferred nitrogen sources, while 2) those sources that do lead to derepression of the alternative pathways are considered to be

non-preferred. The advantage of this criterion is that differences are more easily observed (Magasanik and Kaiser, 2002; Cruz et al., 2003).

The transport of amino acids across the plasma membrane involves specific permeases for certain amino acids, a general amino acid permease of broad-spectrum specificity, as well as feedback inhibition effects resulting from the composition of intracellular amino acids (Cruz et al., 2002; Cruz et al., 2003). *Saccharomyces cerevisiae* encodes 19 amino acid permeases identifiable as members of a diverse family of transporters responsible for the uptake of nitrogen sources from the medium. Some of these transported also act as receptors signalling the availability of their substrate to the interior of the cell. The amino acid permeases are integral membrane proteins with 12 predicted transmembrane domains which are delivered by the secretory pathway to the plasma membrane where they function to take up amino acids for protein synthesis and for use as sources of nitrogen. These amino acid permeases (AAP) can be divided into two classes according to their regulation and function: 1) the nitrogen-regulated permeases include Gap1p, which transports all naturally occurring amino acids, and Put4p, which transports only proline. Both of these permeases are co-ordinately derepressed during yeast growth on poor nitrogen sources, this allows the cell to take up a broad range of amino acids and related compounds from the medium as they become available. 2) The other class of permeases are expressed even when cells are grown on a preferred source of nitrogen. Most of these permeases are specific for particular amino acids, or chemically related sets of amino acids, such as the histidine permease (Hip1p), the basic amino acid permease (Can1p), tryptophan permease (Tat2p), the ammonia permeases (Mep1 and Mep2), glutamine permease (Gnp1) and a recently described tyrosine permease (Tat3p). These classes of permeases are thought to transport amino acids that are available in the growth medium for use in protein

synthesis (André, 1995; Magasanik and Kaiser, 2002; Omura et al., 2007; Garrett, 2008).

Thus, within the AAP family members, Gap1p, the proline permease, Put4p and the acidic, Dip5p, are subjected to nitrogen catabolite repression (NCR), by which their transcription is repressed when preferred nitrogen sources are available. On the other hand, the expression of some low-capacity, relatively specific and high affinity permeases like the branched chain Bap2p and Bap3p, the tyrosine Tat3p, tryptophan permeases Tat2p, and the histidine permease Hip1p, are transcriptionally induced by sensing extracellular amino acids via the Ssy1p-Ptr3p-Ssy5p sensor system (Forsberg and Ljungdahl, 2001; Omura et al., 2007).

Yeast cells employ multiple mechanisms to control Gap1 activity in response to different nitrogen sources. Transcription of Gap1 and other nitrogen-repressible genes are controlled by the GATA type transcription factors (Gln3p, Nil1p, Nil2p and Dal80p) in response to the presence of glutamine or asparagine in the medium. With respect to posttranslational regulation, Gap1 activity is downregulated by endocytosis and vacuolar degradation when a preferred nitrogen source is available. Cells growing on glutamate, do synthesise Gap1p, but the protein is transported directly to the vacuole and never reaches the plasma membrane (Omura et al., 2007; Garrett, 2008).

The capability of *Saccharomyces cerevisiae* cells to rapidly respond and adapt to changing environmental conditions is based on their ability to sense and subsequently transduce information regarding the extra- and intracellular environments. Sensor-initiated signals are used to make dynamic adjustments in patterns of gene expression and protein turnover, processes that enable cells to express the necessary components appropriate for prevailing conditions. Ssy1p, Ptr3p, and Ssy5p, are known components of a yeast plasma membrane system that sense the presence of amino acids in the extracellular environment. Each of the three sensor components adopts conformations

and modifications that are dependent upon the availability of amino acids and on the presence of the other two components. Thus, they function as part of a sensor complex localized in the plasma membrane. Consistent with a sensor complex, the over expression of SSY1 or the unique N-terminal extension of this amino acid permease homologue inactivates the amino acid sensor in a dominant-negative manner. Each of the components of the Ssy1p-Ptr3p-Ssy5p (SPS) signalling system undergoes rapid physical changes. The rapid physical alterations and reduced levels of sensor components are consistent with their being downregulated in response to amino acid availability (Forsberg and Ljungdahl, 2001; Wu et al., 2006).

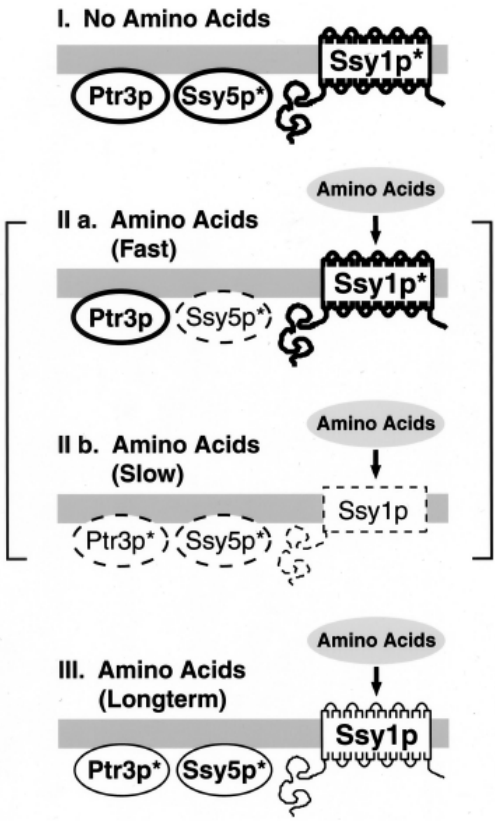


Fig. 2.6.3 Dynamic characteristics of the SPS sensor component interactions (from Forsberg and Ljungdahl, 2001).

A schematic diagram summarizing the dynamic characteristics and interactions of the SPS sensor components is given in Fig. 2.6.3. State I of the sensor is the complex present in cells grown in the absence of amino acids. SPS components present at high levels are represented by heavy outlines. The state I conformation may represent a preactivation complex. States IIa and IIb are transient complexes that rapidly form when cells grown, in the absence of specific amino acids, are induced by other amino acids. The components in the transient states, IIa and IIb, that are undergoing dynamic changes in expression levels, are represented by the dashed outlines in Fig 2.6.3. The state III conformation is the downregulated complex, with the diminished levels of the components are represented by light outlines (Forsberg and Ljungdahl, 2001).

SSY1-mediated signals are also required for full transcriptional repression of the general amino acid permease (GAP1) on ammonium-based media in the presence of amino acids (Forsberg and Ljungdahl, 2001; Perpète et al., 2005).

The beer fermentation process can provide larger brewing yeast cells with different types of stress, e.g. lower temperature, higher osmolarity of wort, higher ethanol concentration, and lower pH at the end of fermentation. It is plausible that having differently regulated permeases can alleviate the destruction of amino acid uptake system when the signalling-based system is inactivated. A better understanding of the functions, of the as yet uncharacterized AAPs, in relation to beer brewing conditions, will help improve the quality of beer production (Omura et al., 2007).

In summary, nitrogen metabolism in *S. cerevisiae* can be understood to be the result of three distinct elements: 1) the enzymes responsible for the synthesis and interconversions of nitrogenous compounds, 2) the permeases for uptake of nitrogenous compounds, and 3) the transcription factors and membrane trafficking proteins that regulate the activity of the enzymes and permeases. It must be pointed out that the elements are interlinked such that each one can exert powerful effects on the other two. For example, the activity of transcription factors governs the relative activities of the enzymes responsible for the interconversion of α-ketoglutarate, glutamate, and glutamine, thus setting the concentrations of these key compounds. Changes in intracellular glutamate and glutamine concentrations can, in turn, produce signals that will alter the activity of permeases leading to changes in the rate of uptake of the precursors to glutamate and glutamine. The existence of the many possible feedback loops, setting the activity of transcription factors, small molecule concentrations and activities of permeases, makes it difficult to understand nitrogen regulation as a whole (Magasanik and Kaiser, 2002; Wu et al., 2006; Garret, 2008). In order to improve the quality and efficacy of beer production, it is crucial to understand in detail the cellular

uptake of nutrients, of which amino acids form a very important group. A first step towards clarification of the complex network of gene expression underlying amino acid accumulation by brewing yeast is to identify and characterize all participating AAPs (Omura et al., 2007).

Sugar metabolism

Standard brewery wort contains approximately 90% carbohydrates (as a percentage of wort solids). This fraction consists principally of the sugars; fructose, glucose, maltose and maltotriose together with dextrin material (Table 2.6.1). In a normal situation, brewing yeast is capable of utilizing: sucrose, fructose, maltose and maltotriose in this approximate sequence although sometimes the order changes. Maltotetraose and other dextrins remain unfermented (Gibson et al., 2008). Altered patterns of sugar utilisation among yeast strains have been reported, and for example, yeast strains have been selected that rapidly and efficiently utilise all wort sugars, a characteristic, vital to industrial fermentation (Cruz et al., 2003).

Table 2.6.1 Typical sugar spectrum of an all malt wort (adapted from Stewart and Russell, 1993).

	Percent Composition
Glucose	10-15
Fructose	1-2
Sucrose	1-2
Maltose	50-60
Maltotriose	15-20
Dextrins	20-30

The generalized sugar uptake pattern initiates with hydrolysis of sucrose, causing an increase in glucose and fructose concentrations. The uptake then follows the route of simplest sugars (the monosaccharides glucose and fructose) first, followed in increasing order of complexity by disaccharides (maltose) and trisaccharides (maltotriose) but excluding maltotetraose and other dextrins (Lodolo et al., 2008).

The pattern of sugar utilization by the yeast has also been studied in detail. The transport of fermentable carbohydrates into the cell is of critical importance in

determining yeast cell metabolism. The initial step in the sugar utilization by yeast is usually either its passage intact across the cell membrane or its hydrolysis outside the cell membrane, followed by the entry into the cell (Fig. 2.6.4). The major superfamily of transporters includes 20 proteins which are encoded by several genes. Maltose and maltotriose pass intact across the cell membrane by active transport; on the other hand, the uptake of monosaccharide such us glucose and fructose are taken up passively (no energy is required). Furthermore, studies show that brewer's yeasts probably possess two independent uptake mechanisms (permeases) to transport maltose and maltotriose across the cell membrane. Once inside the cell, both sugars are hydrolyzed to glucose by α-glucosidase enzyme (Stewart, 2006; Gibson et al., 2008).

The transport, hydrolysis and fermentation of maltose are particularly important in brewing, since maltose is the major sugar component of wort. A major limiting factor in wort fermentation is the repressing influence of glucose upon maltose and maltotriose uptake. Only after approximately 60% of the wort glucose has been taken up by the yeast, will the uptake of maltose commence (Fig. 2.6.5) (Stewart and Russell, 1993)

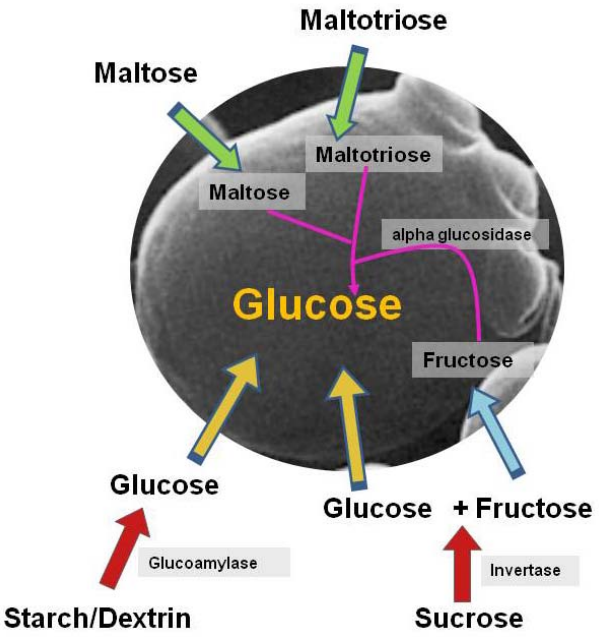


Fig. 2.6.4 Uptake of wort sugars by the yeast cell (adapted from Stewart, 2006).

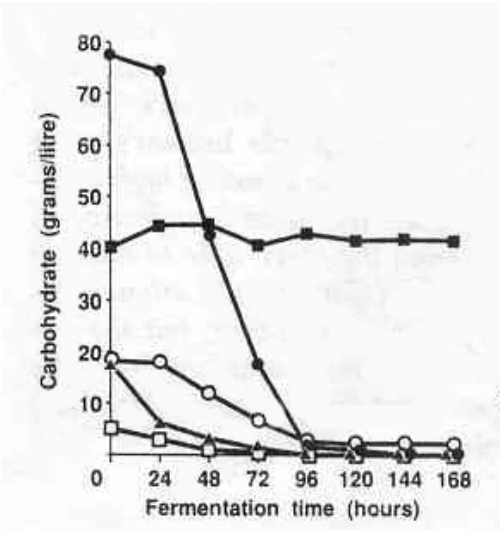


Fig. 2.6.5 Uptake of sugars during fermentation of a 16ºP wort. Glucose (▲), fructose (□), sucrose (Δ), maltose (●), maltotriose (○), dextrins (■) (from Stewart and Russell, 1993).

In summary, high concentrations of glucose or sucrose at early stages of fermentation can cause a significant and long-term decrease in fermentation performance. Thus, the reduction of glucose and sucrose might be advantageous. Indeed, although yeast cells seem to prefer glucose and sucrose over other carbohydrates, offering them too much of this 'fast-food' negatively affects their general fitness (Verstrepen et al., 2004).

Lipid metabolism

Lipids consist of a diverse group of molecules which, in the form of phospholipids make up the membranes around the yeast cell and around organelles within the cell. Since yeast propagate during fermentation to four to six times their own mass, a corresponding amounts of lipid must be built up. The yeast absorb fatty acids from the wort which otherwise would require synthesis with a corresponding need for oxygen.

Thus, the uptake of sterols and unsaturated fatty acids from wort has the potential to reduce the requirement for wort oxygenation (Boulton and Quain, 2001; Kunze, 2004).

Saccharomyces cerevisiae are able to take up fatty acids at low concentrations using a facilitated diffusion system. At high concentrations entry is via simple diffusion. It is assumed that diffusion of such molecules into the cells is facilitated by the lipophilic nature of the membrane. Free fatty acids are powerful detergents and immediately after entry into the cell they are esterified to coenzyme A, to reduce their potential for non-specific enzyme inactivation (Boulton and Quain, 2001).

Mineral metabolism

Minerals are required in the micromolar or the nanomolar range as trace elements necessary for growth. In nature, the trace minerals can be found in wort in levels adequate for fermentation performance (Lodolo et al., 2008). Minerals required by yeast include: phosphorous, sulphur as well as a number of metal ions.

Trace levels of zinc are essential for the structure and function of 4300 enzymes such as alcohol dehydrogenase. It is also the critical component of structural motifs such as zinc fingers. In *S. cerevisiae*, zinc homeostasis is regulated by zinc uptake transporters in the plasma membrane and transporters responsible for intracellular zinc compartmentalization. The activity of these transporters is regulated at both transcriptional and posttranscriptional levels in response to zinc. Zinc deficiency leads to sensitive fermentation problems; the requirement is about 0.10 – 0.15 mg zinc/L of wort. Zinc is the trace element where deficiency symptoms occur most frequently because a majority of malt zinc remains in the spent grains (Jin et al., 1996; Kunze, 2004; Lodolo et al., 2008).

2.6.3 Yeast genotype

The genotype of the particular strain of yeast used is critical to the outcome of fermentation. Yeast strain selection is based on: 1) their potential to produce beer with a suitable composition, and 2) the response to sugars, assimilable nitrogen and oxygen as brewing yeast strains have limited respiratory capacity and are subjected to carbon and nitrogen catabolite repression (Boulton and Quain, 2001).

Phenotypic expression of yeast genotype is modulated by the conditions experienced during fermentation. In large vessels in particular, the yeast is subjected to multiple stresses such us high hydrostatic pressure, elevated concentrations of ethanol and carbon dioxide, low pH, reduced water activity and a lack of nutrients. All of these have the potential to elicit specific biochemical responses by the yeast (Boulton and Quain, 2001; Verstrepen et al., 2004).

While the yeast is in the fermenter it must achieve a physiological condition in which it is capable of enduring starvation during storage. Regulation of carbon flow during fermentation between glycolysis and gluconeogenesis is of significance. Thus, carbohydrate reserves accumulated during fermentation provide energy during the storage phase and possibly carbon for lipid synthesis during the aerobic phase; utilization of exogenous carbon into gluconeogenic pathways is favoured by the high ratio of carbohydrate to other wort components (Boulton and Quain, 2001; Thiele et al., 2007).

Brewer's yeast contain four major polysaccharides: 1) Mannan, made up of mannose units, located in the cell wall plays and important role in yeast flocculation; 2) Glucan, made up of glucose units, also located in the cell wall and a major factor in determining the shape and rigidity of this structure; 3) Glycogen, intracellular storage polysaccharide, providing the cell with an immediate supply of energy during the lag

phase of fermentation; 4) Trehalose, an intracellular disaccharide, that increases the yeast's resistance to stress conditions (Stewart and Russell, 1993).

Ethanol and temperature can cause a number of inhibitory effects on yeast during fermentation; including inhibition of cell growth, reduced cell viability and poor fermentation performance. Inhibition of cell growth and viability has been observed to increase with increasing ethanol concentrations, whereas high fermentative capacity was only inhibited at very high ethanol concentration. The promotion of yeast growth produced by elevating the temperature stimulates the fermentation, and is thought to be suppressed by the application of pressure, which decrease yeast growth (Inoue and Kashihara, 1995).

One of the major target sites of ethanol is the plasma membrane of the yeast, as well as the membranes of the various cell organelles (Fig. 2.6.6). Ethanol is thought to interact with membranes by insertion into their hydrophobic interior, increasing the polarity of this region, weakening the hydrophobic barrier to the free exchange of polar molecules and affecting the positioning and type of membrane components. Changes in the chain length and level of unsaturation of the fatty acids of phospholipids, and decrease in the total lipid content of the cell membrane have all been shown to be induced by ethanol. Thus, the structural

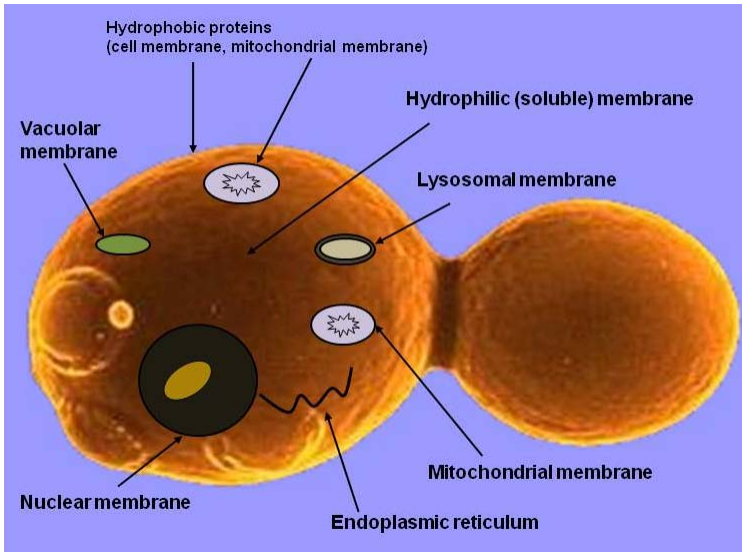


Fig. 2.6.6 Possible target sites of ethanol in yeast cells (adapted from Stewart and Russell, 1993).

alteration in the plasma membrane may be one of the mechanisms underlying ethanol toxicity (Stewart and Russell, 1993; Kunze, 2004).

The impact of the pitching rate on fermentation and beer quality parameters has not been studied systematically. Verbelen et al. (2008) found that fermentation rate increased extensively and the net yeast growth was lowered with increasing pitching rate even though the viability and vitality of the yeast population were not significantly affected. They found FAN consumption was dependent on the pitching rate with the level of total FAN uptake being enhanced at higher pitching rates. On the other hand, the build-up of unsaturated fatty acids in the initial phase of the fermentation was repressed when higher yeast concentrations were pitched. They found that the concentration of trehalose was higher with increased pitching rates, suggesting a moderate exposure to stress in case of higher cell concentrations. The influence of pitching rate on aroma compound production was rather limited, with the exception of total diacetyl levels, which strongly increased with higher pitching rate. These results demonstrated that most aspects of the yeast physiology and flavour balance are not significantly or negatively affected when the pitching rate is changed, although more studies are needed to optimise the conditions for brewing beer with high cell density populations.

2.6.4 Phenotypic yeast expression - formation of flavours compounds

Fermentation has the most significant impact on flavour development. The essential character of any beer is determined by the variety of yeast metabolites that arise during fermentation. The balance of flavour metabolites is largely a consequence of the combination of yeast strain and wort composition. The level and composition of wort assimilable nitrogen have a major influence on flavour compounds, such as higher alcohols, esters, carbonyls and various sulphur compounds. Studies have ranked, in order of importance, positive flavour compounds as follow: ethanol, hop bitterness,

carbon dioxide, isoamyl acetate (banana esters), ethyl acetate (apple esters) and fusel alcohols. In terms of flavour defects (off-flavours) the ranking was: sulphury (DMS+ H_2S), “toffee/butterscotch” (diacetyl) and “stale” (2-trans-nonenal) (O’Connor-Cox and Ingledew, 1989; Boulton and Quain, 2001).

The period of maximum production of extracellular by-products of yeast metabolism occurs when there is a high rate of sugar uptake and dissimilation, coupled with uptake of FAN and other nutrients (Fig. 2.6.7) (Boulton and Quain, 2001).

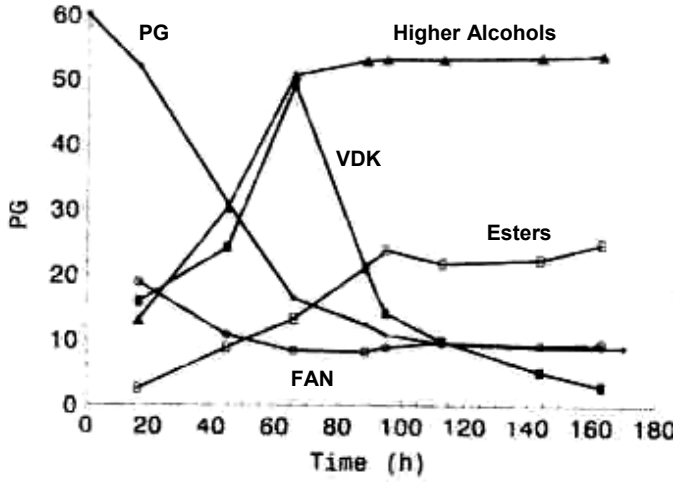


Fig. 2.6.7 Patterns of formation of important groups of flavours active yeast metabolites during the course of lager fermentation. PG: present gravity, VDK: vicinal diketone (adapted from Boulton and Quain, 2001).

A number of flavour active compounds are influenced by levels of nitrogenous components. In nitrogen deficient worts, the catabolism of proteins leads to formation of excessive H_2S, and the inverse relationship also exist. Limiting nitrogen concentrations also leads to abnormal use of the less favoured amino acid groups, altering the time course of flavour precursor production and therefore changing the flavour of the final beer (O’Connor-Cox and Ingledew, 1989).

Carbonyl compounds

Vicinal Diketones (VDK) are considered to impart unpleasant flavours to beer, variously described as “buttery”, “honey”, “toffee-like” or “butterscotch”. The two most important members of this group are: diacetyl and pentane-2-3-dione. The breakdown of these VDK occurs parallel to other maturation reactions during the beer conditioning process and is nowadays regarded as essential for the maturation of a beer. Understanding the yeast metabolism, specially the amino acid biosynthetic pathway, has led to the conclusion that yeast metabolism results in diacetyl and pentane-2,3-dione formation in fermenting wort. In particular, both of these compounds are formed outside the cell by oxidative decarboxylation of α-acetolactate and α-acetohydroxybutyrate respectively. α-Acetohydroxy acids are intermediates in the biosynthesis of leucine and valine (acetolactate) as well as isoleucine (acetobutyrate) and are released into the wort by the yeast during fermentation (Fig. 2.6.8). The diacetyl and pentane-2,3-dione are eventually reabsorbed by the yeast and converted to acetoin or butanediol by yeast reductases (Fig. 2.6.9). Thus, the final concentration of diacetyl in beer is the net result of three separate steps: i) synthesis of and excretion of α-acetohydroxy acids by yeast, ii) oxidative descarboxylation of α-acetohydroxy acids to their diketones and iii) reduction of diacetyl and pentane-2,3-dione by yeast (Stewart and Russell, 1993).

The presence of diacetyl in beer at levels above threshold (70-150 ug/L), occurs when α-acetolactate has decomposed to give diacetyl at a time when yeast cells are either absent or have lost their ability to absorb and reduce diacetyl to acetoin (Petersen et al., 2004). The fault commonly arises when yeast flocculate prematurely. High diacetyl levels can also occur when beer is warmed, during packaging or pasteurization, allowing production of diacetyl from α-acetohydroxy acids when yeast are absent and the diacetyl cannot be converted to acetoin. Diacetyl levels can be controlled by ensuring that there is sufficient active yeast in contact with the beer at the end of fermentation allowing time

for diacetyl to be reduced to acetoin. Diacetyl formation from α-acetolactate has been shown to be dependent upon: pH, concentration of α-acetolactate, fermentation temperature, presence of oxygen, vigour of the fermentation and certain metal ions (Stewart and Russell, 1993).

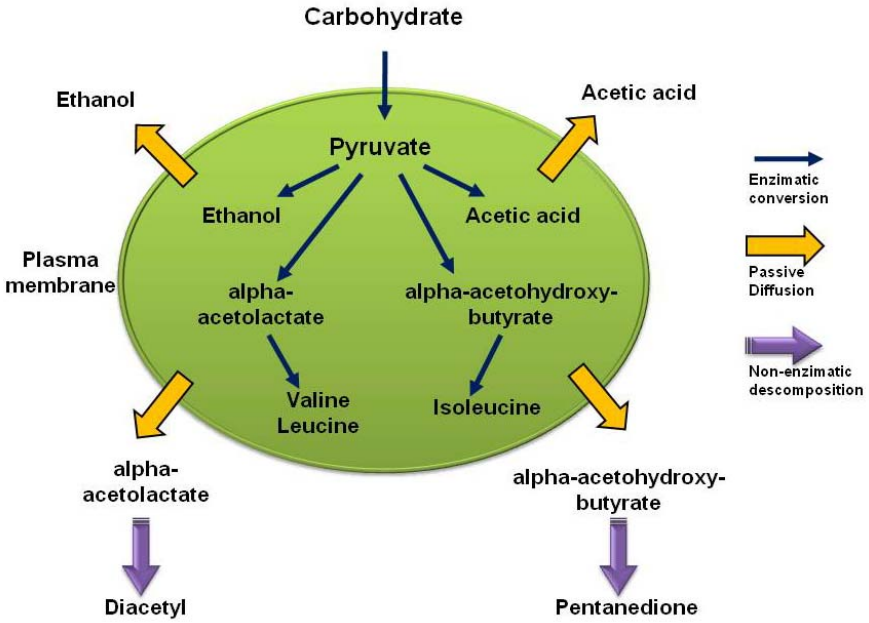


Fig. 2.6.8 Mechanism of diacetyl production in beer by yeast (adapted from Stewart and Russell, 1993).

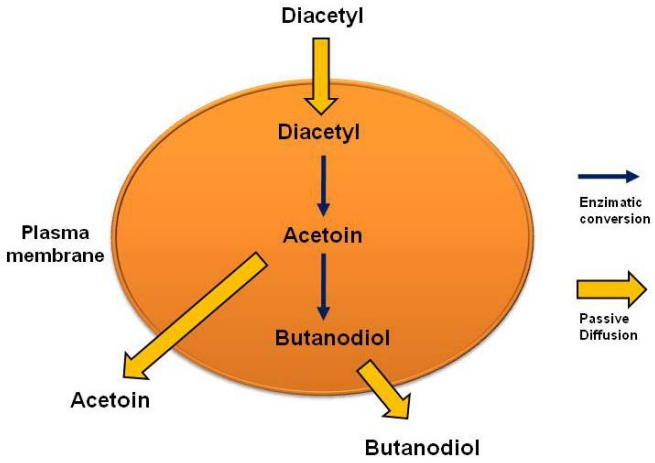


Fig. 2.6.9 Mechanism of diacetyl reduction in beer by yeast (adapted from Stewart and Russell, 1993).

Lowered levels of amino acids in wort brewed with dark malts has a significant effect on the formation of flavour, as several important flavour compounds are dependent on amino acid metabolism by yeast. Although, VDK levels increased with beer colour, beer contained less diacetyl and 2,3-pentanedione than wort. Coghe et al. (2005) showed diacetyl curves for Scarlett, Melanoidin and Cara-aroma wort and they were all clearly distinguishable from the curve of wort brewed with roasted malt (Fig. 2.6.13). For these three malt samples, only one extended diacetyl peak was observed early in fermentation. Conversely, the profile obtained with roasted malt revealed two peaks. The first was small and coincided with the appearance of the single peak seen for the other wort samples, whereas the second peak was sharper and larger. These results are in line with previous studies that indicate that two diacetyl peaks result from low FAN contents. They proposed that the first increase in diacetyl is attributed to valine biosynthesis at the beginning of fermentation when specific amino acids, categorised as group A and not including valine, are consumed. When wort is depleted of group A amino acids (glutamic acid, aspartic acid, serine, threonine, lysine, arginine), yeast cells start to take up valine, which leads to an inhibition of its biosynthesis and a decrease in the level of vicinal diketones due to yeast reductases. Only low FAN levels can give rise to valine depletion, resulting in a renewed biosynthesis and the formation of the second diacetyl peak. They concluded that roasted malts and adjuncts materials produce lower levels of wort FAN because of the depletion

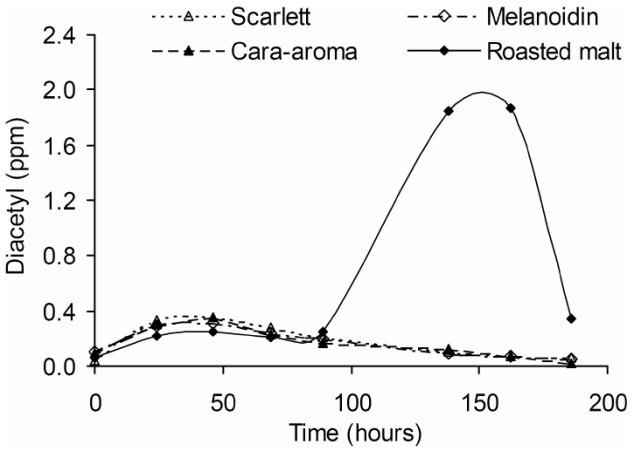


Fig. 2.6.10 Evolution of diacetyl levels during fermentation of wort prepared with 100% Scarlett or with 50% Scarlett and 50% of dark malt (Melanoidin, Cara-aroma and roasted malt) (from Coghe et al., 2005).

of amino compounds through the Maillard reaction or resistance of adjunct proteins to barley malt proteases, respectively (Coghe et al., 2005 & 2006).

Higher alcohols

Apart from ethanol, fermenting yeast cells also produce higher alcohols (fusel alcohols) imparting alcoholic, solvent-like or flowery flavours. Although individual alcohols normally occur at levels below their threshold, fusel alcohols may contribute to the overall aroma of fermented wort due to synergistic effects. The higher alcohols have the important secondary role of providing precursors for esters synthesis. Usually, 3-methylbutanol, 2-methylbutanol and glycerol are the most abundant. Factors that increase the higher alcohol content are high wort oxygen content and increased fermentation temperatures, both of which favour yeast growth (Boulton and Quain, 2001; Coghe et al., 2005).

Of importance is the relationship between fusel alcohol formation and the sequential amino acid uptake. The aliphatic alcohols, isobutanol, isoamyl alcohol and amyl alcohol, are by-products of group B amino acids (valine, leucine and isoleucine), whereas the aromatic alcohols 2-phenylethanol, tyrosol and tryptophol are derived from group C amino acids (phenylalanine, tyrosine and tryptophan). Formation of isoamyl alcohol and isobutalnol is hardly influenced by the malt type used. Similarly, a decrease of FAN by the use of adjuncts has little effect on alcohols derived from group B amino acids. The formation of aromatic alcohols, however, was significantly enhanced by FAN dilution, especially for top-fermented beers (Inoue and Kashihara, 1995; Coghe et al., 2005).

Esters

Esters are possibly the most important set of flavour active beer components, and to a large extent determine its aroma. Higher ester concentrations can, however, give a

beer an unpleasant bitter, fruity taste. The main volatile esters are ethyl acetate (solvent-like flavour), isoamyl acetate (fruity, banana flavour) and phenyl ethyl acetate (flowery, roses, honey). These acetate esters frequently occur in levels above the threshold value. As for fusel alcohols, the presence of different esters can have a synergistic effect. Esters may potentially arise via two routes: 1) reactions between an alcohol, either ethanol or a higher alcohol, and a fatty acyl-CoA ester, or 2) by esterases working in reverse direction (Boulton and Quain, 2001; Coghe et al., 2005).

2.6.5 Factors that affect wort composition and fermentability

Malt fermentability refers to the degree to which a wort, prepared from the malt in a standard way, will ferment. Distillers require maximum wort fermentability to maximize spirit yield. Brewers also require high fermentability values when making low carbohydrate beers. For most beers, brewers require moderate, but reproducible fermentability values unless low-alcohol beers are to be produced, in which case low fermentabilities are advantageous. It is possible to prepare malts with good extract but poor fermentabilities. Fermentability depends not just on the fermentable sugars:dextrin ratio, but also on the supply of other yeast nutrients, such us amino acids, peptides, ions, sulphur, phosphorous, zinc and many other elements. Fermentabilities that are too low can be improved by increasing the modification of the green malt or by maximizing enzyme survival by reducing kilning temperatures. Conversely, fermentability can be depressed by stewing the malt on the kiln and by increasing the curing temperature. The brewer can decrease wort fermentability by increasing the mashing temperature (Briggs, 1998).

Free amino acids

Adequate levels of FAN in wort ensure efficient yeast cell growth and hence, an appropriate fermentation performance. FAN is believed to be a good index for potential yeast growth and efficiency; however, FAN is only a general measurement for setting wort and, ultimately malt specifications (Lekkas et al., 2007). Optimum wort FAN concentration is dependent on the amount of yeast growth required to produce beer with the desired flavour profile in a specific period of time. Worts containing high FAN levels will result in increased diacetyl production, extended lagering time, and produce higher levels of FAN in beer, which has been reported to negatively affect drinkability and beer stability. In contrast, worts with low FAN levels will result in slow fermentations, reduced yeast crop, and high diacetyl levels at the end of fermentation. Ideally, wort should contain the least amount of FAN required for normal yeast growth and minimum amount of diacetyl production (O'Connor-Cox and Ingledew, 1989; Pugh et al., 1997). Worts without sufficient FAN content result in stuck or sluggish fermentations (Ingledew, 2000).

Lekkas et al., (2007) showed that during lager fermentations, the FAN concentration decreased sharply until 43 h and then remained constant (Fig. 2.6.11). During the first 44 h, the pH decreased from 5.07 to 3.78 and then remained constant until the end of fermentation. Wort gravity gradually decreased until the end of the process. In consequent with this decease in harmony, the specific gravity, pH and FAN concentration are considered related.

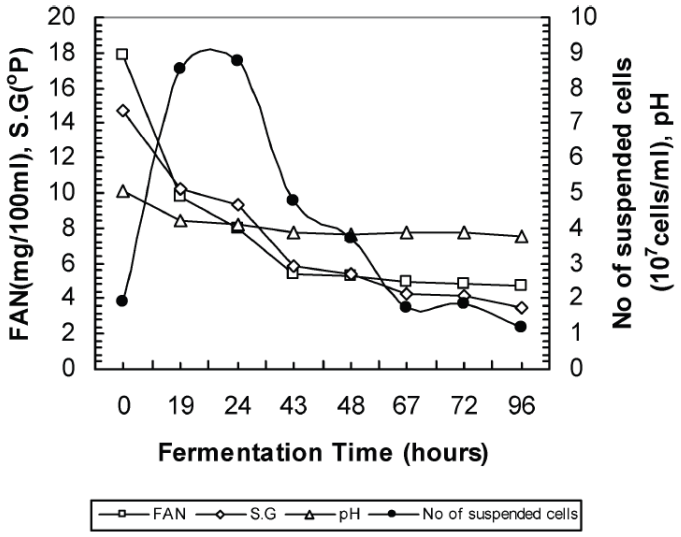


Fig. 2.6.11 Lager fermentation profile, S.G.: specific gravity (from Lekkas et al., 2007).

Edney and Langrell (2005) found that initial levels of individual amino acids had no apparent affect on fermentability. Thus, they concluded that since all samples had acceptable levels of FAN, individual amino acids were not limiting in the worts studied. However, hulless malts with significantly lower levels of some individual amino acids, as well as less alpha-amylase, and, compared with covered malt, had significantly lower fermentabilities.

Proteases

Ingledew (2000) established that brewers yeast demonstrate no appreciable proteolytic activity in wort. Although, they do produce enzymes inside the cell, yeast do not excrete proteases and as a result there is no protein degradation taking place in wort during fermentation. On the other hand, Lekkas et al. (2007) proposed that extracellular yeast proteolytic enzymes are responsible for the degradation of lager wort peptides into smaller ones providing yeast cells with more available assimilable nitrogen sources. They found the pattern of protease activity (Fig. 2.6.12) was similar for both types of fermentations (lager and ale), with a rapid increase in activity between 24 and 48 h and a peak activity at 48 h. This pattern is consistent with the requirement for new sources of assimilable nitrogen as levels of initial FAN become minimal.

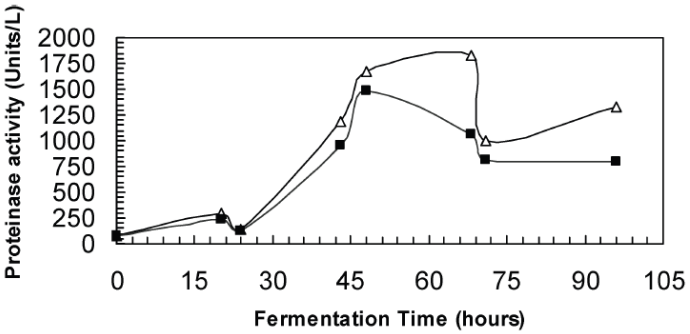


Fig. 2.6.12 Proteinase activity for lager (Δ) and ale (■) fermentations (from Lekkas et al., 2007).

Peptides

Although some peptides and more complex nitrogenous materials are formed during fermentation, the mashing process is quantitatively the most important factor governing the composition of peptides in beer from all malt wort. Fractionation of the soluble nitrogen components of malt, wort and beer revealed that most of the polypeptides with molecular weights between 1500 and 5000 were formed during mashing. Furthermore, due to the complexity of wort, up to 400 dipeptides and 8000 possible tripeptides may exist (Clapperton, 1971; Patterson and Ingledew, 1999).

Clapperton (1971) stated that peptides can act as effective yeast nutrients in maintaining satisfactory fermentation performance. They demonstrated a selective uptake of wort peptides during fermentation, mainly peptides produced during mashing. While some dipeptides were used in initial stages of growth, the majority of dipeptides were utilized as the yeast approached stationary phase. Lekkas et al. (2007) demonstrated, that peptide uptake from wort occurred rapidly for the first 19-20 h fermentation. The wort oligopeptide concentration then started to increase until 24 h into the fermentation. Between 24 and 67 h of fermentation, oligopeptide levels in wort decreased gradually but then started to accumulate again until the end of fermentation (Fig 2.6.13). The fact that nitrogen sources were utilized so quickly, provides evidence that the utilization of small peptides may actually precede or occur at the same time as the uptake of the group A or group B amino acids. The supply of a diverse set of nutrients, such as a combination of free amino acids and peptides, yeast can expend less energy to synthesize the required components for cell metabolism and growth faster (Patterson and Ingledew, 1999).

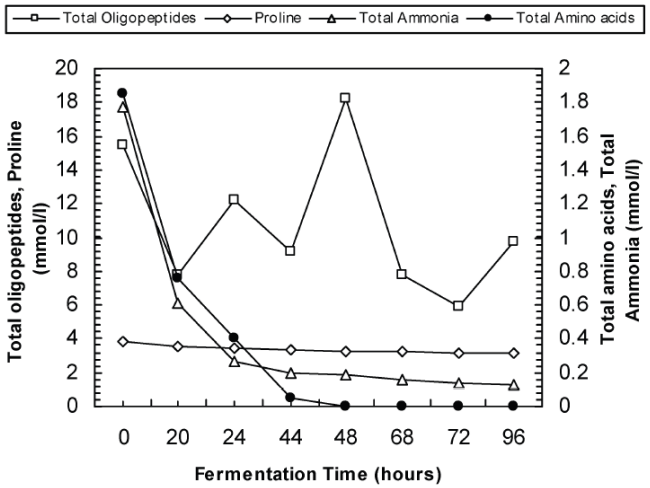


Fig. 2.6.13 Total nitrogen fermentation absorption profile for lager fermentations (from Lekkas et al., 2007).

Endosperm modification

Edney et al. (2007a) established that adequate endosperm modification was the most important factor to achieve good fermentability levels. Endosperm modification, as discussed previously, is affected by a range of barley characteristics including genetics, environmental and processing conditions. Good modification ensures adequate levels of amino acids and sugars for yeast nutrition. They found that levels of starch degrading enzymes appeared to affect fermentability only when malts were adequately modified. However, some studies stated that over-modification could also limit fermentability due to increased levels of soluble protein and reduced levels of fermentable sugars due to elevated malting losses. Viscosity and β-glucan also affected fermentability. Viscosity had the strongest effect, restricting enzyme movement and thus, reducing starch hydrolysis. The β-glucan appeared to affect fermentability in a number of ways including restriction of enzymes movement due to high viscosities, late release of starch from under-modified endosperm as well as increased levels of glucose due to complete β-glucan hydrolysis. If cell wall components and storage proteins are not sufficiently hydrolysed throughout the endosperm during malting, starch kernels will remain well

embedded within the protein matrix of endosperm cell walls. In this state, the starch granules are incompletely gelatinised during mashing, hence, reducing their accessibility to amylolytic enzymes (Gunkel et al., 2002).

Highly modified malts present greater levels of soluble proteins and peptides, which can thermally protect thermolabile enzymes such as β-amylase and limit dextrinase. It is well known that protein thermostability is increased by the presence of sugars and other solute components (Evans et al., 2008). Levels of starch degrading enzymes (α-amylase, β-amylase, and limit dextrinase) were often considered the first limiting factor to the fermentability of malt. However, fermentable sugars, the products of starch-degrading enzymes, only partially explain differences in fermentability (Edney and Langrell, 2005). An essential requirement for efficient starch hydrolysis during mashing is starch gelatinization, which typically occurs between 59 and 63°C. Given that α-amylase is substantially more thermostable than β-amylase and limit dextrinase, it had been reasonably concluded that α-amylase is generally not the limiting diastatic power enzyme during mashing. This suggests that β-amylase, its thermostability, and limit dextrinase are important determinants of wort fermentability. Knowledge of the levels of these enzymes would allow maltsters to potentially blend their malt batches more precisely within or between varieties to balance the levels of DP enzymes and better satisfy brewers' fermentability expectations. Based on this theory, Evans et al. (2009) proposed that the level of α-amylase, β-amylase and total limit dextrinase activities, KI and the thermo-stability of β-amylase in combination are better predictors of commercial wort fermentability than AAL.

β-Amylase thermostability

Studies demonstrate that variations in fermentability are essentially affected by genotype-dependent differences in β-amylase thermostability, which are caused by

polymorphisms of the corresponding gene β-amy1 (Gunkel et al., 2002; Edney et al., 2004; Gómez et al., 2005).

Kilning

During thermal treatment of malt, as mentioned before, Maillard reactions are initiated by the reaction between reducing sugars and amino compounds, it can be deduced that wort brewed with considerable levels of dark malt contains lower levels of carbohydrates and amino acids. This undoubtedly affects several aspects of the fermentation process, including attenuation and fermentation rate. Since the amino acid metabolism of brewers' yeast is closely associated with the formation of fusel alcohols, esters and vicinal diketones, lower amino acid levels in wort brewed with dark malt might substantially affect the generation of these important flavour-active compounds (Coghe et al., 2005).

Despite the similar original extract content the course of the fermentation curves markedly depended on the malt type (Fig. 2.6.14). Although Melanoidin wort contained as much fermentable material as the reference wort, the apparent extract consumed by yeast after 190 h of fermentation was significantly lower (8.26 versus 10.22°P). This supports the suggestion that not only the level of fermentable sugars, but also the presence of Maillard compounds can affect the rate of extract conversion (Coghe et al., 2005).

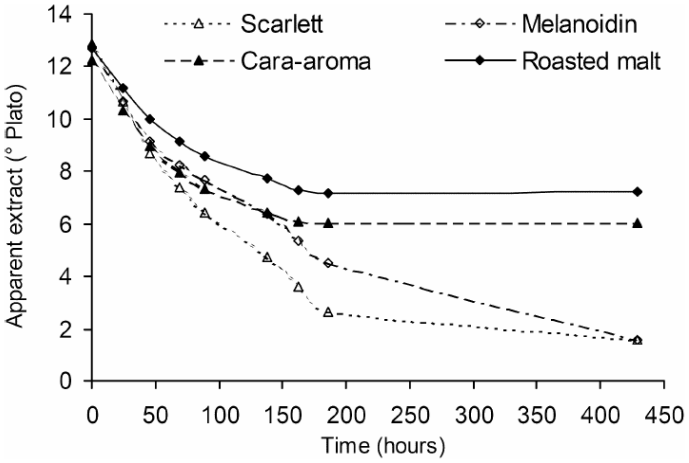


Fig. 2.6.14 Evolution of the apparent extract content during the fermentation of wort brewed with 100% Scarlett or with 50% Scarlett and 50% of dark malt (Melanoidin, Cara-aroma and roasted malt) (from Coghe et al., 2005).

Furthermore, Coghe et al. (2005) studied the different levels of saccharides in Congress worts brewed with 50% pilsner malt and 50% dark malt. They observed that level of maltose, the most abundant sugar in wort, decreased with increasing wort colour and its concentration in the darkest wort sample was nearly half the concentration in the reference wort. For the rest of the sugars the relation was less clear.

Darker malts have a lower extract yield, thus higher quantities of these malts are needed to obtain an original extract of about 12°P. Remarkably, although all worts were more or less of the same original extract; attenuation and ethanol levels were lower while density and real extract were higher for the darker beers. This suggests that dark wort samples contain less fermentable sugars or that Maillard compounds inhibit the metabolism of yeast cells (Fig 2.6.15). Less fermentable extract might be partially due to a lowered malt enzymatic activity which could lead to less hydrolytic reactions during

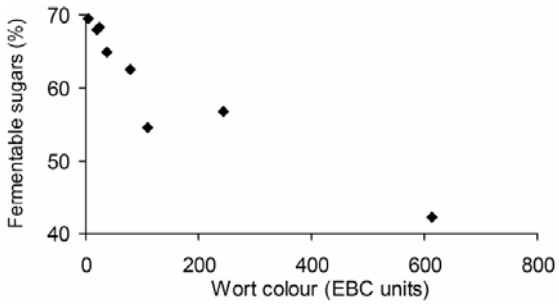


Fig. 2.6.15 Percentage of extract consisting of fermentable sugars as a function of the colour of Congress wort (from Coghe et al., 2005).

mashing and less fermentable extract. A lower fermentable extract content might also be attributed to Maillard reactions rendering sugars unavailable during the production of dark malt (Coghe et al., 2005).

A study in model system in the presence and absence of Maillard reaction products and the formation of ethanol as a marker for the progression of fermentation (Tauer et al., 2004) showed that increasing amounts of Maillard products reduced the formation of ethanol up to 80%. This happened in dependence on the pH value during the Maillard reaction, reaction time as well as the carbohydrate and amino acid component used for the generation of the Maillard reaction products. However, the nature and concentrations of Maillard products found in beer production can differ from those applied in this model system. Therefore, it still remains to investigate if Maillard products from malting mash show similar effects on yeast fermentation.

High gravity brewing

Additional concerns with the nutritional status of brewer's wort arose with the advent of high gravity brewing. High gravity brewing became a common practice during the 1980s as a means of increasing plant efficiency and capacity as well as decreasing labour, energy, and capital costs. In the initial attempts to ferment high gravity wort, the limiting factors implicated in many incomplete fermentation were attributed largely to ethanol toxicity, together with the inhibitory effect of high osmotic pressure inhibiting the uptake of maltose, maltotriose and glucose. Supplementation of high gravity brewing wort with complex lipid compounds and nitrogen constituents avoided incomplete fermentations, thus allowing the production of high levels of ethanol and the preservation of yeast viability. In addition to nitrogen supplementation, fermentation improvement was also dependent on the presence of the correct blends of amino acids in the wort (Cruz et al., 2003; Batistote et al., 2006; Stewart, 2006). In general, the addition of nutrients can

improve alcohol yield, reduce fermentation time, enhance yeast viability and vitality, and increase diacetyl removal, as well as control undesirable flavour compounds (Van Zandycke and Fischborn, 2008).

2.6.6 Malt fermentability assays

Measuring fermentation consistently and reliably has always been difficult. Fermentation is a biological process that is significantly affected by: wort composition, strain and age of the yeast used, as well as by the conditions of fermentation (temperature, oxygenation of the wort, stirring and length of fermentation) (Edney et al., 2007b). As mentioned in previous sections, when fermentability is measured directly, it is generally based on attenuation or on reduction of an extract's gravity, which results from the yeast's metabolization of sugars and amino acids in the extract and the formation of ethanol.

Several methods exist for measuring apparent attenuation limit (AAL) described in the recommended methods of EBC (European Brewing Convention), ASBC (American Society of Brewing Chemists) and MEBAK (Central European Brewing Technology Analysis Commission). The main differences between the standard methods are yeast pitching rate, fermentation temperature and length of fermentation procedure. The methods are further complicated by the fact that each brewery and laboratory tends to use its own strain of yeast. It is well known that different strains and ages of yeast attenuate wort differently, making comparisons difficult.

Laboratory wort attenuations correlated poorly with microbrew attenuations, but in general provided an indication of the extent of fermentability likely to be achieved. The lack of correlation may be due to the variations of wort components and size and shape of the fermenter utilized (Nischwitz et al., 1999). In 1999, Hagen and Schwarz, from the EBC Analysis Committee, compared the EBC 4.11 reference method with the rapid

ASBC method (Wort-5) for their abilities to predict fermentability (final attenuation limit) of both laboratory and commercial worts. The collaborative study showed that repeatability and reproducibility for the reference method (24 hours) were acceptable for both the laboratory and industrial worts despite the fact each laboratory used its own in-house yeast. The rapid method (fermentation time 5 to 7 hours) had a tendency to give worse precision values and resulted in lower attenuation limits. Although some brewers are dissatisfied with the AAL measurement, malt factors identified as good predictors of AAL, also predict brewery fermentation, likely because both are integrally involved with hydrolysis of starch into fermentable sugars (Evans et al., 2005). Furthermore, Gunkel et al. (2002) found that AAL level correlated closely with the maltose concentration in the wort but was not affected by other fermentable sugars or by the total carbohydrate content.

Typically fresh yeast preparations are used for the AAL and limit gravity determinations are made. In the brewery, the supply of yeast is relatively simple as fresh yeast is consistently available. However, many brewers brew at different locations both nationally and internationally complicating the supply of yeast for fermentation testing. One alternative for improving the reproducibility of determining lab fermentability is the use of a common dried yeast strain. The advantages of using dried yeast compared to fresh yeast include: ease of handling (no need to culture), convenience of use (storage), consistency of quality (age and lack of contamination), and improved uniformity among laboratories located in separate geographic regions. However, the manufacturing process for dehydrating yeast and its subsequent re-hydration results in significant stress on the yeast. Modern dehydration strategies, such as conditioning the cells with trehalose, amino acids, growth phase selection and use of favourable dehydration kinetics combined with storage conditions under low temperatures, vacuum and an

atmosphere of nitrogen, have resulted in higher dry yeast viability (Evans and Hamet, 2005).

Despite the attractions of the methods used to determine fermentability, they are relatively time-consuming and suffer from the use of mashing, lautering, and fermentation conditions that are not typical of commercial production. In addition, several aspects of the laboratory procedures are different such as: 1) the method by which a malt is milled (particle size) affecting starch hydrolysis, 2) wort oxygenation, 3) cellar temperature programs, and 4) tank pressure. Consequently, Evans et al (2009) established that routine measurement of the levels of α-amylase, β-amylase, and limit dextrinase provided a more accurate prediction of potential malt fermentability than AAL, with the latter not always providing an accurate prediction of commercial fermentability (Evans et al., 2008). Furthermore, in 2009 they found that the Kolbach index (KI) of protein modification and relative thermostability of different β-amylase types (thermolabile) were also important in predicting fermentability. Recently, Evans (2008) developed a new protocol to determine DP enzymes. The scaling down of original enzyme assays, resulted in the use of between one-quarter and one-fifth the amount of substrate per assay, providing substantial cost savings. The increased labour and cost efficiencies should allow the use of this protocol as a routine assay by malting quality evaluation laboratories, especially for breeding programs.

Premature yeast flocculation is a problem that occurs sporadically in the brewing industry, and it can be qualitatively defined as the early flocculation of yeast from the wort before total usage of all available fermentable sugars, leaving the wort under-attenuated (Speers et al., 2006). As mentioned previously, if yeast flocculates early, many problems ensue in the final product, including irregularities in flavour profiles, increased chance of microbial infections, increased levels of diacetyl, lower alcohol content, lower carbonation levels, and low yeast cell counts at the end of fermentation.

Accordingly, Lake et al. (2008) developed a fermentation assay that was especially sensitive to malt implicated with the potential for premature yeast flocculation. They found that both fermentor height and fermentation speed were major factors for determining CO_2 evolution and agitation within the fermenting wort. Thus, when they downsized their fermentation assay by reducing the fermentor height, they had to increase the rate of fermentation to maintain the adequate shear rates required to keep the yeast in solution.

Chapter III

Materials & Methods

3.1 Barley samples

Barley was selected for investigation with the aim of maximizing variability in profiles of individual amino acids and in endoprotease activity of malts, as suggested by general malt quality. Therefore, a series of barley varieties from Uruguay and Canada that had been grown at multiple sites, were selected (Table 3.1). Varieties from Uruguay included AC Madi, a variety belonging to Ackermann & Co. who is represented in Uruguay by Malteria Oriental S.A (MOSA); and the Musa 936 which belongs to Maltería Uruguay S.A.(MUSA)–AmBev. Both varieties were grown in two locations in Uruguay, one in the south of the country and the second in the north. Canadian barley varieties were: CDC Helgason, CDC Copeland and a hulless variety CDC McGwire. These varieties were grown in two different harvest years in Saskatchewan and were provided by Brian Rossnagel (Crop Development Centre, Saskatoon). AC Metcalfe was the internal check at the Grain Research Laboratory (GRL).

Table 3.1 Barley varieties used in the study

Nº Sample	Barley variety	Growth location/ Year	Abbreviation
1	Musa 936	South	M936S
2	Musa 936	North	M936N
3	AC Madi	South	ACMS
4	AC Madi	North	ACMN
5	CDC Helgason	2006	Helg06
6	CDC Helgason	2005	Helg05
7	CDC McGwire	2006	McG06
8	CDC McGwire	2005	McG05
9	CDC Copeland	2006	Cop06
10	AC Metcalfe	2006	Metc

3.2 Malting methods and schedules

Two different sets of malting equipment were used in the study: an automatic Phoenix micromalting system (Adelaide, SA, Australia) and a 7kg pilot malting plant. The two maltings were located in the Applied Barley Research Unit of the GRL, (Winnipeg).

3.2.1 Automatic Phoenix micromalting

The Phoenix Micromalting System was designed to handle twenty-four samples of 500 g of barley per run. Samples described in Table 3.1 were malted under five different malting treatments:

- *Standard*, performed following the standard malting schedule described in Table 3.2
- *Addition of* GA_3, performed following the standard malting schedule described in Table 3.2, but with addition of 0.25ppm GA_3 sprayed on each sample at 2 hours of germination.
- *Freeze drying*, performed following the steeping and germination program of the standard malting schedule (Table 3.2), but samples were removed prior to kilning, frozen immediately and held at -55ºC until they were freeze dried. Freeze drying was performed on a VirTis bench top freeze dryer (Wizard 2.0) for five days starting at minus 45ºC and ending at 25ºC.
- *Higher kilning final temperature (FT)*, performed following steeping and germination program of the standard malting schedule and then kilned with the higher kilning FT schedule described in Table 3.2.
- *Longer kilning,* performed following steeping and germination program of the standard malting schedule and then kilned with the longer kilning schedule described in Table 3.2.

Preparation of 0.25ppm GA_3 solution:

GA_3 concentrated solution was prepared by dissolving 0.056g of GA_3 (89% purity) in 20mL ethyl alcohol and topped off with pure water to 100mL. The solution was further diluted 50-fold with pure water (milliQ grade) resulting in a 10ppm GA_3 solution. The diluted solution is only good for 24 hours. To add 0.25ppm of GA_3 to green malt (500 g dry matter), 12.5mL of the diluted GA_3 solution were sprayed on each sample.

Table 3.2 Malting schedules applied in the Phoenix micromalting system

	Malting Schedules					
	Standard		**Higher Kilning FT**		**Longer Kilning**	
Step	Hours	T (ºC)	Hours	T (ºC)	Hours	T (ºC)
Wet 1	7	13	7	13	7	13
Air Rest 1	14	13	14	13	14	13
Wet 2	7	13	7	13	7	13
Air Rest 2	12	13	12	13	12	13
Germ	84	15	84	15	84	15
Kilning 1	5	50	10	55	6	30-48
Kilning 2	7	60	6	65	16	48
Kilning 3	6	65	2	75	8	48-66
Kilning 4	2	75	4	85	10	66
Kilning 5	4	85	2	90	2	66-80
Kilning 6	-	-	-	-	6	80
Total Hrs	**148**		**148**		**172**	

Moisture content was measured, by weight difference, at steep out and at the end of germination for all treatments.

An extra malting run was performed following the malting schedule described in Table 3.3 (same as used in the pilot plant) in order to remove samples at different stages of the malting process. The selected stages were: 1) end of first air rest, 2) end of second air rest, 3) end of first day of germination, 4) end of second day of germination, 5) end of third day of germination, 6) end of germination (4^{th} day), 7) end of 55ºC (kilning 1), 8) end of 65ºC (kilning 2), 9) end of 75ºC (kilning 3) and 10) end of 85ºC (kilning 4).

Collected subsamples plus raw barley and finished malt were immediately frozen and stored at minus 55ºC until freeze dried.

3.2.2 Pilot plant

Three barley samples, ACMN, M936N and Metc, were selected, based on initial malt quality analysis, for additional malting in the 7kg pilot malting plant. Five kilograms of each sample were malted following the schedule described in Table 3.3. Samples were weighted at steep out to calculate moisture content and 300g of water were add to each sample to achieve 44% moisture.

Table 3.3 Malting schedules applied in Pilot Plant

	Pilot Plant	
Step	Hours	T (ºC)
Wet 1	7	13
Air Rest 1	14	13
Wet 2	7	13
Air Rest 2	12	13
Germ	84	15
Kilning 1	12	55
Kilning 2	6	65
Kilning 3	2	75
Kilning 4	4	85
Total Hrs	**148**	

3.3 Barley and malt analyses

Barley and malt analyses were performed according to GRL - Barley Applied protocols based on the ASBC methods, American Society of Brewing Chemists, Nineth Edition, (2007). Analytical results for barley and malt were reported on a dry weight basis.

3.3.1 Barley analysis

Moisture content of barley

Moisture content of barley was predicted using NIR equipment that had been calibrated by the standard ASBC method (ASBC Barley 5C).

Protein content (N x 6.25)

Protein content was predicted on dockage-free barley using NIR equipment that had been calibrated by Combustion Nitrogen Analysis (CNA). Results were reported on a dry matter basis (ASBC Barley 7C).

Germination energy

Germination energy was determined by placing 100 kernels of barley on two layers of Whatman #1 filter paper, in a 9.0 cm diameter Petri dish, and adding 4.0 mL of purified water. Samples were controlled at 20ºC and 90% relative humidity in a germination chamber. Germinated kernels were removed after 24 and 48 hours and a final count was made at 72 hours (ASBC Barley 3C, IOB, and EBC procedure).

Water sensitivity

Water sensitivity was determined exactly as described for germination energy, except that 8.0 mL of purified water was added to each Petri dish (ASBC 3C, IOB and EBC procedure). The actual water sensitivity value was the numerical difference between the 4mL and 8mL tests.

3.3.2 Malt analysis

Malt mills

Fine-grind malt was prepared with a Buhler-Miag disc mill set to fine-grind. The setting for fine grind was calibrated quarterly, based on the screening of a ground ASBC standard check malt (ASBC Malt-4).

Moisture content of malt

Moisture content of malt was determined on a ground sample at 104°C for 3 hours in a convection oven (ASBC Malt-3).

Fine-grind extract

Extract was prepared using an Industrial Equipment Corporation (IEC, Melbourne, Australia) mash bath and the Congress mashing procedure from 45°C to 70°C. Specific gravity was determined at 20°C with an Anton Paar DMA 5000 (Graz, Austria) digital density meter (ASBC Malt-4).

Free Amino Nitrogen (FAN)

Free amino nitrogen was determined on the fine extract according to the official ASBC method Wort-12, automated to run on a Skalar segmented flow analyzer.

Kolbach index (ratio S/T)

Kolbach index was calculated from the formula, (% wort-soluble protein/% Malt protein) x 100.

Wort Viscosity

Viscosity was measured on fine grind Congress wort using an automated Schott AVS 500 Micro-Ubbelodhe glass capillary viscometer, which had been calibrated according to ASTM method D-445 (ASBC Wort-13).

Wort-soluble protein

Wort-soluble protein was determined spectrophotometrically using ASBC method Wort-17.

Wort ß-Glucan content

ß-Glucan content was determined in malt extract by Skalar segmented flow analysis using Calcofluor staining of soluble, high molecular weight ß-glucan (ASBC Wort-18).

Diastatic power

Diastatic power was determined on a Skalar segmented flow analyzer, using an automated neocuproin assay for reducing sugars, which was calibrated using malt standards analysed using the official ferricyanide reducing sugar method, (ASBC Malt 6A).

α-amylase activity (DU)

α-Amylase activity was determined using ASBC method MALT 7B automated to run on a Skalar segmented flow analyser, using ASBC dextrinized starch as the substrate, and calibrated with standards that had been determined by method ASBC Malt 7A.

3.4 Enzyme analysis

Amylolytic enzyme analyses were performed on malt samples (15g) ground with a Perten Falling Number Laboratory Disc Mill Model 3303. The ground malt was extracted according to individual procedures as described by Megazyme (Wicklow, Ireland) for each enzyme.

α-amylase method by Megazyme

The Amylazyme red tablets α-amylase method (T-AMZRD200) employs red-crosslinked amylose material which hydrates in water but is water insoluble. Hydrolysis by α-amylase produces red dye fragments that are water soluble, and the rate of solubilization, measured by the increase in absorbance at 510nm, can be related directly to enzyme activity. This substrate allows specific measurement of α-amylase in presence of large excess of the other amylolytic enzymes β-amylase and α-glucosidase present in malt (Megazyme method data booklet).

β-amylase method by Megazyme

The Betamyl β-amylase method (BETA 9/2003) employs p-nitrophenyl-α-D-maltopentaose (PNPG5) and high purity α-glucosidase which ensures maximum

sensitivity of the assay. This method can be used in the presence of α-amylase. α-Amylase can hydrolyse PNPG5 but only at a slow rate compared with β-amylase which rapidly hydrolyzes the substrate. β-amylase hydrolyses PNPG5 to PNPG3 and maltose and then PNPG3 is immediately cleaved to glucose and free p-nitrophenol by α-glucosidase. Thus the rate of release of p-nitrophenol is directly related to the rate of release of maltose by β-amylase. Finally the reaction is stopped using Trizma base solution and the phenolate colour is developed (Megazyme method data booklet).

Limit dextrinase method by Megazyme

The limit-dextrizyme method (LDZ 7/98) utilises Azurine-crosslinked-pullulan substrate which is hydrolyzed by limit dextrinase and pullulanase, but is resistant to attack by the rest of amylolytic enzymes present in malt. Hydrolysis by limit dextrinase produces water soluble dye fragments and the rate of release of these fragments, measured by increase in absorbance at 590nm, can be related directly to enzyme activity (Megazyme method data booklet).

3.5 Amino acid analysis

The ACQUITY UltraPerformance LC (UPLC®) separation system from Waters® (Fig 3.1) was used to quantify individual free amino acids in barley, malt and wort. The UPLC system uses relatively new technology to achieve excellent separation of over 29 amino acids in less than 10 minutes between injections. The main unique aspect of the ACQUITY UPLC technology is the use of stationary phase particles measuring 1.7 μm in diameter which, for a given column length, increases backpressure inversely with the square of the particle diameter. In another inverse relation, the column's optimum linear velocity increases as particle size decreases. Combined, these effects mean the pressure needed to maintain optimum linear velocity increases to the third power of the particle size. A first-generation UPLC column therefore requires a pressure of about 25

times higher than that required for a 5-µm HPLC column of the same length. The binary solvent manager and injector can produce pressures up to 103421 kPa (1034 bar, 15000 psi) and can generate high-pressure gradients with minimal gradient delay. Under isocratic conditions, the UPLC column is three times faster than the HPLC column, and the plate count is three times greater. Performance differences between UPLC and HPLC stand in even greater contrast when you run the columns at the same linear velocity. The upper limit of the flow rate range is 2 mL/min. Small-particle chemistries used with the UPLC system generate narrow peaks but to maintain these narrow peaks, extra bandspreading must be controlled by lower detector cell volume, minimized tubing volumes, and specialized fittings (ACQUITY UPLC System Operator's Guide, 2008).

The system was operated following the procedure described in:

- ACQUITY UPLC System Operator's Guide (2008) 71500082502 / Revision C from Waters Corporation.
- UPLC Amino Acid Analysis Application Solution, System Guide, 71500129702 / Revision A from Waters Corporation.
- Overview and Description of the UPLC™ Amino Acid Analysis Application Solution Project Template EmpowerTM Build 1154 or Higher (June 2006).
- ACQUITY UPLC System Quick Reference Card, 71508250006rA from Waters Corporation.

3.5.1 Setting conditions for the UPLC system

The system must be powered up in the proper sequence: 1) first Sample manager (SM), 2) Binary solvent manager (BSM), 3) TUV detector and 4) start the Empower operating software. Options selected from the Empower pro Chromatography Data System operation software to run samples were:

1) Project: AccQTag_Ultra_FreeAminoAcids0
2) Chromatographic system: UPLC_TUV
3) Application: Cell Culture
4) Instrument method: Ultra Cell Culture A2 at59
5) Run samples options: a) Run mode: “Run only”; b) Suitability modes: “Continue and default”; c) Shutdown method: “Shutdown Matt”
6) Processing method: Ultra_Cell Culture Sep9 08
7) Reporting method: Component Summary 3

3.5.2 Sample preparation

Wort extraction

Wort samples were filtered using acrodisc syringe filters, 0.2µm GH Polypro (GHP) membrane disc. Filtered wort was diluted 1:10 by adding 100µl of wort to 900µl of pure fresh water.

Barley and malt extraction

Barley or malt samples (15g) were ground with a Perten Falling Number Laboratory Disc Mill (Model 3303). Barley or malt was weighted (0.5g) into a tube and 4mL of boiling pure water were added. Tubes were thoroughly mixed and let stand at room temperature for 30 min with

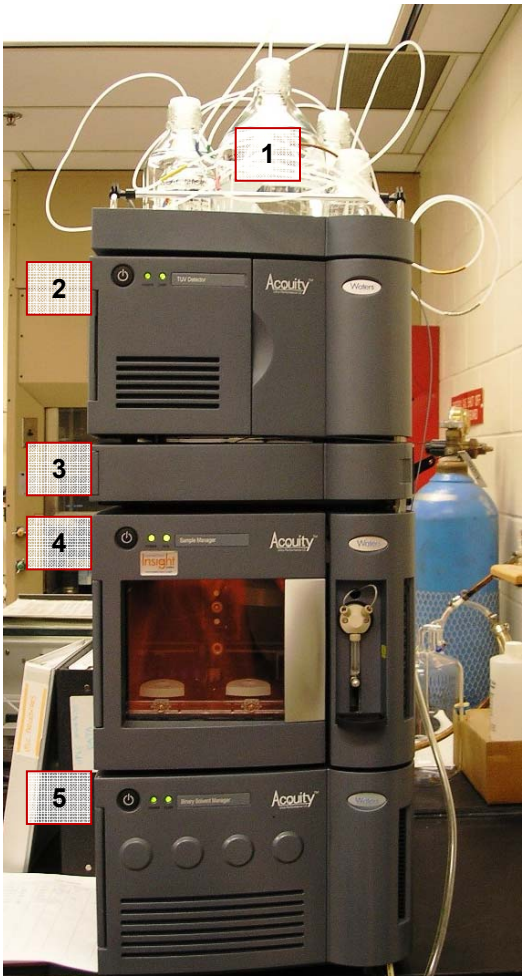


Figure 3.1 **The** UPLC system at the GRL-Applied barley Unit. 1-solvent tray; 2-TUV detector; 3-column heater; 4-sample manager (SM); and 5- binary solvent manager (BSM) (By Blanca Gómez G)

vortexing every 10 min. Samples were then centrifuged at 8000rpm for 10 min. The supernatant was filtered, like wort, using the acrodisc syringe filters. Filtered samples were also diluted 1:10 with pure fresh water.

Amino acid standard solution

Waters supplied ampoules containing their Amino Acid H standard Mix, a mix of 17 amino acids all at 2.5 mM concentrations except Cysteine which was at 1.25 mM. Sealed ampoules were stored in a freezer until further use. To use, ampoule contents were transferred to a 1.5 mL eppendorf (maximum shelf life of opened ampoules was one month at minus 20ºC). Solutions of the four amino acids not supplied in the Waters ampoules were prepared according to instructions in Table 3.4 to produce individual stock solutions of 100 mM concentration. Listed weights were added to 5 mL of 0.1N HCl and then vortexed for 10 sec. The shelf life of each these individual amino acid solutions was 6 month at minus 20ºC.

Figures in Table 3.4 were calculated as follows:

Required final concentration = 100 mM

Required final volume = 5 mL

MW of Asn (example) = 131.12 g/L (mg/ mL)

Molar concentration required in final 5 mL:

100 mmoles (per L) = 0.5 mmoles /5mL

Weight required in final 5mL: 0.5mmoles x 131.12 g/mole = 65.56 mg/5 mL

Table 3.4 Weight of individual amino acid (mg) required to prepare 100mM solutions

Amino Acid	MW	Weight of amino acid	Volume of 0.1 NHCl	Stock Concentration
Glutamine (Gln)*	146.14	73.07	5 ml	100mM
Asparagine (Asn)	131.12	65.56	5 ml	100mM
γ- amino-n-butyric acid (GABA)	103.12	51.56	5 ml	100mM
Tryptophan (Trp)	204.23	102.12	5 ml	100mM

*L-glutamine is very stable as a dry powder but degrades relatively rapidly in liquid media or stock solutions. Therefore it is recommended that the solution be frozen into separate aliquots. Each aliquot is good for a single use. The pure amino acids were from Fluka.

To obtain a standard amino acid solution with each amino acid at 1000pmol/µl concentration, the volumes described in Table 3.5 were transferred to a microcentrifuge tube and vortexed for 10 seconds.

Table 3.5 Amino acids volumes to prepare 1000pmol standard amino acid solution

Amino Acid	Volume of Standard
H Standard	400 µL
Gln	10 µL
Asn	10 µL
GABA	10 µL
Trp	10 µL
Water	550 µL
Total Volume	**1000 µL**

To prepare standard amino acid solutions at different concentrations (e.g. 100pmol, 200pmol), aliquots from the 1000pmol/ul solution were removed and diluted with pure fresh water. Diluted aliquots (20µL) were kept frozen until analysis. Each aliquot was good for a single use.

3.5.3 Derivatization procedure

Standards and samples were derivitized, prior to UPLC separation, with AccQ•Fluor from Waters. The procedure required dilution with borate buffer, reaction with AccQ•Fluor followed by inactivation of excess derivitization agent.

Reconstituting the AccQ•Fluor reagent

The vial containing the AccQ•Fluor reagent powder (vial 2A) was lightly tapped before opening to ensure all AccQ•Fluor reagent powder was at the bottom of the vial. Micropipette was rinsed and cleaned by drawing and discarding 1mL of AccQ•Fluor reagent diluent from Vial 2B. This rinsing step was repeated and then 1.0mL of the AccQ•Fluor reagent diluent from vial 2B was transferred to the AccQ•Fluor reagent powder in Vial 2A, capped and mixed for 10 seconds. Vial 2A was heated **on top** of the heating block until the AccQ•Fluor reagent powder was dissolved. Notes: The reagent **should not be heated for longer than 10 minutes.** The reconstituted AccQ•Fluor reagent can be stored in a desiccator at room temperature for up to one week but the vial must be tightly sealed because AccQ•Fluor reagent reacts with atmospheric moisture. It is recommended to **not refrigerate the reagent.**

Preparing the derivatization blank

AccQ•Fluor Borate buffer (reagent 1) (80µL) was placed in a vial. Reconstituted AccQ•Fluor reagent (reagent powder 2A) (20µL) was added to the buffer. The vial was capped and vortexed for several seconds. To hydrolyze the excess of reagent to AMQ the vial was stood for at least one minute at room temperature. Then the vial was heated for 10 minutes at 55 °C in a preheated heating block. After it was removed from heating block the blank vial was placed in position 1:A,1 of plate 1 in the ACQUITY UPLC Sample Manager.

Preparing the derivatization standard and samples

AccQ•Fluor Borate buffer (reagent 1) (70µL) was placed in a vial. A sample of amino acid standard or filtered sample (10µL) was carefully pipetted down the wall of the vial. The vial was capped and vortexed for several seconds with special attention to proper mixing of the sample drop on the wall and the borate buffer solution. Formation of bubbles at the bottom of the vial has to also be avoided. When several samples were analysed together, the borate buffer and the filtered worts were mixed for all samples prior to addition of the next reagent. Reconstituted AccQ•Fluor reagent (reagent powder 2A) (20µL) was added to vial with the borate and sample vial, the vial was capped and vortexed for several seconds. Vials stood for at least one minute at room temperature, to allow hydrolysis of excess reagent to AMQ. Vials were heated for 10 minutes at 55 °C, removed from the heating block, cooled and were placed in consecutive positions in the ACQUITY UPLC Sample Manager plate. Derivatized samples could be stored at room temperature for up to one week.

3.5.4 Setting the sample set table

The sample set table was developed following the instructions described in Empower Manual page 54-55. Two standard solutions, with different concentrations (100pmol and 150pmol) were run for calibrations purposes. Standard solutions were also included as unknowns in the sample set table allowing one to monitor quantification performance of the UPLC. For best results, the sample set table was started with the Equilibrate function followed by the Conditioning column function. Boxes at the bottom of the sample set screen indicated amounts of the eluent volume (A and B) required for the described run and it was essential to ensure that amounts of eluents in excess to the figure were prepared.

3.6 Endoprotease analysis

Two endoprotease assays performed in this study differed mainly with respect to their substrate which was either Azogelatin or Azo-casein.

3.6.1 Solutions

- Ammonium acetate buffer (NH_4Ac) 0.1M pH 4.8 and pH 5.5. NH_4Ac (0.773g) was dissolved in 90mL of pure water (milliQ). The pH was adjusted to 4.8 or 5.5 with 12M acetic acid and diluted 1:10 with pure water. The volume was adjusted to 100mL.
- Sodium citrate buffer (NaCitrate) 0.1M pH 6.0. NaCitrate (2.941g) was dissolved in 90mL of pure water. The pH was adjusted to 6.0 with 1M HCl. The volume was adjusted to 100mL.
- Sodium phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$) 0.1M pH 7.0. $Na_2HPO_4 \cdot 2H_2O$ (1.78g) was dissolved in 90mL of pure water. The pH was adjusted to 7.0 with 1M HCl. The volume was adjusted to 100mL.
- Cysteine 0.1M. L-cysteine (Sigma C-7880) (0.386g) was dissolved in 50mL of pure water.
- Trichloroacetic acid (TCA).15% TCA: TCA (7.5g) was dissolved in 50mL of pure water. 5%TCA: TCA (2.5g) was dissolved in 50mL of pure water.

3.6.2 Pure enzymes

- Cysteine enzyme class: Papain from Carica papaya, Fluka-76218, enzymatic activity 10.5U/mg, EC 3.4.22.3. It was recommended to store at -18ºC.

- Serine enzyme class: Trypsin Type III from Bovine pancreas, Sigma T-8253, enzymatic activity 10,500U/mg, EC 3.4.21.4. It was recommended to store at less than 0ºC.
- Aspartic enzyme class: Pepsin, Pepsin A from Porcine Stomach Mucosa, Sigma P-7000, enzymatic activity 539U/mg, EC 3.4.23.1. It was recommended to store at room temperature.
- Metalloenzyme class: Protease Type IX from *Bacillus polymyxa*, Sigma P6141, enzymatic activity 1U/mg, EC 3.4.24. It was recommended to store between 2-8ºC.

All the standard enzymes solutions were prepared by dissolving 1mg of pure enzyme in 1mL of pure water.

3.6.3 Azogelatin assay

Azogelating substrate synthesis

The azogelatin was synthesised following the method described by Jones et al. 1998 and through personal contact with the author. The specific procedure is described below.

Solution A

To 275mL of pure water containing 4g dissolved $NaHCO_3$ (sodium bicarbonate), were added, in small portions, 20g of gelatin (porcine skin type A, 300 bloom, from Sigma-Aldrich). The solution was well stirred and held at 54ºC until all was dissolved

Solution B

To 30mL of pure water containing 1.25mL of 8M NaOH (16g of NaOH dissolved in 50mL pure water to make 8M) were added 1732mg (0.01 mole) of sulfanilic acid. Then, 690mg of $NaNO_2$ (sodium nitrite) were added, followed by the addition of 10mL of 2M

HCl (20 milliequivalent HCl). The solution was stirred for 2min, or until it became cloudy. Finally, 2.5mL of 8 M NaOH were added and the solution was stirred again.

Reaction

Solution B was stirred for 5 seconds, mixed with all solution A and stirred briskly for 5min resulting in a red-orange coloured solution.

The reaction mixture was dialyzed against 4L of 0.01% sodium azide for 5 hrs followed by a change of sodium azide and a second dialysis against 4L of overnight. Finally, it was dialyzed against 4 L of pure water for 8 hr. Since this is a gelatin protein, it became a gel at room temperature. For this reason, dialyses were carried out at 40ºC. The dialysis tubing used in this study were Spectra/Por6 membrane with a cut-off MW (MWCO) of 3,500.

After dialysis, the reaction mixture was freeze dried. The freeze dried azogelatin was ground to a powder in a Waring Blender and stored in a well sealed jar in a refrigerator.

A working azogelatin solution was prepared by dissolving an appropriate amount of substrate (1% solution w/v) in NaCitrate buffer pH 6.0. The substrate solution was heated to 40ºC prior to use. The solution was stable for several weeks.

Enzyme extraction

Ground malt (0.38g) was suspended in 1500µl of sodium citrate buffer pH 6.0, mixed and held at 4ºC for 30min, vortexing every 10min. The preparation was then centrifuged at 14000rpm for 20min. The supernatant was removed and either analysed immediately or frozen at -20ºC until analysis.

Enzymatic reaction

1. Enzyme extract (670µl) and NaCitrate buffer (1670µl) were pre-equilibrated for 10min at 40ºC.

2. Pre-equilibrated azogelatin solution was added (2200µl) to the enzyme solution and mixed. The first aliquot (600µl) was removed at 0min (considered blank reaction). Additional aliquots were removed at 10, 25, 45 and 60min reaction time.
3. Aliquots (600µl) were dispensed in 900µl of 15% TCA to stop the reaction.
4. The tube was vortexed and incubated in an ice water bath for at least 30min.
5. Samples were centrifuged at 11500g for 8min.
6. The absorbance of the supernatant was measured at 440nm.

3.6.4 Azo-casein assay

Azo-casein substrate

Azo-casein was provided by Megazyme (S-AZCAS 12/2007) (Megazyme data booklet).

Dissolution: 1g of azo-casein was weighted into a beaker and 2mL of ethanol was added. The mixture was stirred with a magnetic stirrer to disperse lumps and then 48mL of sodium citrate (NaCitrate) buffer pH 6.0 was added. The resulting suspension was vigorously stirred with a magnetic stirrer until the substrate was completely dissolved (more than 10min). The solution was stored in a well sealed glass bottle and was stable for several weeks when stored at 4ºC.

Enzyme extraction

Ground malt or barley (1.0g) was suspended in 3.0mL of sodium citrate buffer pH 6.0, and held for 15min at room temperature; vortexing every 5min. The preparation was then centrifuged at 12000g for 15min. The supernatant was removed and either analysed immediately or frozen at -20ºC until analysis.

Enzymatic reaction

1. Enzyme extract (415µl), NaCitrate buffer (pH 6.0) (319µl) and cysteine 100mM (100µl) were pre-equilibrated for 10min at 40ºC.
2. Pre-equilibrated 2%azocasein solution (834µl) was added to the enzyme solution tube, stirred and incubated at 40ºC for 30min.
3. The reaction was stopped and non-hydrolyzed azo-casein was precipitated with the addition of 3.0mL (5%) TCA and vortexing vigorously for 5 second.
4. The reaction tubes were allowed to equilibrate at room temperature for 5 min and then the contents were filtered through Whatman Nº1 filter circles.
5. The absorbance of filtrates was read against the reaction blank at 440nm.

Blank reaction was prepared by adding 3.0mL of TCA to 100µl of cysteine and 319µl of sodium citrate buffer, mixed and then 415µl of enzyme extract was added.

Calculations

One endoprotease unit was defined as the amount of enzyme required to hydrolyze (and remain soluble in TCA) one micromole of tyrosine equivalents per minute from soluble casein under standard assay conditions (pH 7.0 and 40ºC). The reference protease assay uses casein as substrate, involves a TCA precipitation step and is standardised against tyrosine (Megazyme data booklet).

Endoprotease activity was determined by reference to a standard curve. Papain standard enzyme calibration curve is showed in Figure 4.12 from where it was extracted the regression equation and enzyme activity was calculated as follows:

mU/assay = [(Abs 440nm – Blank reaction)* 143.1634] + 22.74447

$$\text{Total mU} = \left[\frac{\text{mU/ assay}}{\text{Aliquot assayed (mL)}}\right] * \text{Extract volume buffer (mL)}$$

$$\text{mU/g (dry basis)} = \left[\frac{\text{Total mU}}{\text{Weight malt (g)}}\right] * \frac{1}{(100 - \%\text{Moisture})*100}.$$

An example of calculation table is showed in chapter IV (Table 4.8).

3.7 Fermentability analysis

Three different fermentability tests were used in this study. 1) AAL measurement following the EBC method. 2) Tall tubes fermentations. 3) AAL measurement following a modified EBC method that used broth solutions prepared by mixing high maltose syrup with Congress wort.

3.7.1 AAL EBC method

Apparent attenuation limit (AAL) was measured following the EBC 4.11.1 method (Analytica-EBC, 4th ed. 1998) using baker's fresh compressed yeast supplied by Fleischmann. The industrial nature of the fresh yeast produced more reproducible results which helped to remove yeast as a source of variability. The pitching rate recommended by the EBC method was used: 7.5g in 100mL of wort. Fermentation was carried out at 20ºC for 24 hours with stirring.

3.7.2 Micro-brewing

ACMN, M936 and Metc barley varieties were malted in a malting pilot plant mentioned earlier and then brewed in a micro-brewery at the Canadian Malting Barley

Technical Centre (CMBTC) (Fig. 3.2). Brewing performance of the three varieties and assay conditions are shown in Table 3.6.

Fermentation was carried out in tall tubes (1.5L each) at 15ºC in duplicate for one week (Fig. 3.3). The pitching rate was 6.6g in 1.5L of boiled wort. A lager yeast slurry (in-house strain) was provided by CMBTC. An overnight fermentation was also performed with 250 g of the same yeast type and 1.5L of wort which determined the lowest gravity that could be achieved during fermentation.

Figure 3.2 Micro-brewing at the CMBTC. 1: mash mixer; 2: lauter container; 3: kettle (boiling) (By Blanca Gómez G).

Figure 3.3 Four fermentation tall tubes at CMBTC (1.5L) (By Blanca Gómez G).

Table 3.6 Brewing conditions and quality parameters of three malted varieties.

	Varieties		
Brew performance	**ACMN**	**M936N**	**Metc**
Malt parameters			
Ground malt by weight (kg)	1.5	1.5	1.5
Mash mixer			
Calcium chloride (g)	2.0	2.0	2.0
Malt mash-in water (L)	5	5	5
Malt mash-in temperature (ºC)	48	48	48
Malt mash hold (min)	30	30	30
Malt mash first raise (ºC)	65	65	65
Malt mash hold (min)	30	30	30
Mash-off temperature (ºC)	77	77	77
Time to iodine negative (min)	18	17	16
Lautering parameters			
Underlet volume (L)	0.25	0.25	0.25
Underlet water temperature (ºC)	82.5	87.0	81.0
Rest (min)	5	5	5
Time to clear (min)	3.0	2.0	1.5
First wort (ºP)	18.84	17.80	18.17
First wort time (min)	1.0	1.4	7.0
Sparge temperature (ºC)	81.6	87	85.1
Sparge volume (L)	6	6	6
Runoff-time (min)	44	60	34
Last runnings (ºP)	2.43	2.58	2.29
Bioling parameters			
Kettle full (ºP)	10.62	9.99	9.98
Boil time (min)	60	60	60
Kettle full volume (L)	10.6	11.2	11.5
Kettle cast volume (L)	8.0	8.0	8.2
Kettle KO (ºP)	12.96	11.845	12.051
Wort pH	5.13	5.60	5.08
Wort colour	3.53	3.31	3.13
Fermentation parameters			
Yeast pitching rate (g)	6.6	6.6	6.6
Forced fermentation (ºP)	2.28	2.37	1.96
Hops-GRL-gms 60min	2 nugget	2 nugget	2 nugget

Microscope yeast cell counting was carried out on the lager yeast slurry from CMBTC following the method described by ASBC Yeast-4. Lager yeast slurry (2.4g) was weighted into a volumetric flask with 1L of pure water. Aliquots from the stirred solution

were used to count the yeast cells in a hemocytometer. Calculations were as described in the ASBC method.

Subsamples of mash and wort were removed at different stages during the brewing process. During mashing and after boiling the subsamples were removed to determine only individual free amino acids. During fermentation, on the other hand, gravity and individual free amino acids were determined.

Sampling schedule:

Mashing

- 16min at 48ºC
- 25min at 48ºC
- 0min at 65ºC
- 20min at 65ºC
- 0min at 77ºC

Boiling

- After boiling

Fermentation

- 24 hours (day1)
- 48 hours
- 72 hours
- 96 hours
- 120 hours
- 144 hours
- End of fermentation (day7)
- Overnight fermentation

3.7.3 Broth preparation and AAL measurement

In this study, broths were prepared from different ratios of high maltose solutions and Congress wort.

The high maltose solution had to have the same ºP as Congress wort in order to limit dilution effects during mixing. The high maltose solution was prepared by adding 100.8g of high maltose syrup to 1L of pure water to achieve a solution gravity of 8.5ºP.

The three varieties (ACMN, M936N, and Metc) malted in the pilot plant were used to prepare Congress worts (W), which were then mixed with the high maltose solution (HMS) to prepare the broths. Several broths were prepared for each wort sample by selecting different ratios as follows: 1) 100% high maltose solution syrup (HMS); 2)

80%HMS:20%Wort (W); 3)60% HMS:40%W; 4) 40% HMS:60%W; 5) 20% HMS:80%W; 6) 100%W.

Broths were fermented and AAL was determined following a procedure similar to the EBC method 4.11.1. Broth (100 mL) was brought to a boil, cooled to 25°C, and the volume corrected. Boiled broths were aerated by swirling flasks, and 0.47 g of fresh yeast (baker's fresh compressed yeast, Fleischmann) was added. Flasks were shaken in a controlled chamber temperature at 20°C for 24 hr. Fermented broth was centrifuged for 5 min at 10,000 × g, followed by filtering through fluted paper (32 cm, grade 802 fluted; Whatman, Clifton, NJ), and density was measured. Fermentations were performed in duplicate for each broth solution. Subsamples were removed during the fermentation process to quantify changes in levels of individual free amino acids. The selected stages were: 1) before pitching, 2) at four hours fermentation, and 3) at the end of the fermentation.

3.8 Experimental design and statistical analysis

Statistical analyses were performed using randomized complete block as the experimental design with variety considered as a random effect. Although barley varieties were not selected as random, the random option was used to run SAS (Statistical Analysis Software, SAS® 9.1.2, SAS Institute Inc.) due to the lack of barley sample duplicates in the malting treatment experiment. Thus, experiments were designed to look into differences in malt quality parameters among malting treatments with different barley varieties only increasing variability in malt quality. Treatment effects were studied in pairs, contrasting each treatment against the standard schedule. Furthermore, no interaction effects among treatments were studied. The SAS program used for statistical analysis was:

```
Data One;
Set 'C:\Documents and Settings\Blanca\My Documents\Universidad\Master-
PhD\Thesis\Statistics\QUALITYAAENDOWOUTHULLES';
proc Mixed data=One Method=type3;
  title1 'Effect of All Trt on Steep Out Moisture';
  class Variety Trt;
  model Steep=Trt / outp=OneResid;
  Random Variety/solution;
  Lsmeans Trt / diff=control('C');
*                     A  B  C  D  G;
  Contrast 'G-C' Trt  0  0 -1  0  1;
  Contrast 'A-C' Trt  1  0 -1  0  0;
  Contrast 'B-C' Trt  0  1 -1  0  0;
  Contrast 'D-C' Trt  0  0 -1  1  0;
Proc Univariate Plot normal Data=OneResid;
  Var Resid;
Proc Plot Data=OneResid;
  Plot Resid*Pred;
Quit;
```

Correlation studies were carried out using the SAS program described below.

```
Data One;
Set 'C:\Documents and Settings\Blanca\My Documents\Universidad\Master- PhD\Statistics\thesis';
Proc Corr Data=One;
 Var NH3 His Asn Ser Gln Arg Gly Asp Glu Thr Ala GABA Pro Cys Lys Tyr Met Val Ile Leu Phe Trp
Yield Moi Fri Fine SP MP Kolbach Glucan Visc DP DU ASBC EBC pH FAN AAL Alpha Beta LD Papain
Totalaa;
Quit;
```

Chapter IV

Results & Discussion

4.1 Introduction

The study investigated differences in amino acid profiles and endoprotease activities in a series of barley samples that were malted under a range of conditions. The release of amino acids during malting, mashing and their consumption during fermentation was also investigated. In each case impacts on fermentability of amino acid levels, endoprotease activity and malt quality were investigated. Methods for analysis of individual free amino acids in wort and the assay of endoprotease activity required modification, refinement and verification before experimental analysis could commence. A unique “broth method” was developed for investigating effects of micro nutrients, such as amino acids, on fermentablity.

4.2 General presentation on UPLC amino acids analysis

Initial work with the UPLC indicated small changes in pressure occurred during a run as the UPLC works at very high pressures (>10,000 psi). Changes in pressure were found to affect retention times of each amino acid interfering with proper identification of each amino acid peak in chromatograms. To handle this situation, a sample run table was created with standard samples, at two different concentrations, bracketing six to eight unknown samples. Bracketing minimized the effects of retention time variation and improved calibrations.

Accuracy and precision tests were performed using amino acid standard solutions at two different concentrations, 100 and 150 pmol for each amino acid with the exception of cysteine which had a concentration of 50 and 75 pmol respectively. Analyses of 22 standard solutions for each concentration were performed in one week and the results are presented in Table 4.1. From these results it can be seen that the majority of the amino acids present a coefficient of variation (CV) lower than 4.0% with the exception of the glutamine and alanine standards at the 150 pmol concentration. Ammonium (NH_3) is

another amino compound that presented CV values higher than 4.0 (Table 4.1). This result was in accordance with chromatogram plots, in which NH_3 peaks did not achieve complete baseline resolution, causing greater variation in the peak area determination compared with other peaks (Fig. 4.1). Based on this observation, it was decided to not report NH_3 values in this study.

Table 4.1 Amino acid analyses from 22 samples of each of the 100 and 150 pmol amino acid standard solutions.

Amino	Standard Amino acid solution					
	100 pmol			150 pmol		
Compound	Mean	Std	CV	Mean	Std	CV
Ala	98	3.2	3.3	152	6.3	4.1
Arg	98	3.2	3.3	152	5.6	3.7
Asn	98	3.0	3.0	152	5.9	3.9
Asp	98	3.2	3.3	151	6.1	4.0
Cys	49	1.4	2.8	76	2.6	3.4
GABA	98	3.3	3.3	151	5.9	3.9
Gln	99	3.1	3.1	152	7.0	4.6
Glu	97	3.1	3.2	151	5.7	3.8
Gly	98	3.3	3.3	151	5.7	3.8
His	98	2.9	2.9	151	5.4	3.6
Ile	98	3.1	3.2	151	5.6	3.7
Leu	98	3.4	3.4	151	5.8	3.9
Lys	98	3.1	3.1	151	5.6	3.7
Met	98	3.0	3.1	151	5.1	3.4
NH3	100	5.1	5.1	148	6.3	4.3
Phe	98	3.4	3.5	151	5.9	3.9
Pro	98	3.1	3.2	152	6.0	4.0
Ser	98	3.2	3.3	152	5.9	3.9
Thr	98	3.1	3.1	151	5.5	3.6
Trp	97	3.5	3.6	151	6.0	4.0
Tyr	98	3.1	3.2	151	5.4	3.5
Val	98	3.0	3.1	151	5.6	3.7

4.2.1 Wort amino acids analysis

Wort amino acid precision tests were performed measuring repeatability and reproducibility of five different worts prepared the same day from the same malt barley variety. Results are presented in Table 4.2 including: average concentrations for all amino acids from the five different worts, the standard deviations and calculated CV values (in %). As expected, the CV values from wort were higher than the CV values for the standard amino acid solutions, due to the greater source of error associated with wort preparation and the presence of other compounds in worts. However, results were considered acceptable.

Several concentrations of amino acids in calibration standards were investigated for their ability to produce a calibration curve which properly quantified amino acids (ug/ml) in wort samples. The concentrations assayed were 100, 150, 200, 250, 300, 400 and 500 pmol (Fig. 4.2). The final concentrations selected for the amino acid calibration standards, were 100 and 150 pmol and these were both used for all subsequent amino

acid analyses. Although using standards with these concentrations allowed for the achievement of consistent results, it would be recommended that higher pmol concentrations be used in the future. For instance, amino acid standard solution of 150, 400 and 700 pmol would assure better coverage of the concentration ranges expected in wort samples.

Table 4.2 Amino acid concentrations in five different worts prepared in a single day from the same malt.

	Wort reproducibility test (ug/ml)							
Amino acid	**1**	**2**	**3**	**4**	**5**	**Mean**	**Std**	**CV**
Ala	75.3	82.7	81.6	82.7	78.9	80.2	3.2	4.0
Arg	82.8	90.4	89.1	91.6	87.5	88.3	3.4	3.9
Asn	91.3	102.3	97.3	100.9	94.3	97.2	4.6	4.7
Asp	48.1	53.2	51.8	52.8	50.1	51.2	2.1	4.2
Cys	nd	nd	nd	nd	nd	nd	nd	nd
GABA	66.5	72.9	71.5	72.1	68.7	70.3	2.6	3.8
Gln	169.2	185.1	181.0	185.5	175.3	179.2	7.0	3.9
Glu	26.6	29.3	29.4	29.7	28.4	28.7	1.3	4.4
Gly	28.4	31.0	30.4	31.1	29.6	30.1	1.1	3.7
His	36.6	41.4	39.3	40.4	38.3	39.2	1.9	4.7
Ile	51.1	56.0	54.7	56.3	53.3	54.3	2.1	3.9
Leu	106.5	116.6	114.7	117.6	111.9	113.4	4.5	3.9
Lys	70.0	76.8	75.4	76.9	73.1	74.4	2.9	3.9
Met	22.5	24.6	24.2	24.8	23.7	24.0	0.9	3.8
Phe	85.3	93.8	91.6	93.9	89.6	90.8	3.6	3.9
Pro	374.0	415.9	400.6	411.0	390.5	398.4	16.8	4.2
Ser	65.3	69.6	70.3	72.1	68.5	69.2	2.5	3.6
Thr	43.7	47.6	46.7	48.2	45.5	46.4	1.8	3.9
Trp	41.0	44.7	44.1	44.8	43.0	43.5	1.6	3.6
Tyr	65.1	71.6	70.3	71.8	68.4	69.4	2.7	4.0
Val	82.6	91.0	88.8	91.0	86.4	88.0	3.6	4.0
TotalAA	1632	1797	1753	1795	1705	1752	70.5	4.0

Std: Standard deviation of the method; nd: not detected

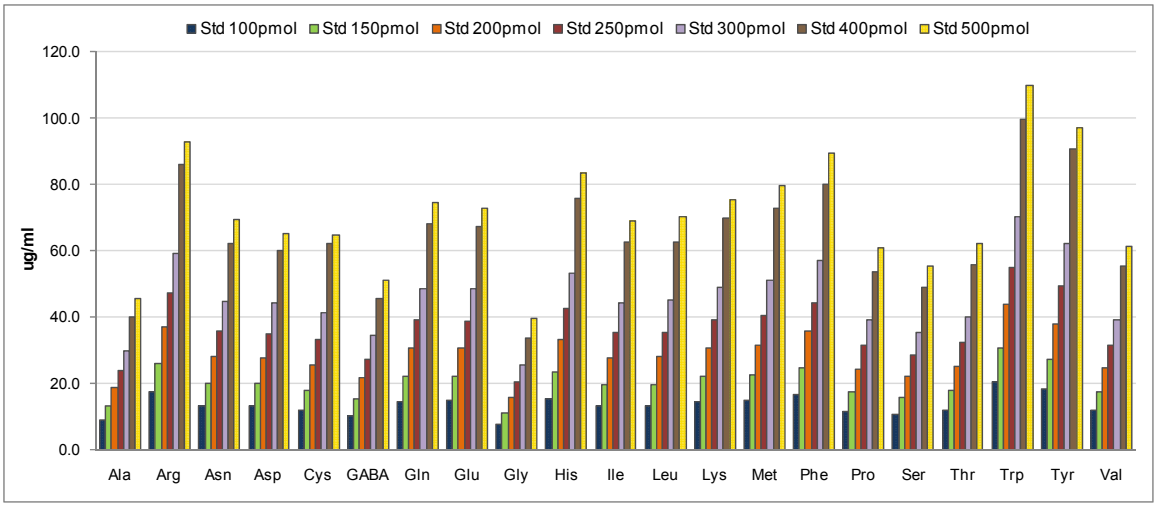


Figure 4.2 Relationship between pmol and ug/ml of the amino acid standard solution at different concentrations.

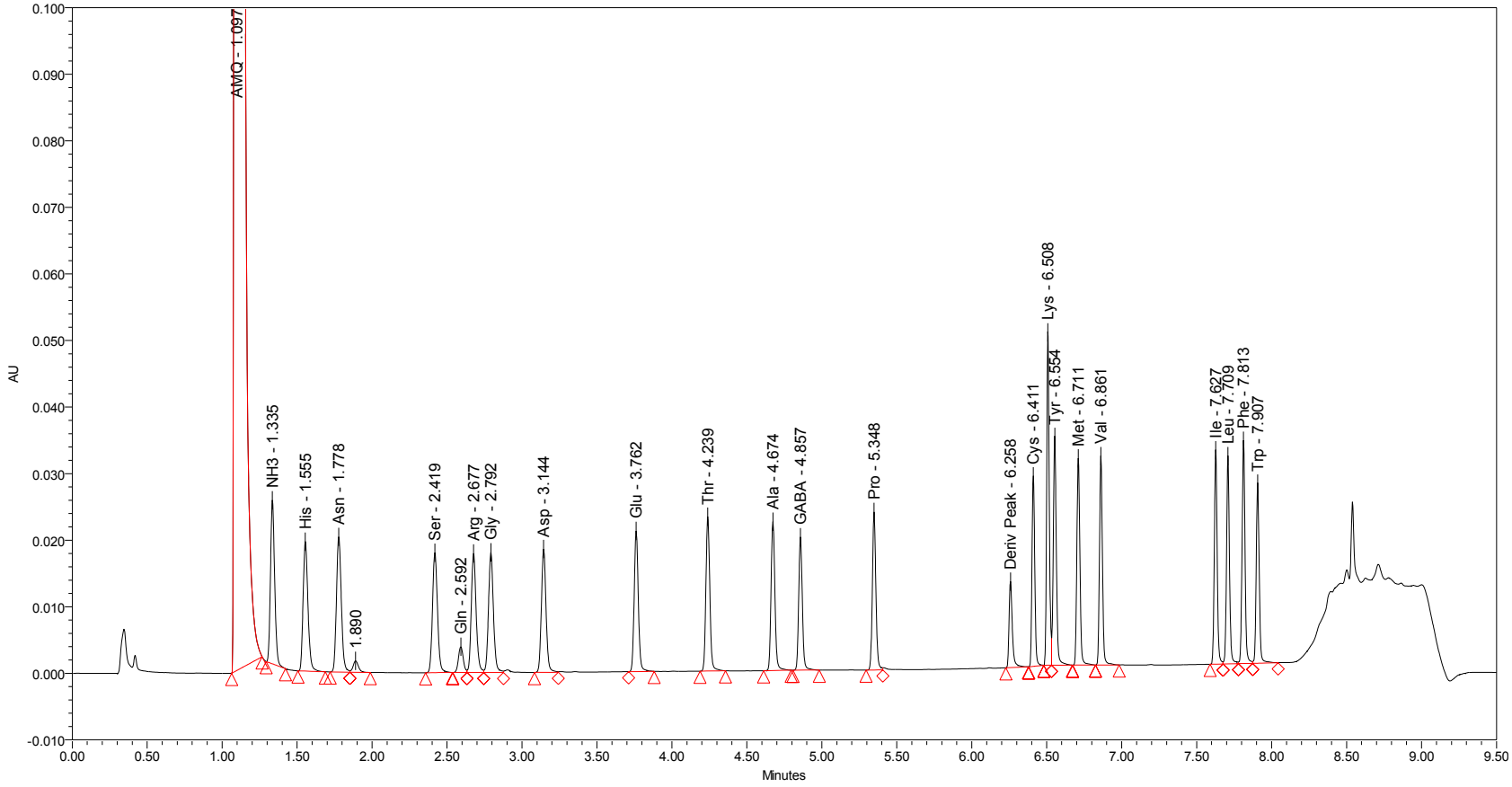


Figure 4.1 Chromatogram of amino acid standard solution of 100pmol as produced with the UPLC system

4.2.2 Barley and malt amino acids analyses

Different procedures were investigated for extracting all the free amino acids from a ground malt sample without allowing for liberation of additional free amino acids by proteases. Boiling water was found to be the best extraction solution for producing reproducible results (Table 4.3).

Table 4.3 Reproducibility of extracting free amino acids with boiling water. Four different extractions were performed on the same malt sample.

	Malt reproducibility test (ug/ml)						
Amino acid	**1**	**2**	**3**	**4**	**Mean**	**Std**	**CV**
Ala	56.3	51.3	50.6	52.1	52.6	2.5	4.8
Arg	66.1	60.1	61.5	62.4	62.5	2.6	4.1
Asn	90.4	81.6	85.3	88.7	86.5	3.9	4.5
Asp	40.6	36.5	38.4	38.7	38.5	1.7	4.4
Cys	nd	nd	nd	nd	nd	nd	nd
GABA	42.8	39.0	32.7	33.6	37.0	4.8	12.9
Gln	186.9	171.3	179.1	181.9	179.8	6.5	3.6
Glu	30.2	27.9	36.1	37.2	32.8	4.5	13.7
Gly	19.7	17.6	17.1	17.2	17.9	1.2	6.8
His	31.5	28.9	29.6	30.3	30.1	1.1	3.7
Ile	38.6	35.1	35.6	36.2	36.4	1.5	4.2
Leu	73.9	67.1	66.5	67.9	68.9	3.4	4.9
Lys	41.7	37.7	36.3	37.2	38.2	2.4	6.2
Met	14.7	13.3	13.6	13.6	13.8	0.6	4.7
Phe	68.4	62.6	63.5	65.0	64.9	2.6	4.0
Pro	381.4	357.2	369.6	380.3	372.1	11.3	3.0
Ser	51.7	46.7	46.8	48.0	48.3	2.3	4.8
Thr	31.9	28.6	28.6	29.5	29.6	1.5	5.2
Trp	36.8	33.9	35.4	36.1	35.5	1.3	3.6
Tyr	49.7	44.5	45.2	46.6	46.5	2.3	4.9
Val	65.7	59.6	59.7	61.1	61.5	2.9	4.7
TotalAA	1428	1310	1341	1373	1363	50.5	3.7

Std: Standard deviation of the method; nd: not detected

The reproducibility of the extraction of the amino acids Glu and GABA was poorer than other amino acids. Despite this high variability with Glu and GABA, the quantification was considered acceptable and the extraction method was used for analysis of free amino acids in malt. More work should, however, be done with the extraction method to achieve better results.

Based on results from malt extraction, barley extractions with boiling water were found to produce repeatable quantification of free amino acids from barley.

4.3 General presentation on endoprotease enzymatic method

Assays for endoprotease activity are dependent on selection of an appropriate protein-type substrate. Two substrates, azogelatin (Jones et al. 1998) and azo-casein (Megazyme), have received the most attention.

4.3.1 Azogelatin substrate

Preparation of azogelatin in the present study was based on the methodology described by Jones et al. (1998). Reaction conditions for assays of endoproteases vary widely in the literature. As a result, a decision was made to use, in this study, assay conditions that represent the conditions found inside malt grain and wort, where the endoproteases actually act. Average pH values for malt were found to be 5.50 and an average of 6.00 for worts (Table 4.9). Consequently, it was decided to perform all malt extractions at pH 5.50 with measurements of wort endoprotease activities at pH 6.00.

The pure cysteine class enzyme, papain, was used to develop standard calibration curves and adjust conditions for the assay. An increase in absorbance with different concentrations, of papain and over a range of reaction times (Fig 4.3), proved that papain hydrolysed the azogelatin substrate allowing for measurement of cysteine type endoprotease activity as expected. However, absorbance at 0 min reaction time (blank reaction) was high, presenting values from 0.332 to 0.386. This high blank could be related to the instability of the substrate at 40ºC; although, the amount of time left at 40°C was limited as the time between the first sample assayed and the last was only 9 min. As well the absorbance after 25 min reaction time was also a concern with enzyme concentrations from 26.3 mU/ml to 157.5 mU/ml. Absorbance actually decreased,

compared to that observed after a 10 min reaction and in some cases the 25 min absorbance was lower than the reaction blank (0min) (Fig.4.3 red arrow). This result was not expected as the activity at 45 min continued increasing, showing some inconsistency in the method. The method did demonstrate a positive linear relationship between enzyme concentration and 440nm absorbance at 45 and 60min reaction. Furthermore, higher enzyme concentrations were assayed to prove the linearity at higher concentrations and also to obtain higher absorbance values (Fig.4.4).

The results with higher concentration were better and more promising than previous attempts and a decision was made to continue studying measurement of endoprotease activity with this substrate. Further research on standardized conditions for the "in solution" methodology of Jones et al. (2000, 2002 and 2003), indicated the need for a substrate and enzyme ratio greater than 1:1 to assure good results. The best ratio was found to be 1.32:1.

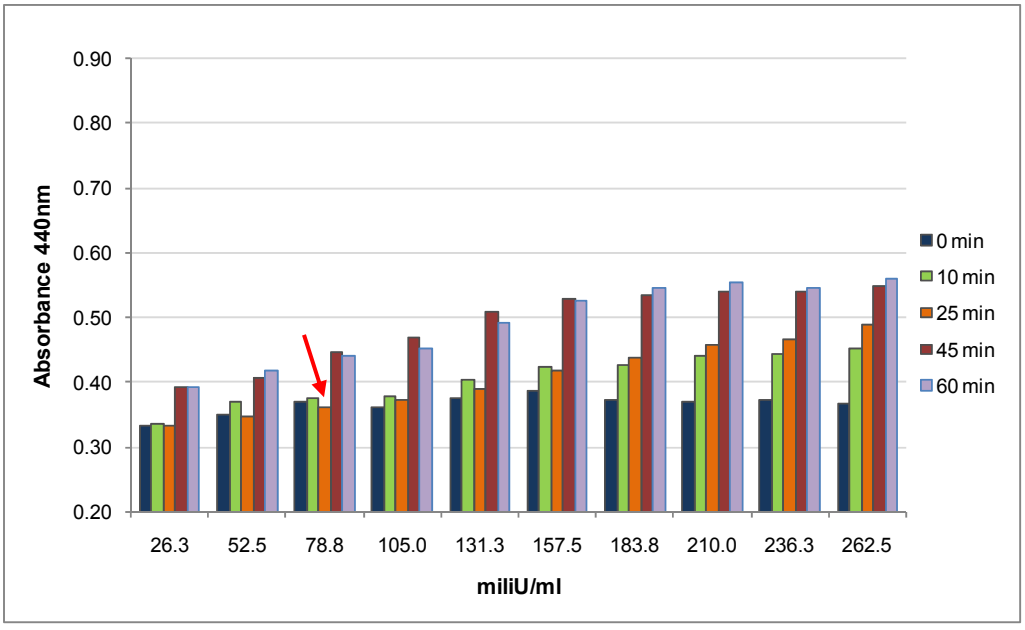


Figure 4.3 Endoprotease activity, as measured by increased absorbance after a range of reactions times, in a series of papain solutions at lower concentrations. Red arrow indicates an example of unexpectedly low absorbance after the 25 min reaction time.

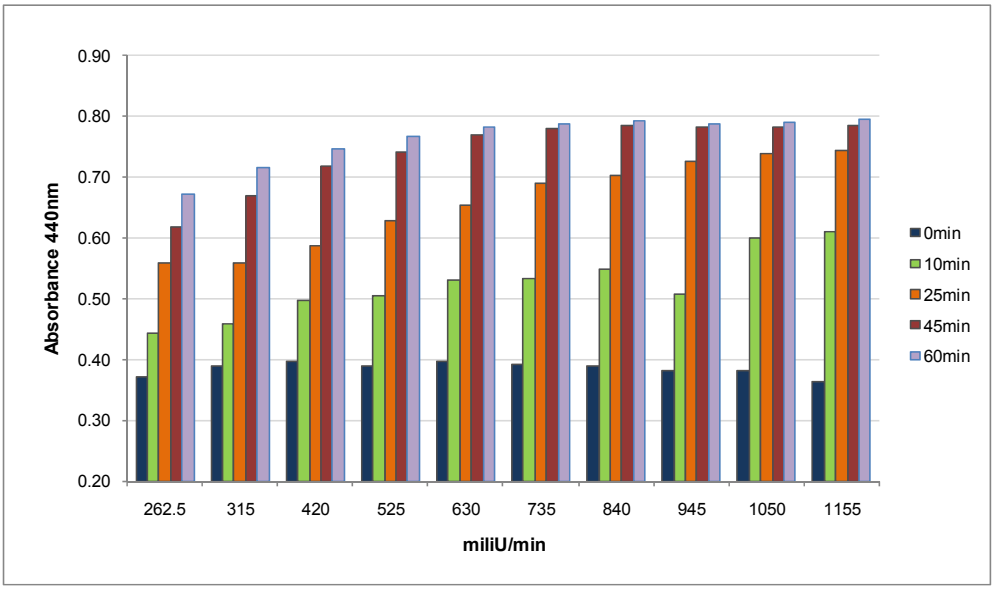


Figure 4.4 Endoprotease activity, as measured by increased absorbance after a range of reactions times, in a series of papain solutions at higher concentrations

Conditions for extracting endoproteases from malt were investigated to establish the best assay of malt endoproteases. Conditions included malt (enzyme) concentration, time and temperature of extraction. Several malt concentrations were tested and 0.38 grams of malt in 1.5 ml of extraction buffer was the preferential concentration and was determined most appropriate producing absorbance values around 0.50 after 25 min reaction time.

The most suitable extraction times and conditions were established using two different malts with results presented in Fig. 4.5. Conditions of 15 min extraction time at room temperature were found to produce the most consistent results. Similarly to Fig 4.3 absorbance after a 25 min reaction time were sometimes seen to be lower than after the 10 min reaction time. However, the results presented in Fig 4.5 had less variability in the reaction blank.

Based on malt quality results, Harrington was expected to exhibit higher endoprotease activity as the Harrington malt contained higher FAN values (189 mg/L)

than the AC Metcalfe malt (161 mg/L). However, AC Metcalfe in fact had higher endoprotease activity which may have reflected higher KI values.

The high absorbances observed in blanks when using azogelatin as a substrate for assaying endoprotease activity, were a major concern which made it difficult to obtain adequate absolute differences in absorbance for test samples. Furthermore, with some samples, differences in absorbances of the blank and the test sample were negative demonstrating the inconsistency of the method. Several methods were investigated for reducing the absorbance of the blank reaction but no successful results were obtained.

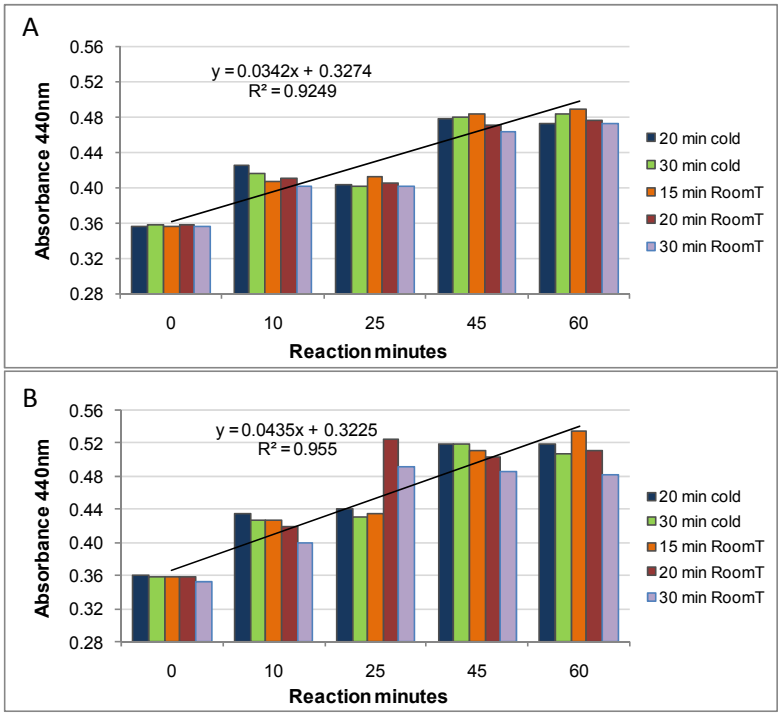


Figure 4.5 Endoprotease activity after a range of reaction times, of extracts from two malt samples (A Harrington malt variety; B AC Metcalfe malt variety) as extracted under different malt extraction conditions (time and temperature).

The problem of high blank absorbances was further investigated with pure enzymes and with a range of malt concentrations. Results still showed variable absorbances for the blanks (Table 4.4) with values even higher than previous runs. Absorbance for the reactions were similar (no data presented) to previous runs, but because the blanks were higher, differences were actually lower resulting apparently in less activity.

Table 4.4 Blank reaction absorbance variability. Blanks were prepared from different enzyme extraction sources.

Enzyme extraction	Blank reaction absorbance 440nm
Low malt concentration	0.408
Middle malt concentration	0.424
High malt concentration	0.411
Pure enzyme (papain)	0.488

Inconsistencies between 10 and 15 min reaction times (Fig 4.3 and 4.5) were further investigated with ten malt samples that were selected based for their range in FAN values. Malt 1 had the lowest FAN (98 mg/L) while malt sample 9 had the highest (256 mg/L). Results, using both the 10 and 25 min reaction times (Fig. 4.6), further demonstrated the inconsistency of the method with some malt samples once again producing higher absorbance values at 10 min than at 25 min. Absorbance values also did not reflect the malt quality of the samples, with no relation found between absorbance with either FAN values nor KI values.

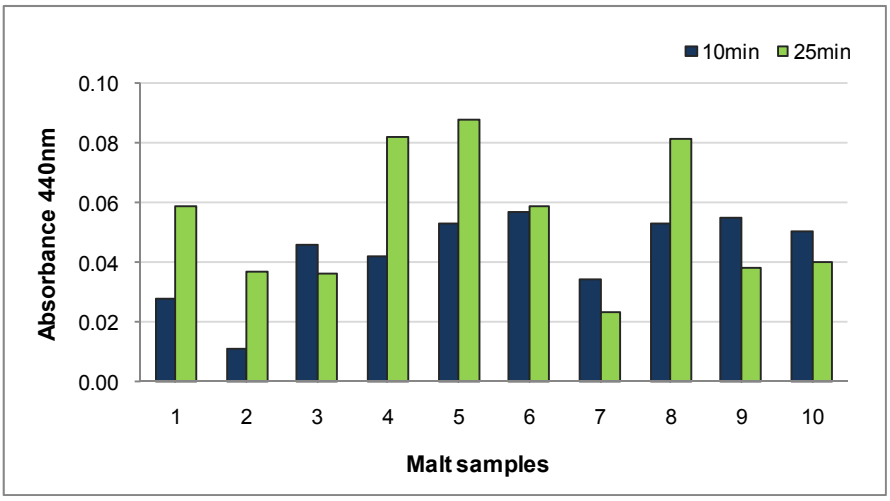


Figure 4.6 Malt endoprotease activity using azogelatin as substrate at pH 6.0.

Based on these results it was decided to discontinue the use of azogelatin substrate for assaying endoprotease activity in malt samples. Personal communication with Dr. Barry McCleary confirmed difficulties in standardizing an “in solution” assay with azogelatin substrate. Consequently, further studies on endoprotease activity switched to the use of azo-casein as substrate.

4.3.2 Azo-casein substrate

Investigations on the use of azo-casein substrate, purchased from Megazyme, followed methodology described by the company. Enzymatic reactions were carried out at pH 7.0 for 10 min. Pure standard enzymes Papain (cysteine class), Pepsin (aspartic class) and Protease (metallo class) were assayed. Results showed that low blank absorbances at 440nm (0.074), were possible to achieve with azo-casein, in contrast to azogelatin and that there were different absorbances among enzyme classes (Table 4.5). Absorbance values for the enzymes were low and a second run was performed at higher enzyme concentrations resulting in significantly higher absorbances for Protease (metallo class) but with no effect on Papain and Pepsin proving that metallo proteases are more active at the higher pH.

Assay conditions were to approximate conditions expected in malt grain, to mimic malting conditions, or wort pH, to mimic mashing conditions. Studies investigated extraction buffers at pH 5.5 and pH 6.0 and dilution buffers at pH 7.0 and 6.0 (Table 4.5 from third to fifth columns). Protease activity decreased slightly when the extraction buffer pH was changed to 5.5, but significantly decreased to almost zero when the pH of the dilution buffer was lowered to 6.0, with no effect by addition of cysteine. Furthermore, Papain presented a slight decrease in absorbance value at extraction pH 5.5, which was not expected given its pH preference. However, when the pH of the dilution buffer was lowered 6.0 the absorbance increased slightly. When cysteine was included with the pH

6.0 dilution buffer, a dramatic increase in absorbance (>1.0) was observed. Pepsin was not affected by a change in extraction buffer pH but higher absorbances with the pH 6.0 dilution buffer were seen but no change with the addition of cysteine. These results demonstrate the ability of the substrate to detect different endoproteases class, and how diverse pH conditions affect endoproteases activity.

Table 4.5 Endoproteases absorbance using azo-casein substrate under different assay conditions.

Conditions/ Enzymes	**Absorbance 440nm – 10 min reaction**				
Extraction buffer	pH 7.0	pH 7.0	pH 5.5	pH 6.0	pH 6.0
Dilution buffer	pH 7.0	pH 7.0	pH 7.0	pH 6.0	pH 6.0
Enzyme volume	100ul	200ul	100ul	100ul	100ul
Cysteine added	-	-	-	-	2mM
Papain	0.067	0.073	0.024	0.050	2.672
Pepsin	0.010	0.000	0.000	0.102	0.099
Protease	0.074	0.766	0.498	0.007	0.010

As well as investigating the pH of the buffers used for malt extraction (Table 4.5), the relation between malt concentration and absorbance values was investigated. Results (Fig 4.7A) revealed an excellent positive linear relationship; however, the absorbance range was low. As a result, higher malt concentrations were tested and results (Fig. 4.7B) continued to demonstrate a positive linear relationship, with somewhat higher absorbance values but not yet as high as expected.

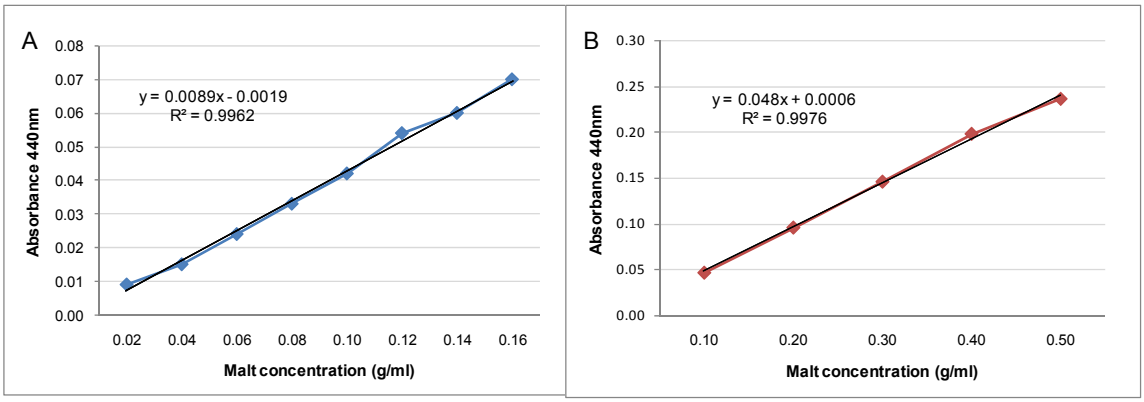


Figure 4.7 Malt endoprotease activity using azo-casein as substrate at pH 6.0 (dilution and extraction buffers) for 10 min. A) Lower malt concentrations. B) Higher malt concentrations.

To increase malt absorbance values different reaction conditions were assayed (Table 4.6). Results showed that absorbance increased proportionally with the addition of higher concentrations of cysteine for all malt concentrations tested. Furthermore, a longer reaction time allowed for even higher absorbance values, both with and without the addition of cysteine. Consequently, a combination of cysteine addition and longer reaction times was selected as the best strategy for obtaining absorbance values within the normal absorbance range (from 0.1 to 1.0).

Table 4.6 Malt endoproteases absorbance using azo-casein substrate under different assay conditions

Conditions	**Reaction pH 6.0**									
Malt conc.	0.02 (g/ml)		0.14 (g/ml)				0.16 (g/ml)			
Reaction time	10min	10min	10min	10min	10min	10min	10min	10min	25min	25min
Cysteine add.	-	2mM	-	2mM	8.3mM	25mM	-	8.3mM	-	8.3mM
Abs. 440nm	0.011	0.017	0.063	0.099	0.116	0.138	0.072	0.127	0.151	0.260

Experiments with different reaction times and with the addition of cysteine were carried out using pure enzymes (Table 4.7) and malt extracts (Fig. 4.8) to further investigate optimum reaction conditions.

Table 4.7 Effect of reaction time on activities of three pure enzymes as measured with azo-casein substrate and a 6.0 pH dilution buffer.

Conditions	**Reaction pH 6.0 with Cys 25mM**					
Enzymes	Papain		Pepsin		Protease	
Enzyme Vol.	50ul		50ul		50ul	
Reaction time	20min	40min	20min	40min	20min	40min
Abs. 440nm	1.841	2.469	0.129	0.142	0.018	0.013

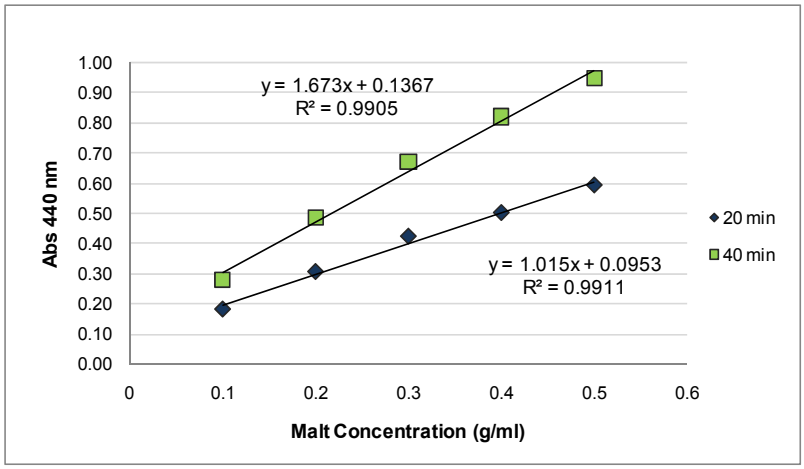


Figure 4.8 Malt endoprotease activity using azo-casein as substrate at pH 6.0, with addition of 25mM Cys at two different reaction time 20 and 40 min.

The results led to a decision to investigate decreasing concentrations of added cysteine and increasing the reaction time to one hour. Results (Fig 4.9 & 4.10) demonstrated that at pH 6.0 (wort pH) and reduced conditions (added cysteine), Papain enzyme showed the best performance. Papain presented a significant positive linear relationship with absorbance during one hour reaction, although the absorbance values were outside the normal range (Fig. 4.9 A). Protease (metallo class) also presented a positive linear relationship between absorbance and reaction time (Fig. 4.9 B); however, the absorbance values were low with a small range. These results were expected as the optimum pH for metallo class of enzymes is higher than the pH 6.0 used. Pepsin, on the

other hand, did not present a high determinant coefficient value (Fig. 4.9 C) indicating that these assay conditions were not suitable for determining the activity of aspartic class proteases.

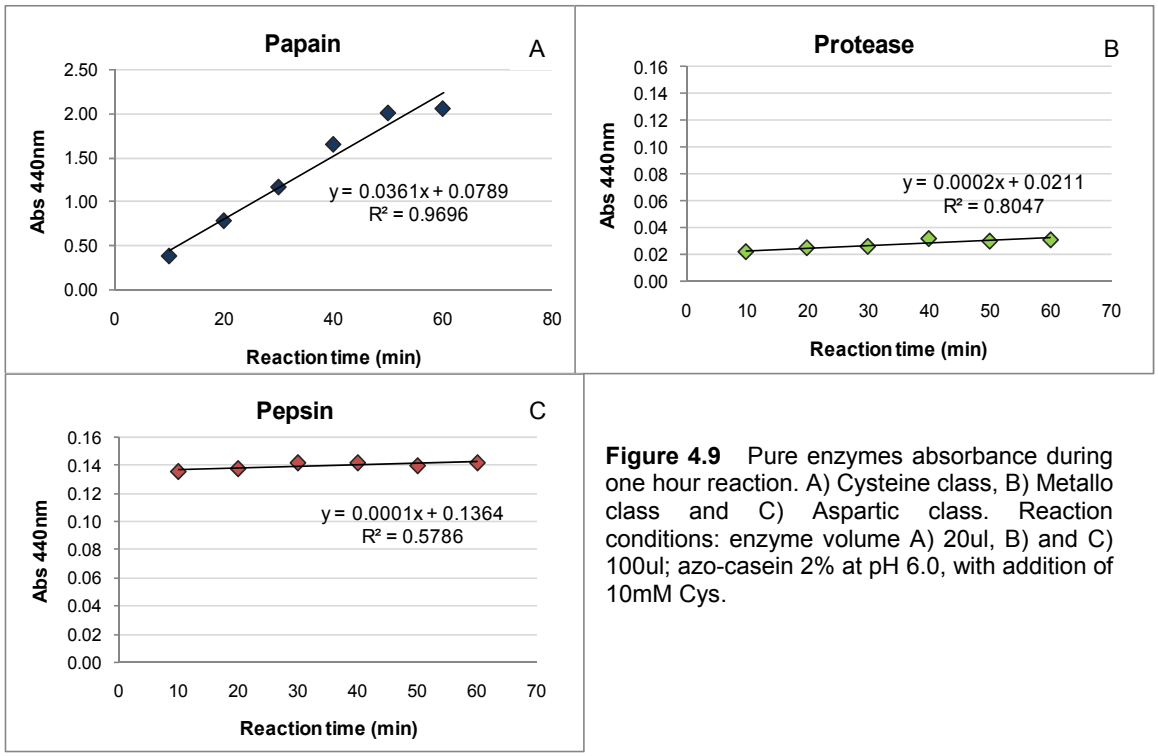


Figure 4.9 Pure enzymes absorbance during one hour reaction. A) Cysteine class, B) Metallo class and C) Aspartic class. Reaction conditions: enzyme volume A) 20ul, B) and C) 100ul; azo-casein 2% at pH 6.0, with addition of 10mM Cys.

Endoprotease activity was measured, using the conditions outlined in Figure 4.10, in extracts made from malt with varying quality. Measured absorbances, over a range of reactions times, presented a positive linear relationship with high determinant coefficient value (Fig. 4.10). A good indication of the relevancy of the method was its ability to distinguish between malts with high FAN levels (Malt 2: 257 mg/L and Malt 3: 164 mg/L) and a malt with a low FAN value (Malt 1: 93 mg/L). This is an important characteristic which should allow for classifying barley malt varieties based on their endoprotease activity.

Reaction time was fixed in 30 minutes as it produced absorbance values in the middle of the desired range.

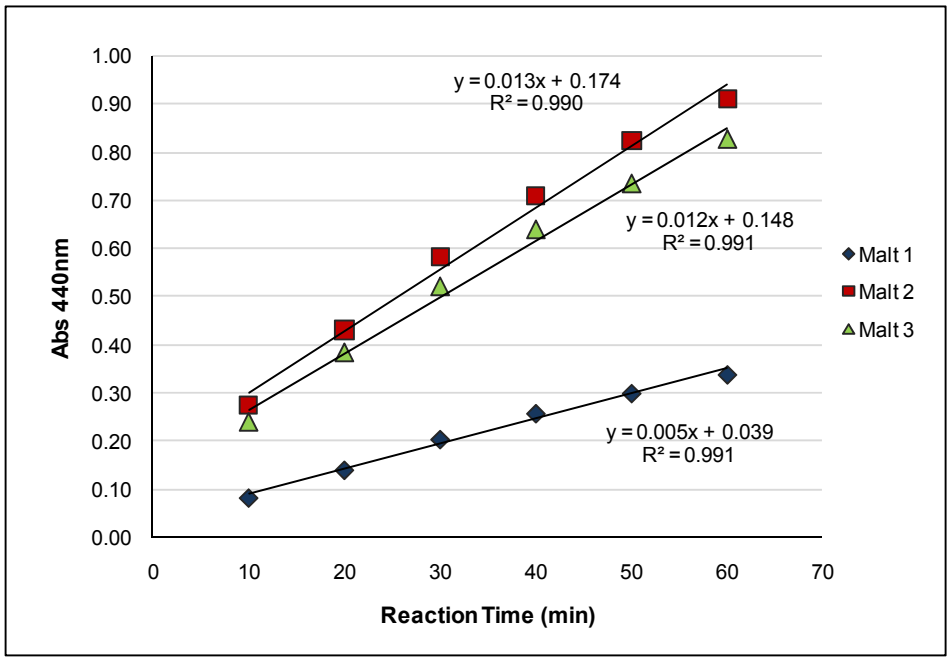


Figure 4.10 Absorbances malt endoproteases, as extracted from three different malt samples with varied malt quality. Reaction conditions: malt concentration 0.33g/ml, azo-casein 2% at pH 6.0, with addition of 10mM Cys over a one hour reaction time.

Trypsin was used to determine if the azo-casein assay detected the last of the four endoprotease classes found in malt, the serine endoprotease class. A standard calibration curve was constructed and results are shown in Fig 4.11. Results demonstrated that Trypsin presented a high positive association between enzyme concentration and absorbance value, although absorbance values were all higher than 0.6. Lower enzyme concentrations would be required to obtain a calibration curve with a lower absorbance range.

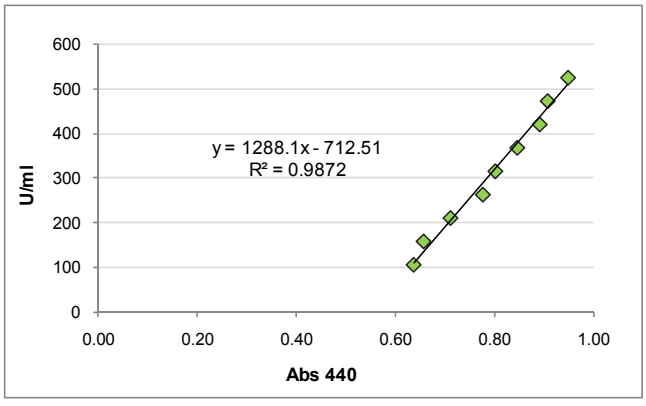


Figure 4.11 Trypsin standard calibration curve. Reaction conditions: enzyme concentration 1mg/ml, azo-casein 2% at pH 6.0, with addition of 10mM Cys during 30 min reaction.

The results supported the use of Papain as the pure enzyme for standardizing the method and constructing the calibration curve (Fig. 4.12).

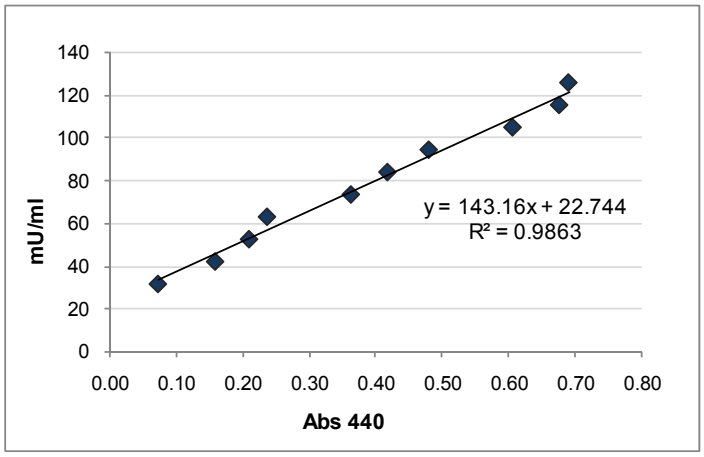


Figure 4.12 Papain standard calibration curve. Reaction conditions: enzyme concentration 1mg/ml, azo-casein 2% at pH 6.0, with addition of 10mM Cys during 30 min reaction.

The same malt sample was extracted and analysed ten times to measure the repeatability and reproducibility of the malt endoprotease test (Table 4.8). Standard deviation and the maximum and minimum control limits of endoprotease activity of the malt check were determined from the results.

Barley samples were assayed following the same methodology as malt, although there were no measurements made to optimize extraction pH or reaction conditions.

Wort endoprotease activity was also assayed to prove the scope of the method (Fig. 4.13). Samples were removed every ten minutes from the start of Congress mashing. Endoprotease activity decreased with increasing mashing time. This was expected, given the temperature increase (45ºC to 70ºC) that occurs as mashing proceeds and which would inactivate the proteases.

Table 4.8 Malt reproducibility and repeatability test

Sample ID	Sample	#	Weight of Malt (g)	Extraction Buffer (ml)	Aliquot Assayed (ml)	Absorbance @440nm					mU/assay	mU/ml	total mU	mU/g sample	Moisture %	mU/g dry sample
						Sample 1	Sample 2	Average	Blank	Difference						
Metcafle	1		1.0806	3	0.42	0.579	0.587	0.583	0.062	0.521	97.333	234.536	703.609	651.128	5.29	687
Metcafle	2		1.0776	3	0.42	0.590	0.598	0.594	0.062	0.532	98.907	238.331	714.993	663.505	5.29	701
Metcafle	3		1.0285	3	0.42	0.576	0.587	0.582	0.062	0.520	97.118	234.019	702.057	682.603	5.29	721
Metcafle	4		1.0442	3	0.42	0.576	0.585	0.581	0.062	0.519	96.975	233.674	701.022	671.348	5.29	709
Metcafle	5		1.0396	3	0.42	0.579	0.594	0.587	0.062	0.525	97.834	235.744	707.231	680.292	5.29	718
Metcafle	6		1.0337	3	0.42	0.589	0.587	0.588	0.062	0.526	98.048	236.261	708.784	685.676	5.29	724
Metcafle	7		1.0587	3	0.42	0.590	0.605	0.598	0.062	0.536	99.408	239.538	718.615	678.772	5.29	717
Metcafle	8		1.0041	3	0.42	0.581	0.580	0.581	0.062	0.519	96.975	233.674	701.022	698.159	5.29	737
Metcafle	9		1.0471	3	0.42	0.600	0.592	0.596	0.062	0.534	99.194	239.021	717.063	684.809	5.29	723
Metcafle	10		1.0969	3	0.42	0.606	0.619	0.613	0.062	0.551	101.556	244.713	734.139	669.285	5.29	707
					Average	0.587	0.593	0.590		0.528						
					STD	0.010	0.011	0.010		0.010						
					2*STD	0.021	0.023	0.020		0.020						
					3*STD	0.031	0.034	0.031		0.031						

Variations among extractions	Average	714
	Std	14
	Min	686
	Max	742
Variation within extractions	Average	716
	Std	8
	Min	700
	Max	733

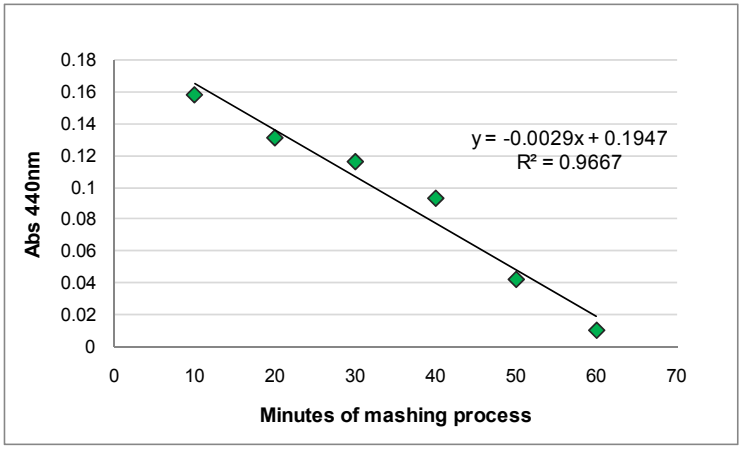


Figure 4.13 Wort samples removed at different minutes from the Congress mashing. Reaction conditions: enzyme concentration 1mg/ml, azo-casein 2% at pH 6.0, with addition of 10mM Cys during 30 min reaction.

In summary, a new method was developed to measure endoprotease activity in malt, barley and wort using reducing conditions (10mM cysteine), pH 6.0 (wort pH), for 30 min reaction time and azo-casein as substrate. Good levels of accuracy and precision were achieved with the described methodology. Cysteine class endoprotease were preferentially identified under the conditions defined in this study, although changing the pH and reducing conditions could be used to assay activity of the other three classes of endoproteases, aspartic, serine and metallo, as was demonstrated. More work would be required to optimize conditions of the assay if it were to be used to assay these other classes.

4.4 Differences in malt quality among barley samples

The research required a series of malt samples with a range in malt quality in order to better understand the effects of protein modification on fermentability. Canadian and Uruguayan barley varieties were selected based on their potential for protein modification during malting. The selected varieties came from different crop years and locations in Canada and Uruguay. The samples were all malted under different process

conditions to further increase variability in malt quality. A standard malting schedule produced significant differences in malt quality (Table 4.9) among barley varieties used (Table 4.10), resulting in a wide malt quality spectrum as required for the study.

The majority of the malt quality results (Table 4.9) were outside commercial specifications. However, the focus of this experiment was to obtain a maximum range in malt quality in order to more easily identify treatment effects on levels of endoproteases and wort amino acids. Based on results from the standard malting schedule, barley varieties were classified by their malt quality performance. AC Metcalfe (Metc), AC Madi South (ACMS) and AC Madi North (ACMN) showed good malt quality based on: a) good cytolytic modification level (low β-glucan levels and high friability values), b) adequate proteolytic modification (good KI and adequate FAN levels), c) high amylolytic enzyme contents (high DP, α-amylase, β-amylase and limit dextrinase) and d) high fermentability levels. Musa936 South (M936S), Musa 936 North (M936N) and Copeland 06 (Cop06) showed poorer overall malt quality compared to the first group of samples. The second group had: a) poor cytolytic modification (high β-glucan levels), b) regular proteolysis (adequate KI, low FAN levels), c) low amylolytic enzyme contents (low DP) and d) low fermentability levels, especially apparent with M936 South and North. Helgason 06/05 (Hel06, Hel05) and the hulless variety CDC MacGwire (McG05, McG06) represented a third group. Helgason had high β-glucan levels, poor friability, poor proteolytic modification (low KI, low FAN levels) and low amylolytic enzyme content (low

Table 4.9 Differences in malt quality produced with the standard malting schedule among barley samples. Red numbers indicate the maximum value for each parameter among the varieties. Blue numbers indicate the minimum value for each parameter among the varieties.

	Parameter																		
Malt Variety	Yield (%)	SOMoi (%)	Moi (%)	Fine (%)	Fri (%)	Glucan (ppm)	Visc (cps)	MP (%)	SP (%)	KI (%)	FAN (mg/L)	Colour (ASBC)	Colour (EBC)	pH	DP (ºL)	Alpha (DU)	AlphaM (U/g)	Beta (U/g)	LD (mU/g)	AAL (%)
Metc	93.7	44.9	5.8	80.2	**75.2**	99	**1.43**	11.9	4.4	37.0	172	1.51	3.0	5.95	**168**	**66.6**	**55.7**	**1245**	421	84.2
ACMS	93.5	46.6	6.7	79.5	62.5	73	1.46	13.8	**4.9**	35.2	**192**	1.59	3.1	**5.90**	124	55.0	43.1	805	**472**	**84.3**
ACMN	94.2	45.5	7.6	81.1	66.7	**53**	1.46	12.5	4.7	**37.8**	181	**1.83**	**3.6**	5.99	131	61.0	49.6	832	461	83.5
M936S	**95.3**	43.9	6.7	80.3	61.1	266	1.53	**10.6**	3.9	36.5	123	1.50	3.0	**6.09**	**84**	43.2	30.5	**507**	321	**77.8**
M936N	94.6	**46.7**	6.2	79.5	64.8	137	1.49	11.1	4.1	36.4	130	1.57	3.1	6.05	86	46.5	39.9	531	327	78.9
Cop06	93.6	**43.3**	**5.7**	79.8	63.5	252	1.48	12.4	4.5	36.2	167	1.55	3.1	5.99	139	54.5	35.3	782	352	81.2
Helg06	93.9	44.0	5.8	**79.2**	50.5	309	1.48	12.9	4.1	31.6	145	1.78	3.5	6.01	130	58.3	50.0	860	302	82.5
Helg05	93.7	44.1	5.8	79.8	37.3	593	1.61	12.7	4.2	33.1	147	1.46	2.9	6.03	117	60.9	54.4	658	312	79.6
McG06	90.7	44.1	**11.9**	83.3	58.7	637	1.69	**14.6**	**3.8**	**26.1**	**115**	**1.35**	**2.7**	6.00	158	**37.7**	**23.4**	970	178	83.1
McG05	**90.6**	43.6	7.5	**84.4**	**31.5**	**964**	**1.93**	13.1	3.8	29.4	124	1.52	3.0	6.01	118	43.6	29.9	802	**142**	79.2
Std	0.70	1.39	0.05	0.13	2.00	1.70	0.01	0.13	0.06	0.75	3.05	0.05	0.05	0.06	3.35	1.70	1.23	23.80	9.80	0.50
Mean	94.2	45.2	6.5	80.1	65.6	147	1.48	12.0	4.4	36.5	161	1.59	3.1	6.00	122	54.5	42.4	784	392	81.7
CV	0.74	3.08	0.78	0.16	3.05	1.16	0.68	1.08	1.36	2.05	1.90	3.14	1.60	1.00	2.75	3.12	2.90	3.04	2.50	0.61

SOMoi: Steep out moisture; Moi: Moisture; Fine: Fine Extract Level; Fri: Friability; Glucan: β-Glucan; Visc: Viscosity; MP: Total malt protein; SP: Soluble protein; KI: Kolbach Index; FAN: Free amino nitrogen; DP: Diastatic power; Alpha: Alpha amylase; AlphaM: Alpha amylase by Megazyme method; Beta: β-amylase; LD: Limit dextrinazyme; AAL: Apparent attenuation limit; Std: Standard deviation of the method

Table 4.10 Barley quality profile

	Barley quality				
Variety	**Germination Capacity (%)**	**Water Sensitive (%)**	**Plump (%)**	**Moisture (%)**	**Protein (%)**
Metc	99	0	nd	9.1	13.1
ACMS	94	13	85.8	12.1	13.4
ACMN	98	11	98.0	12.1	12.4
M936S	98	3	92.7	12.1	10.7
M936N	97	6	93.8	12.4	11.2
Cop06	100	1	95.5	10.8	12.8
Helg06	100	0	97.1	11.0	13.0
Helg05	99	15	98.4	10.5	12.9
McG06	99	0	99.1	10.7	14.2
McG05	99	0	99.5	10.8	13.1

DP); however, it had acceptable fermentability levels. CDC McGwire was expected to deliver a different malt quality because of its hulless characteristic. The lack of hull leads to quicker water absorption, compared with the covered ones. This variety showed poor quality with: a) low yield, b) extremely poor cytolytic modification (high β-glucan levels, high viscosity and low friability), c) extremely poor proteolytic modification (low KI, and FAN levels), d) low amylolytic enzymes content (especially α-amylase) but e) high levels of malt extract and f) good fermentability. The McGwire sample from 2006 (McG06) showed especially poor quality with the lowest levels of FAN and amylolytic enzymes among the samples tested.

4.5 Differences in malt quality among processing conditions

Barley samples (Table 4.10) were malted under five different malting schedules (Chapter III, 3.2.1).

Results on the quality of hulless barley malt were removed from overall discussion. Preliminary statistical analysis with SAS demonstrated that the malting behaviour of hulless barley was extremely different from that of the covered samples, causing the loss of normal statistical behaviour in the majority of parameters analysed. Consequently, the statistical analysis of malt quality data, as presented in Table 4.11, involved only the eight covered barley varieties.

Table 4.11 presents data on the statistical significance of malting treatment on malt quality. The major significant effect of GA_3 addition was on protein modification as indicated by significant increases in levels of SP, KI and FAN. Fine, Fri, alpha and AAL values were also significantly higher. The effects of GA_3 are in accordance with Briggs et al. (1998) who explained the effect as increased stimulus by GA_3, during germination, of enzyme synthesis in the aleurone layer. Moreover, the lack of response of β-amylase to

GA_3 was not surprising, supporting the results of Evans et al. (2009) which indicated that β-amylase is synthesised during grain development, not during germination.

Table 4.11 Effect of malting treatment on malt quality of eight covered malting barley varieties

		Treatment samples mean					
Paramter	**Units**	**Standard**	**Add GA_3**	**Freeze Dry**	**Higher KilnFT**	**Longer Kiln**	**SE**
Yield	(%)	94.0	92.5***	92.3***	93.7	94.3	0.3
SOMoi	(%)	44.9	44.6	44.7	44.6	43.3***	0.4
Moi	(%)	6.3	6.0	2.8***	4.5***	4.2***	0.1
Fine	(%)	79.9	80.5***	80.6***	79.8	79.8	0.2
Fri	(%)	60.2	68.8**	94.4***	64.0	65.0	3.7
Glucan	(ppm)	223	162	292	232	547***	77
Visc	(cps)	1.49	1.47	1.54**	1.50	1.59***	0.02
MP	(%)	12.2	12.3	12.0**	12.3	12.4	0.3
SP	(%)	4.3	4.9***	4.4	4.5	4.4	0.1
KI	(%)	35.5	40.0***	36.4	36.4	35.9	0.7
FAN	(mg/L)	157	196***	167*	163	152	10
Colour	(ASBC)	1.60	1.92	1.49	2.22***	1.83	0.08
Colour	(EBC)	3.1	3.8	2.9	4.4***	3.6	0.1
pH		6.00	5.93***	6.10***	6.00	5.99	0.02
DP	(ºL)	122	129	132*	94***	97***	10
Alpha	(DU)	55.8	69.2***	62.7***	55.0	55.3	3.1
AlphaM	(U/g)	44.8	55.2**	52.7**	38.7*	51.5*	3.5
Beta	(U/g)	778	824	709	520***	470***	78
LD	(mU/g)	371	405	400	179***	338	26
AAL	(%)	81.5	83.0**	81.6	79.7***	80.2**	1.0

SE: Standard error of treatment sample mean. Significance level of treatment effect as compared to the Standard, for each quality parameter: (P<0.001) ***; (P<0.01)**; (P<0.05)*

The freeze drying treatment had less effect on malt quality than the GA_3 with no significant effect on protein modification. The major significant effects were with friability and α-amylase because of the reduced effects of kilning. However, wort colour was surprisingly not significantly affected despite an expected drop with no kilning. Furthermore, no fermentability effect was observed although the levels of Fine, FAN and α-amylase activity were increased.

Results from the treatment of higher final kilning temperature were similar to reports in the literature (Karababa et al., 1993; Briggs, 1998; Coghe et al. 2005) with increased wort colours, lower levels of amylolytic enzymes, due to high temperature inactivation, which led to a decrease in fermentability. Although a significant decrease in FAN level

was expected due to increased Maillard reaction, a small increase (non significant) was obtained with this treatment.

The longer kilning treatment, which was twice as long as the Standard schedule, significantly increased β-glucan levels which resulted in higher wort viscosities. Furthermore, amylolytic enzymes were significantly affected by this treatment with increased α-amylase activity but decreased β-amylase activity. Consequently, with high β-glucan levels, low DP and low β-amylase activity, fermentability levels were significantly decreased in value. These results could be explained by a combination of longer kilning times at temperatures below 66°C and above 66°C. With the additional time at lower temperatures, amylolytic and proteolytic activity along with endosperm modification would have been promoted. In contrast additional time at the higher temperature would have inactivated enzymes and stopped endosperm modification. The overall effect would have been a compromise with a decrease in DP and β-amylase but an increase in the more heat stable α-amylase. The significant increase in wort β-glucans could be explained by inactivation of β-glucanase and ß-glucosidase between 70-80ºC (Briggs 1998) and thus their unavailability during mashing. Measurements of β-glucanase and β-glucosidase activities would be necessary to prove this hypothesis. Measurement of malt β-glucan content could also have indicated how cell wall modification had proceeded during germination and the first stages of kilning.

In summary a range of processing conditions was used to produce malts with different quality profiles, some with rich proteolytic modification and others with low amylolytic enzyme activity and poor cytolytic modification. Fermentability was both significantly decreased and significantly increased by different treatments with most effects as expected, although, the lack of effect with freeze drying was unexpected.

4.6 Individual wort amino acid profiles

The UPLC system was used to measure levels of individual wort amino acids in worts made from malts produced with the different malt processing conditions. Levels of individual amino acids were then compared to FAN levels in the worts studied.

4.6.1 Individual wort amino acid profiles among malt barley varieties

Levels of wort amino acids among malt barley varieties and under the Standard malting schedule are shown in Table 4.12. There was considerable range in levels of total amino acids going from close to 1200 ug/ml in hulless malt (McG05) and M936S to over 2000 ug/ml in ACMN and ACMS. Figure 4.14 presents wort amino acid composition in percentage where it can be appreciated that Pro, Gln, Asn and Arg were the most abundant amino acids found in wort but these four amino acids also presented the greatest variation among varieties. None of the other 17 amino acids represented more, individually, than 6% of the total content. The results agree with numbers published by Samaras et al. (2005) who found the same three amino acids, Pro, Gln and Asn, to be most abundant in green, lager and pale malt. On the other hand, Coghe et al. (2005) found Pro, Leu and Arg to be the most abundant amino acids in wort prepared from Scarlett barley, although, malts were prepared in a pilot plant.

It is also important to note that the methodology used in those two studies did differ in several ways from the present study, such as detection and derivitization, although all three studies used the same HPLC principle of separation.

Table 4.12 Wort amino acid profiles among malt barley varieties

Malt	Amino acids (ug/ml)																					
Variety	His	Asn	Ser	Gln	Arg	Gly	Asp	Glu	Thr	Ala	GABA	Pro	Cys	Lys	Tyr	Met	Val	Ile	Leu	Phe	Trp	Total AA
Metc	35.3	115.4	71.0	223.5	110.7	33.6	53.1	44.8	47.1	71.1	47.5	313.0	6.6	66.5	68.1	22.4	86.5	56.9	111.4	91.1	30.8	1706
ACMS	46.6	162.7	79.4	240.8	149.5	37.8	72.1	65.8	56.3	96.0	70.8	329.3	9.1	84.9	76.2	28.3	101.8	65.2	119.5	106.0	41.2	2039
ACMN	46.3	189.2	72.4	237.2	170.5	39.3	76.4	62.8	53.7	87.3	65.0	363.8	11.0	82.9	73.7	27.3	99.8	61.9	110.5	102.8	35.1	2069
M936S	27.4	108.0	51.0	121.2	76.0	23.7	50.0	31.9	32.9	54.0	46.4	218.6	5.3	49.5	47.1	15.9	56.1	35.2	65.8	58.2	32.9	1207
M936N	34.0	110.0	60.5	145.8	96.3	28.5	53.4	36.3	40.9	66.6	58.1	241.9	6.6	61.5	57.4	20.1	69.6	43.4	82.7	69.4	42.5	1425
Cop06	36.6	139.1	69.0	232.9	105.2	34.2	56.0	44.0	48.6	77.7	51.0	316.5	6.3	64.1	66.7	23.0	83.4	54.2	106.5	90.8	31.5	1737
Helg06	34.1	168.7	65.5	192.4	106.2	31.1	55.5	39.5	44.7	71.1	54.8	265.1	5.7	56.0	57.3	20.9	77.7	53.2	90.3	76.6	27.5	1594
Helg05	33.6	140.7	68.7	199.1	111.1	32.2	61.2	35.8	46.2	74.4	61.5	258.5	5.5	63.1	63.2	22.7	79.3	53.7	98.3	84.7	28.4	1622
McG06	31.8	126.1	54.0	99.9	106.4	24.4	44.4	72.1	32.8	59.7	36.4	244.4	5.0	55.1	47.3	14.3	60.3	34.1	67.5	64.6	25.8	1306
McG05	27.5	89.0	49.1	132.3	89.7	25.3	31.9	32.1	31.0	54.6	40.4	250.0	6.1	48.5	45.8	14.6	54.7	33.3	66.7	66.1	23.0	1212
Std	1.9	4.6	2.5	7.0	3.4	1.1	2.1	1.3	1.8	3.2	2.6	16.8	nd	2.9	2.7	0.9	3.6	2.1	4.5	3.6	1.6	70.5
Mean	35.3	134.9	64.1	182.5	112.2	31.0	55.4	46.5	43.4	71.3	53.2	280.1	6.7	63.2	60.3	21.0	76.9	49.1	91.9	81.0	31.9	1592
CV	5.3	3.4	3.9	3.8	3.1	3.6	3.8	2.7	4.2	4.5	5.0	6.0	nd	4.6	4.6	4.4	4.6	4.3	4.8	4.4	4.9	4.4
Max	46.6	189.2	79.4	240.8	170.5	39.3	76.4	72.1	56.3	96.0	70.8	363.8	11.0	84.9	76.2	28.3	101.8	65.2	119.5	106.0	42.5	2069
Min	27.4	89.0	49.1	99.9	76.0	23.7	31.9	31.9	31.0	54.0	36.4	218.6	5.0	48.5	45.8	14.3	54.7	33.3	65.8	58.2	23.0	1207

Std: Standard deviation of the method

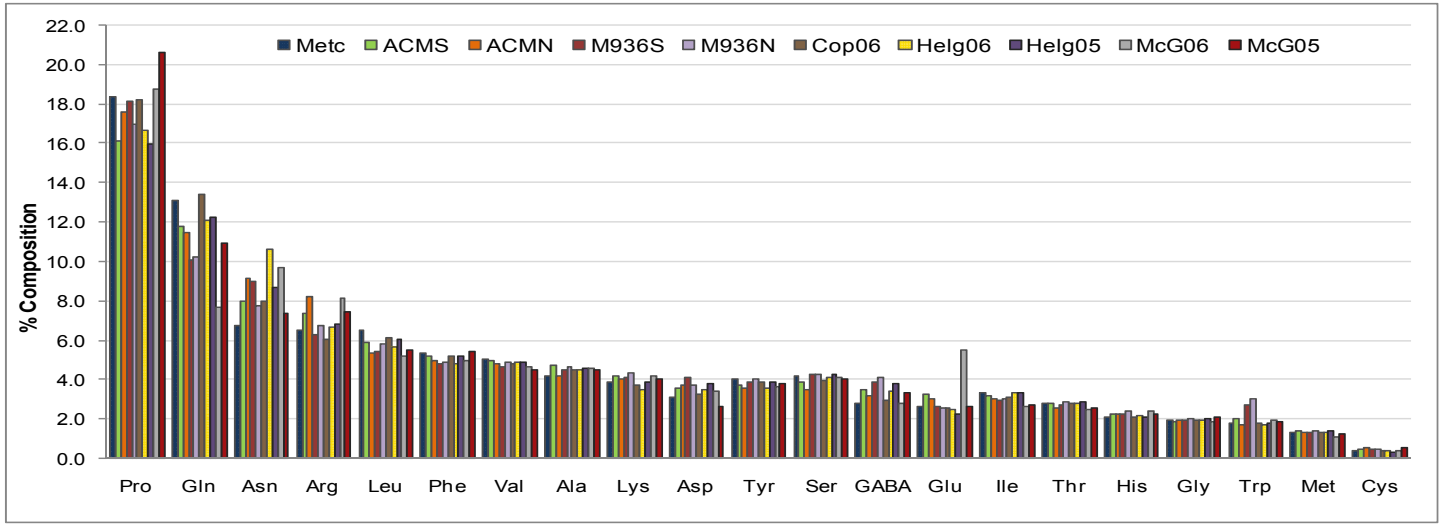


Figure 4.14 Variation of wort amino acid composition among malt barley varieties.

4.6.2 Individual wort amino acid profiles among malt processing treatments

Three malt varieties, with contrasting levels of amino acids, were selected to investigate effects of malting treatment on amino acid composition (Fig. 4.15). The figure shows that Pro, Gln, Asn and Arg were the amino acids that varied the most among treatments, with the same tendency observed for each of the three malt varieties. These results, in conjunction with previous data (Fig 4.14), suggested that levels of these four amino acids could be used to represent variability of all the amino acids, thus, simplifying the study of genotypic effects in breeding programs and, of malt quality as affected by processing conditions. Future work on genetic mapping association of wort amino acids in populations of doubled haploid lines, for example, could be significantly simplified by considering levels of just these four amino acids.

Table 4.13 Treatment means, across the eight covered malts, of free amino acid wort levels including the statistical significance of each treatment as compared to the Standard

Amino	Treatment samples mean (ug/ml)					
Acid	Standard	Add GA_3	Freeze Dry	Higher KilnFT	Longer Kiln	SE
Ala	74.8	86.1***	64.6**	72.4	61.0***	4.2
Arg	115.7	134.7***	116.5	111.4	113.5	9.7
Asn	141.7	157.4**	108.7***	147.8	130.4*	9.1
Asp	59.7	65.4*	52.8**	56.8	55.7	3.2
Cys	7.0	7.3	8.8**	0.0	3.9***	0.7
GABA	56.9	61.2	67.2***	49.2**	59.4	2.5
Gln	199.1	260.5***	282.4***	177.1*	198.1	17.5
Glu	45.1	49.5	36.7**	38.4**	28.0***	4.2
Gly	32.6	35.5*	27.0***	29.7*	28.7***	1.6
His	36.7	41.3	45.3**	37.2	46.6***	2.3
Ile	53.0	67.7***	62.4***	51.3	52.7	3.8
Leu	98.1	128.5***	121.6***	96.3	99.3	7.0
Lys	66.1	75.6**	80.1***	60.1*	60.1*	4.2
Met	22.6	27.8***	26.7***	22.3	22.3	1.4
Phe	85.0	111.3***	98.5**	82.5	86.6	6.3
Pro	288.3	357.5***	306.9	282.8	354.2***	19.3
Ser	67.2	76.3**	86.2***	60.6*	65.5	3.5
Thr	46.3	54.8***	52.1**	44.2	43.9	2.5
Trp	33.7	38.5***	35.1	32.3	33.0	2.0
Tyr	63.7	80.6**	71.9**	61.4	62.3	3.7
Val	81.8	100.2***	96.2***	82.3	78.4	5.6
Total AA	**1675**	**2017*** **	**1848****	**1596**	**1684**	**96**

SE: Standard error of treatment sample mean; ns: No significant; (P<0.001) ***; (P<0.01)**; (P<0.05)*.

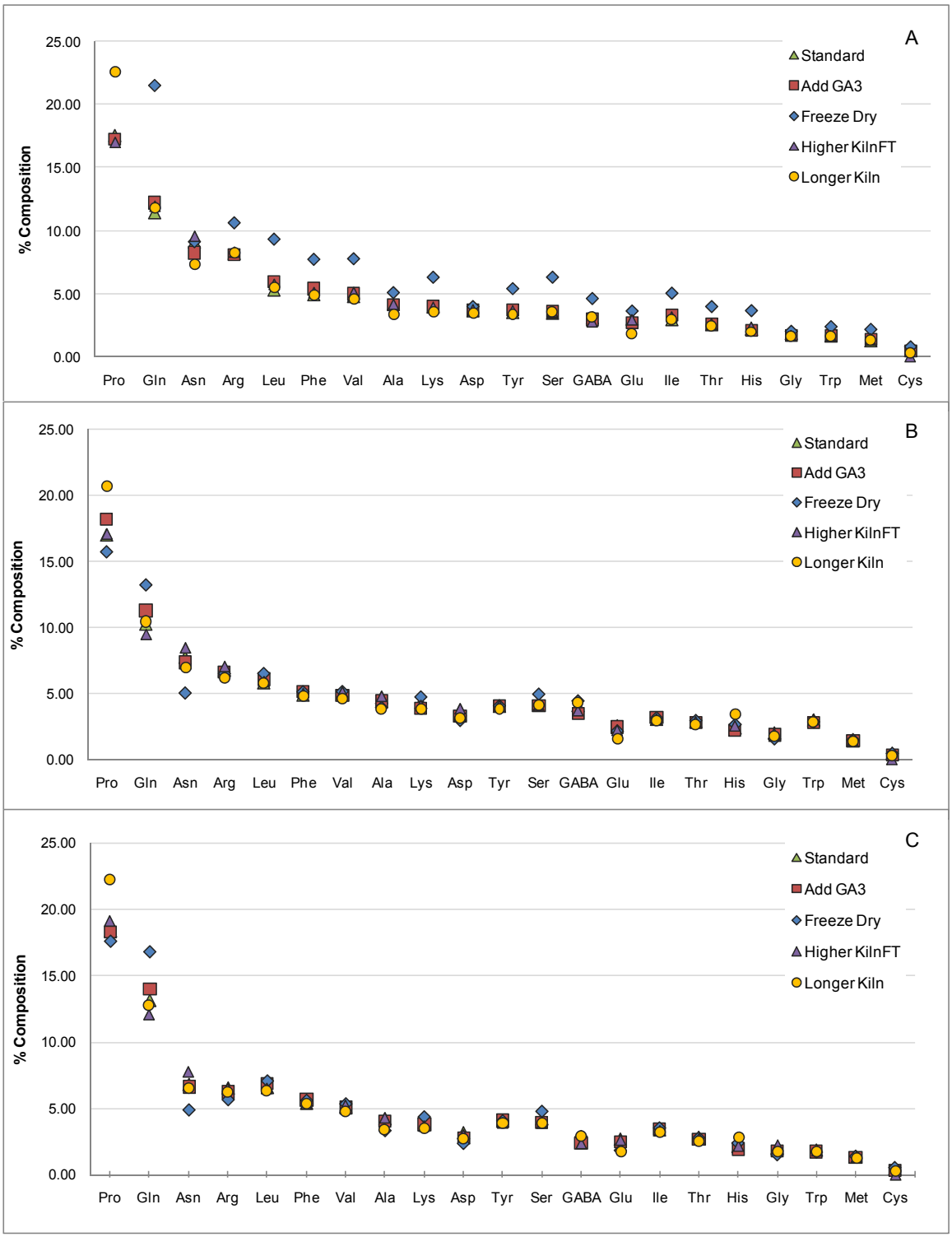


Fig. 4.15 Variation of wort amino acid composition from three different barley varieties malted under different malting conditions. A: ACMN; B: M936N; and C: Metc

Mean levels of individual amino acids, as affected by treatment, are compared to the Standard in Table 4.13. The GA_3 and freeze drying treatments had the greatest effect with significant effects on the majority of amino acids. However, with GA_3 all the significant changes were positive, while with the freeze drying treatment differences were both positive and negative. This variation in effects of the freeze dried treatment was a reflection of two competing forces that occur during kilning, to which the freeze dried samples were not exposed. During the lower temperature stages of kilning, endosperm modification continued, releasing additional amino acids. However, during the later stages of kilning, when higher temperatures were encountered, levels of free amino acids would have decreased due to their participation in the Maillard reaction. As well not all amino acids would have been affected to the same extent by the Maillard reaction. Freeze dried samples would have had no further endosperm modification after germination, thus lowering amino acid levels, but they also would not have been exposed to Maillard conditions leading to increased levels of some amino acids. So, variation in treatment effect on amino acids was not unexpected as some amino acids were released and some were consumed or tied up.

The higher final kilning temperature treatment only affected six amino acids significantly but all six levels were lower than levels with the Standard treatment which would be expected given higher kilning temperatures should promote the Maillard reaction. The longer kilning time treatment, in contrast, showed increased levels of certain amino acids, His and Pro, and significant decreased levels of other amino acids, Ala, Asn, Cys, Glu, Gly and Lys, as compared to the Standard. The reason for these inconsistencies, both increase and decrease in levels of amino acids, was likely similar to the effect seen with the freeze dried treatment where there was a compromise between increased release of amino acids with increased times at low temperatures and increased opportunities for Maillard reactions with the increased times at higher

temperatures. Interestingly the two treatments, freeze drying and longer kilning, had opposite opportunities for the Maillard reaction with the freeze drying not exposed to any conditions favouring the reaction while the longer kilning treatment was exposed to more conditions for promoting the Maillard reaction. This was demonstrated with Lys, the amino acid most susceptible to the Maillard reaction, which had significantly increased levels, compared to the Standard, with the freeze drying treatment and significantly decreased levels with the longer kilning treatment. Overall, however, effect for both of these treatments would have been a balance of increases and decreases of particular amino acids depending on specificity of proteases and susceptibility to the Maillard reaction. The total amino acid level may not be affected because of the plus and minus effect, which was observed with both kilning treatments where total amino acid content was similar to the Standard treatment. In contrast the freeze drying treatment showed a significant increase in total amino acids which could be explained by this treatment having no opportunity for the Maillard reaction.

Levels of all individual amino acids in worts were summed and compared among treatments and barley samples to detect any general tendencies brought about by malting treatment (Fig 4.16). Addition of GA_3 increased the total amino acid content across varieties; however, the extent of the increase differed among varieties. It was surprising, for example, that M936S and M935N were less affected by GA_3 than most other varieties despite having the lowest levels of amino acids under the Standard treatment. The results suggested that secretion of GA_3 was not the limiting factor to the release of free amino acids during germination.

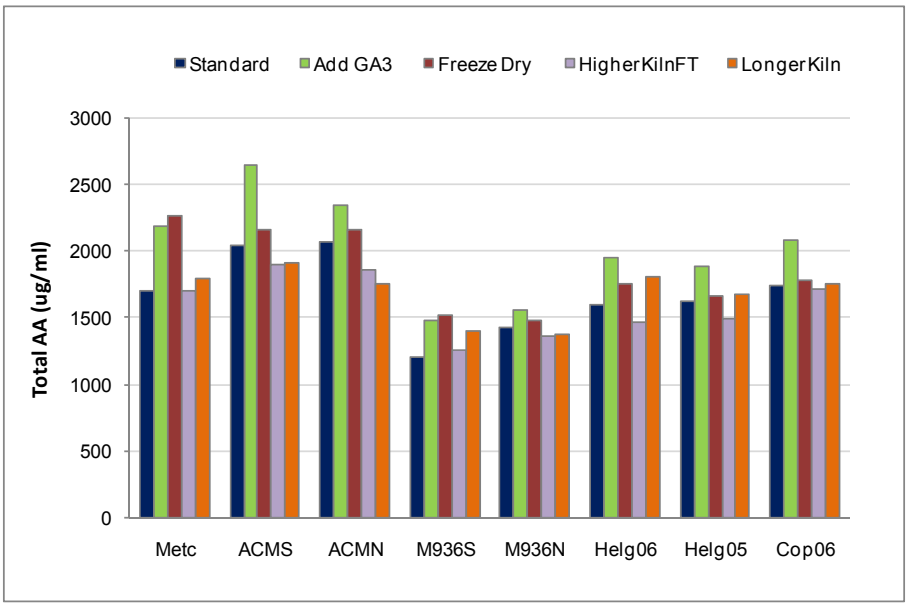


Fig. 4.16 Variation of total wort amino acid content under different malting conditions among malted barley varieties.

The freeze drying treatment increased total amino acids in the majority of varieties but not in Cop06 and Helg06, However, Helg05, the same variety as Helg06 but grown in different year, had increased levels of amino acids with freeze drying which could not be explained. As expected the hulless barley varieties (McG06, McG05) were most affected by the freeze drying treatment. Reports in the literature (Edney et al., 2004) suggest hulless barley is more susceptible to heat during kilning which could limit protease activity or increase vulnerability to the Maillard reaction. Freeze drying would have removed the effects of high temperatures at kilning leading to higher levels of amino acid either through greater release or less breakdown by the Maillard reaction.

The two kilning treatments were found to show the greatest variation among genotypes in their effects on total amino acid levels. In general the higher final kilning temperature and longer kilning treatments had limited effects on levels of total amino acids, although, both increased and decreased levels were observed among varieties, and even within varieties grown in different locations. A possible reason for differences

was the balance between increased release of amino acids and increased susceptibility to Maillard as discussed at the top of the page.

In summary, Pro, Gln, Asn and Arg were the most abundant amino acids found in wort, and they showed the greatest variability among varieties. Glycine and Lysine were the only two amino acids affected by all malting treatments. Tauer et al. (2004) did establish the importance of Lys and Gly in the formation of Maillard products which helps explain the variation of these amino acids in kilning treatments. Tauer et al also indicated the potential for Maillard products from Lys and Gly to cause an inhibition of ethanol formation further emphazising the importance of the observed variation. Lysine, as well, is one of the essential amino acids so its variation was of even greater commercial significance. According to Jones and Pierce (1967a) Lys was classified as an essential amino acid (class 3) for fermentability, as deficiencies of this amino acid in wort may result in major changes in nitrogen metabolism of yeast, affecting the final quality of the beer. Gly was classified as critical (class 2) since in the latter stages of fermentation the major sources of their carbon skeletons came from the synthesis of the keto analogues from sugars in the medium.

In contrast, Gln, Asn, Ser, Glu and Ala, which were affected by three of the four treatments, were all classified as class 1 amino acids. The initial concentration of class 1 amino acids in wort are not critical since the by products of their synthesis are being produced in significant quantities at the end of the fermentation. As a result the variation of these five amino acids was not considered a concern.

4.6.3 Individual wort amino acid profiles versus FAN

Total AA content and FAN (Fig 4.17) were found to be highly associated as would be expected given they both measure free amino acids in wort. The coefficient of determination with the greatest significance was with the GA_3 treatment with an R^2 of

0.9963, followed by higher final temperature (0.975); standard treatment (0.907); longer kilning treatment (0.888) and finishing with the freeze drying treatment (0.876).

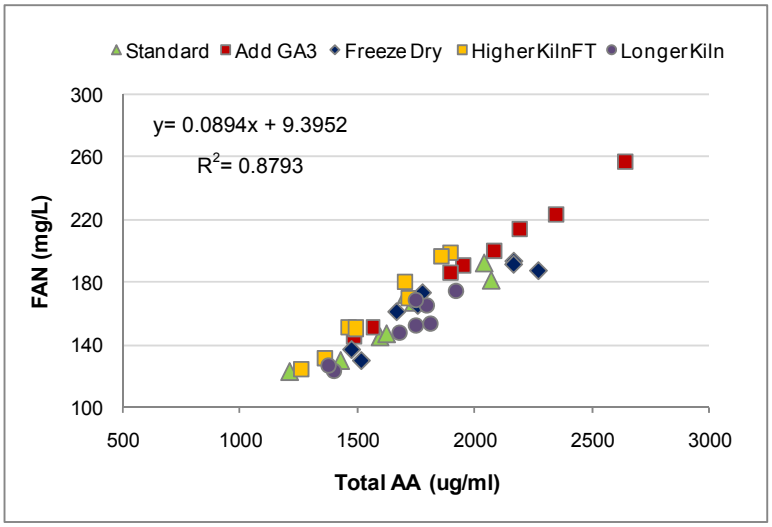


Fig. 4.17 Relationship of total free amino acid content and FAN among malting treatments.

However, when total AA content were compared to FAN with individual varieties, the patterns for each variety among treatments presented differences (Fig. 4.18). One reason for discrepancies could be that FAN quantifies free amino acids, ammonia and peptides, where total AA content quantifies only free amino acids and ammonia, although in some cases differences were too high to be only related to peptide content.

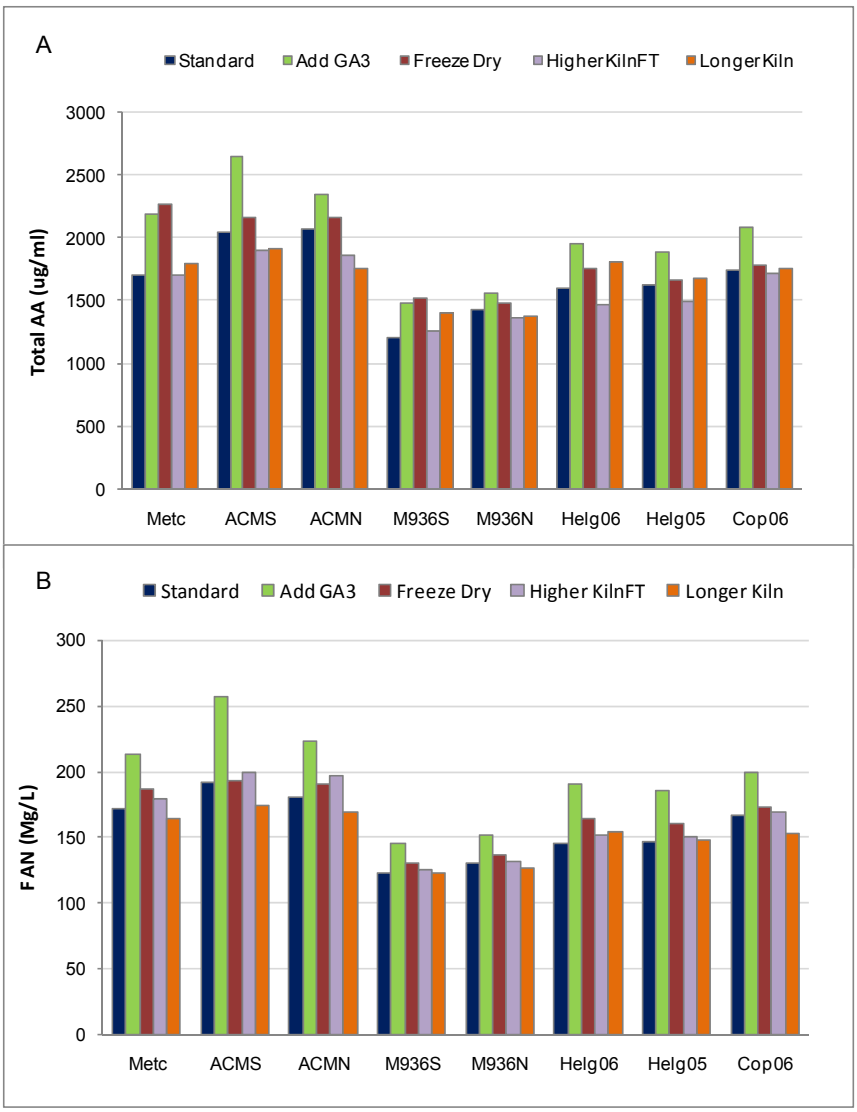


Fig. 4.18 A: Total free AA content among malt varieties. B: FAN levels among malt varieties.

In comparing individual amino acids to levels of FAN, Gly was found to have a coefficient of determination across all treatments (Fig. 4.19) that was not as high as that obtained with total AA content and FAN (Fig. 4.17). In looking at treatment effects (Fig. 4.20) it can be observed that GA_3 gave the highest correlation value between Gly and FAN with a positive linear relationship. Longer kilning treatment also presented a positive linear relationship, although the correlation value was not as high as with the GA_3 treatment.

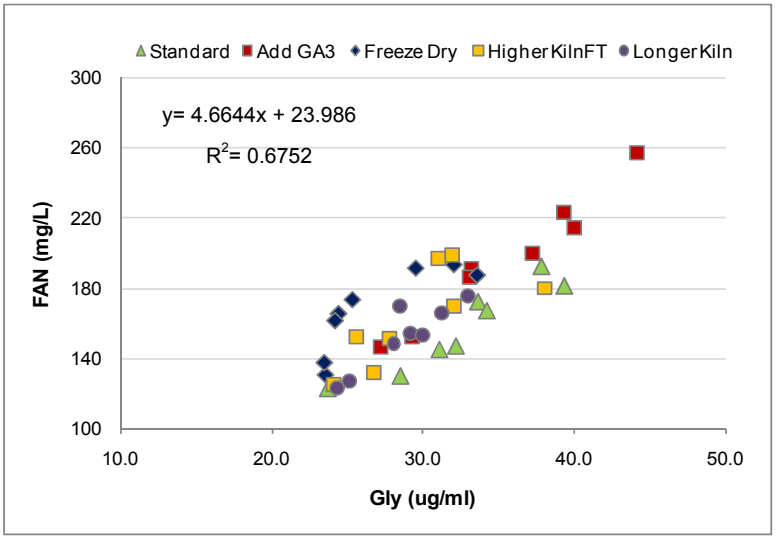


Fig. 4.19 Relationship of FAN and Gly among malting treatments

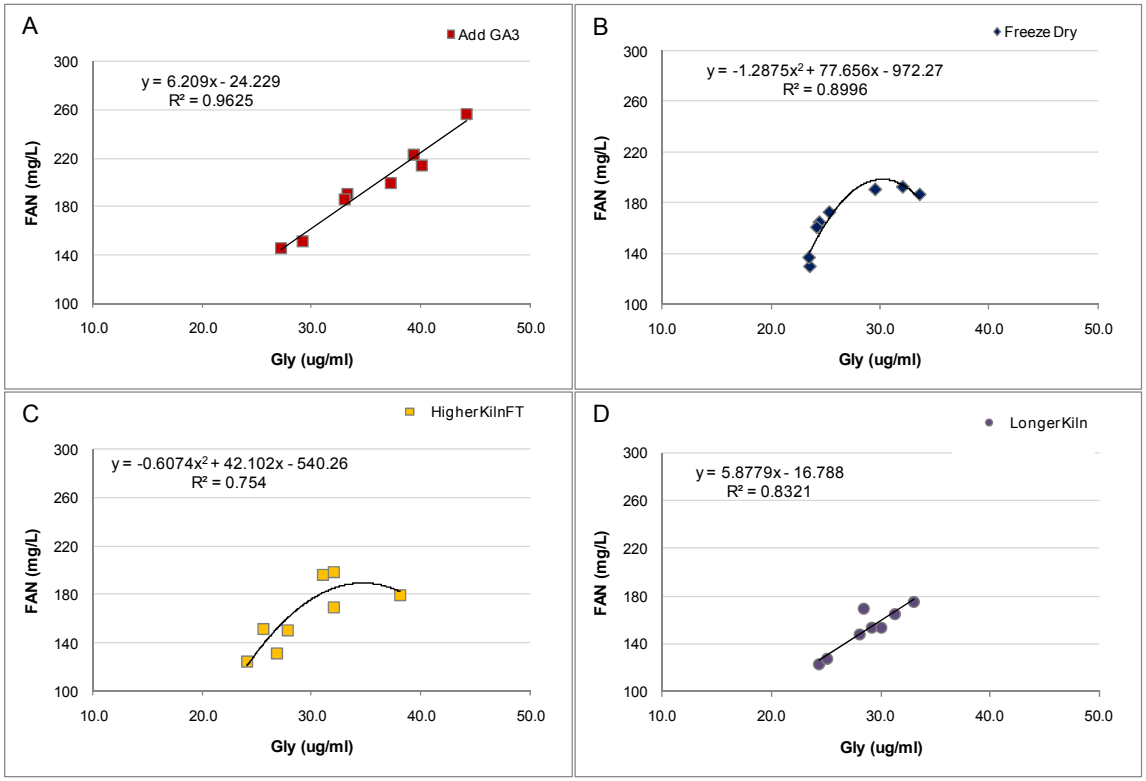


Fig. 4.20 Relationship of FAN and Gly in each malting treatment among varieties. A: GA_3; B: Freeze drying; C: Higher kilning final temperature; and D: Longer kilning

On the other hand, the freeze drying and higher final kiln temperature treatments presented polynomial grade 2 relationships between FAN and Gly. As the number of

samples was small, more samples should be assayed under the same conditions to verify the no linear relationships.

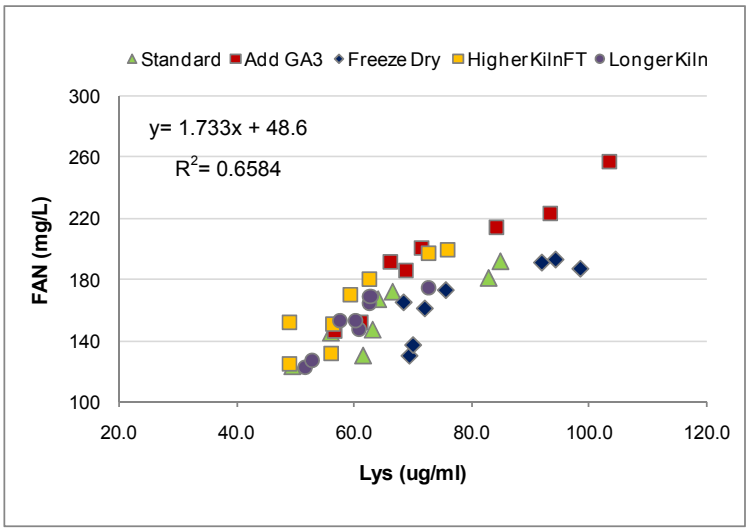


Fig. 4.21 Relationship of FAN and Lys among malting treatments

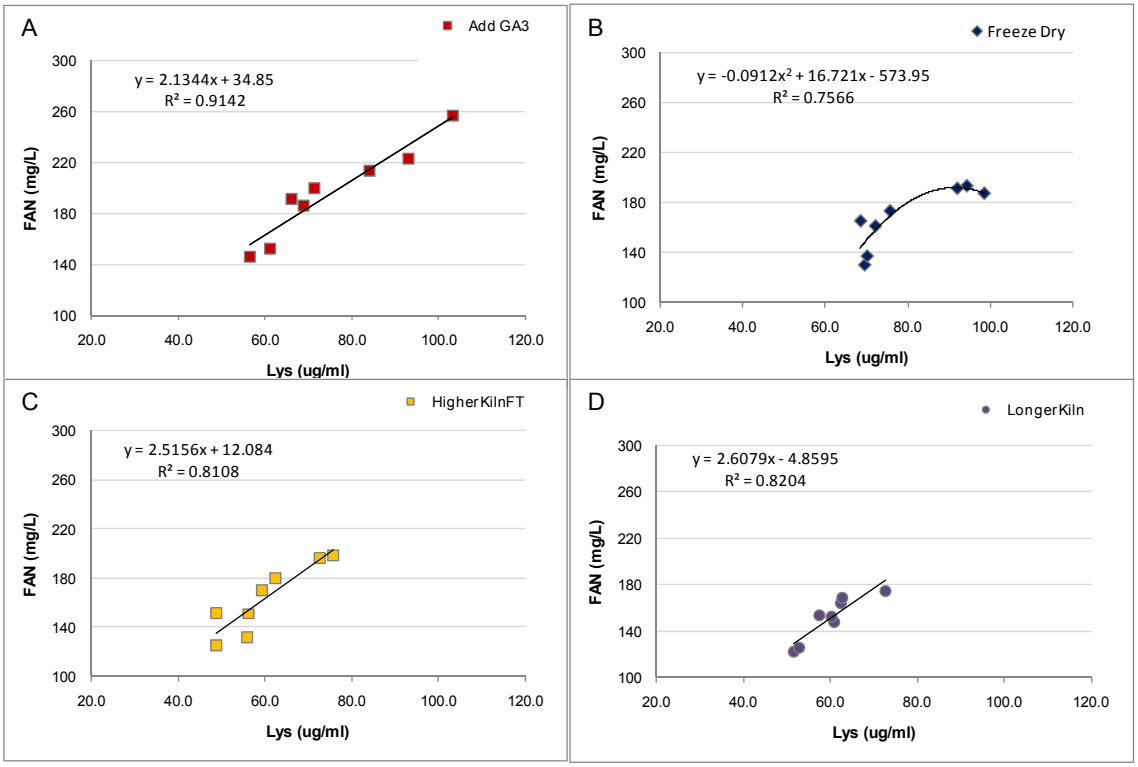


Fig. 4.22 Relationship of FAN and Lys in each malting treatment among varieties. A: GA_3; B: Freeze drying; C: Higher kilning final temperature; and D: Longer kilning

In the case of Lys the correlation value obtained in relation to FAN (Fig. 4.21) was even lower than that obtained with Gly. In Fig 4.22 it can be observed that only the freeze drying treatment presented a polynomial relationship with FAN.

In summary, Gly and Lys were associated with FAN levels although in some treatments the relationship was not linear. The lack of linearity suggested a need for the monitoring of Lys, rather than FAN, given the importance of this amino acid for fermentability.

4.7 Endoprotease activities among malt barley varieties and malt processing conditions

Endoprotease activities in the malts of the 10 barley samples, as malted with the Standard schedule, were found to vary both among the varieties and within varieties coming from different environments (Table 4.14). Metc produced the highest activity while the hulless barley (McG06, McG05) producing the least activity. Differences in endoprotease activity based on growing location were sometimes greater (ACMN versus ACMS) than differences among varieties, but some varieties were more consistent across growing locations (M936N versus M936S).

Effects of treatment on endoprotease activity are summarized in Table 4.15. Treatment means were based on only the 8 covered malt samples as statistical analysis had indicated hulless results were too extreme for inclusion. Supplementation with GA_3 significantly increased endoprotease activity, compared to the Standard, due to the promotion of enzyme synthesis by GA_3 during germination. The significant effect of freeze drying on endoprotease activity was related to the avoidance of high temperatures during kilning and the associated inactivation of enzymes. On the other hand, the treatments of high final kilning temperature and longer kilning times had no

significant effects, agreeing with previous studies (Jones et al., 2000; Osman, 2002 and Kihara et al., 2002).

Table 4.14 Malt endoprotease activity among the 10 barley varieties as malted with the Standard schedule.

Endoprotease activity	
Variety	**(mU/g)**
Metc	700
ACMS	693
ACMN	561
M936S	643
M936N	682
Cop06	536
Helg06	486
Helg05	549
McG06	372
McG05	400
Std	14.0
Mean	562.2
CV	2.5

Std: Standard deviation of the method

Table 4.15 Statistical comparison of malt endoprotease activity of the four experimental malt schedules and the Standard schedule. Values averaged across the eight covered barley varieties

	Samples mean
Treatment	**Endoproteases (mU/g)**
Standard	606
Add GA_3	677***
Freeze Dry	671***
Higher KilnFT	621
Longer Kiln	613
SE	25

SE: Standard error of treatment sample mean;
significance level: (P<0.001) ***; (P<0.01)**; (P<0.05)*.

The effects of treatment on endoprotease activity of individual barley samples are illustrated in Fig 4.23. The varieties tended to show similar tends with respect to treatment effect except for the hulless samples. These samples (McG06 and McG05) were more severely affected by higher kilning temperature, showing proportionally greater increase in endoprotease activity with freeze drying. This apparent effect of kilning on enzyme activity supports the idea that enzymes in hulless barley are more susceptible to heat inactivation because of the lacking insulation of a hull during kilning.

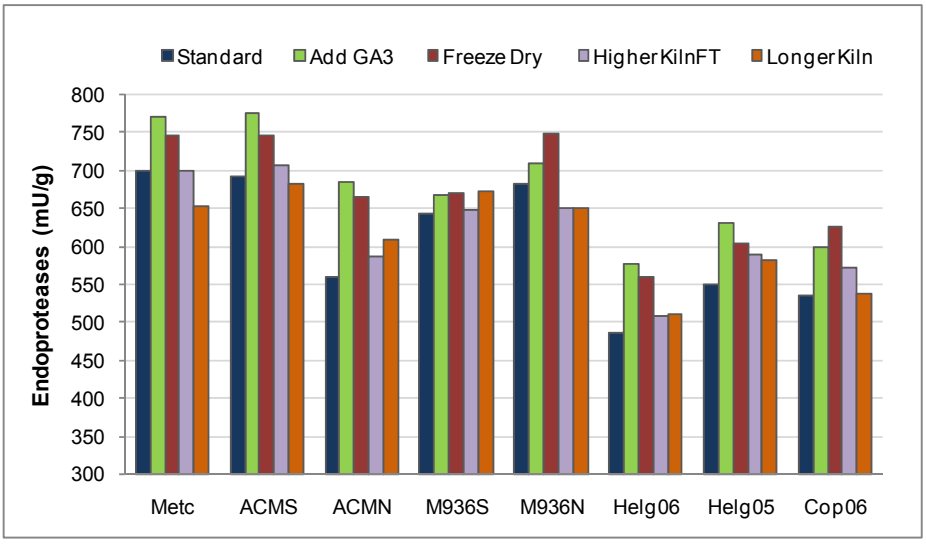


Fig. 4.23 Malt endoprotease activity among barley varieties malted under different malting treatments.

The association of endoprotease activity and malt quality was examined with correlation analysis. Results (Table 4.16) supported the expected association between Kolbach Index and endoprotease activity. Kolbach Index is an indicator of proteolytic modification which should be closely associated with endoproteases and protein solubilization. The effect was only found under addition of GA_3 and freeze drying treatment, agreeing with previous results published by Kihara et al. (2006). Furthermore,

Table 4.16 Correlation values (r) between endoprotease activity and malt quality parameters under different malting treatments.

	Treatments				
Parameter	**Standard**	**Add GA**	**Freeze Dry**	**Higher Kiln**	**Longer Kiln**
Yield	0.20	-0.01	0.62	0.15	0.46
SOMoi	0.42	0.67	0.49	0.54	0.59
Moi	0.17	0.51	0.48	0.17	0.66
Fine	0.06	-0.11	0.06	-0.15	0.24
Fri	0.59	0.57	0.26	0.38	0.43
Glucan	-0.54	-0.61	-0.69	-0.48	-0.37
Visc	-0.32	-0.22	-0.42	-0.30	-0.22
MP	-0.30	-0.16	-0.32	-0.22	-0.44
SP	0.11	0.29	0.21	0.15	-0.29
KI	0.55	0.79*	0.79*	0.45	0.53
FAN	0.10	0.40	0.11	0.19	-0.09
Colour	-0.46	0.89**	0.45	-0.48	-0.49
pH	-0.23	-0.53	0.20	-0.12	0.08
DP	-0.12	0.03	-0.17	-0.02	-0.37
Alpha	-0.21	0.04	-0.24	-0.07	-0.56
AlphaM	-0.14	-0.10	-0.31	-0.18	-0.50
Beta	0.09	0.17	-0.09	0.08	-0.31
LD	0.43	0.32	0.10	0.07	-0.03

Level of significance: (P<0.001) ***; (P<0.01)**; (P<0.05)*

wort colour under GA_3 treatment also showed a strong association with endoprotease activity. On the other hand, higher FAN and SP correlation values were expected as had been previously published (Jones and Budde, 2005). However, the associations reported in the literature were based on endoprotease activity as measured in wort extractions after traditional mashing in contrast to the present study where specialised extracts of malt were used. Furthermore, the methodology applied to quantify endoproteases also differed with respect to substrate and reaction conditions.

In summary, malt endoprotease activities were found to vary significantly among varieties and with some processing conditions, specifically the GA_3 supplementation and freeze drying treatments. Furthermore, highly significant associations between malt endoprotease activity and KI were observed under the GA_3 and freeze drying treatments.

4.8 Changes in the endoprotease activities and amino acid profiles during the malting process

Changes in endoprotease activity and amino acid profiles were followed during the malting process by removing sample at specific points during the process in a Phoenix micro-maltings. Activity was monitored at 12 points during the malting process (Fig. 4.24). Three covered barley samples were selected from the eight studied, for use in this experiment. The samples were selected based on their malt quality, especially FAN level, with a range in quality desired. The ACMN and M936N samples were selected for their contrasting malt quality, while Metc was selected as a malt check that has been widely grown and studied in Canada.

4.8.1 Endoproteases activities during malting

Endoproteolytic activity in the raw barley was shown to be quite low; however, differences were obtained among samples. Endoproteolytic activity continued to remain low at the end of the second steep (Fig. 4.24), but began to increase during the first day of germination due either to *de novo* formation and/or activation. Maximal activity was observed at day 3 for the ACMN and M936N samples, and after day 4 with the Metc sample. A marked reduction in endoproteolytic activity was observed in samples taken on completion of the 55° kilning step. Further samples taken during kilning showed only minor changes in activity. These results are in accordance with published results (Jones et al. 2000, Jones 2005a).

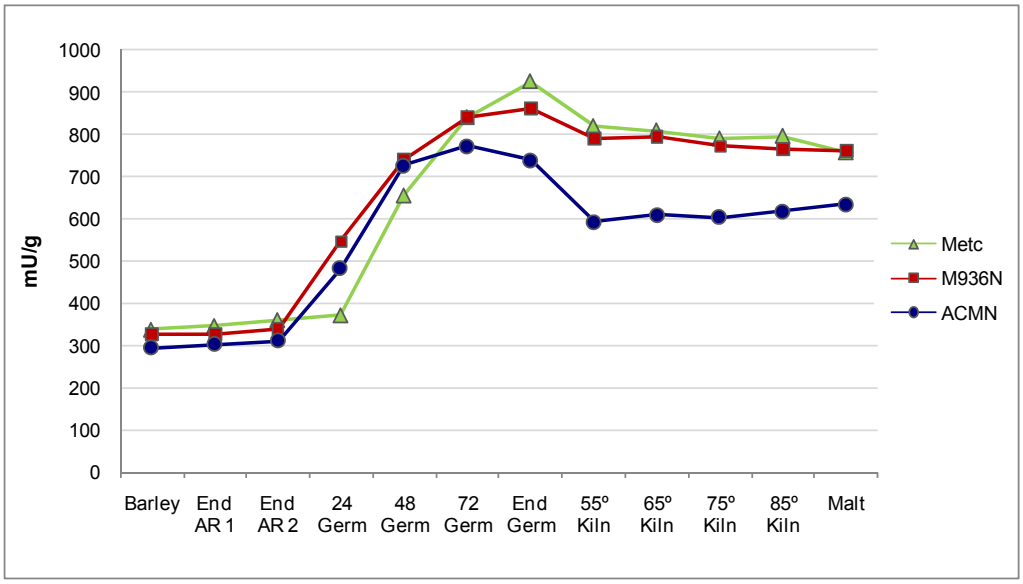


Fig. 4.24 Endoprotease activities of three barley varieties as sampled during the malting process.

4.8.2 Amino acids during malting

Table 4.17 indicates levels of individual free amino acids in samples of the three varieties as removed during the malting process. Total amino acid content was found to vary significantly between ACMN and the other two varieties in the barley grain and in samples taken after steep 1 and 2. Individual amino acid profiles in barley and steeped

samples are illustrated in Fig 4.25. Asparagine was found to be the most abundant free amino acid in the barley samples of all three varieties assayed, although levels varied among the varieties. In contrast His and Cys were not detected in the barley or the steeped barley of any varieties. Met was also not detected at all in Metc and only appeared afterAR1 in ACMN and after AR2 in M936N. Arg and Ile only appeared in the AR2 samples of both Metc and M936N while Thr appeared in both at the AR1 stage. Tyr was first seen after the AR1 in M936N and after AR2 in Metc. Phe was not detected in Metc until the end of AR1. Gln was not detected in the barley of M936N, despite being a significant free amino acid in the ACMN barley. Pro content was also very low in M936N and Metc compared to a high content in ACMN. However, Pro levels did double in both varieties by the end of AR2, whereas the level of free Pro in ACMN changed little during steeping.

A massive degradation of reserve proteins apparently occurred during germination leading to significant increases in free amino acid content of green malt compared to the raw barley (Table 4.17). Total amino acid patterns showed similar changes during germination for both ACMN and M936N. There was a quick increase at the start of germination followed by a limited increase in free amino acids towards the end of germination. Metc, in contrast, showed low levels at the beginning of the

Table 4.17 Total amino acid content of three barley varieties as sampled during the steeping and germination phases of malting.

Total amino acid content (ug/ml)			
	ACMN	**M936N**	**Metc**
Barley	497	175	157
Steeping			
End AR1	559	250	215
End AR2	604	410	364
Germination			
24hr. Germ	1009	729	701
48hr. Germ	1348	999	1076
72hr. Germ	1656	1090	1513
End Germ	1645	1134	1595

Steeping stages: End AR1 (end of air rest one) and End AR2 (end of air rest two). End of Germ: 96 hr germ. Standard deviation of the method: 51 ug/ml.

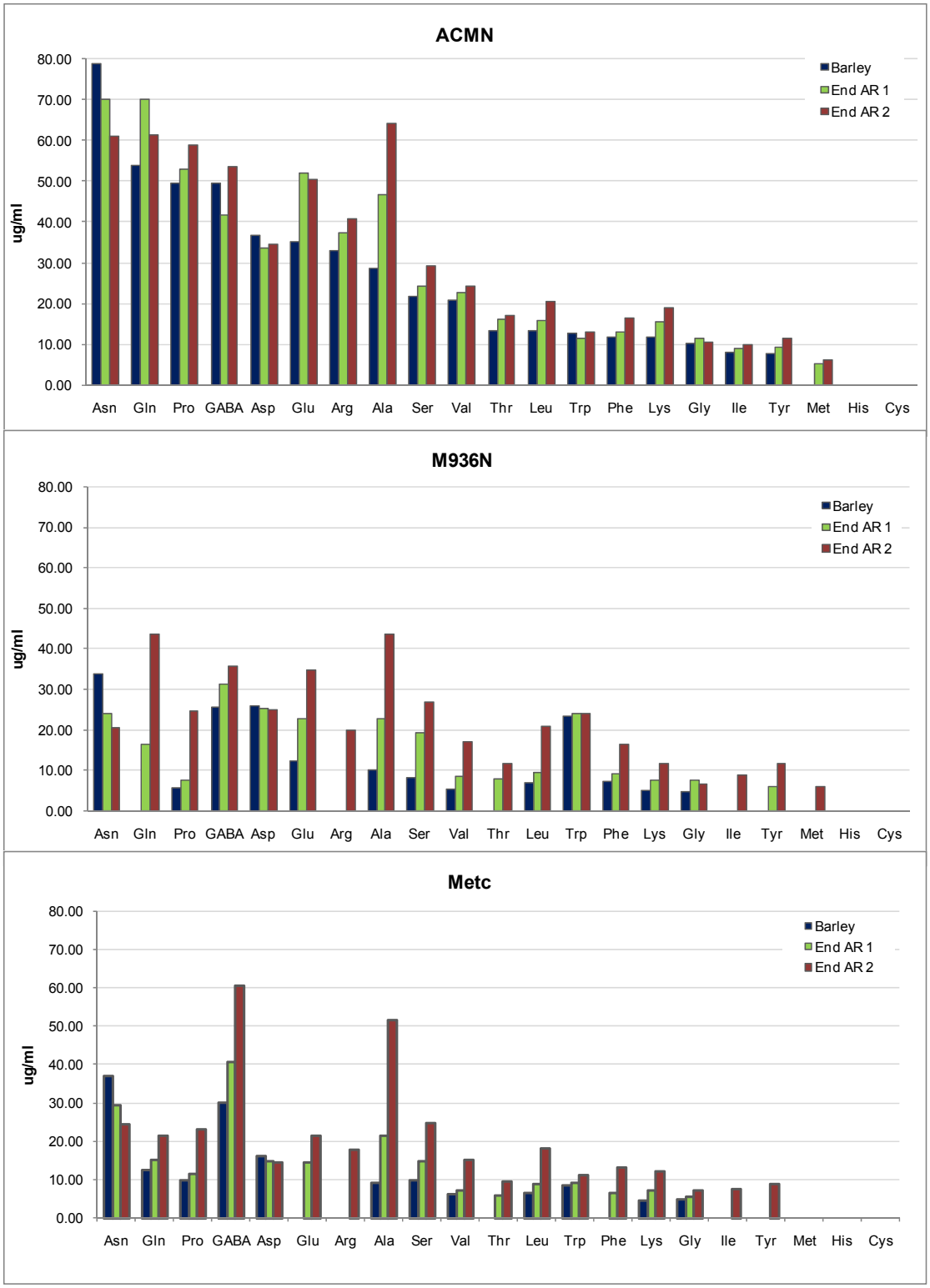


Fig. 4.25 Free amino acid profiles for raw barley and two steeping stages (AR1 - end air rest one; AR2 - end of air rest 2). The three varieties are illustrated.

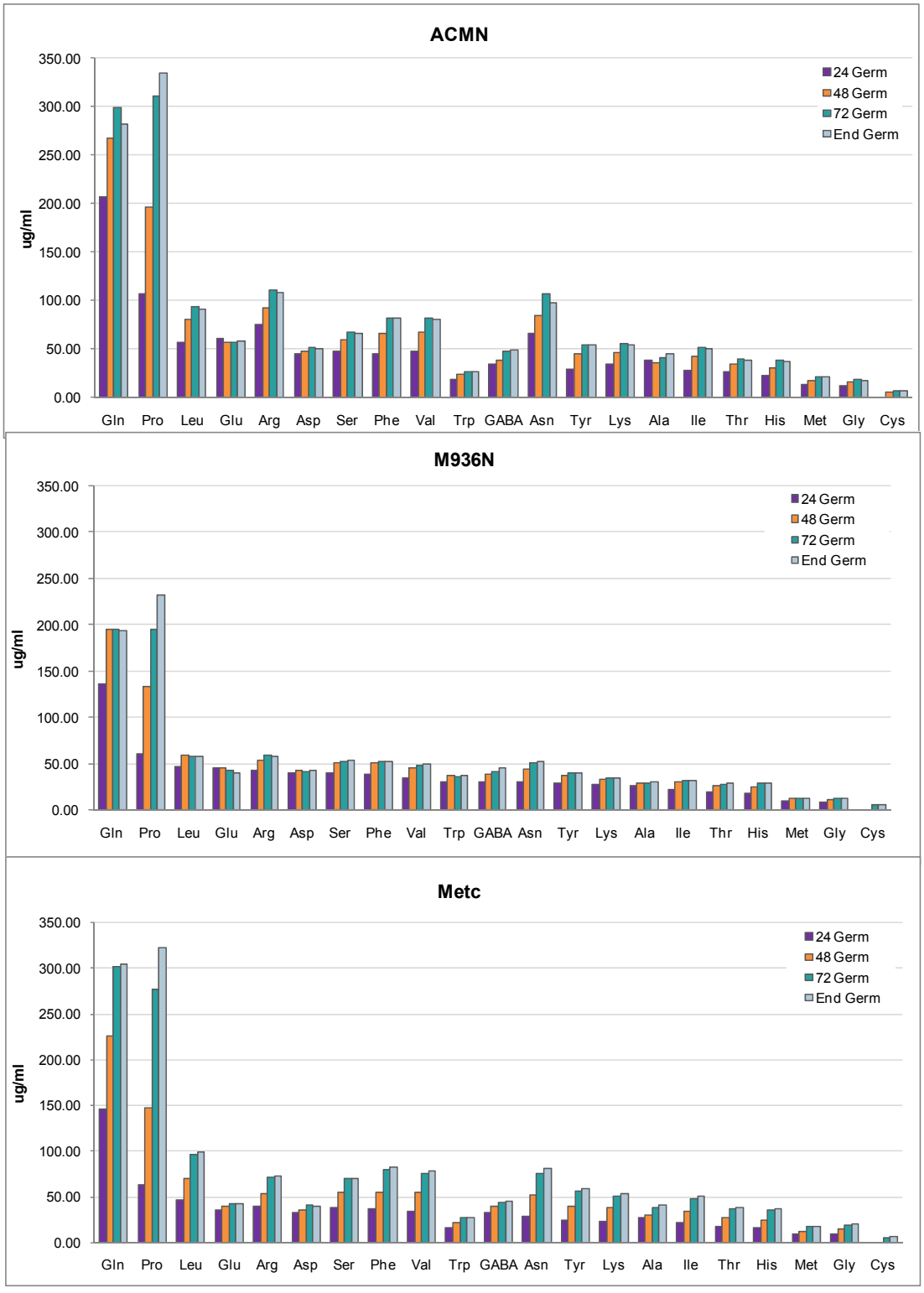


Fig. 4.26 Changes in free amino acid profiles during the four days germination.

process followed by a marked increase during the second and third day of germination, eventually demonstrating this variety's great potential for protein modification.

The three varieties all showed increased levels, to some extent, of all the individual free amino acids as germination proceeded (Fig 4.26). Gln and Pro were the most abundant amino acids at the start of germination with the abundance of both becoming more pronounced as germination proceeded. Interestingly, the Pro increase continued steadily throughout germination while the increase of Glu began to level off towards the end and even decreased in the ACMN and M936N samples. Cys only began to be detected after the second day of germination in ACMN and after the third day in M936N and Metc. The levels of several amino acids remained relatively constant during germination (Glu, Asp, Gly) across varieties.

The general amino acid profiles did vary among the varieties. Mect and ACMN profiles showed a steady increase for the majority of the amino acids while, in contrast, M936N showed an increase in levels of several amino acids (Gln, Ser, Lys, Tyr, Ile, Leu and Trp) during the first day of germination but their values then remained constant for the duration of germination. The difference among varieties could be related to malt modification performance, as M936N, with the lowest levels of amino acids, also showed the worst malt modification performance, as indicated by high wort β-glucan content and low DP.

Table 4.18 Changes in Total amino acid contents of three barley varieties during kilning process.

Total amino acid content (ug/ml)			
	ACMN	**M936N**	**Metc**
55ºC	1866	1187	1882
65ºC	1886	1103	1745
75ºC	1750	1157	1619
85ºC	1751	1150	1651
Malt	2039	1059	1742

Standard deviation of the method: 51 ug/ml.

Kilning

Samples removed during kilning showed increased levels of total amino acid content

during the first stage of kilning, up to 55ºC, followed by a gradual decrease (Table 4.18). A marked decrease was expected, especially with curing temperatures (>82ºC), where Maillard reactions were expected. However, data showed an increase during the final two hours at 85ºC. The only explanation was related to endoprotease activities which did not decrease during kilning process and in fact increased in some varieties (ACMN). Although no direct relationship between free amino acid content and endoprotease activity was demonstrated, endoproteolytic enzymes that remained active during the kilning process would be expected to promote free amino acid production. To prove that the observed increase of total amino acid content in malt was not related with moisture content of the samples, calculations of total amino acid content in dry basis was done and similar results were obtained.

Fig. 4.27 illustrates changes in levels of all the individual amino acids during the kilning process, going from green malt to finished malt. Only two amino acids, (Gln and GABA) showed a general trend, across varieties, of decreasing as kilning advanced. Arg was the only amino acid that showed an increase in concentration among all varieties as kilning progressed. Levels of several amino acids (Pro, Asn, Glu) showed variable levels across the kilning times and across varieties.

The Metc and ACMN presented a similar profile of amino acid change across the kilning process while the profiles for M936N showed limited change in levels of most amino acids during the kilning process. This situation might have been due to the poor endosperm modification associated with this variety.

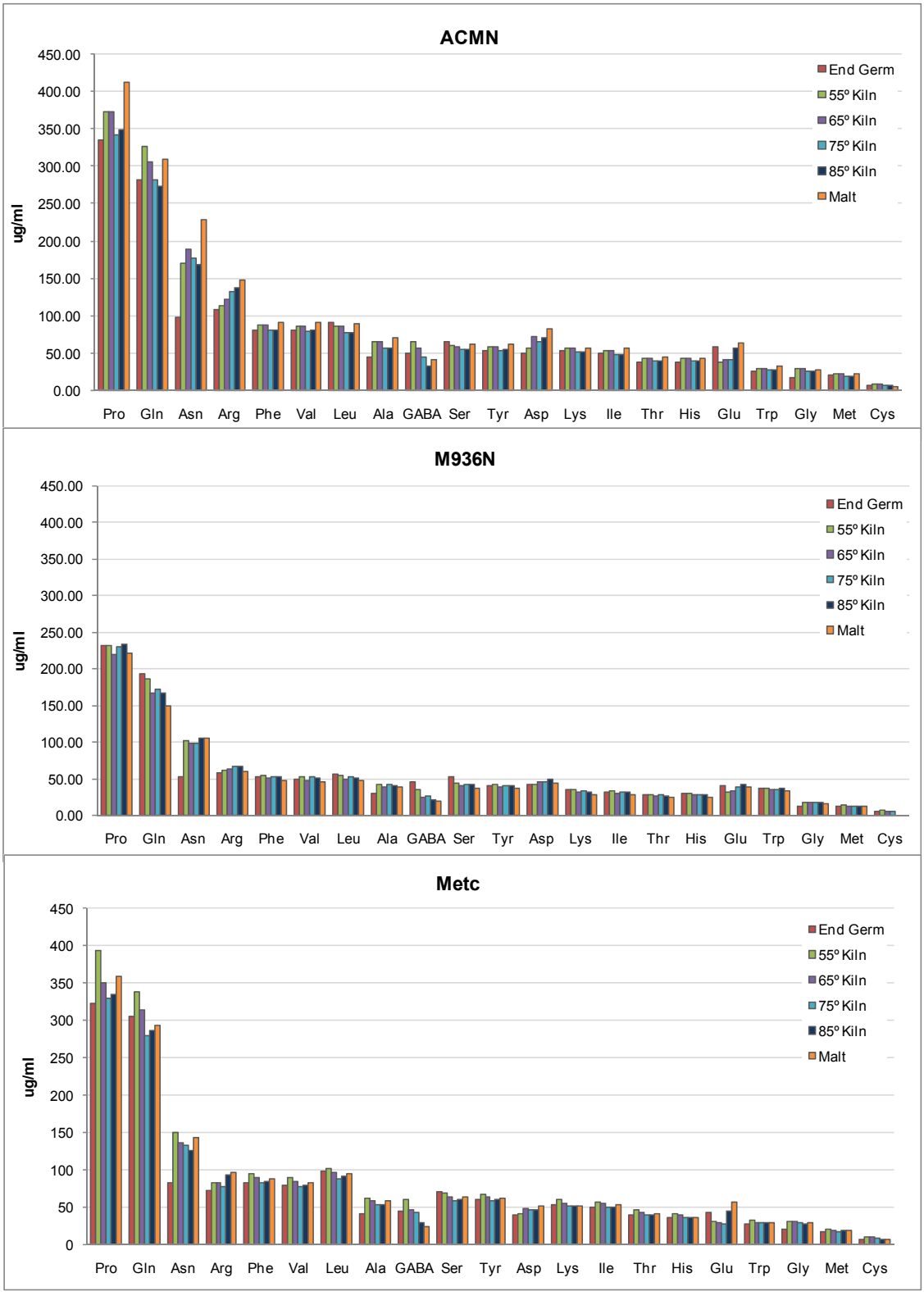


Fig. 4.27 Changes in levels of amino acids during kilning.

The five most abundant amino acids (Pro, Gln, Asn, Arg, and Val), were monitored over the entire malting process (Fig. 4.28). Gln increased the fastest after steeping achieving its maximum level at the third day of germination after which it maintained a constant level. Levels of Pro continued to increase after germination day 3 until Pro became the most abundant amino acid in green malt. Asn was the most abundant amino acid in barley but started to decrease during steeping and only after the first day of germination did it gradually start to increase. During the first stage of kilning, Asn experienced an important increase becoming the third most abundant amino acid in malt. Arg and Val showed an almost identical curve, presenting a steady increase during the overall process but with only a small increase in content compared to the starting values. The other amino acids, not represented in Fig. 4.28, showed curves similar to those of Arg and Val.

In summary, individual amino acid profiles of the raw barley and of steeped samples were markedly different among the three varieties but the profiles became more similar during germination. At this stage, amino acid production greatly increased achieving its maximum on the third or fourth day of germination depending on variety. During kilning amino acid content decreased, but not as much as expected and the final malt in fact showed an increase without any clear explanation. Metc was the variety that showed the greatest amino acid production during malting as it contained the lowest amino acid contents in barley grain but some of the highest in malt. Results indicated that total free amino acid content in barley grain was not related at all with total free amino acid levels in the final malt, which was expected given the effects the malting process has on endosperm physical structure and biochemistry, such as protease development.

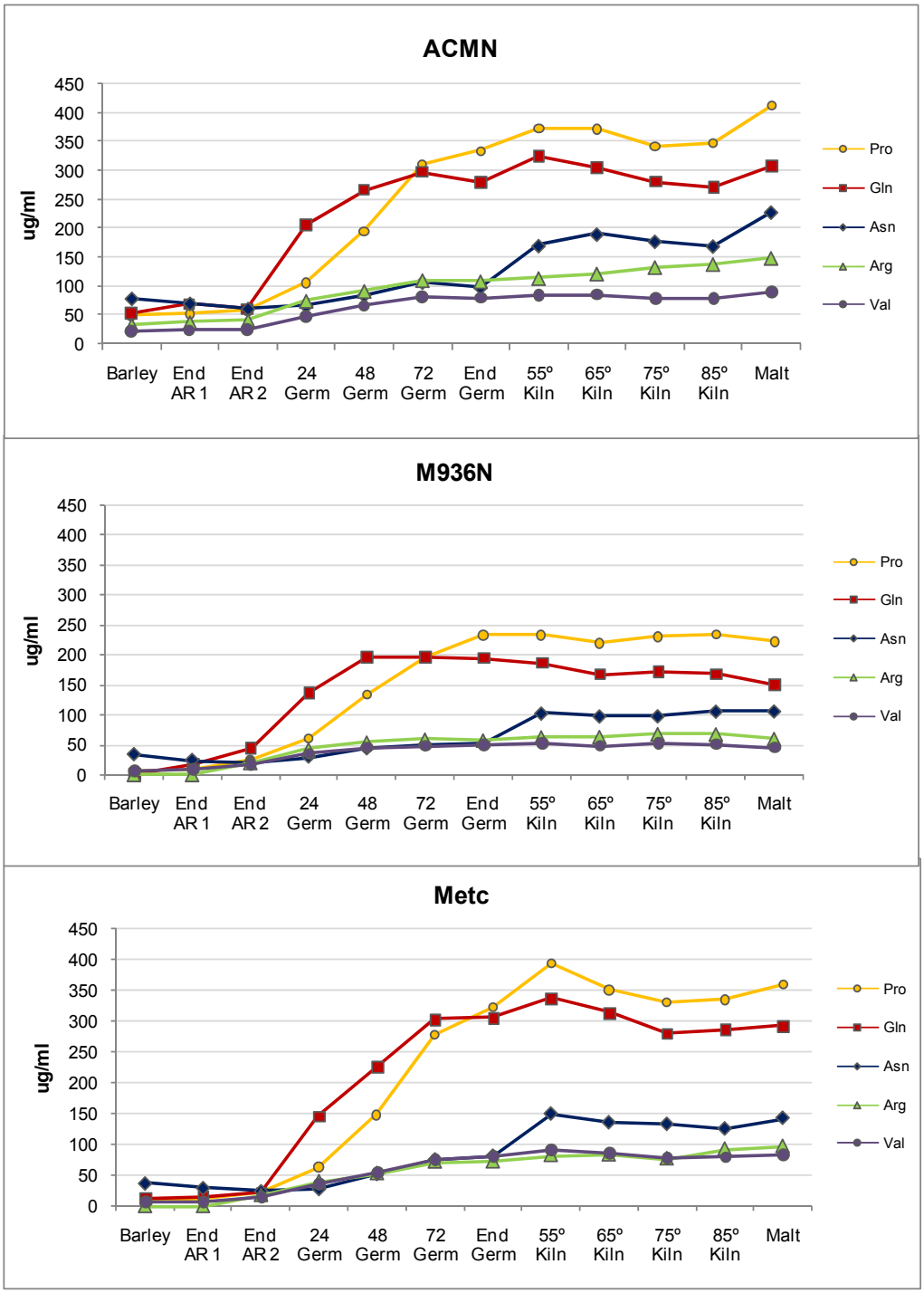


Fig. 4.28 Overall changes in levels of specific amino acids during the malting process for the three varieties tested.

4.9 Relationships between levels of endoproteases and amino acids

Endoprotease activity followed a similar pattern of change to free amino acids during the malting process (Fig. 4.29). Activities began to increase after steeping and continued to increase during the first two to three days of germination. A maximum was reached which, with some varieties then declining, agreeing with published results (Jones and Pierce, 1963). Endoprotease activities were very similar among the three varieties even though total amino acid content varied significantly. Figure 4.29 suggests limited relationship between endoprotease activity and total amino acid content. The variety with the highest total AA content, ACMN, actually had the lowest endoprotease activity.

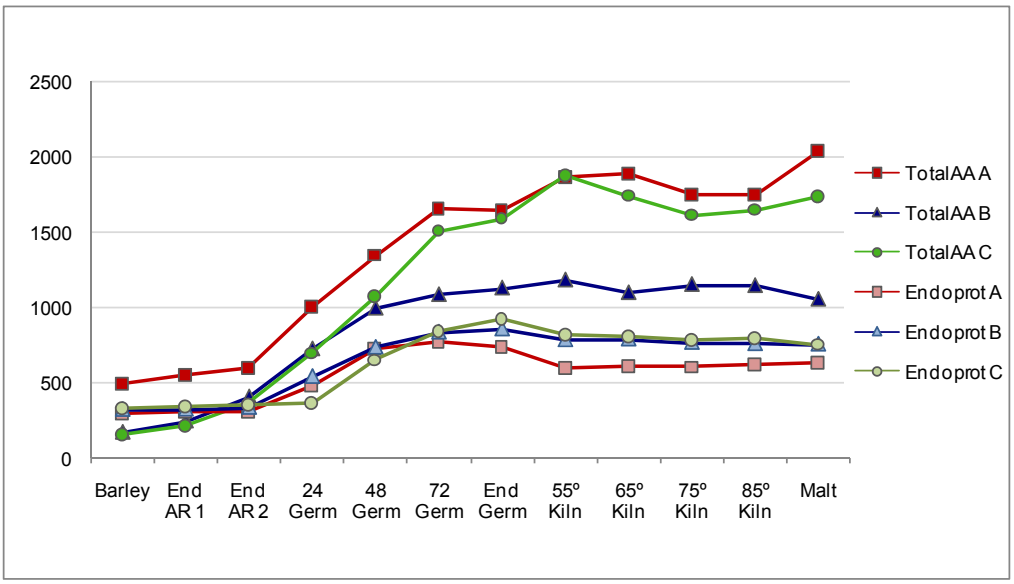


Fig. 4.29 Changes in total amino acid content and endoprotease activities during the malting process. Varieties: A: ACMN (squares, red lines); B: M936N (triangle, blue lines) and C: Metc (circles, green lines). TotalAA: total AA content (ug/ml); Endoprot: endoprotease activity (mU/g)

The relationship between endoprotease activity and individual amino acids was further investigated with correlation analysis. The analysis used previously reported data, obtained from the malting of the eight covered barley samples under different processing conditions (Table 4.13 and 4.14). Results (Table 4.19) agreed with the

indications in Figure 4.29, demonstrating weak associations between endoprotease activities and total amino acid content among malting treatments. Trp was the only amino acid that presented a consistent high association with endoprotease activity. Asn levels did show a strong negative association with endoprotease activity under longer kilning treatment but this was in contrast to all the positive relationships observed with Trp.

Table 4.19 Correlation coefficients (r) between endoprotease activity and individual amino acids from eight covered malt varieties among different malting treatments.

Amino	Treatments				
Acid	Standard	Add GA	Freeze Dry	Higher Kiln	Longer Kiln
His	0.06	0.48	0.50	0.25	-0.48
Asn	-0.54	-0.20	-0.32	-0.57	-0.73*
Ser	0.01	0.42	0.43	0.42	-0.27
Gln	-0.17	0.25	0.12	0.07	-0.40
Arg	-0.05	0.40	0.25	0.22	-0.10
Gly	-0.10	0.46	0.55	0.45	-0.09
Asp	-0.08	0.35	0.13	0.17	-0.36
Glu	0.15	0.65	0.55	0.41	0.26
Thr	-0.06	0.37	0.39	0.15	-0.32
Ala	-0.03	0.49	0.62	0.28	-0.10
GABA	0.01	0.32	0.39	0.07	0.36
Pro	-0.04	0.38	0.33	0.20	-0.16
Cys	0.14	0.45	0.30	0.00	-0.05
Lys	0.21	0.58	0.57	0.44	0.14
Tyr	0.10	0.46	0.45	0.35	-0.29
Met	0.00	0.42	0.41	0.23	-0.19
Val	0.00	0.43	0.34	0.20	-0.20
Ile	-0.09	0.37	0.23	0.10	-0.39
Leu	0.05	0.44	0.35	0.27	-0.29
Phe	0.00	0.41	0.32	0.26	-0.35
Trp	0.67	0.80*	0.91**	0.68	0.73*
Totalaa	-0.07	0.39	0.32	0.19	-0.30

Level of significance: (P<0.001) ***; (P<0.01)**; (P<0.05)*

Associations have been expected between endoprotease activity and Pro and/or Glu as Jones and Pierce (1967a) established that hordeins are rich in glutamic acid and proline. Endoproteases were expected to actively degrade storage proteins leading to increased levels of these two amino acids. However, it might be possible that

endoproteases from other classes, besides the cysteine-type endoprotease class monitored in the developed assay, would have shown better relationships with amino acids. It was also possible that exopeptidases played a stronger role in releasing these particular amino acids. Future studies should consider exopeptidases in conjunction with endoprotease activities to better understand the release of amino acids during malting and mashing. Results in Table 4.19 would suggest that malting treatments had limited effect on the relation between endoprotease activities and individual amino acids.

4.10 Changes in the amino acid profile during brewing process

Malts from the three varieties, ACMN, M936N and Metc, were brewed in a microbrewing plant to study effects of mashing, wort boiling and fermentation on free amino acid levels. Malts for this experiment were malted in a malting pilot plant under standard processing conditions. Mashing and fermentation process in the micro-brewing plant also were performed under standards processing conditions.

4.10.1 Effects of mashing and wort boiling on amino acid profile

Changes in amino acid profile during the brewing process were monitored by removing samples, for each of the three malts, at 6 times during brewing. Table 4.20 shows that the three malt samples already had high levels of total amino acids present at the first sampling point (16 min at 48°C). Total amounts showed

Table 4.20 Total amino acid content during mashing and after wort boiling in the pilot plant brewery.

Total amino acid content (ug/ml)			
	ACMN	**M936N**	**Metc**
16min at 48ºC	4543	3443	4615
25min at 48ºC	5000	3575	4950
0min at 65ºC	4881	3439	4532
20min at 65ºC	4600	3575	5099
0min at 77ºC	4440	3330	4563
After boiling	2726	2044	2762

Standard deviation of the method: 71 ug/ml.

no consistent trends at other points in the mashing process, remaining relatively constant but values did drop significantly after wort boiling. The six most abundant amino acids in mashes and worts were: Pro, Gln, Asn, Leu, Arg, and Val. This was consistent among the three varieties analysed, although actual levels were different for each variety. Results agreed with Jones and Pierce (1963), who found free amino acids increased slightly during initial periods of mashing followed by a steady decline. The present study did find that individual amino acids performed differently (Fig 4.30) with some amino acids showing a steady decline such as the amides (Gln and Asn) as well as Pro. In contrast other amino acids showed steady increases such as the basic amino acids (Lys and Arg) and Leu.

The boiling phase of the brewing process significantly reduced free amino acids levels, particularly Gln, which decreased to almost 30% compared to the last stage of mashing (Fig. 4.30). In a manner similar to kilning, the Maillard reaction was enhanced by high temperatures that occurred during boiling, producing melanoidins and, thus, decreasing amino acid content.

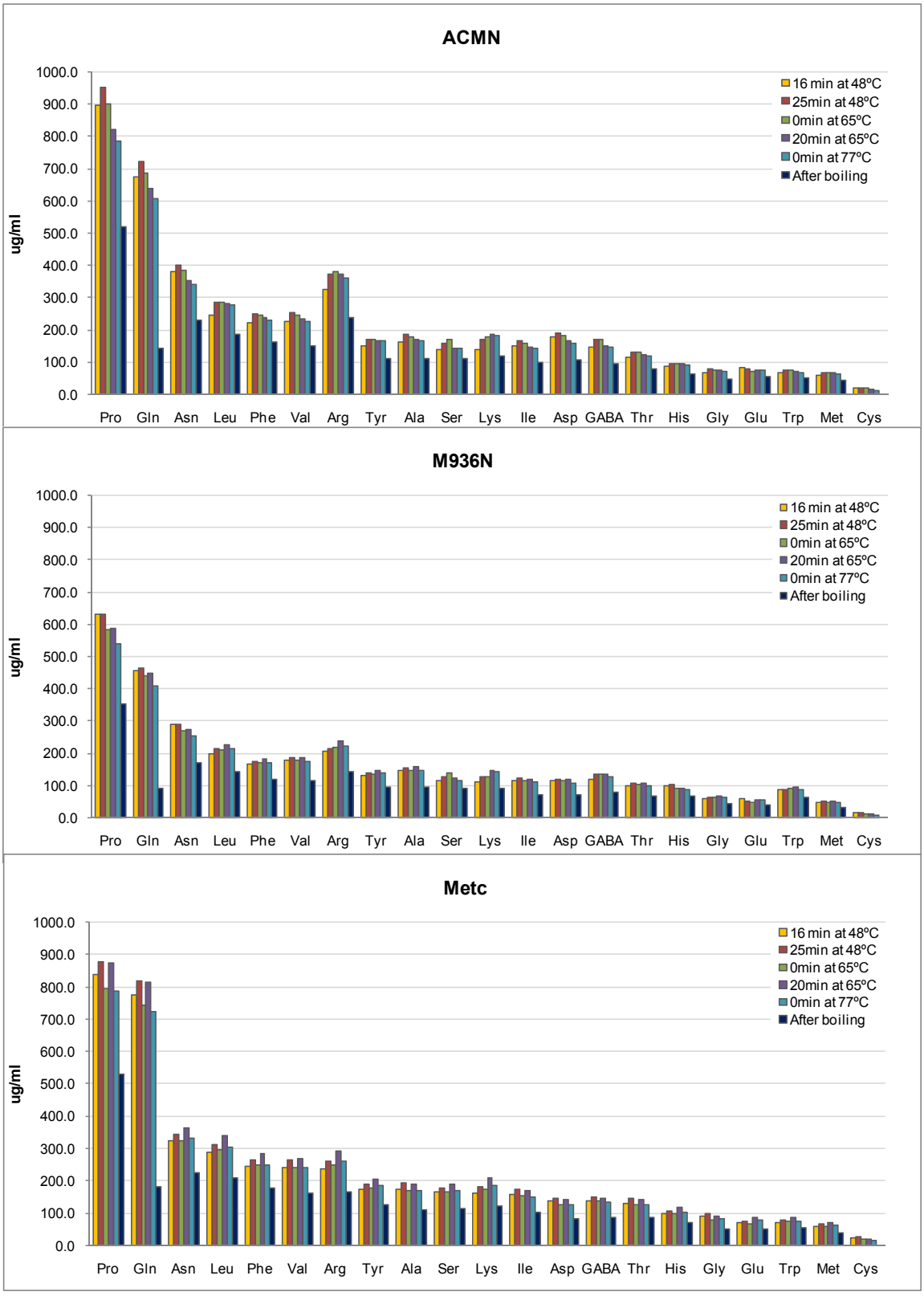


Fig. 4.30 Percentage of amino acid content during the mashing process and after the boiling phase of micro-brewing.

Total amino acid content was found to show an interesting relationship with wort pH (Fig. 4.31). The two varieties with the higher total amino acid content also had lower wort pH values compared to the third variety. The results were in accordance with Jones and Pierce (1967c) and Mikola et al. (1971) who suggested a lower pH was more optimal for activities of carboxypeptidases which then led to higher levels of amino acids. The idea further supports work on carboxypeptidases and their relation to free amino acids.

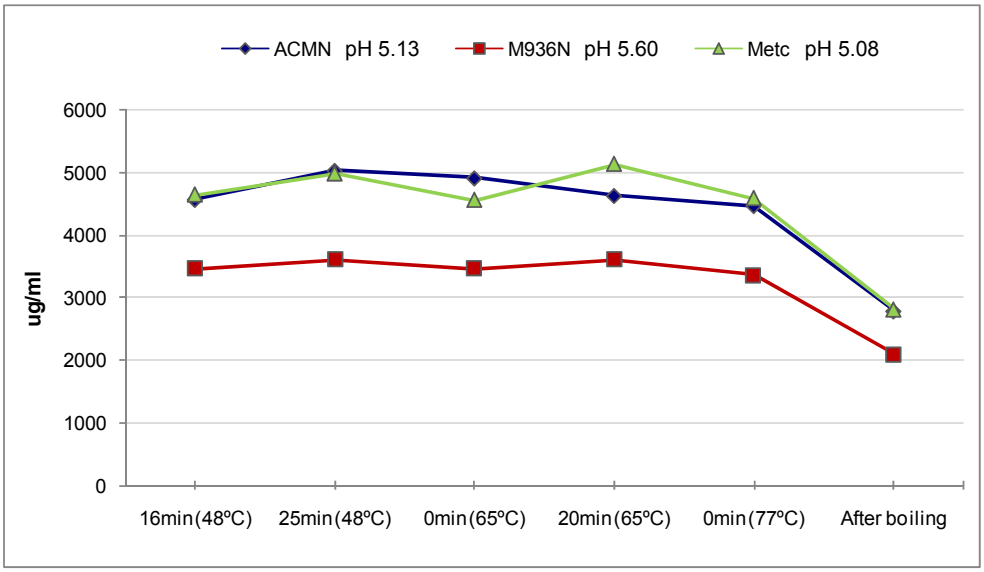


Fig. 4.31 Total amino acid content during mashing and after boiling at a micro-brewing.

4.10.2 Changes in amino acid profiles during fermentation

Fermentation was performed in duplicate using tall tubes. Results of total amino acid content and wort gravity (Fig. 4.32) were consistent between duplicates, although amino acid contents varied more than the gravity. The three varieties all started with similar gravities but total levels of amino acids did vary with Metc and ACMN containing similar but higher amounts than M936N.

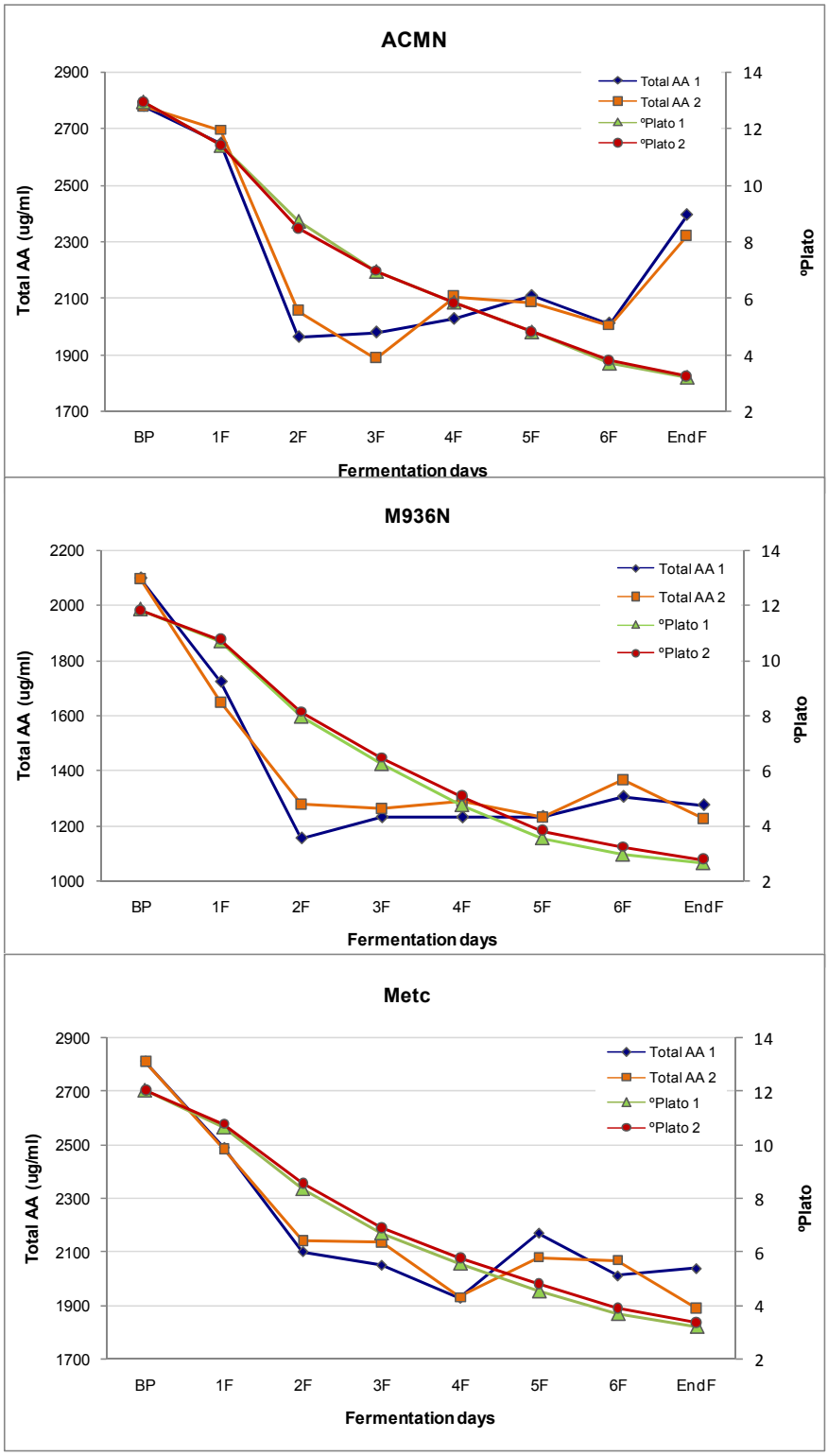


Fig. 4.32 Measurements of total amino acid content and ºPlato at different stages of fermentation in tall tubes at 15ºC. BP: before pitching, 1F – 6F: days of fermentation and EndF: end of fermentation (7th day).

Gravity and amino acids are seen to drop during fermentation; however, gravity did not drop as steeply at the beginning of fermentation as expected. Poor oxygenation of the wort, prior to yeast pitching, was suspected to have limited fermentation, causing a delay in the loss of gravity. Gravity did decrease over time and this decrease was mirrored by a decrease in total amino acid content. Interestingly the drop in total amino acid content proceeded the drop in gravity for all three varieties, likely a reflection of the need for amino acids during the growth phase of the yeast and prior to uptake of fermentable sugars. The decrease in amino acid contents eventually levelled off in all three varieties but at different days of fermentation. The ACMN and M936N varieties achieved minimum levels after two days of fermentation while Metc required four days to reach a minimum. The minimum levels of total amino acids remained relatively constant for the remainder of fermentation for the M936N and Metc samples. However, levels of amino acids in the ACMN sample increased significantly at the end of fermentation. Generally, increases in amino acids at the end of fermentation are related to yeast stress and yeast metabolism. There was no apparent reason for an increase with ACMN, but duplicates were consistent and the lack of effect in the other samples suggested a difference in ACMN's wort content.

Changes in levels of individual amino acids during fermentation are shown in Fig 4.33. The three varieties all showed losses of some amino acids but M936N, the variety with the lowest total amino acid content (Table 4.20), was the only variety that showed complete loss of several amino acids (Asn, Ser, Lys, Met and Thr). The other two varieties also showed marked losses of these five amino acids but not completely. Pro and Ala were the only amino acids that increased during the fermentation process and this was consistent among the three varieties. These results were in agreement with Magasanik and Kaiser (2001) who showed that yeast, grown under conditions with limited sources of nitrogen, are subjected to nitrogen catabolite repression which

activates pro permease (Put4p). This allows the yeast to preferentially use Asn, an amino acid that was completely consumed during the first two days of fermentation in the present study. Most other amino acids remained constant during fermentation (Tyr, GABA, Asp, His, Trp, Gly and Glu) or remained constant after an initial loss (Leu, Gln, Phe, Arg, Val and Ile). Cys levels were small or nonexistent with no marked change during fermentation.

It is important to point out that for none of the varieties were all the amino acids consumed during fermentation and only with M936N were some individual amino acids consumed completely. These results differed from those reported by Lekkas et al. (2007) where total amino acid consumption was achieved in 48 hrs of fermentation. However, Lekkas et al (2007) used a higher fermentation temperature, (18ºC), a higher gravity wort containing 30% high maltose syrup (HMS) and addition of $ZnSO_4$ 0.2 (mg/L). Higher fermentation temperatures are known to accelerate yeast metabolism which increase amino acid consumption. The use of HMS dilutes amino acids from wort possibly to a limiting extent. Zinc supplementation is also known to stimulate amino acid uptake (Jin et al. 1996).

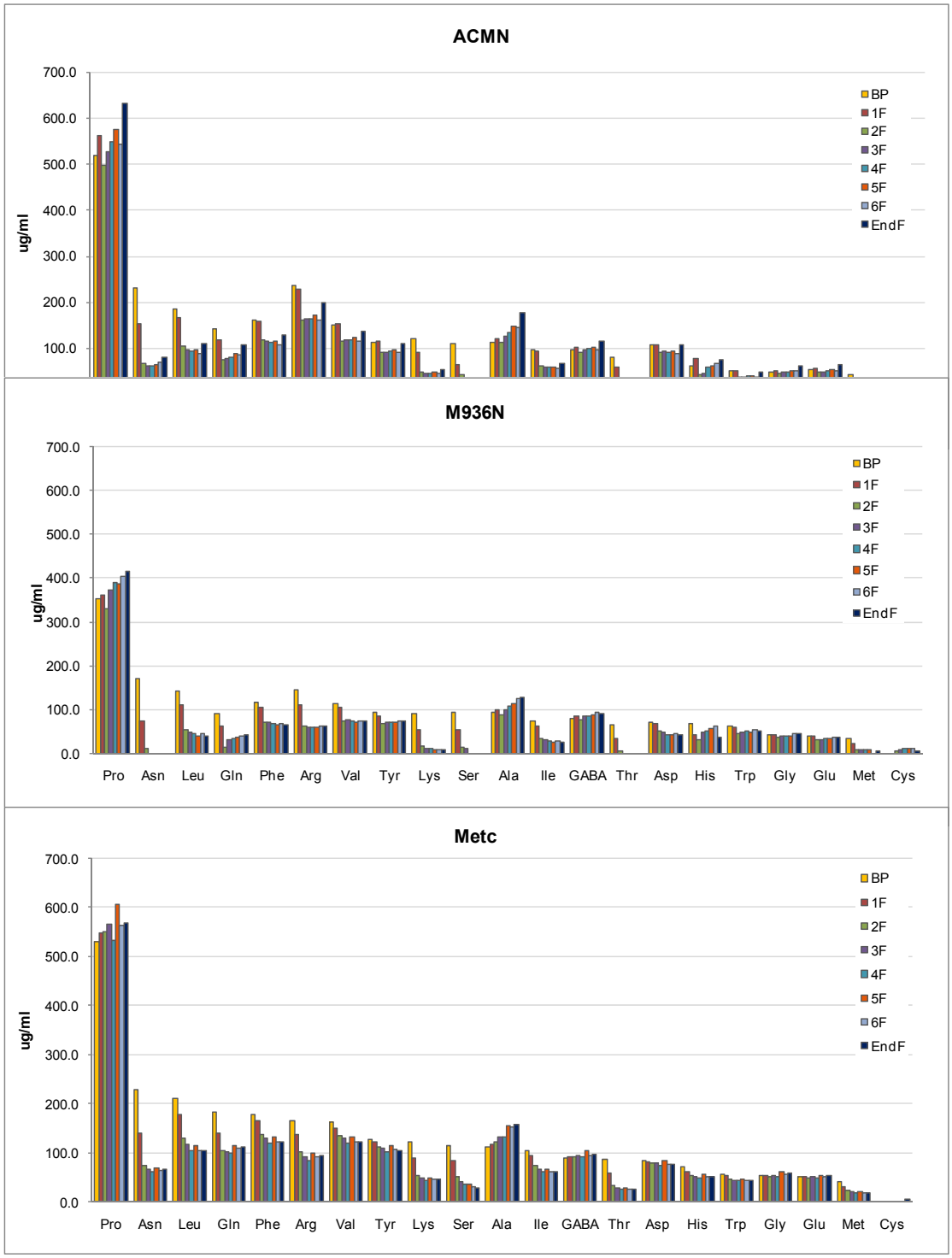


Fig. 4.33 Changes in amino acid profiled during fermentation in tall tubes at 15ºC. BP: before pitching, 1F to 6F: days of fermentation and EndF: end of fermentation (7^{th} day).

4.11 Relationships between fermentability and amino acids

To study the influence of different malt processing conditions on fermentability levels and wort free amino acids relationship, correlation coefficients were calculated between individual amino acids and AAL (Table 4.21).

Table 4.21 Correlation (r) values between AAL (Standard EBC conditions) and levels of individual amino acids for the eight covered varieties as malted under different malting conditions

Amino Acid	Treatments				
	Standard	**Add GA**	**Freeze Dry**	**Higher Kiln**	**Longer Kiln**
His	0.76*	0.86**	0.59	0.64	0.06
Asn	0.61	0.81*	0.91**	0.65	0.67
Ser	0.85**	0.93***	0.61	0.73*	0.87**
Gln	0.85**	0.91**	0.80*	0.84**	0.97***
Arg	0.74*	0.84**	0.66	0.60	0.80*
Gly	0.83*	0.91**	0.56	0.74*	0.89**
Asp	0.57	0.71*	0.54	0.27	0.69
Glu	0.80*	0.74*	0.36	0.66	0.73*
Thr	0.83*	0.90**	0.71	0.79*	0.89**
Ala	0.75*	0.78*	0.49	0.62	0.74*
GABA	0.37	0.52	-0.04	-0.08	-0.10
Pro	0.84**	0.88**	0.62	0.80*	0.86**
Cys	0.63	0.88**	0.64	nd	0.64
Lys	0.73*	0.85**	0.51	0.56	0.76*
Tyr	0.82*	0.87**	0.60	0.71*	0.98**
Met	0.78*	0.86**	0.65	0.70	0.87**
Val	0.88**	0.91**	0.71*	0.81*	0.94***
Ile	0.89**	0.92**	0.78*	0.88**	0.91**
Leu	0.86**	0.89**	0.68	0.79*	0.93***
Phe	0.84**	0.87**	0.72*	0.76*	0.94***
Trp	0.00	0.24	-0.45	-0.43	-0.49
Total AA	0.84**	0.91**	0.72*	0.78*	0.95***

Level of significance: (P<0.001) ***; (P<0.01)**; (P<0.05)*

Results showed that individual amino acids in GA_3, standard and longer kilning treatment presented higher association with AAL for almost all amino acids. The only amino acid with no association with AAL under any of the treatments was Trp. The high AA associations could be related to the number of samples assayed in this experiment, as only eight samples per treatment were assayed, correlation values were higher.

Future studies with bigger sample population will help to confirm the higher associations between amino acid content and fermentability levels.

Wort is a very complex medium containing a range of nutrients, predominately fermentable sugars. Micronutrients, such as amino acids and minerals, are seldom limiting, especially in all malt worts, complicating research on relationship of fermentability with micronutrients. To study more directly the relationship between amino acid profile and fermentability, therefore, a broth was created as a controlled model medium. The broth consisted of a mixture of wort and high maltose syrup (HMS) with the aim of having excess fermentable sugars and limited amounts of free amino acid. This broth allowed studying the direct effect of free amino acid levels on fermentability performance. Several ratios of HMS:wort were investigated to optimize the test.

4.11.1 Broth experiment

A broth was required that simulated standard analysis conditions and that, therefore, had a gravity (ºPlato) similar to that of Congress wort. As a result high maltose syrup was diluted with water to achieve a solution with 8.5ºPlato gravity. This solution was then mixed at six different ratios with Congress wort (Table 4.22). Resulting broths were fermented in duplicate following the standard EBC method for AAL; 7.5g of yeast/100ml of broth, 20°C fermentations for 24 hours with continuous stirring. Fermentability results (Table 4.22) demonstrated that at the standard yeast pitching rate, good fermentations occurred no matter the level of nitrogen compounds present as illustrated with the 100% HMS broth with no nitrogen. There apparently was enough yeast in the medium to ferment all the sugars present without the need for yeast reproduction and the corresponding requirement for assimilation of nitrogen compounds. As a result, ranges of pitching rates were studied with the aim of limiting fermentability of broths with no free amino acids.

Four different pitching rates were assayed with the selection based on: 1) amount used in the EBC method (7.5 g/100 ml), 2) amount used with tall tubes in micro-brewing (0.45 g/100 ml), 3) amount used in rapid overnight fermentation tests (16.7 g/100 ml) and 4) an intermediate value between the lowest and the EBC method (3.0 g/100 ml). In this experiment, only the 100% HMS broth was investigated. Results (Table 4.23) showed that the objective, limited fermentation of the broth, was best achieved with the 0.47g/100ml pitching rate.

Table 4.22 Fermentability of broths containing mixtures of wort and HMS as fermented with the using EBC conditions including pitching rate.

Broth HMS:W	Initial Gravity °P	Final Gravity °P	AAL EBC %
100:0	8.934	0.806	91.0
100:0	8.934	0.798	91.1
80:20	8.871	0.866	90.2
80:20	8.871	-	-
60:40	8.815	0.962	89.1
60:40	8.815	0.972	89.0
40:60	8.756	1.068	87.8
40:60	8.756	1.068	87.8
20:80	8.695	1.231	85.8
20:80	8.695	1.231	85.8
0:100	8.642	1.388	83.9
0:100	8.642	1.378	84.1

(-) no data, densimeter problems

Table 4.23 Fermentability test of the 100% HMS broth using EBC conditions but different pitching rates.

100%HMS	Initial Gravity °P	Final Gravity °P	AAL EBC %
7.5 g yeast	8.696	0.852	90.2
7.5 g yeast	8.696	0.843	90.3
0.47 g yeast	8.696	8.475	2.5
0.47 g yeast	8.696	8.478	2.5
16.7 g yeast	8.696	0.942	89.2
16.7 g yeast	8.696	0.925	89.4
3.0 g yeast	8.696	5.127	41.0
3.0 g yeast	8.696	4.850	44.2

The amino acid profile of the 100%HMS broth was analysed before and after fermentation with a range of pitching rates (Table 4.24). The original 100% HMS broth unexpectedly contained a significant level of Ser (20.63 ug/mL). Fermented worts contained various amount of His, Ser, Lys and Val depending on the pitching rate. In the case of the sample that did not ferment (0.47g) Ser was not consumed but His and Val were produced. In contrast samples with the higher fermentability levels (7.5g and 16.7g) consumed all of the original Ser and produced higher amounts of His and some Lys. The

wort fermented with the 3.0g pitching rate produced results intermediate to these observations. This amino acid production might be related to fusel alcohol production pathways as well as central role of pyruvate and acetyl-CoA in the utilization of broth nutrient and formation of flavour-active metabolites.

The selected pitching rate (0.45g/100mL) was used on four of the broth compositions presented in Table 4.22 to prove that at higher free amino acid contents higher fermentability levels could be achieved. Results shown in Table 4.25 demonstrated that the fermentability of broths improved with increasing wort proportions. The results validated the broth model and supported its use to study effects of free amino acid on fermentability.

Table 4.24 Amino acid profile of 100% HMS Broth and fermented samples under different pitching rates.

	Amino acid content (ug/ml)			
Sample	**His**	**Ser**	**Lys**	**Val**
100% HMS		20.63		
Fermented broth's				
7.5 g yeast	44.11	0.00	8.85	0.00
7.5 g yeast	46.70	0.00	9.08	0.00
0.47 g yeast	26.37	23.27	0.00	19.90
0.47 g yeast	26.30	24.56	0.00	19.95
16.7 g yeast	55.94	0.00	9.21	0.00
16.7 g yeast	72.07	0.00	9.27	0.00
3.0 g yeast	37.69	10.90	6.00	0.00
3.0 g yeast	27.44	0.00	0.00	0.00

Table 4.25 Fermentations, at the low pitching rate (0.45g/100ml), of four broths with different compositions.

Broth HMS:W	**Initial Gravity °P**	**Final Gravity °P**	**AAL EBC %**
80:20	8.656	5.257	41.4
60:40	8.670	3.161	65.6
40:60	8.684	1.808	80.6
20:80	8.698	1.428	84.8

4.11.2 Amino acid contents in broths with varying ratios

The three malts used in micro-brewing experiments were used to study effects of amino acids on fermentability as determined with the broth method. Broths of varying composition were prepared for each malt, with six different broths prepared for each malt as described in Table 4.22. Total amino acid contents of the different broth compositions

are illustrated for each variety in Fig 4.34. As was expected, ACMN broths contained the highest free amino acid content, especially in broths with higher wort concentrations, followed by Metc broths and finishing with M938N.

One concern with the broth model, was the consistency of the amino acid composition among broths with varying HMS:Wort ratios. Consistency was with respect to relative amounts of amino acids to each other which was monitored with percentage of total amino acids for each individual amino acid. Results (Figure 4.35) indicated that the percentage of composition was maintained among broths with different HMS:Wort (HMS:W) ratios. However, Ser presented some variation due to its presence at a high concentration in the pure HMS solution. As a result Ser percentage decreased as wort concentration in the broth increased.

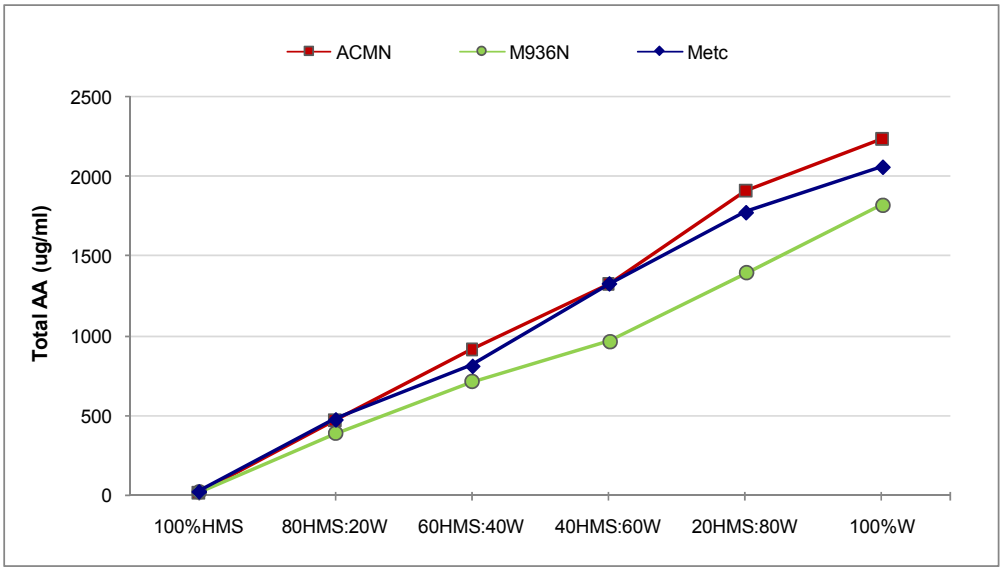


Fig. 4.34 Total amino acid content of the three malted barley varieties among broths with different compositions.

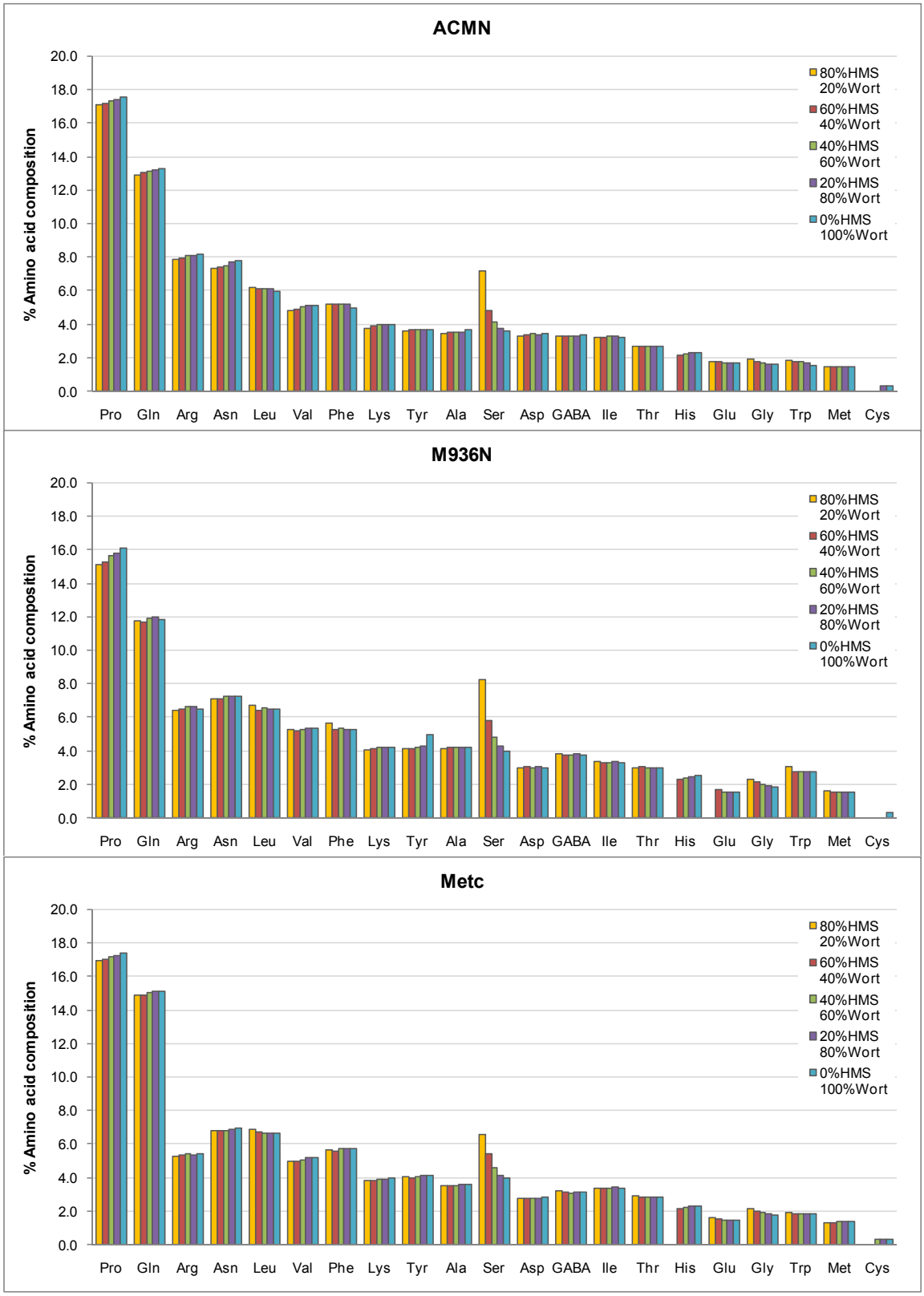


Fig. 4.35 Percentage of amino acid composition of three malted barley varieties among broths of different compositions.

Although the percentage of amino acid composition was maintained across broths, differences did exist among the varieties which were expected given the differences observed previously (Fig. 4.27, Malt). Abundance of amino acids, were similar to what had been observed in previous experiments. The only major difference from previous experiments was a lack of His in the 80HMS:20W broths for all varieties and a lack of Glu in the same broth for M936N.

4.11.3 Using the broth method to investigate effects of amino acids on fermentability

Fermentability was measured in broths of varying HMS:W ratios for each of the three malt varieties, using standard EBC conditions for AAL but with the lower pitching rate. Results (Table 4.26) found the highest fermentability levels were obtained with the 20:80 (HMS:W) broth composition for all varieties assayed. Although these results were not expected as one would expect the 100% wort sample to malt the best, they could be explained. Free amino acids were most likely not limiting in broths with more than 50% wort (40:60, 20:80 and 0:100) and effects of fermentable sugars would have begun to play a greater role. The 0:100 and 20:80 broths, which had the same original gravity, would have contained a mixture of fermentable and non-fermentable sugars; however, the 0:100 would have contained more non-fermentable because the HMS would have been 100% fermentable sugars. Therefore, the 100% W did not achieve the same fermentability level as 20:80 broth. Eventually, however, other nutrients would have

Table 4.26 Apparent attenuation limits (EBC method) for the three varieties as determined with broths of varying HMS:W ratios.

Broth	AAL (%)		
HSM:W	**ACMN**	**M936N**	**Metc**
80:20	40.3	38.0	46.2
60:40	72.4	62.3	72.1
40:60	84.8	78.5	85.0
20:80	85.2	82.5	86.2
0:100	83.4	80.1	84.6

become limiting (60:40 and 80:20 broths) which would have masked differences in fermentable and non-fermentable sugars. In conclusion, when free amino acids were adequate for yeast nutrition, sugars become limiting and defined fermentability.

Fig 4.36 illustrates total amino acid consumption during the fermentation of the broths from the three varieties. Broths with lower initial levels of total amino acids quickly consumed the amino acids as fermentation proceeded. The observation was constant for all three varieties tested.

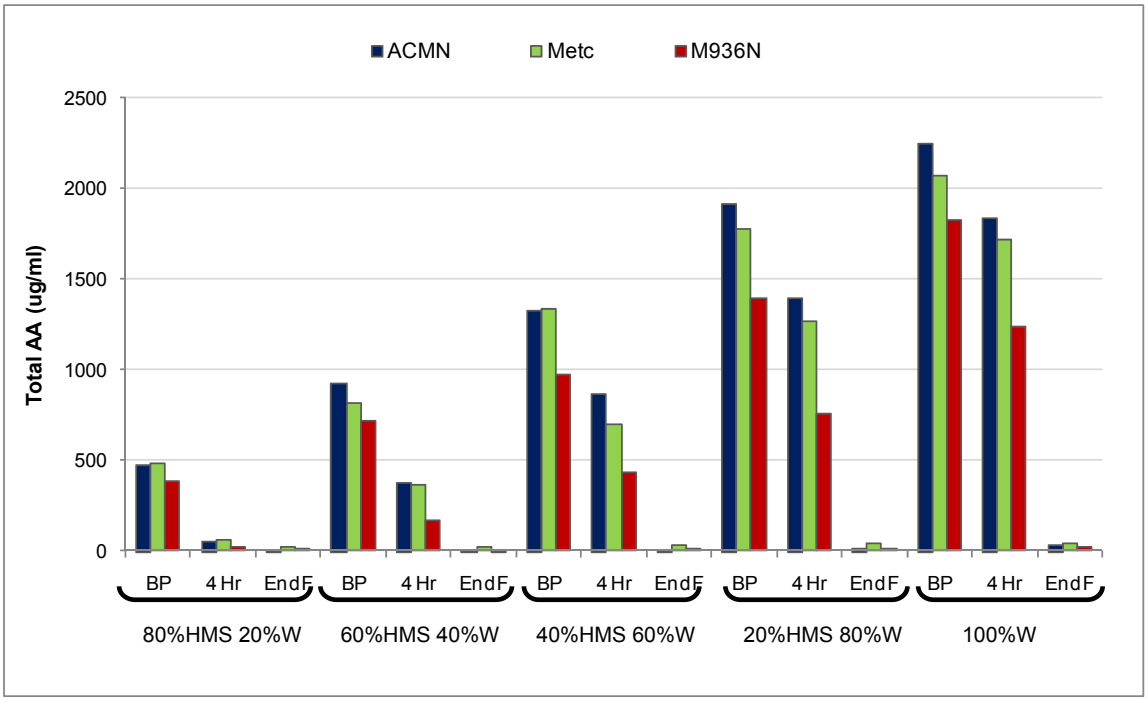


Fig. 4.36 Total amino acid consumption during fermentation of broths from the three malted barley varieties. Fermentation followed standard EBC conditions but at the low pitching rate (0.45g/100ml). BP: before pitching; 4Hr: sample removed after 4 hours fermentation started; EndF: end of fermentation, 24 hours.

In particular, when a broth contained low levels of total free amino acids (80:20 broth), approximately 480ug/ml, 90% of the amino acids were consumed in the first 4 hrs fermentation (Fig 4.36). When broth amino acid content increased to 900ug/ml (60:40 broth), 55% of the amino acids were consumed in the first 4 hours of fermentation. The 20:80 broth contained 1300ug/ml of total amino acids and it consumed 40% of the amino

acids in the first 4 hours. The last two broths, 20:80 and 0:100, contained 1800ug/ml and 2243 ug/ml respectively, and had 32% and 18% consumption, respectively, in the first 4 hrs of fermentation. All of the broths achieved over 97% amino acid use by the end of fermentation.

The individual amino acids that remained at the end of the fermentation varied depending on the wort concentration of the broth. At 80:20 (HMS:W) Ser was the only amino acid that remained for all the varieties. From 60:40 (HMS:W) to 0:100 (HMS:W) broths, Tyr was the only amino acid that remained at the end of fermentation for all varieties. The amount of Tyr remaining was proportional to the concentration of free amino acids in the original broth. This result could be due to the relation established by Forsberg and Ljungdahl (2001) and Omura et al. (2007) that high affinity permeases like Tyr (Tat3p) are transcriptionally induced by sensing extracellular amino acids via the SPS signalling system which are being downregulated in response to amino acid availability. ACMN and M936N contained some Gln at the end of the fermentation, but only in the 0:100 (HMS:W) broth. In the case of Metc, an increase of Cys at the end of fermentation was observed among all broths, although Cys amounts were lower than 10ug/ml (Fig. 4.37 e.g.M936N).

Changes in levels of individual amino acid during the first four hours fermentation were monitored in the broths made from the three malt varieties, but only results from M936N (Fig 4.37) are presented. This variety contained the lowest amino acid contents which caused an increase in speed of amino acid consumption by yeast compared with the other two varieties. The figure illustrates the total or near total consumption of amino acids after four hours fermentation, and at the end of the fermentation, especially when concentrations of amino acids in the original broths were low (Fig 4.37 A and B). In broths with higher concentrations of amino acids originally (Fig.4.37 B and C), the amino acids, Gln, His, Asn, Arg, Asp, Glu, Thr and Met were preferentially consumed by yeast.

In the broth with composition 20:80 (HMS:W), free amino acids were not limiting (Fig 4.37 D) only His and Glu were totally consumed. There were other amino acids that were scarcely consumed during the first four hours of fermentation, Pro, Ala, GABA, Val, Tyr and Trp. In summary, the series of plots on consumption of amino acids over the first four hours and at the end of fermentation, once again demonstrated that speed of amino acid absorption decreased in broths with increasing levels of free amino acids in the original broth.

Jones and Pierce in 1964 classified amino acids according to their speed of absorption by yeast during fermentation. The present experiment was not designed to verify their classification as samples were only removed at one point during fermentation. However, amino acid loss after the four hours, results from the present experiment, were compared to Jones and Pierce's classification (Table 4.27). The present study found, Glu, Asp, Asn, Gln, Thr, Arg, His and Met were the first amino acids to be consumed, which agreed somewhat with Jones and Pierce's (1964) Class A amino acids, Glu, Asp, Asn, Gln, Ser, Thr, Lys and Arg. The only exceptions being in the present study the early consumption of Met and His and with no early consumption of Ser and Lys. The Ser discrepancy could be related to the extra supply of Ser from the HMS. The Group B amino acids (Val, Met, Leu, Iso, His) of Jones and Pierce (1964) were difficult to classify in the present experiment due to the one-time sampling at only 4 hours. Group C (Gly, Phe, Tyr, Trp, Ala and NH_3) and D (Pro) are slowly assimilated by yeast (Pro, Ala, GABA, Val, Trp and Tyr) which could not be monitored with the present experimental setup.

Table 4.27 Classification of amino acids according to their speed of absorption from wort under brewery conditions (adapted from Pierce, 1964).

Group A	Group B	Group C	Group D
Glu	Val	Gly	Pro
Asp	Met	Phe	
Asn	Leu	Tyr	
Gln	Ile	Trp	
Ser	His	Ala	
Thr		Ammonia	
Lys			
Arg			

The amino acid profiles after four hours fermentation in the bench-top experiment were compared to first-day results from the tall tube fermentation. Despite experimental differences, some amino acids (Pro, Gln, Asn, Arg, Thr, Met, Ala and GABA) showed similar profiles with the two fermentation methods. However, amino acid profiles in these two experiments were totally different at the end of fermentation where yeast stress, continuous stirring and nutrients remaining in the wort, undoubtedly played an important role.

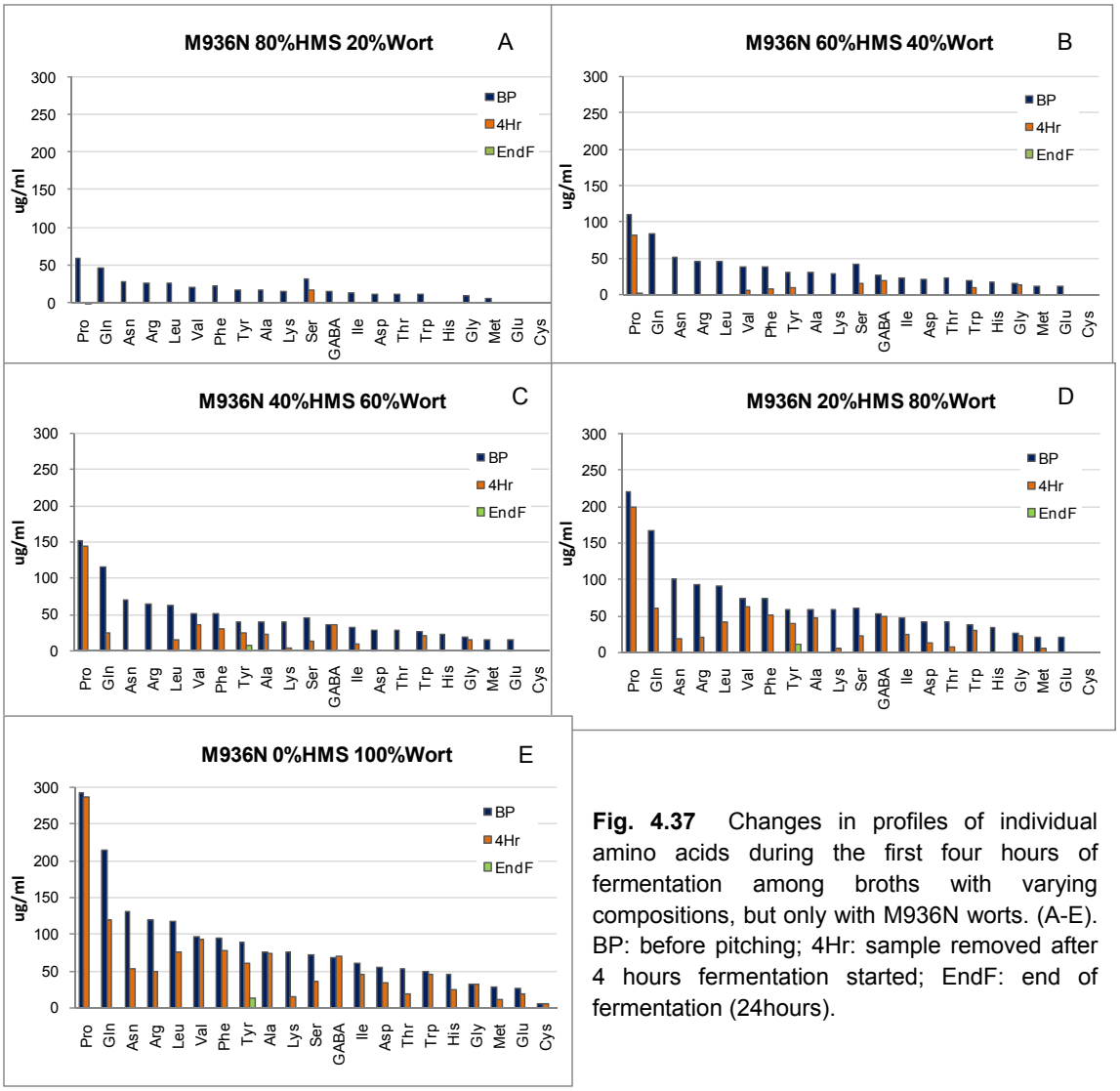


Fig. 4.37 Changes in profiles of individual amino acids during the first four hours of fermentation among broths with varying compositions, but only with M936N worts. (A-E). BP: before pitching; 4Hr: sample removed after 4 hours fermentation started; EndF: end of fermentation (24hours).

4.11.4 Different pitching rates experiment

To prove the effect of different pitching rates on amino acid profile, experiments at small scale fermentation using two different pitching rates were performed with 100% worts.

Small scale fermentation

Effects of pitching rate on amino acid profile were compared using bench top fermentability tests. Fermentation conditions were based on the standard EBC AAL method, 20ºC with stirring for 24 hours, but with two pitching rates , the standard 7.5 g/100 ml and the micro brewery rate of 0.45 g/ 100 ml. One Congress wort was prepared for each variety and aliquoted for the two pitching rates. Amino acid profiles were determined at three stages: before pitching, after four hours of fermentation and at the end of fermentation (24 hours).

Attenuation limits, shown in Table 4.28, were somewhat higher with the standard pitching rate (7.5g/100ml) as compared to the low pitching rate (0.45 g/100ml). Furthermore, Metc produced a higher fermentability levels, with both pitching rates, than ACMN, despite the latter having higher total amino acid content. This indicated once again that when total free amino acid content in wort was not limiting, the degree of fermentation was affected by other wort nutrients, likely fermentable sugars.

Yeast was found to begin consumption of amino acid at a faster rate (Figure 4.38) with the higher pitching rate than with the lower rate. The later consumed less than 20% of free amino acids after four hours fermentation, compared to over 90% at the high rate. However, amino acid levels at the end of fermentation were higher at high pitching rate than the low ones for at least some amino acids, Pro, Tyr, GABA and Glu.

Comparing the varieties with their different total amino acid contents (Table 4.28) showed that the variety with the lowest total free amino acid content (M936N) (Fig 4.38

A-B) had quicker amino acid consumption than varieties with higher wort total free amino acid contents (ACMN) (Fig 4.38 C-D). In contrast, varieties with similar total free amino acid contents, like Metc (Fig. 4.39) and ACMN (Fig 4.38 C-D), showed similar changes in amino acid profiles during fermentation, with only small differences at the end of fermentation; for example Gln present only in ACMN and Glu present only in Metc.

In summary, results suggested that changes in amino acids during fermentation were highly influenced by the total free amino acid content of the original wort and less dependent on barley genotype. Different genotypes with similar total amino acid contents showed similar changes in amino acid profile, but this did not necessarily mean they would ferment the same.

Table 4.28 Total AA content and AAL values among varieties.

	ACMN	**M936N**	**Metc**
Total AA(ug/ml)			
Congress Wort	2242	1825	2064
AAL (%)			
Low PR	83.4	80.1	84.6
High PR	84.0	81.5	86.2

Std of the method: 0.5%. High pitching rate: High PR, Low pitching rate: Low PR.

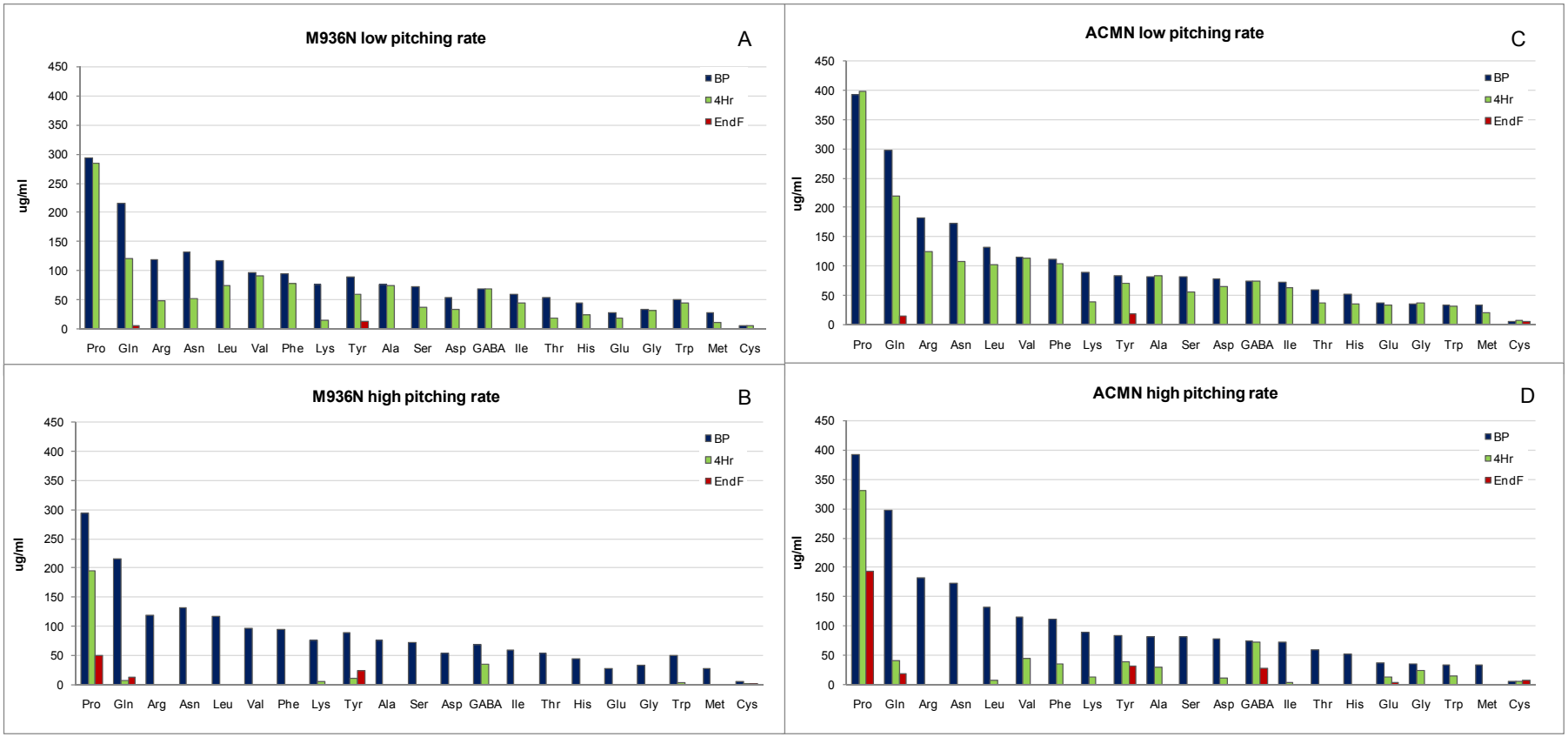


Fig. 4.38 Changes with ACMN and M936N malts, in individual amino acid profiles during fermentation with low (0.47g/100ml) and high (7.5g/100ml) pitching rates. BP: before pitching; 4Hr: sample removed after 4 hours fermentation started; EndF: end of fermentation (24 hours).

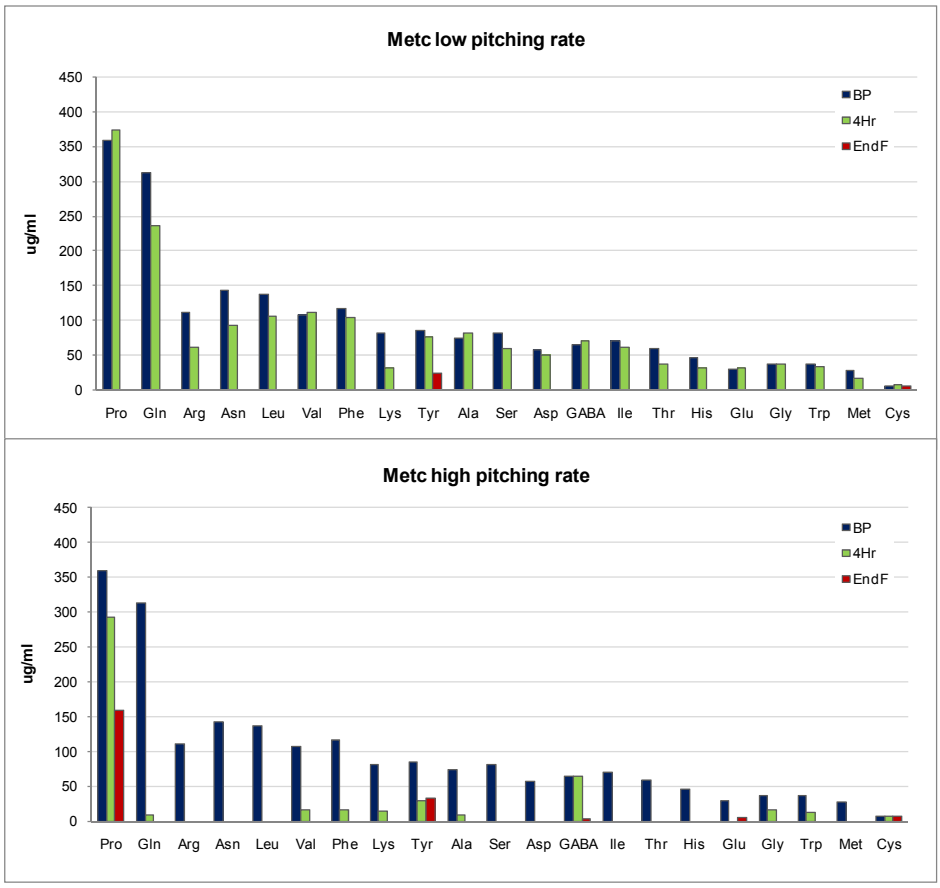


Fig. 4.39 Changes, with Metc malt, in individual amino acid profiles during fermentation with low (0.47g/100ml) and high (7.5g/100ml) pitching rates. BP: before pitching; 4Hr: sample removed after 4 hours fermentation started; EndF: end of fermentation (24 hours).

4.12 Relative importance of amino acids, starch-degrading enzymes and endosperm modification in explaining fermentability

The present study did not demonstrate a major effect of amino acid levels on fermentability. Results demonstrated (Table 4.26) that the variety with the highest free amino acid content (ACMN) did not achieve the highest fermentability level, even in the broth experiments where fermentable sugars were in excess due to the HMS. The highest fermentability was obtained with Metc, which presented high total amino acid content but not the highest. The results suggested that other wort nutrients were playing an important role in fermentability.

Malt quality did show some interesting differences among the three varieties that were tested extensively (Table 4.29). Metc had the highest levels of DP and alpha amylase activity, lower viscosity and higher KI and SP content that the other two varieties. Viscosity and KI are closely associated with degree of endosperm modification, already demonstrated by Edney et al. (2007a), and that could have a significant effect on fermentability. Furthermore, correlation analysis between AAL (Standard EBC) and malt quality results from the eight covered barley varieties malted under different malting conditions (Table 4.30), supported the importance of FAN, SP, β-glucan, DP, amylolytic enzyme activities and pH values in determining fermentability. The higher levels of starch-degrading enzymes in Metc would expect to be a major factor in explaining its superior fermentability. However, Metc still fermented better (Table 4.26) than ACMN in the broth with the highest HMS:wort ratio (80:20) where higher levels of enzymes would expect to have had limited effect. Therefore, Metc's higher fermentability was most likely related to its better modification.

Table 4.29 Malt quality profile among varieties malted in a Pilot Plant under standard program conditions.

		Variety		
Parameter	**Units**	**Metc**	**ACMN**	**M936N**
Yield	(%)	92.6	93.4	93.8
SOMoi	(%)	40.8	40.9	39.4
Moi	(%)	5.4	4.8	4.7
Fine	(%)	79.9	81.4	79.8
Fri	(%)	80.1	82.3	60.2
Glucan	(ppm)	96	74	213
Visc	(cps)	1.41	1.45	1.49
MP	(%)	11.8	12.1	11.1
SP	(%)	5.0	4.8	4.2
KI	(%)	42.3	39.6	38.2
FAN	(mg/L)	193	198	160
Colour	(ASBC)	1.79	2.15	1.84
Colour	(EBC)	3.52	4.23	3.62
pH		5.95	5.95	5.90
DP	(ºL)	169	107	78
Alpha	(DU)	82.8	60.6	53.6
Endoproteases	(mU/g)	971	860	911
AAL	(%)	86.2	84.0	81.5

Table 4.30 Correlation (r) values between AAL and malt quality profile among eight covered varieties malted under different malting conditions.

	Treatments				
Parameter	**Standard**	**Add GA**	**Freeze Dry**	**Higher Kiln**	**Longer Kiln**
Yield	-0.70	-0.87**	-0.60	-0.69	-0.83*
SOMoi	0.71*	0.73*	0.49	0.47	0.44
Moi	0.15	0.72*	0.37	0.49	-0.41
Fine	0.11	0.30	-0.08	0.20	0.05
Fri	0.38	0.57	0.73*	0.08	0.50
Glucan	-0.53	-0.76*	-0.10	-0.54	-0.54
Visc	-0.71	-0.91**	-0.41	-0.74*	-0.60
MP	0.70	0.67	0.95***	0.64	0.82*
SP	0.80*	0.89**	0.85**	0.74*	0.83*
KI	0.06	0.63	-0.45	0.14	-0.74*
FAN	0.89**	0.90**	0.88**	0.82	0.93***
Colour	0.46	-0.02	-0.55	0.61	0.88**
pH	-0.91**	-0.90**	-0.87**	-0.86**	-0.91**
DP	0.81*	0.83*	0.73*	0.91**	0.83*
Alpha	0.74*	0.79*	0.72*	0.91**	0.72*
AlphaM	0.56	0.68	0.57	0.92**	0.72*
Beta	0.83*	0.85**	0.70	0.87**	0.87**
LD	0.77*	0.92**	0.87**	0.93***	0.89**
Endproteases	0.06	0.29	-0.27	-0.07	-0.38

Level of significance: (P<0.001) ***; (P<0.01)**; (P<0.05)*

Chapter V

Conclusions

5.1 Conclusions

Individual free wort amino acid profiles showed variable patterns during malting and brewing production depending on malting conditions and barley variety. Results generally agreed with previous studies, however, the use of a sophisticated UPLC system allowed for more comprehensive characterization of 20 individual amino acids. The result showed some interesting discrepancies. Pro, Gln, Asn and Arg were found to be the most abundant amino acids during the processes. Gly and Lys were the only two amino acids that varied significantly among the malt processing conditions investigated. Lys appeared to be more susceptible to Maillard reaction during kilning which was significant given the importance of this amino acid for yeast nutrition. Another identified concern with this amino acid was the lack of linear relationship with FAN suggesting FAN has some inadequacies as a standard method of malt analysis.

A new endoprotease method was developed to quantify barley, malt and wort endoprotease activity. Endoproteases, mainly cysteine class, presented the same general trends during malting as total AA content, although there were no significant associations between endoprotease activity and levels of individual amino acids. Future studies on relationships between amino acids and proteases should investigate other classes of endoproteases as well as the exoproteases.

A modified fermentability test was developed based on the use of high maltose syrups. The new test, in combination with low pitching rates, was able to demonstrate the importance of amino acids, a relationship which was not as obvious with the standard EBC fermentability test (AAL). Results showed that amino acids were first consumed by yeast prior to any decrease in gravity demonstrating yeast started growing prior to significant fermentation of sugars. Relationships between amino acids levels and fermentability might be better studied with model solutions, where levels of each of these amino acids were controlled, could better indicate their importance for fermentability.

Studies on non-fermentable sugars will be important as results showed that when free amino acids were adequate for yeast nutrition, sugars become limiting and defined fermentability level. Endoprotease activities (cysteine class) did not significantly affect malt fermentability level under any of the malting conditions studied. Malt modification was found to be essential for good fermentability. Varieties with good protein modification (adequate levels of amino acids) and adequate enzymes were found to still have poorer fermentability than varieties that produced low wort viscosities and had high Kolbach indexes. These modification factors probably need to be better controlled in order to better demonstrate the necessity of amino acids for good fermentation.

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