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In vitro digestion of starch in barley

Effect of boiling, fermentation and enzymatic treatment

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Abstract

Bioactive compounds such as phenolic acids have resulted in the widespread acceptance of barley as a functional food ingredient. Food processing methods such as fermentation and boiling can result in a release of phenolic acids from the barley matrix. This is due to the fact that various physical and biological processes are initiated in the barley during processing which subsequently affects the bioavailability of phenolic compounds.

In the present study the effects of fermentation and boiling on the free phenolic acid content in hulled barley, barley flakes and barley flour was investigated. Fermentation of barley flour with *Lactobacillus reuteri* SD2112 and *Lactobacillus acidophilus* LA-5 resulted in a significant increase in the levels of free caffeic acid (CA), *p*-Coumaric acid (*p*-CA), and ferulic acid (FA in addition to the level of total free phenolic acids. These effects were significantly enhanced by the addition of the commercial xylanase (pentopan). With respect to boiling, a significant increase in the level of total free phenolic acids was only observed in boiled hulled barley. However there was a significant increase in the level of total free phenolic acids in boiled barley flour following the addition of pentopan.

Previous *in vitro* digestion studies have shown that phenolic acids can have an inhibitory effect on the enzymatic activity of pancreatic α-amylase. This hypothesis is not supported by the current study. However, the results suggest that the presence of lactic acid or acetic acid in barley products may exert an inhibitory effect on the digestion of starch. This effect appears to be partially counteracted by the addition of pentopan. In addition to fermentation starch digestion also appears to be hindered in by thermal processing such as boiling. This is the case for both hulled barley and barley flakes.

A synopsis of the study shows that processing of barley has the potential to increase the level of phenolic acids in addition to decreasing starch digestion. However there is not always a correlation between these effects.

Sammendrag

Grunnet et høyt innhold av bio-aktive komponenter som fenoliske syrer, blir bygg mer og mer benyttet som en funksjonell matingrediens. Prosessering, som fermentering og koking, kan føre til en frigjøring av disse fenoliske syrene fra byggmatriksen. Dette fordi ulike fysiske og biologiske prosesser foregår i matvaren under prosessering noe som igjen kan påvirke biotilgjengeligheten av fenoliske komponenter.

I denne studien undersøkes effektene av fermentering og koking på frie fenoliske syrer i byggproduktene avskallet bygg, byggflak og byggmel. Fermentering av byggmel med *Lactobacillus reuteri* SD2112 and *Lactobacillus acidophilus* LA-5 førte til en signifikant økning i nivå av fri kaffesyre (CA*), p*- kumarinsyre (*p*-CA) og ferulsyre (FA) i tillegg til nivå av summen av frie fenoliske syrer. Tilsetning av pentopan førte til en signifikant økning i disse resultatene. Når det gjelder koking ble det kun observert en signifikant økning i summen av frie fenoliske syrer i kokt avskallet bygg. Etter tilsetning av pentopan ble det en signifikant økning i summen av frie fenoliske syrer i byggmel.

Tidligere *in vitro* fordøyelse-studier har vist at fenoliske syrer kan ha en hemmende effekt på enzymatisk aktivitet av pankreatisk α-amylase. Denne hypotesen blir ikke bekreftet i denne studien. Allikevel, resultatene tyder på at melkesyre og eddiksyre i byggprodukter kan virke hemmende på fordøyelse av stivelse. Denne virkningen kan delvis motvirkes av tilsetting av pentopan. I tillegg til fermentaing kan koking ha en hemmende effekt på fordøyelse av stivelse. Dette er tilfelle for avskallet bygg og byggflak, men ikke byggmel.

Studien kan oppsummeres med at prosessering av bygg har potensial til å øke nivået av fenoliske syrer i tillegg å redusere fordøyelse av stivelse. For øvrig er det ikke alltid en sammenheng mellom disse virkningene.

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Overview of tables and figures

Abbreviations

CA: Caffeic acid

p-CA: *p*-Coumaric acid

FA: Ferulic acid

SA: Sinapic acid

HPLC: High Performance Liquid Performance

RPLC: Rapid Separation Liquid Performance

UV: Ultraviolet

DAD: Diode array detector

1 Introduction

During the past few years *in vitro* digestion models have been widely employed to investigate the digestibility of particular food components under simulated gastrointestinal conditions. One of the most frequently investigated food components is starch, in particular starch in plant based food samples (Hur et al. 2011). Starch is regarded as the most important carbohydrate in the world (Anker-Nilssen et al. 2006) and is the predominant polysaccharide in whole grain cereals such as barley (*Hordeum vulgare* L.) (Asare et al. 2011). In addition to starch, barley contains high levels of bioactive compounds such as phenolic acids. These phenolic compounds are regarded as having considerable health benefits (Abdel-Aal & Rabalski 2013) and have been linked to the prevention of degenerative diseases such as cardiovascular diseases, cancer and neurodegenerative diseases (Tsao 2010).

Due to the potential effects of consuming barley, one of the primary objectives of this thesis was to investigate the phenolic acid content in various barley products. Furthermore the effect of processing and enzymatic treatment on the level of phenolic acids was also studied. This was due to the fact that various physical and biological processes are initiated in a foodstuff during processing which may affect the bioavailability of potentially beneficial compounds. Bioavailability is the absorption, distribution and subsequent effect of the ingested compound upon the target tissue (bioactivity). Therefore a change in the bioavailability of potentially beneficial compounds may subsequently affect the potentially bioactive properties of a particular foodstuff (Kwaku 2011).

Phenolic acids have been shown to have an inhibitory effect on the digestive actions of pancreatic α-amylase (McCue et al. 2005). Therefore having determined the phenolic acid content in barley products, the possibility of an impact on *in vitro* digestion of starch was investigated. The principle aim was to establish if an increase in free phenolic acids resulted in a corresponding decrease in the digestion of starch.

This chapter begins with a description of the structure, types, chemical composition and use of barley. This is followed by an account of phenols, polyphenols and phenolic acids. The various enzymes which are involved in the release of phenolic acids are also described. An account of the human digestion system precedes the final section in this chapter which contains background information on the various methods used.

1.1 Barley

1.1.1 Structure of barley grain

Barley belongs to the grass family, Gramineae, which produces dry one-seeded fruits. This fruit is called *caryopsis* but is more commonly referred to as the barley kernel or the barley grain. The *caryopsis* develops within floral envelopes which essentially constitute the hull of the barley. In addition to the hull, the *caryopsis* consists of a *pericarp* (fruit coat) and a seed. The *pericarp* adheres tightly to the *testa* (seed coat) which in turn surrounds the seed. The seed itself is composed of the *testa*, the nucellar epidermis, the embryo and the endosperm. The endosperm cells are packed with starch embedded in a protein matrix. Approximately 70% of the cell wall of the endosperm is made up of ß-D-glucan while the remainder is composed of arabinoxylan (20%), protein and mannan (Delcour & Hoseney 2010). At the periphery of the endosperm, two to three layers of cells make up the aleurone layer (Becraft 2007). The aleurone cell well is made up of 85% arabinoxylan which has a β-1-4 xylan backbone with 33% of the xylosyl residues at positions 2 and 3 substituted with single arabinofuranosyl groups (McNeil et al. 1975). One of the key enzymes in the aleurone wall is xylanase which hydrolyses arabinoxylan in addition to facilitating the release of other hydrolytic enzymes (Benjavongkulchai & Spencer 1986). These hydrolytic enzymes contribute to the depolymerisation of starch stored in the cells of the endosperm. In order to access this starch, degradation of the walls in the endosperm cells is necessary. Breakdown of the endospermic cell walls occurs in various stages where the first step involves the dissolution of ß-D-glucan. Once in solution ß-D-glucan is subsequently attacked by the endoenzymes (1-3,1-4)-β-glucanases (Georg-Kraemer et al. 2004) leading to the disintegration of the cell wall.

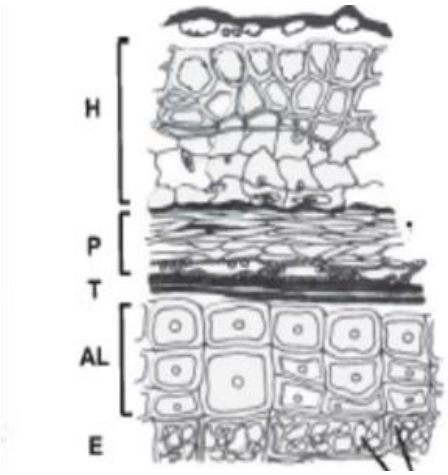

Figure 1.1: Outer layers of the caryopsis with indication of the Hull (H), the Pericarp (P), the Testa (T) the Aleurone layer (AL) and the Endosperm (E).(Delcour & Hoseney 2010)

1.1.2 Barley types

1.1.2.1 Covered barley

Covered barley has a tough, inedible outer hull which surrounds the *caryopsis* or barley grain. This inedible hull must be removed before barley can be used for human consumption (*Types of Barley* 2013).

1.1.2.2 Hulled barley

Hulled barley is covered barley that has been processed in order to remove the tough inedible outer hull. This processing is minimal and must be carried out carefully in order that the *pericarp*, aleurone layer and endosperm is left intact (*Types of Barley* 2013)

1.1.2.3 Barley flakes

Barley flakes are hulled barley which has been steamed, rolled and dried. Steaming increases the surface area of the barley flakes, thus causing them to cook faster (*Types of Barley* 2013).

1.1.2.4 Barley flour

Hulled barley is available in several forms including flakes, grits and flour. Barley flour is hulled barley which has been ground. Barley flour may also be referred to as barley meal (*Types of Barley* 2013).

Figure 1.2: In the figure above the four different barley products which were used in this thesis are shown. Top (left to right): Covered barley and hulled barley. Bottom (left to right): Barley flakes and barley flour. The images were taken by Hanne Zobel (Nofima) using the program DinoCapture 2.0 (Dino-Lite Europe/ IDCP B.V. The Netherlands)

1.1.3 Chemical composition of barley grain

Between 60 – 80% of the barley grain is composed of carbohydrates with starch being the most abundant. In addition to carbohydrates barley consists of 9 – 13% protein, 1-2% fat and 10- 15% water (Asare et al. 2011). The low water content in barley accounts for its stability during storage (Delcour & Hoseney 2010).

1.1.3.1 Starch

Starch is the most predominant carbohydrate in barley (Asare et al. 2011) and is generally regarded as the most important carbohydrate in the world (Anker-Nilssen et al. 2006). Photosynthesis in green leaves produces transitory starch which is transported to the endosperm where it serves as the plants main energy reserve. In the endosperm starch is laid down in the endosperm tissue and stored as water insoluble granules. Two types and sizes of these water insoluble starch granules are found in the barley endosperm. The first type is a large lens-shaped granule which is initially formed by the amyloplasts that are found in barley. These lens shaped granules form outgrowths which separate from the amyloplast and form the second type of granule: a small spherical granule (Delcour & Hoseney 2010).

1.1.3.1.1 Composition of starch

Most common starches are predominantly composed of polymeric carbohydrate material. This polymeric material is built up of monomeric α-D-glucopyranosyl units which are linked to their neighboring glucose via glycosidic bonds. These glycosidic bonds have the α orientation and are linked to either position 4 or position 6 on the glucose molecule. In general α-1,4 bonds are thought to result in linear structures while α-1,6 bonds result in a branched structure (Delcour & Hoseney 2010).

The two main types of polymers found in starch are amylose and amylopectin (Zobel 1988). Amylose constitutes 20-25% of starch and is composed of a linear chain of α-D-glucose units linked together by α-1,4 bonds (Nybraaten 2004). These α-1,4 bonds give rise to a gradual left handed twist in the amylose chain, resulting in a spiral or α-helix formation (Zobel 1988). This α-helix formation allows amylose to form complexes with a variety of chemical compounds such as iodine, fatty acids and alcohols. This is due to the fact that these compounds can position themselves inside the spiral formation and thereby interact with the amylose molecule (Nybraaten 2004). In barley the amylose content can range from 3 to 46 % of the total starch content, with a normal level of approximately 20–30 % (Stevnebø et al. 2006).

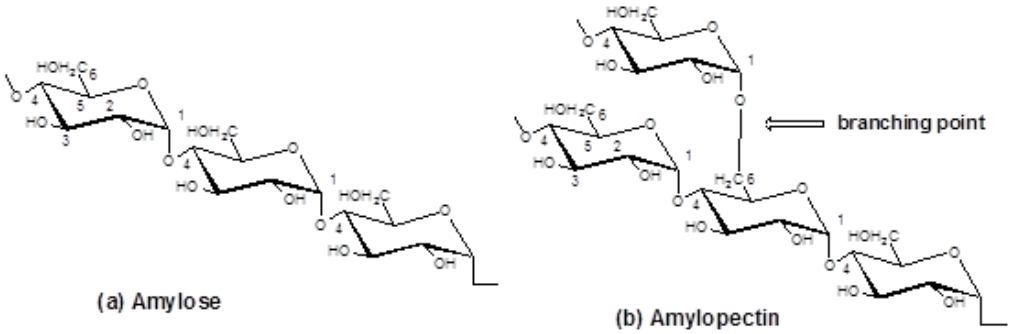

Figure 1.3: The two main types of polymers found in starch; amylose and amylopectin (Hayes)

The second main type of polymer which is found in starch is amylopectin (Zobel 1988). Amylopectin is one of the largest natural polymers (Delcour & Hoseney 2010) and makes up 75-80% of starch. Similar to amylose, amylopectin is composed of a linear chain of α-D-glucose units linked together by α-1,4 glycosidic bonds (Nybraaten 2004). These linear chains are joined via α-1,6 glycosidic bonds and it is these linkages which gives amylopectin its characteristic branched structure. Branching makes the amylopectin molecule very compact. Three types of chains are found in the amylopectin molecule; A-chains, B-chains and C-chains. The A-chains are composed of α-1,4-linked glucose units and are therefore not branched. The B-chains are made up of both α- 1,4 and α-1,6 glucose linked units and therefore carry branches. The C-chains is also branched and is composed of both α- 1,4 and α -1,6 glucose linked units. The C-chain is the only chain containing a reducing end in the amylopectin molecule (Delcour & Hoseney 2010).

1.1.3.2 Protein

Between 9-15% of the dry weight of the barley grain is composed of protein (Asare et al. 2011). The main proteins are the storage proteins hordeins and glutelins (Anker-Nilssen 2007) with the hordeins making up approximately 40% of the protein found in barley. The barley endosperm contains mainly hordein and glutelin proteins while albumin and globulin predominate in the *pericarp,* aleurone layer and embryo (Wang et al. 2010). Protein, which is found on the surface of the starch granule, may act as a physical barrier to the digestion of starch. This is due to the fact that it reduces the contact between the starch granule and the digestive enzymes (Svihus et al. 2005).

1.1.3.3 Lipids

Between 1-2% of the barley grain is made up of lipids (Asare et al. 2011). The lipids which are associated with cereal starches are generally polar and prevalent inside the starch granules. They consist mainly of lysophospholipids and unsaturated fatty acids; with linoleic acid (18:2) and palmitic acid (16:0) being the two most abundant (Delcour & Hoseney 2010).

Some lipids are also found on the surface of the starch granule where they often act as a barrier to the digestion of starch. This is due to the fact that they diminish the contact between the digestive amylases and the substrate (Svihus et al. 2005)

1.1.3.4 Vitamins

The vitamins found in the barley grain are often concentrated in the aleurone layer. Given the low level of lipids which are present in the barley grain, barley is a poor source of the fat soluble vitamins A, D and K. However, barley is an important source of the water soluble B-vitamins such as niacin, riboflavin and thiamin (Delcour & Hoseney 2010).

1.1.3.5 Minerals

The mineral composition of the barley grain includes phosphorus, potassium, calcium, magnesium, iron, copper and manganesium (Delcour & Hoseney 2010). The aleurone layer is the major storage site for the minerals phosphorus, magnesium, potassium, and calcium with over 70% of these minerals accumulating here (Becraft 2007).

1.1.3.6 Enzymes

To release the energy which is stored as starch in the endosperm, the barley grain contains a number of enzymes. The starch-hydrolyzing enzyme α-amylase functions by hydrolyzing the α-1,4 linkages of the starch chain internally. This hydrolysis is more or less random and results in the production of α-limit dextrins, maltose and maltotriose. β-amylase produces the disaccharide maltose and is often referred to as the saccharifying or sugar producing enzyme. The combined activities of α-amylase and β-amylase degrade starch more rapidly and frequently than either of the enzymes working alone. However neither of these enzymes are capable of breaking the α-1,6 linkages present in amylopectin and therefore do not completely degrade starch. In general about 85% of starch is converted to sugar by a mixture of α-amylase and β-amylase. In addition to hydrolyzing the α-1,4 linkages at the non-reducing ends of the starch molecules, the exoamylase glucoamylase can break down the α-1,6 bonds in the amylopectin molecule. In theory glucoamylase can completely convert starch to β-D-glucose (Delcour & Hoseney 2010).

1.1.3.7 Bioactive compounds

Barley contains a high level of bioactive compounds including phenolic compounds such as benzoic and cinnamic acid derivatives, pro-anthocyandines, quinones, flavonols, chalcones, flavones, flavanones and amino phenolic compounds. The majority of these compounds are present in both free and bound forms but it is primarily the bound form which predominates in barley. The free phenolic compounds which have been identified in barley include

proanthocyandines and flavonoids while the bound phenolic compounds consist mainly of ferulic acid and its derivatives (Bonoli et al. 2004).

1.1.4 Use

Barley is an ancient and important cereal grain which was primarily used as human food but which has gradually evolved into a feeding, malting and brewing crop (Baik & Ullrich 2008). Despite ranking fourth in terms of cultivation and utilization behind maize, rice and wheat, barley is now the least utilized grain for human food consumption. In recent years however, the high levels of bioactive compounds present in barley have resulted in a widespread acceptance of barley as a functional food ingredient (Asare et al. 2011). These bioactive compounds including phenolic compounds such as free and bound phenolic acids (Bonoli et al. 2004).

Given the fact that barley contains number of phenolic compounds, the following sections will take a look at the structure of phenols and polyphenols. This will be followed by a brief account of phenolic acids.

1.2 Phenols

1.2.1 Structure of phenols

Phenols are molecules which have a hydroxyl group directly attached to their aromatic ring. Phenolic molecules tend to form hydrogen bonds with one another and therefore have relatively high boiling points. These high boiling points results from the fact that breakdown of the hydrogen bonds is necessary in order for vaporization of phenols to occur. Breakdown of these bonds requires a substantial amount of energy in the form of heat. Heat is also required to vaporize the molecules following breakdown of the bonds (Hart et al. 2003).

1.2.1.1 Phenols ability to act as acids

Phenolic molecules have an ability to act as a weak acid, with their hydroxyl group acting as a proton donor. They ionize in aqueous solutions producing H^+ ions. Dissociation (shown in figure 1.2) occurs in a similar manner to that of water (Hart et al. 2003).

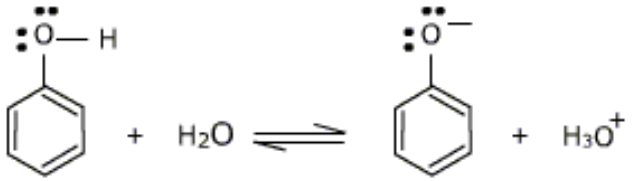

Figure 1.4: Dissociation of a phenolic molecule. The molecule ionizes in water producing H^+ ions (SHRESTHA)

In comparison to alcohols phenols are much stronger acids. This is primarily due to resonance stabilization. The negative charge on an alkoxide ion is concentrated on the oxygen atom, while the negative charge on a phenoxide ion can de delocalized to other positions on the aromatic ring. This delocalization is achieved through resonance and has a stabilizing effect on the phenolic molecule, thereby making phenols much stronger acids than alcohols (Hart et al. 2003) .

1.2.1.2 Antioxidant function of phenols

In addition to their ability to act as acids, phenolic compounds are also capable of functioning as antioxidants. They react with and destroy peroxy (ROO·) and hydroxy (HO·) radicals and thus prevent the oxidation of substances which are sensitive to air oxidation. Given that human beings use antioxidants for protection against peroxy- and hydroxyl radicals, phenols, in particular polyphenols, have awakened an interest in the field of human nutrition (Hart et al. 2003) .

1.2.2 Polyphenols

Polyphenols are molecules which have several hydroxyl groups attached to their aromatic ring. They constitute the active substances found in many medicinal plants and modulate the activity of a wide range of enzymes and cell receptors (Manach et al. 2004). Rich sources of polyphenols include fruit, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine (Tsao 2010). Whole grains in particular are recognized as an important source of phenolic compounds and have been linked to the prevention of chronic illnesses such as cancer and cardiovascular disease (Abdel-Aal & Rabalski 2013). This is due to the strong antioxidant properties of polyphenols, which combined with other dietary reducing agents, help protect the body's tissues against oxidative stress thereby reducing the risk of oxidative stress related diseases (Scalbert & Williamson).

1.2.3 Structure of polyphenols

Molecules containing several hydroxyl groups on aromatic rings are said to have a polyphenol structure. Polyphenols may be classified into different groups according to the number of phenol rings that they contain and the number of structural elements that bind these rings to one another (Manach et al. 2004). The majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at various positions in their polyphenol skeleton (Tsao 2010). They arise biogenetically from one of the two main synthetic pathways: the shikimate pathway or the acetate pathway (Bravo 1998). Due to the diversity and wide distribution of polyphenols in plants, various ways of categorizing these naturally occurring

compounds has arisen (Tsao 2010). In this thesis classification of polyphenols will be carried out according to the chemical structure of the non-sugar group of the glycoside.

1.2.3.1 Phenolic acids

Phenolic acids are polyphenolic compounds which can be divided into two main classes: benzoic acid derivatives and cinnamic acid derivatives. These two main classes are defined according to the nature of their carbon skeleton with benzoic acid derivatives having a C_1-C_6 structure while derivatives of cinnamic acid have a C_3-C_6 backbone (Tsao 2010). The four phenolic acids investigated in this thesis are derivatives of cinnamic acid and their structure is shown in figure 1.5 (caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid).

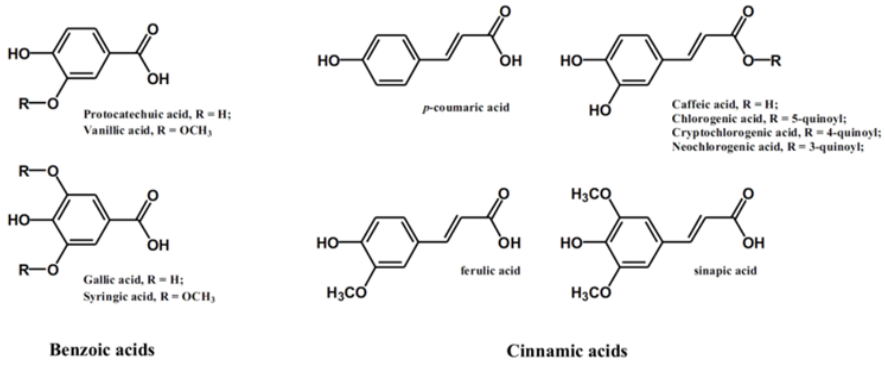

Figure 1.5: The two main classes of phenolic acids: derivatives of benzoic acid and derivatives of cinnamic acid (Tsao 2010)

Phenolic acids occur primarily in conjugated form with one or more sugar residues linked to their hydroxyl groups. These sugars can be present as monosaccharides, disaccharides or even as oligosaccharides with glucose being the most common associated sugar. Phenolic acids can be found in both free and bound forms. This is due to the fact that their corresponding methyl esters, ethyl esters and glycosides commonly occur in both free and bound forms (Bravo 1998). It is primarily the bound form which predominates in barley although free phenolic compounds have also been identified (Bonoli et al. 2004).

Ferulic acid and *p*-coumaric acid are the two most abundant phenolic acids found in the barley grain. They accumulate in the outer layers of the *caryopsis* (hull, testa, and aleurone layer) where they are present as ester-linked cell wall polymers (Gamel & Abdel-Aal 2012; Hernanz et al. 2001). Ferulic acid is linked to arabinoxylan which is an important constituent of the cell walls of the aleurone cells (Nordkvist et al. 1984). *p*-CA on the other hand forms linkages with lignins (Higuchi et al. 1967) which are most abundant in the cell wall of the hull than in other parts of the grain (Salomonsson et al. 1978). Due to the fact that no mammalian endogenous enzymes can release these bound cinnamic acids, xylanases and esterases are required to carry out this task (Kroon et al. 1997).

1.3 Enzymes

1.3.1 Xylanases

Xylanases are hydrolytic enzymes which specifically target and depolymerize arabinoxylan (Selinheimo et al. 2006). Arabinoxylan is one of the major non-starch polysaccharides present in barley (Izydorczyk & Dexter 2008) and makes up approximately 20% of the cell wall of the barley endosperm (Delcour & Hoseney 2010). Arabinoxylan is composed of a linear chain backbone of β-D-xylopyranosyl residues linked together by glycosidic linkages (Izydorczyk & Dexter 2008). It can be divided into water extractable and water un-extractable arabinoxylan, with water extractable arabinoxylan considered to be the precursor to water un-extractable arabinoxylan (Selinheimo et al. 2006). Pentopan, is an xylanase, which belongs to the glycosyl hydrolase family (Li et al. 2013). It cleaves the glycosidic linkages in the arabinoxylan molecule thereby converting water un-extractable arabinoxylan into water extractable arabinoxylan (Selinheimo et al. 2006). Pentopan Mono BG is the commercial xylanase which was added to a number of the samples investigated in this master thesis.

1.3.2 Feruloyl esterases

Due to the heterologous nature of arabinoxylan several enzymes are required in order to break down the polysaccharide. In addition to being degraded by xylanases, arabinoxylan is also be broken down by feruloyl esterases (Faulds et al. 2006).

Arabinoxylan chains are often cross linked by di-ferulic bridges which arise as a result of the dimerization of ferulic acid (Renger & Steinhart 2000). These di-ferulic bridges fortify the plant cell wall while simultaneously impeding the release of arabinoxylan. Feruloyl esterases cleave the ester link joining the β-D-xylopyranosyl residues on arabinoxylan and the di-ferulic or ferulic acid (Faulds et al. 2006) This results in the release of the ester-linked ferulic acid from the cell wall.

Naturally occurring feruloyl esterases have been identified in the barley grain where they cleave methyl esters of cinnamic acid derivatives. These enzymes are active upon the methyl esters of sinapic, *p*-coumaric ferulic and caffeic acids being most effective upon methyl esters of ferulic acid and sinapic acid (Sancho et al. 1999).

1.3.3 Acetic acid esterases

Acetic acid esterase activity have been detected in both the endosperm and aleurone cells in the barley grain (Engel 1947). The linear chain of β-1,4-xylopyranose units, which constitutes the backbone of arabinoxylan molecules, can be substituted at carbons 2 and 3 with acetate groups linked by ester bonds. This acetylation stiffens the chain of β-1,4-xylopyranose units

so that they can adopt a more extended two-fold helical conformation in solution. This two-fold helical formation is believed to stabilise the xylan molecule (Humberstone & Briggs 2000) as well as increasing its solubility in water (Poutanen et al. 1990). Acetic acid esterase cleaves the acetyl groups substituted at carbons 2 and 3 of the xylan backbone of arabinoxylans (Latha & Muralikrishna 2007) . Given that acetyl substitution is believed to impede the action of endoxylanases on the xylan backbone, this esterase plays a central role in the breakdown of the acetyl arabinoxylan (Tenkanen 1998).

1.4 Digestion

As mentioned in the introductory paragraph of this chapter, the relationship between free phenolic acids and starch digestion was one of the aspects investigated in this master thesis. This is due to the fact that phenolic acids have been shown to have an inhibitory effect on pancreatic α-amylase. A brief account of human digestion is therefore deemed appropriate. This section opens with a definition of glycemic index. This is followed by an account of the human gastrointestinal tract and a description of the enzymes involved in the digestion of starch will be given

1.4.1 Glycemic index

Glycemic index (GI) is a classification system whereby the glycemic responses of foods are indexed against a standard (white bread). Different glycemic responses are produced by different foods and studies suggest that these differences are often related to the rate at which foods are digested (Wolever 1990). Carbohydrates with a high glycemic index for example rapidly raise blood glucose levels. This is due to the fact that they are quickly digested and absorbed into the bloodstream. Elevated blood glucose levels stimulate a high demand for insulin therefore increasing the risk of obesity and type 2 diabetes mellitus. Ideally carbohydrates with a low glycemic index should be consumed. These carbohydrates are digested more slowly and therefore raise blood glucose more gradually. Examples of carbohydrates with a low glycemic index include the carbohydrates found in fruit, vegetables, grainy bread and pasta (Saladin 2012) Food processing methods may also influence the rate at which food is digested and thereby the glycemic response (Wolever 1990).

1.4.2 Gastrointestinal tract

The gastrointestinal tract is a flexible muscular tube which extends from the mouth, through the esophagus, stomach, small intestine, large intestine and rectum to the anus. Nutrients enter the body by penetrating the wall of the gastrointestinal tract, while non-nutrient materials pass through the tract and are subsequently eliminated from the body (Whitney & Rolfes 1999). In

order to be able to use the nutrients which penetrate the wall of the gastrointestinal tract, it is necessary to break them down into smaller particles. The primary purpose of the digestive system is to break nutrients down into forms which may be used by the body. This aim is carried out with the aid of both mechanical and chemical digestion. Mechanical digestion can be defined as the physical breakdown of food into smaller particles. The cutting and grinding action of the teeth as well as the churning action of the stomach and the small intestine are both examples of mechanical digestion. By breaking the food into smaller particles, mechanical digestion increases the surface area of the food thus exposing more of the ingested food to the digestive enzymes. Chemical digestion is carried out by digestive enzymes and involves the breaking down of carbohydrates, proteins and lipids into their respective components. Digestive enzymes are produced in the mouth, the stomach, the pancreas and the small intestine (Saladin 2012).

1.4.3 Digestive enzymes

1.4.3.1 Salivary amylase

In the mouth the teeth are responsible for crushing the food and breaking it down into smaller particles. Vigorous mastication stimulates the production of saliva which lubricates the food thereby making it easier to swallow (Whitney & Rolfes 1999). Saliva contains the starch-digesting enzyme amylase which hydrolyses starch into short oligosaccharides of up to eight glucose residues long. These oligosaccharides are subsequently broken down into the disaccharide maltose, which is finally degraded to glucose. Salivary amylase functions best at pH 6.8-7.0 and is quickly denatured upon contact with stomach acid. However by concealing itself in the center of the food mass, amylase can escape contact with the stomach acid and thereby continue digesting starch for up to 2 hours after entering the stomach. As pepsin, acid and the churning actions of the stomach break up the food mass, amylase is denatured and subsequently digested by pepsin. This is due to the fact that amylase is a protein (Saladin 2012).

1.4.3.2 Pancreatic amylase

About 50% of dietary starch is digested before it reaches the small intestine. In the small intestine starch digestion is carried out by pancreatic amylase. Pancreatic amylase breaks starch down into small oligosaccharides and the disaccharide maltose. The enzymes dextrinase and glucoamylase hydrolyze the small oligosaccharides, into glucose, while maltase hydrolyzes maltose into its glucose constituents. The monosaccharide glucose is absorbed by the epithelial cells and used as a source of energy in the body (Saladin 2012).

1.4.3.3 Porcine pancreatic α-amylase

Due to the fact that the inhibitory effects of phenolic acids on pancreatic α-amylase were investigated in this thesis, oral α-amylase was not used. This paragraph gives a brief description of the porcine pancreatic α-amylase used in this study in addition a brief account of the inhibitory effects of phenolic compounds.

Porcine pancreatic α-amylase is a glycoprotein which is composed of a single polypeptide chain of ~ 475 residues. It randomly hydrolyzes the α-D-(1,4)-glycosidic bonds present in amylose and amylopectin. The pH range for this activity is between 5.5– 8.0 with an optimum pH of ~6.9 (Ishikawa et al. 1990). Many plant extracts including phenolic compounds have been shown to inhibit the enzymatic activity of pancreatic α -amylase. These phenolic compounds interact with protein and inhibit the enzymatic breakdown of starch, thereby helping to modulate glucose levels (McCue et al. 2005)

1.4.3.4 Pepsin

Digestion of protein begins in the stomach and is carried out by enzymes called proteases or peptidases. In the stomach, any peptide bond between tyrosine and phenylalanine is hydrolyzed by the enzyme pepsin and dietary protein is broken down into shorter polypeptides and a small amount of free amino acids. Pepsin has an optimal pH of 1.5-3.5 and can digest up to 15% of dietary protein. The alkaline environment of the duodenum (pH 8) inactivates pepsin and protein digestion is subsequently taken over by trypsin and chymotrypsin (Saladin 2012). These enzymes however will not be discussed further in this master thesis.

1.4.3.5 Bile

Bile is a green fluid which consists of minerals, cholesterol, neutral fats, phospholipids, bile pigments and bile acids. Bile acids are steroids which are synthesized from cholesterol and which combined with lecithin participate in the digestion of lipids. The large fat globules which enter the duodenum are exposed to lipase only at their surfaces. These globules are broken up into smaller emulsification droplets by a combination of bile acids and bile-lecithin. These two agents have both hydrophilic and hydrophobic regions. The hydrophilic regions attach to the surrounding water while the hydrophobic regions attach to the surface of the fat globules. A combination of agitation produced by intestinal segmentation and a coating of lecithin and bile keeps these fat globules broken up. As a result more of the surface area of the globule is exposed to the digestive action of enzymes such as pancreatic lipase (Saladin 2012).

1.5 Methods

The final section in this chapter aims to provide background information to the methods employed in this thesis.

1.5.1 Moisture determination

Moisture content is defined as the percent water in a sample (James 1995). Accurate determination of the moisture content in foodstuffs is often difficult. This is due to the fact that not all water present in food exists in a form that easily freezes or easily evaporates. Hydrogen bonds, ionic forces of attraction and polar forces of attraction between the water molecules themselves often results in the binding of water, therefore creating difficulties in determining moisture content. One way of determining moisture content in foodstuffs is using an infrared moisture balance. An infrared moisture balance is an example of evaporation methods which are widely used to determine moisture content in food. This is due to the fact that these methods are easy to perform, reasonably accurate as well as requiring little in the form of expensive equipment (James 1995). The principle behind infrared moisture balances is relatively simple. As the sample is heated and dried the weight changes caused by evaporation are recorded. The difference in the pre- and post-drying weights is used to calculate both the moisture content and the dry matter content in the sample. These calculations can subsequently be used to help avoid unjust comparisons of samples. Analytical results can be reported on a "as sampled basis" or on a "dry matter basis". "As sampled basis" gives the results for a sample in its natural state (including water) while "dry matter basis" gives the results for a sample in its dried state (without water). Due to the fact that water has a diluting effect on results, results reported on a "dry matter basis" permit the direct comparison of different samples. (Equi-analyticalLaboratories 2004).

1.5.2 Isolation in Pure Culture

A pure culture consists of a single type of microorganism and may be obtained in a number of different ways. One of the most common ways to obtain a pure culture is by using the streak plate technique. For organisms which grow well on agar plates this method is quick and easy to use. Repeated picking and re-streaking of a well isolated bacteria culture can give rise to a pure culture that may be subsequently transferred to a liquid medium. Furthermore with appropriate incubation facilities it is possible to purify both aerobes and anaerobes using the streak plate technique (Madigan & Martinko 2006)

1.5.3 Fermentation

Fermentation is regarded as one of the oldest food processing methods in the world. Specific micro-organisms are selected and their actions controlled in order to alter a particular characteristic or property in a target foodstuff. Two main groups of fermentations exist; those in which the primary products are carbon dioxide and ethanol and those in which the main products are organic acids. Microorganisms which produce a single main by-product are called homofermentative while those which produce several by-products are called heterofermentative (Fellows 2000) Lactic acid bacteria are examples of homofermentative microorganisms. This is due to the fact that their major by-product is lactic acid.

1.5.4 Boiling

In general cereals are cooked or boiled prior to their consumption. Boiling results in significant changes in chemical composition which in turn affect the bioaccessibility and concentration of compounds such polyphenols (N'Dri et al. 2012). These changes include starch gelatinization, retrogradation and protein denaturation (Gallegos-Infante et al. 2010).

Gelatinization of starch

At some point in time the starch in most cereal foods are heated in the presence of water. Viewed under an optical microscope, individual starch granules dispersed in water lose their high degree of molecular order (birefringence) over a very small range of temperature. Birefringence is first lost at the site where starch biosynthesis is initiated (hilum) and thereafter in the remainder of the starch granule. This transitional process is defined as gelatinization and is a granule by granule event. Gelatinization is an irreversible process which affects all of the structural levels in the starch granule. In addition to disrupting the molecular order, gelatinization results in the swelling of granules, the leaching of soluble polysaccharides, the absorption of water as well as the melting of crystallines (Delcour & Hoseney 2010). Gelatinised starch is more susceptibile to starch degradation in the digestive tract than native starch (Svihus et al. 2005)

Retrogradation

During storage gelatinized starch can undergo a process called retrogradation (Delcour & Hoseney 2010). Retrogradation is the process which occurs when the molecules which make up the gelatinized starch begin to re-associate and develop a more ordered structure. If conditions are favorable, this ordered structure may develop into crystalline forms in an unstructured matrix (Atwell et al. 1988). As amylopectin retrogradation occurs very slowly, it is primarily amylose retrogradation which is considered important. In general high amylose

content starch is associated with reduced digestibility (Svihus et al. 2005). This is due to the fact that retrograded starch is resistant to digestion (Eerlingen et al. 1994).

Protein denaturation

Proteins are composed of chains of amino acids. The primary structure of the protein is defined by the sequence of these amino acids in the chain. The secondary structure is when the chains fold in on themselves locally and wrap around themselves to form the tertiary structure. This tertiary structure has a specific three dimensional shape which is related to the function of the protein. Exposure to heat or the addition of chemicals result in the denaturation of proteins and thereby the loss of this three dimensional structure. In terms of digestion cooking denatures the proteins found in the food, rendering digestion more efficient. This is due to the fact that the denatured protein is more accessible for the enzyme (Nybraaten 2004)

1.5.5 Freeze drying

Freeze drying can be defined as the removal of water from a frozen product based on the principle of sublimation. Sublimation is the process which occurs when a solid goes directly to a vaporous state without passing through the liquid state first. During freeze drying, heat is applied to a frozen sample promoting the removal of water molecules. A vacuum pump creates conditions favorable for the occurrence of sublimation, encouraging the free flow of water molecules. These water molecules are collected and subsequently condensed in a cold trap or condensing system.

In order for freeze drying to be successful pre-freezing of the sample is necessary. This is due to the fact that freeze drying is based on the principle of sublimation and therefore imperative that the sample to be freeze dried is in a frozen or solid state. Following pre-freezing, ice is removed from the frozen product by the process of sublimation. The rate of sublimation is affected by the vapour pressure of the product and the vapour pressure of the ice condenser. The water molecules have a natural affinity to migrate from the high pressure sample to the low pressure ice condenser. Therefore it is important that the pressure in the sample is higher than the pressure in the condenser. As vapour pressure is related to temperature, it is necessary that the product temperature is higher than temperature in the ice condenser. The key to optimal freeze drying is maintaining a balance between the temperature that allows the product to remain structurally intact and the temperature that maximizes the vapour pressure of the product. This is the removal of the bound moisture which is still present in the sample

following the sublimation of ice. Bound water is desorbed from the sample in a process called isothermal desorption. Secondary drying occurs at a higher temperature than primary drying but both pressure and condenser temperature remains the same (LABCONCO).

1.5.6 Extraction, analysis and identification of polyphenols

1.5.6.1 Liquid/liquid extraction

In liquid/liquid extraction, the sample is often dissolved in an organic solvent which extracts the target compound or analyte. The choice of solvent is based on a number of factors including the nature of the analyte. Due to its high polarity and immiscibility with most organic solvents, the majority of solvent systems involve water. It is therefore important that the target analyte is soluble in the chosen organic solvent but simultaneously insoluble in water (Harris 2010).

1.5.6.2 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC), uses high pressure is to force a solvent through a closed column containing fine particles. These fine particles represent the stationary phase and are usually tightly packed into the column. In general highly purified, spherical microporous particles of silica make up the stationary phase. As the size of these particles decreases, the efficiency of the column increases. The solvent which is forced through the column represents the mobile phase and can be more or less polar than the stationary phase. Elution of the analyte can be performed with isocratic elution where a single solvent or constant solvent mixture is used. Alternatively a continuous change of solvent composition may be used and this is referred to as gradient elution. Heating the column generally reduces the viscosity of the solvent thereby promoting faster flow and reducing required pressure. Furthermore increased temperature decreases retention times and improves resolution by accelerating the diffusion of solutes. Introduction of particulate matter into the column is avoided by filtration of the samples before they are loaded into vials. Furthermore the entrance to the main column may be protected by a guard column, which retains fine particles in addition to strongly adsorbed solutes. To avoid column degradation pure HPLC grade solvents are used. These also contribute to minimizing detector background signals and noise (Harris 2010).

1.5.6.2.1 Pump

The quality of the HPLC pump can be determined by how steady and reproducible a flow it can produce. Weak signals can be obscured by a fluctuating flow rate which can create detector noise. A steady flow rate is therefore important (Harris 2010).

1.5.6.2.2 Injection valves

An injection valve can have several interchangeable loops each of which can hold a fixed volume. In the load position, the loop is washed and loaded with a fresh sample using a syringe at atmospheric pressure. The contents of the loop are injected into column at high pressure (Harris 2010)

1.5.6.2.3 UV detector - Diode array detector (DAD)

An ultraviolet (UV) detector using a flow cell is one of the most common HPLC detectors. This is due to the fact that many solutes absorb UV light (Harris 2010) . A diode array detector (DAD) is an example of a UV detector which detects absorption in the ultraviolet-visible (UV-VIS) region and has multiple photodiode arrays. This allows the detector to obtain information over a wide range of wavelengths at any one given point in time. During separation by HPLC, spectra are often measured at intervals of one second or less. If this measurement is carried out at a fixed wavelength, the components may only be identified using their retention time. A minor deviation in retention time can therefore render identification of components difficult. In such a case, a DAD can be used to identify components by a comparison of spectra (*HPLC Basic Course.* 2001)

1.5.6.2.4 Calibration curves

A chromatogram is a graph of detector response as a function of time. However a chromatogram does not tell us which analytes are present. One way of determining this is to measure the individual characteristics of the analytes as they emerge from the column. In general analytes with equal contentrations give different detector responses. The response must therefore be measured for a known concentration of the analyte. A graph of detector response as a function of analyte concentration is called a calibration curve. To construct such a curve, standard solutions containing known concentrations of an analyte are prepared and subsequently injected into the column. The resulting peak height or area under the peak is measured and this is proportional to the quantity of analyte passing through the detector.

Straight lines are drawn through the calibration points and can be used to determine the concentration of the analyte (Harris 2010).

1.5.7 Determination of total starch

Determination of total starch in a sample is based on the principle that the addition of α-amylase hydrolyses starch into soluble branched and unbranched maltodextrins. These maltodextrins are then quantitatively hydrolysed into monomeric D-glucose units by the addition of amyloglucosidase. D-glucose units is subsequently oxidised to D-gluconate and one mole of hydrogen peroxide (H_2O_2) is released. H_2O_2 is quantitatively measured in a colorimetric reaction employing peroxidase and the production of a quinoneimine dye (glucose oxidase). Samples containing resistant starch are pre-dissolved in dimethylsulfoxide (DMSO), while samples containing a high D-glucose and maltodextrins content are washed with aqueous ethanol (*Total starch assay procedure* 2011)

1.5.8 Spectrophotometry

Any technique which uses light to determine the chemical concentration of a sample is called spectrophotometry. Colorimetry is any procedure based on the absorption of visible light. The instrument which is used to carry out this measurement is called a spectrophotometer. While both single-beam and double-beam spectrophotometers are available, it is single-beam spectrophotometers which are of interest to this master thesis. In a single-beam spectrophotometer, a monochromator is used to disperse light into its component wavelengths and selects a narrow band of wavelengths to pass the dispersed light onto a sample or detector. This light usually comes from a source such as a deuterium or halogen lamp. The irradiance from the light striking the detector with a reagent blank (P_0) in the sample compartment is measured first. When a sample of interest replaces the reagent blank, some radiation is usually absorbed and therefore the irradiance striking the detector (P) is less than P_0. Transmittance (T) which is defined as P/P_0 is usually a number between 0 and 1. Absorbance which is proportional to concentration is defined as $\log P_0/P = -\log T$. Baseline absorbance is recorded first and subsequently subtracted from the measured absorbance of the sample. This gives the true absorbance of the sample (Harris 2010) in addition to an indication of the chemical concentration.

1.5.9 *In vitro* digestion models

Digestibility, structural changes and bio-accessibility under simulated gastrointestinal conditions are often investigated using *in vitro* digestive models. Plant based foods are amongst the most prominent food samples investigated, while pancreatin, pepsin lipase and α-amylase are amongst the most frequently used digestive enzymes. One of the most important factors in an *in vitro* digestive system is the characteristics of the digestive enzymes and how these are affected by factors such as pH, temperature and incubation time. Digestive enzymes are usually selected on the basis of the target food component and are added sequentially to the *in vitro* model. Many *in vitro* models for example are based on simulation of the stomach by incubation with pepsin followed by simulation of the small intestine by incubation with pancreatin. The digestive temperature in the majority of *in vitro* models is 37ºC while the digestive time is approximately two hours. Digestive time refers to the amount of time spent in the mouth, the stomach and the intestines and should ideally reflect the digestion times in humans. An important factor influencing digestion time is the size of the target food sample. Large food particles for example move more slowly through the stomach than small food particles. Therefore food containing large particles requires longer incubation time in the stomach than food containing small food particles. In general *in vitro* digestion systems are common and useful tools for the analysis of food digestion. However due to the inherent complexity of the process *in vitro* methods will never be able to reproduce the accuracy of investigating digestion *in vivo.* Despite these shortcomings however *in vitro* conditions can provide a useful and inexpensive alternative to human models (Hur et al. 2011)

1.5.10 Statistical Analysis

1.5.10.1 Analysis of variance (ANOVA)

Analysis of variance (ANOVA) looks at variation in data and attempts to identify where this variation is found. More specifically ANOVA compares the amount of variation within groups with the amount of variation between groups. This variation is called the sum of squares and is used to calculate the F-ratio. The F-ratio is a test statistic which is 1 if the average difference between groups is similar to the average difference within groups. However the more the average difference between groups becomes greater than the average difference within groups, the more the F ratio becomes greater than 1.This F-ratio is used to calculate the probability (P-value) of obtaining the data assuming that all group means are equal. A significant P-value suggests that at least one group mean is significantly different from the others. The P-value is usually set at $P<0.05$. (Hindle 2013).

1.5.10.2 Post hoc tukey tests

On completion of ANOVA, a post hoc test is required in order to determine which groups differ from one another. In the case of Tukey's post-hoc test, the differences between the means of all of groups are first found. This difference is compared to a critical value to determine if the difference is significant. This critical value must be computed and is called the Honestly Significant Difference (HSD) (*Post hoc tests*). An honestly significantly different mean is preferred.

2 Materials and methods

2.1 Materials

2.1.1 Cereal samples

Covered barley, hulled barley, barley flakes and barley flour were the principle materials utilized in this master thesis. The four different barley products were procured from the mill "Ottadalen Mølle" in Lom, a municipality in Oppland County, Norway. Images of the four cereal products are shown below.

Figure 2.1: In the figure above the four different barley products which were used in this thesis are shown. Top (left to right): Covered barley and hulled barley. Bottom (left to right): Barley flakes and barley flour. The images were taken by Hanne Zobel (Nofima) using the program DinoCapture 2.0 (Dino-Lite Europe/ IDCP B.V. The Netherlands)

Commercial ground wheat flour, obtained from Lantmannen Cerealia, Stockholm, was used for comparison studies.

2.2 Methods

2.2.1 Moisture determination in barley products

Determination of moisture content in the four barley products was carried out using a Thermo Control YTC 01 L Infrared moisture balance (Sartorius). Approximately two grams of sample was distributed on an aluminum weighing dish which was placed on the balance. Heat was applied and the sample was dried until it reached a constant weight. Pre and post-weight of the sample was recorded in addition to the loss of moisture measured in percent. Moisture content (%) was used to calculate the dry matter content (%) in the sample which was utilized in later calculations. On completion the sample was disposed of and the above procedure was repeated for the remainder of the samples.

Figure 2.2: Sartorius Thermo Control YTC 01 L Infrared moisture balance. The picture is downloaded from: http://www.biovendis-products.de/deutsch/gebrauchtger%C3%A4te-shop-1/feuchtigkeitsbestimmer/

2.2.2 Fermentation of barley flour

2.2.2.1 Preparation of pentopan stock solution

Pentopan 500 BG (~27mg) was weighed into a 5ml volumetric flask and diluted with type 1 Milli-Q water (Millipore) to a final concentration approximately 5.4 $^{mg}/_{ml}$. Pentopan is known to effectively enhance dough properties such as extensibility and stability.

2.2.2.2 Isolation in Pure Strain

The lactic acid bacteria (LAB) strains used in this thesis were *Lactobacillus johnsonii* LA1, *Lactobacillus reuteri* SD2112 and *Lactobacillus acidophilus* LA-5. Pure strains were isolated from the three LAB using the streak plate technique on MRS agar plates. The LAB, which were stored at -80°C, were streaked out on the plates using a sterilized inoculating loop and subsequently incubated (37°C, anerobic) for 48 hours. Following incubation, colonies were

picked from the various agar plates and inoculated in glass tubes containing 20ml MRS broth. A single colony was selected from each plate and subsequently transferred over to its corresponding glass tube. The tubes were vortexed and subsequently incubated for 24 hours (37°C, anaerobic).After incubation 100µl of the strain was transferred to new glass tubes containing 20ml MRS broth. The tubes were vortexed and incubated (37°C, anaerobic) for a further 24 hours. After 24 hours each of the three LAB strains was transferred to their respective 50ml centrifuge tubes and centrifuged (1000 rpm, 10 minutes). Following disposal of the supernatant, 20 ml distilled water was added to the pellet in each of the tubes. The contents of the tubes were mixed using a vortex mixer and 1ml of each bacterium was transferred to 1.5ml Eppendorf tubes (2 parallels) and these tubes were stored at -20°C. A flow chart for the process described above is given at the end of this chapter in figure 2.7.

2.2.2.3 Sample preparation

Barley flour samples (~18g) were weighed into individual 100ml disposable cups with perforated lids. Distilled water (30°) was added to the two samples which were thoroughly mixed using a metal spatula. Acetic acid, LAB and/or pentopan were added to the samples when applicable. Acetic acid was added to the samples until the pH was ~3.8. With respect to the samples containing LAB, 50µl of the defrosted bacteria strain was first added to a 50ml centrifuge tube distilled water (30°). The contents of the tube were mixed using a vortex mixer and subsequently added to the appropriate sample. In the case of the samples containing pentopan, 100µl pentopan stock solution was added to the samples after addition of distilled water (30°C). This gave a final concentration of ~0.03 $^{mg}/_{g\ flour\ (as\ is)}$ pentopan in the samples. Control samples (T_0 samples), were prepared and immediately placed in the freezer (-20°C) in order to inhibit fermentation. The remainder of the samples was incubated at 37° C for 21 hours. All samples were prepared in duplicates. Fermentation of the barley flour samples is illustrated in a simplified flow chart at the end of this chapter (figure 2.8). A more detailed flow chart is given in appendix 2.

2.2.2.4 pH measurement

A PHM210 standard calibrated pH meter (Radiometer Analytical) was used to record pH in this thesis. The pH meter was calibrated using buffers pH 4 and pH 7. Pre-fermentation pH was measured in the barley flour and water samples in addition to the samples containing acetic acid. Post-fermentation pH was measured in all of the samples. Approximately 0.5g sample was mixed with 4.5ml distilled water before pH measurement.

Figure 2.3: PHM210 standard pH meter. This picture is downloaded from http://spektra.co.id/products/6_radiometer/scientific/ph_bench.htm

2.2.3 Boiling of hulled barley, barley flakes & barley flour

An illustration of the process described below is given at the end of this chapter (figure 2.9). Hulled barley (45g) was weighed into an 8ml weighing dish and subsequently transferred to an Erlenmeyer flask. Distilled water (300ml) was added to the flask which was covered with parafilm and left to soak overnight. The following day the sample was transferred to a saucepan and brought to the boil. The heat was reduced and the sample was allowed to simmer for 15 minutes until occasional stirring. The boiled samples were transferred to disposable cups (100ml) and placed in the freezer (-20°C) until further analysis.
Distilled water (300ml) was added to barley flakes (45g) and mixed using a metal spoon. The mixture was brought to the boil and allowed to simmer under occasional stirring for 15 minutes. Following transferal to disposable cups, the samples were placed in the freezer and stored at -20°C.

Barley flour (45g) was mixed with distilled water (300ml) and brought to the boil. The heat was reduced and the mixture was allowed to simmer for 20 minutes before being transferred to disposable cups. The samples were then placed in the freezer and stored at -20°C.
With respect to the samples containing pentopan, 1.4mg PENTOPAN was added to 45g barley flour (45g) followed by distilled water (300ml). After 1 hour the mixture was slowly brought to the boil and allowed to simmer gently under continuous stirring for 20 minutes. The mixture was then transferred to disposable cups and placed in the freezer (-20°C) until further analysis.

2.2.4 Freeze drying

Following fermentation and boiling approximately 4g of each sample was transferred into new disposable cups with perforated lids and placed in the freezer (-20°C) for three hours. The frozen samples were subsequently freeze dried for a period of four days. The weight of each of the samples was recorded both before and after freeze drying. The difference in the pre- and post-weight was used to calculate the moisture content and the dry matter content in the samples.

Figure 2.4:: Christ alpha 1-2/ LD plus Freeze Dryer. The picture is downloaded from http://www.martinchrist.de/index.php?id=97&L=1

2.2.5 Separation, analysis and identification of free phenolic acids in barley

2.2.5.1 Grinding of samples

Prior to sample preparation, covered barley, hulled barley and barley flakes were ground to flour using a ZM 200 Ultra Centrifugal Mill (Retsch)with a 0.5mm sieve. It was unnecessary to grind the barley flour and freeze dried fermented samples.

Figure 2.5: ZM 200 Ultra Centrifugal Mill .This image is downloaded from http://www.directindustry.com/prod/retsch/ultra-centrifugal-mills-19308-51350.html

2.2.5.2 Preparation of working stock and standard solutions for calibration

Individual stock solutions of caffeic acid(Fluka Chemika), p-Coumaric acid(Aldrich), ferulic acid(Aldrich) and sinapic acid(Fluka Chemika) were prepared by dissolving 10 mg of each of the acids in 25% methanol. The component was weighed into a 20ml volumetric flask and 5ml methanol was subsequently added to the flask. The mixture was vortexed before being diluted up to the graduation mark using type 1 Milli-Q water The final concentration in each of the 4 stock solutions was ~ 500 $^{\mu g}/_{mL}$.

Working solutions of the stock solutions were prepared by pipetting 2ml stock solution into a 10ml volumetric flask and diluting up to the graduation mark with Milli-Q water. The concentration of the working solutions was ~100 $^{\mu g}/_{mL}$. 1ml of each of the four working solutions was pipetted into a 10ml volumetric flask and diluted with Milli-Q water. The concentration of this calibration standard was 10 $^{\mu g}/_{mL}$(calibration standard 4). Calibration standards 1, 2 and 3 with concentrations of 2.5, 5, and 7.5 $^{\mu g}/_{mL}$ respectively were prepared by mixing various volumes of calibration standard 4 with Milli-Q water (see Table 2.1 below).

Table 2.1: Preparation of calibration solutions

Calibration standard	**Amount calibration standard 4 added (ml)**	**Amount distilled water added (ml)**	**Concentration $^{\mu g}/_{ml}$**
1	1	3	2.5
2	1	1	5
3	3	1	7.5

Prior to analysis all of the standards were filtered through a 0.45 µm PVDF filter into a glass vial. An aluminum cap with central hole and septum was subsequently fixed on the vial.

2.2.5.3 Sample preparation

1g sample was weighed into a 50ml centrifuge tube. 10ml 50% MeOH (v/v) was added to the sample and the contents of the tube were mixed using a vortex mixer. The sample was placed in an ultrasonic water bath for 30 minutes. In order to ensure that the temperature in the bath was held at approximately 0°C throughout the duration of the 30 minute period, ice was added to the water. Following subsequent centrifugation (4000g, 15 minutes) the supernatant was transferred to a 100ml glass flask and the residue in the tube re-suspended in 5ml 50% MeOH (v/v). The sample was mixed, centrifuged (4000g, 15 minutes) and the supernatant transferred to the glass flask. This procedure was repeated one more time giving a final volume in the glass flask of ~ 20ml. A rotavapor and water bath (30°C) was used to evaporate the sample. 6M HCL was added to Milli-Q water until the pH reached pH 2.0. 2.5 ml acidified Milli-Q water was added to the glass flask, mixed and subsequently transferred to a 50ml centrifuge

tube. This procedure was performed a total of four times giving a final volume in the centrifuge tube of 10ml. 10 ml ethyl acetate was added to the sample which was shaken (250 rpm, 10 minutes) and subsequently centrifuged (2800 rpm, 10 minutes). The supernatant was transferred over to a new 50ml centrifuge tube and 10 ml ethyl acetate added to the initial tube. This tube was shaken and centrifuged as above and the supernatant transferred over to the new 50ml centrifuge tube. This was repeated two more times giving a final volume of ~40ml ethyl acetate in the new centrifuge tube. The sample was evaporated to dryness using a centrifugal vacuum concentrator. The duration of sample evaporation was approximately four hours. Upon evaporation 250µl MeOH was added to the tube and the contents of the tube were re-suspended in the MeOH using a vortex mixer. 750 µl Milli-Q water was then added to the tube and the contents re-vortexed. 250 µl sample was filtered through a 0.45 µm PVDF filter and subsequently injected to a glass vial containing an insert. An aluminum cap with central hole and septum was then fixed on the vial. An overview of the extraction of free phenolic acids in barley is given at the end of this chapter in figure 2.10

2.2.5.4 Rapid Separation Liquid Chromatography (RSLC)

The samples were analyzed using RSLC (Dionex™ UltiMate™ 3000 Rapid Separation LC System, Thermo Scientific) with photodiode array ultraviolet detector (Dionex™ UltiMate™ 3000 Rapid Separation Diode Array Detector, Thermo Scientific) and a 1.7µm 2.1 x 150 mm column (Acquity UHPLC BEH C8 1.7µm 2.1 x 150 mm column, Waters). An auto-sampler (Dionex™ Ultimate™ 3000 Rapid Separation Autosampler, Thermo Scientific) was used to inject 10µl sample into the system, which was subsequently pumped throughout the system using a binary pump (Dionex™ Ultimate™ 3000 Binary Rapid Separation (RS) Pump, Thermo Scientific). A gradient elution program was employed with 1% acetic acid in Milli-Q water as eluent A and 1% acetic acid in acetonitrile as eluent B. The flow rate was set to 0.450 ml/min, while the temperature in the column oven (Dionex™ Ultimate™ 3000 Rapid Separation Thermostatted Column Compartment,Thermo Scientific) was set to 50°C. The pressure in the system fluctuated between 550 and 600 bar.

2.2.5.5 Series set-up

The samples were analyzed in the following order: first two vials containing filtered Milli -Q water were analyzed. These were followed by calibration standards 1,2 3 and 4. Two parallels of each sample were subsequently analyzed. Following analysis of 10 samples the standard calibration solutions were re-analyzed. They were also re-analyzed at the end of the sample

series. The final sample in the series was a vial containing filtered Milli-Q water from Millipore.

2.2.5.6 Identification and calculation of phenolic acids

The phenolic acids were identified at wavelength 320nm. A calibration curve was set up in Chromeleon Chromatography Data System (Dionex) to identify and quantify the phenolic acids in the samples. The calibration curve consisted of four external standards (see 2.2.5.2) whose retention times formed a basis for the identification of phenolic acids in the samples. In the case of covered barley, hulled barley, barley flakes and barley flour the dry matter content (%) determined was used in calculations.

2.2.6 Separation, analysis and identification of bound phenolic acids in barley

2.2.6.1 Grinding of barley products

The barley products were ground in the same manner as described in section 2.2.5.1 of this chapter.

2.2.6.1 Preparation of working and stock solutions

Preparation of stock solutions and working solutions are described in section 2.2.5.2 of this chapter. Stock solutions, working solutions and calibration standards were prepared in the same way as for determination of free phenolic acids in barley.

2.2.6.1 Sample preparation

0.2g sample was weighed into a 50ml centrifuge tube. 10ml 50% MeOH (v/v) was added to the sample and the contents of the tube were mixed using a vortex mixer. The sample was placed in an ultrasonic water bath for 30 minutes. Ice was added to the bath in order to ensure that temperature in the bath was held at approximately 0°C throughout the duration of the 30 minute period. Following subsequent centrifugation (4000g, 15 minutes) the supernatant was disposed of and the residue in the tube re-suspended in 5ml 50% MeOH (v/v). The sample was vortexed, centrifuged (4000g, 15 minutes) and the supernatant disposed of. This procedure was repeated one more time.. 10ml 2M NaOH (see appendix 1) was added to the sample, which was vortexed and hydrolyzed overnight at room temperature. In order to avoid exposure to light, the samples were covered with black clothing throughout the duration of the hydrolysis period. 6M HCL was used to adjust pH in the samples to pH 1.3-1.5. Following pH adjustment, 10 ml ethyl acetate was added to the sample which was shaken (250 rpm, 10 minutes) and subsequently centrifuged (2800 rpm, 10 minutes). The supernatant was transferred over to a new 50ml centrifuge tube and 10 ml ethyl acetate added to the initial centrifuge tube. This tube was shaken and centrifuged as above and the supernatant

transferred over to the new centrifuge tube. This procedure was repeated two more times giving a final volume of ~40ml ethyl acetate in the new centrifuge tube. The sample was evaporated to dryness using a centrifugal vacuum concentrator. The duration of sample evaporation was approximately four hours. Upon evaporation 500µl MeOH was added to the tube and the contents of the tube were re-suspended in the MeOH using a vortex mixer. 1500 µl Milli-Q water was then added to the tube and the contents re-vortexed. 250 µl sample was filtered through a 0.45 µm PVDF filter and subsequently injected to a glass vial containing an insert. An aluminum cap with central hole and septum was then fixed on the vial. Figure 2.11 at the end of this chapter provides an illustration of the extraction of bound phenolic acids in barley products.

Sample analysis, setup of sample series and identification of phenolic acids are described in sections 2.2.5.4, 2.2.5.5 and 2.2.5.6 respectively of this chapter. The procedures described apply to both free and bound phenolic acids.

2.2.7 Method testing

In the course of this thesis, testing of the method which is used for extraction of free phenolic acids was carried out. Two parallel experiments were performed. The first experiment attempted to determine if the choice of evaporation method had an influence on the level of free phenolic acids extracted. The second experiment involved determining whether using 80% MeOH instead of 50% MeOH would have an impact on the final results.
Six barley flour samples á ~1g were weighed into six 50ml centrifuge tubes. The first two samples were prepared as described in section 2.2.5.3 of this chapter. The second two samples were prepared in a similar manner apart from the fact that sample evaporation was performed using a centrifugal vacuum concentrator only. In the final two samples, 50% MeOH was replaced with 80% MeOH. These samples were also evaporated using a centrifugal vacuum concentrator only.

With regard to both of the tests described above, the samples were analyzed and the phenolic acids identified as described in sections 2.2.5.4 - 2.2.5.6 of this chapter. The calibration standards used were those described in section 2.2.5.2.

2.2.8 Determination of total starch

The total starch content of the four barley products used in this master thesis was determined by Silje Johansen. Prior to sample preparation a number of buffers were made.

2.2.8.1 Preparation of buffers

Preparation of the buffers used below is described in appendix 3.

2.2.8.2 Preparation of reagent solutions/suspensions

29.0ml 50mM MOPS buffer (see appendix 3) was added to 1.0ml thermo stable α-amylase. The solution was vigorously shaken and subsequently divided in to 3.0ml aliquots. The solution was stored in polypropylene tubes at -20°C.

20ml GOPOD Reagent Buffer (see appendix 3) was mixed with GOPOD Reagent Enzymes and subsequently transferred to the volumetric flask. In order to protect the enclosed reagent from light the flask was covered with aluminum foil. The flask was stored at -4°C.

2.2.8.3 Sample preparation

Covered barley, hulled barley and barley flakes were ground to pass a 0.5mm sieve. 2 parallels á ~100mg sample were weighed into glass test tubes and 200µl aqueous ethanol (80% v/v) added to the samples. The tubes were vortexed and 2ml dimethyl sulfoxide (DMSO) was added to the tubes. Following addition of DMSO, the tubes were mixed using a vortex mixer and incubated in a water bath (100°C) for 5 minutes. Following incubation, 3ml thermo stable α-amylase (diluted) was added to the tubes before returning them to the boiling water bath for a further 6 minutes. The tubes were stirred vigorously after 2, 4 and 6 minutes respectively. 1.6ml of the aqueous sample was pipetted over to a new glass test tube and the remainder of the sample was stored in the freezer (-20°C). The tubes were placed in a water bath at 50°C and 2ml 200mM Na-acetate buffer (see appendix 1) was added to the samples. Following stirring, 100µl amyloglucosidase was added to the tubes which were vortexed and subsequently incubated at 50°C for 30 minutes. The entire contents of the test tube were transferred to a 100ml volumetric flask and the contents of the tube were thoroughly rinsed with distilled water. The volume in the flask was adjusted up to the graduation mark with distilled water and subsequently mixed. An aliquot of the sample was centrifuged (3000 rpm, 10 minutes). Duplicate aliquots á 100µl sample were pipetted over to new glass test tubes. 3ml GOPOD Reagent was added to the samples, D-glucose controls and blank solutions. D-glucose controls consisted of 2 parallels á 100µl D-glucose standard solution while Reagent Blank solutions consisted of 100µl distilled water. The tubes were incubated at 50°C for 20 minutes. The absorbance for each sample and the D-glucose control was read at 510nm against the reagent blank. Figure 2.12 at the end of this chapter illustrates the procedure for the determination of total starch in barley products.

Figure 2.6:: UVmini-1240 Spectrophotometer from Shimadzu. This image is downloaded from http://www.news-medical.net/UVmini-1240-Spectrophotometer-from-Shimadzu

2.2.9 *In vitro* digestion of starch in barley products

In vitro digestion of starch was performed in three steps: an oral phase, a gastric phase and a duodenal phase. An illustration of these three steps is provided in figure 2.13. The method employed was based on the static *in vitro* digestion model proposed by EU Cost Action 1005 INFOGEST working group 2 (http://www.cost-infogest.eu/). Some modifications to this protocol have been made. These modifications included the omission of α-amylase in the oral phase. This was necessary in order to investigate the effects of pancreaticα-amylase alone. Furthermore 0.2g sample was used instead of 1g. Due to the high starch content in the samples, it was difficult to measure absorbance in 1g sample.

2.2.9.1 Preparation of digestive fluids used in the in vitro digestion model

Simulated saliva fluid (SSF) was made by combining 10ml KCL, 20ml KH_2PO_4, 4ml $NaHCO_3$, 1ml NaCl and 1ml $MgCl_2(H_20_6)$ in a 500ml volumetric flask. For details of preparation of the components of SSF please see appendix 2. The contents of the flask were diluted up to the graduation mark with distilled water. pH in the SSF was adjusted to pH 7 using 4ml 1M NaOH and 1ml 1M HCL.

Simulated gastric fluid (SGF) was made by mixing 28ml KCL, 0.9ml KH_2PO_4, 6.5ml $NaHCO_3$, 10ml NaCl and 2ml $MgCl_2(H_20_6)$ in a 500ml volumetric flask. Details of preparation of the components of SGF are given in appendix 2. The contents of the flask were diluted up to the graduation mark with distilled water. pH in the fluid was adjusted to pH 3 using 3ml 1M HCL.

2.2.9.2 Sample preparation

One of the aims of this master thesis was to observe *in vitro* digestion of starch in barley products after 5, 60 and 120 minutes in the duodenal phase. In order to achieve this objective, three parallels for each of the above time points were prepared giving a total of 9 glass tubes per sample.

2.2.9.2.1 Oral phase

Approximately 200mg sample was weighed into a glass centrifuge tube. 1ml SSF was added to the sample followed by addition of 5µl $CaCl_2(H_20)_2$ (appendix 2). The sample were mixed using a vortex mixer and incubated at 37°C in a water bath for two minutes. In order to investigate the effects of pancreatic α-amylase alone, oral α-amylase was admitted from the oral phase in this study.

2.2.9.2.2 Gastric phase

Pepsin (1.464mg pepsin/ml SGF) was dissolved in SGF and 2ml of the solution added to the sample. Thereafter 3µl $CaCl_2(H_20)_2$ was added and the contents of the tube were mixed with the aid of a vortex mixer. pH was adjusted to pH 3 by the addition of ~50µl 1M HCL. The samples were placed in an incubator shaker (37°C, 215 rpm) for the duration of two hours.

2.2.9.2.3 Duodenal phase

A duodenal mix was prepared by dissolving bile (59.6 mg/ sample) and pancreatin (9.6mg/sample) in the appropriate volumes of 0.15M $NaHCO_3$ (1,882ml/sample) (see appendix 1) and distilled water (2.118ml distilled water/sample). The mixture was vortexed and subsequently placed on ice. 4ml of the mix was added to each of the samples and the contents of the tubes were mixed using a vortex mixer. pH was measured and adjusted if necessary using 1M NaOH. Following pH adjustment/measurement the samples were placed in the incubator shaker (37°C, 215 rpm) for 5, 60 and 120 minutes respectively. After 5 minutes one-third of the samples were taken out of the shaker After 60 minutes another third of the samples were removed while the remainder of the samples were taken out of the shaker after 120 minutes. Following removal from the incubator shaker the samples were placed in a boiling water bath (100°C, 10 minutes) before being left on ice for 5 minutes. After centrifugation (2000 rpm, 10 minutes) the supernatant was transferred to a new glass centrifuge tube and placed in the freezer (-20°) until the following day.

2.2.9.3 Determination of total starch in the in vitro digested barley products.

The determination of total starch in the *in vitro* digested barley products was carried out according to the procedure described in sections 2.2.8.1, 2.2.8.2 and 2.2.8.3 in this chapter. However minor modifications were made to sample preparation (section 2.2.8.3).
Sample preparation commenced with the transferal of 1.6ml duodenal phase supernatant to a new glass centrifuge tube. 50µl amyloglucosidase was added to the tube as opposed to 100µl. Following incubation (50°C, 30 minutes) the contents of the tube were transferred to a 50ml volumetric flask instead of a 100ml volumetric flask. Figure 2.12 illustrates this procedure.

2.2.10 Treatment of raw data

2.2.10.1 Extraction of phenolic acids

Two parallels of each of the untreated barley products (covered barley, hulled barley, barley flakes and barley flour) were prepared and analyzed. Two parallels of each of the treated samples were prepared and two replicates of each parallel were subsequently analyzed using RSLC. Sub-optimal results caused by interference and noise in the chromatogram, were not included in the statistical analysis of the raw data.

2.2.10.2 In vitro digestion of starch in barley products

Three parallels of each of the three time points for all of the samples were prepared. This gave a total of nine tubes per sample. Two replicates of each of these nine examples were analyzed using a spectrophotometer. In the case of an obvious error in measured absorbance, the results were omitted from statistical analysis of the raw data and therefore not included in the thesis.

2.2.11 Statistical analysis

2.2.11.1 Extraction of phenolic acids in barley products

The experiments were performed in duplicates and the data are expressed as the mean of these duplicates ± standard deviation (SD). For each of the four phenolic acids the effect of the different treatments was analyzed using a mixed model ANOVA (proc glm) in the Statistical Analysis System SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Treatment type was included as a fixed factor, and the two replicates (random factor) were nested within treatment type. When a significant effect of treatment type was determined Tukey's post-hoc test was used to decide which treatments resulted in different amounts of phenolic acids.

2.2.11.2 In vitro digestion of starch in barley products

The experiments were performed in triplicates and the data are expressed as the mean of these triplicates ± SD. For each of the three time points the effects of the different treatments was analyzed using a mixed model ANOVA (proc glm) in the Statistical Analysis System SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Treatment type was included as a fixed factor, and three replicates (random factor) nested within treatment type. When a significant effect of treatment type was determined, Tukey's post-hoc test was used to decide which of the treatments resulted in different amounts of digested starch.

2.2.12 Flow charts

The following flow charts are intended as an accompaniment to some of the methods described in this chapter. More detailed flow charts are found in the appendix 2.

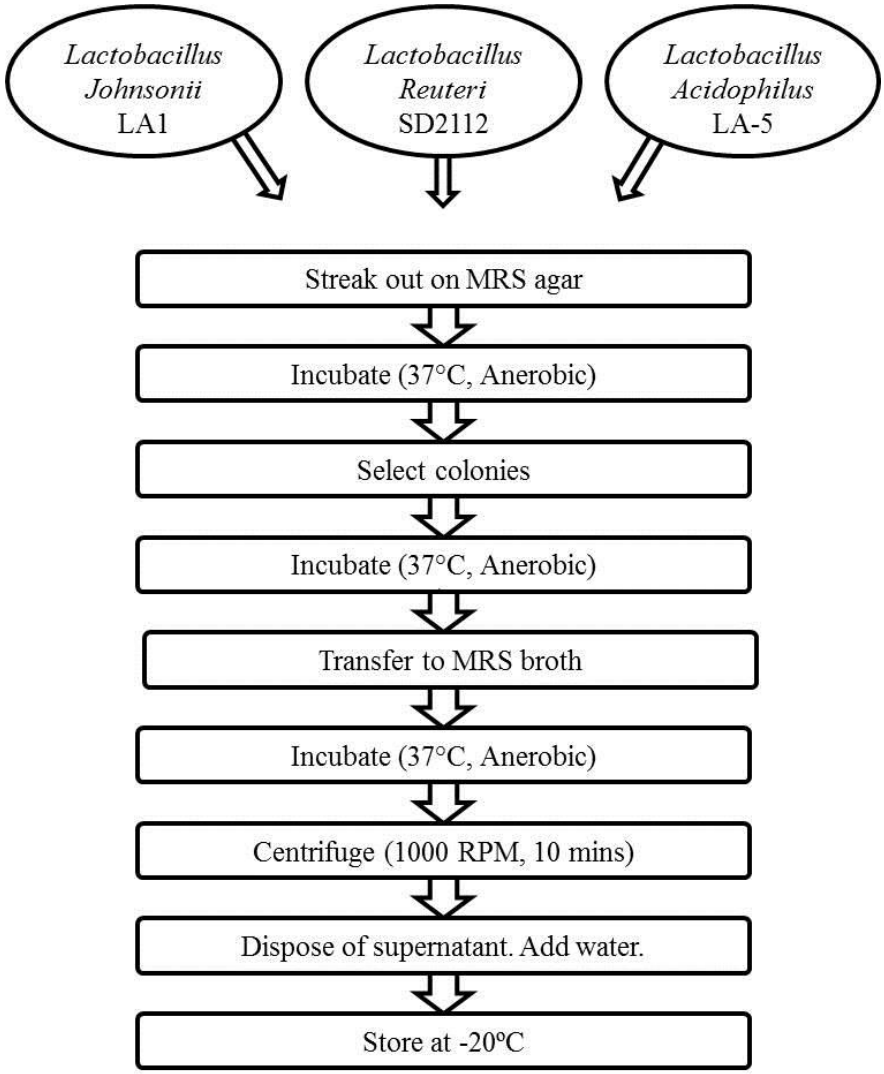

Figure 2.7: A simplified flow chart for isolation in pure strain

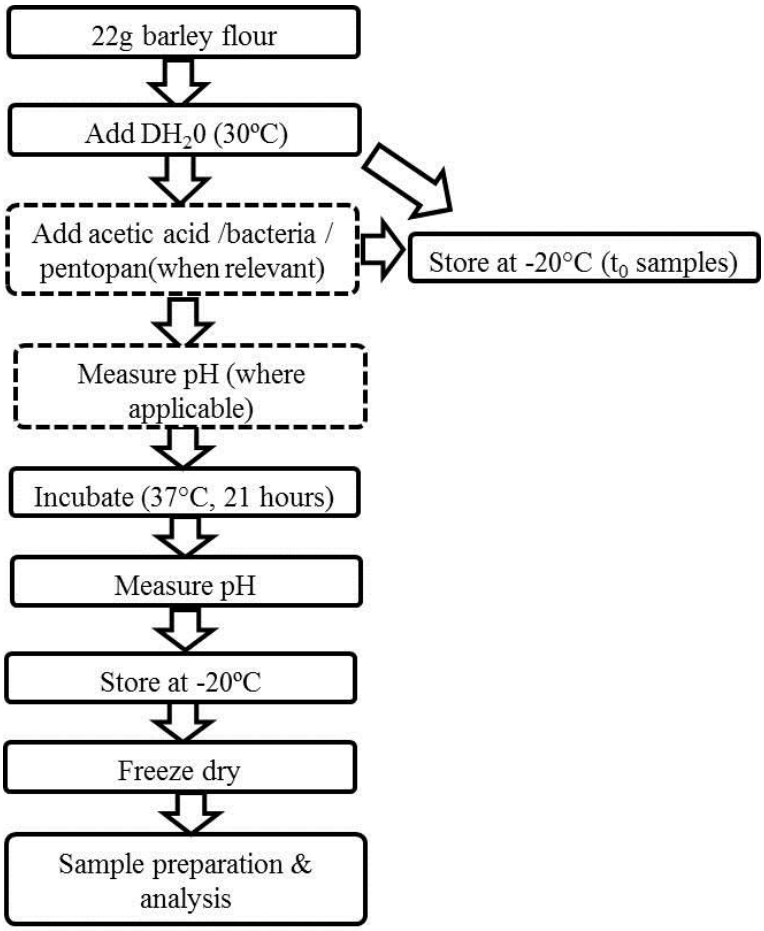

Figure 2.8: A simplified flow chart for fermentation of barley flour.

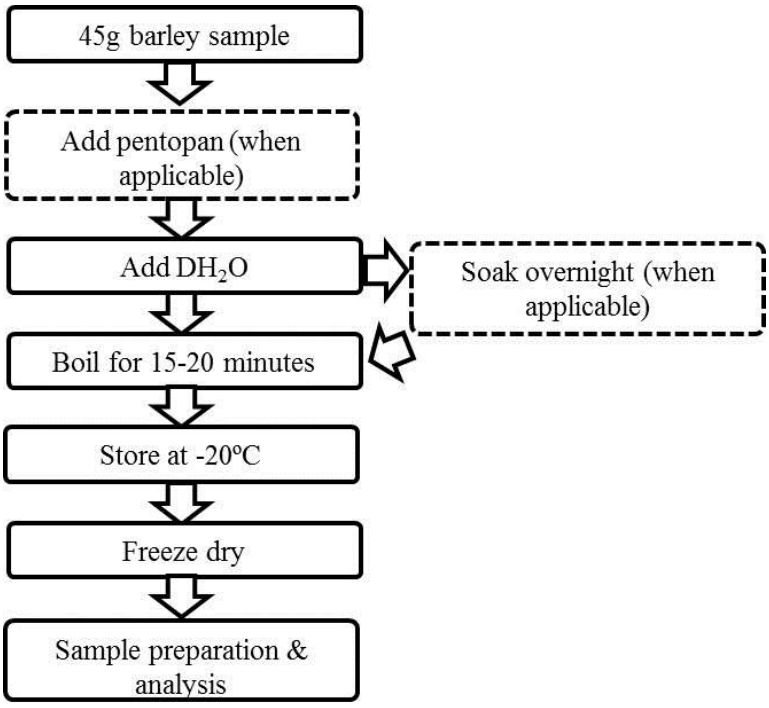

Figure 2.9: A simplified flow chart for boiling of hulled barley, barley flakes & barley flour.

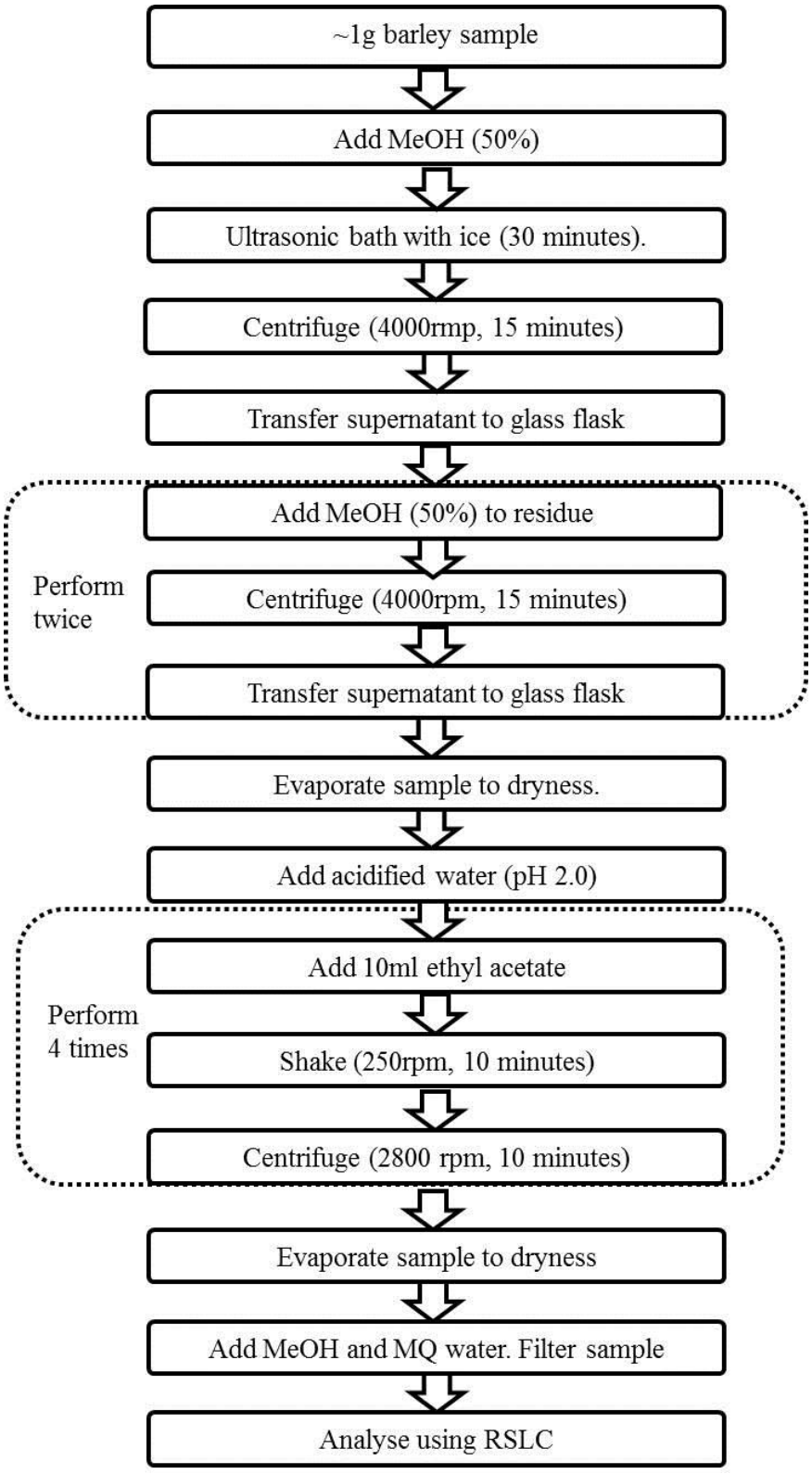

Figure 2.10: A simplified flow chart for extraction of free phenolic acids

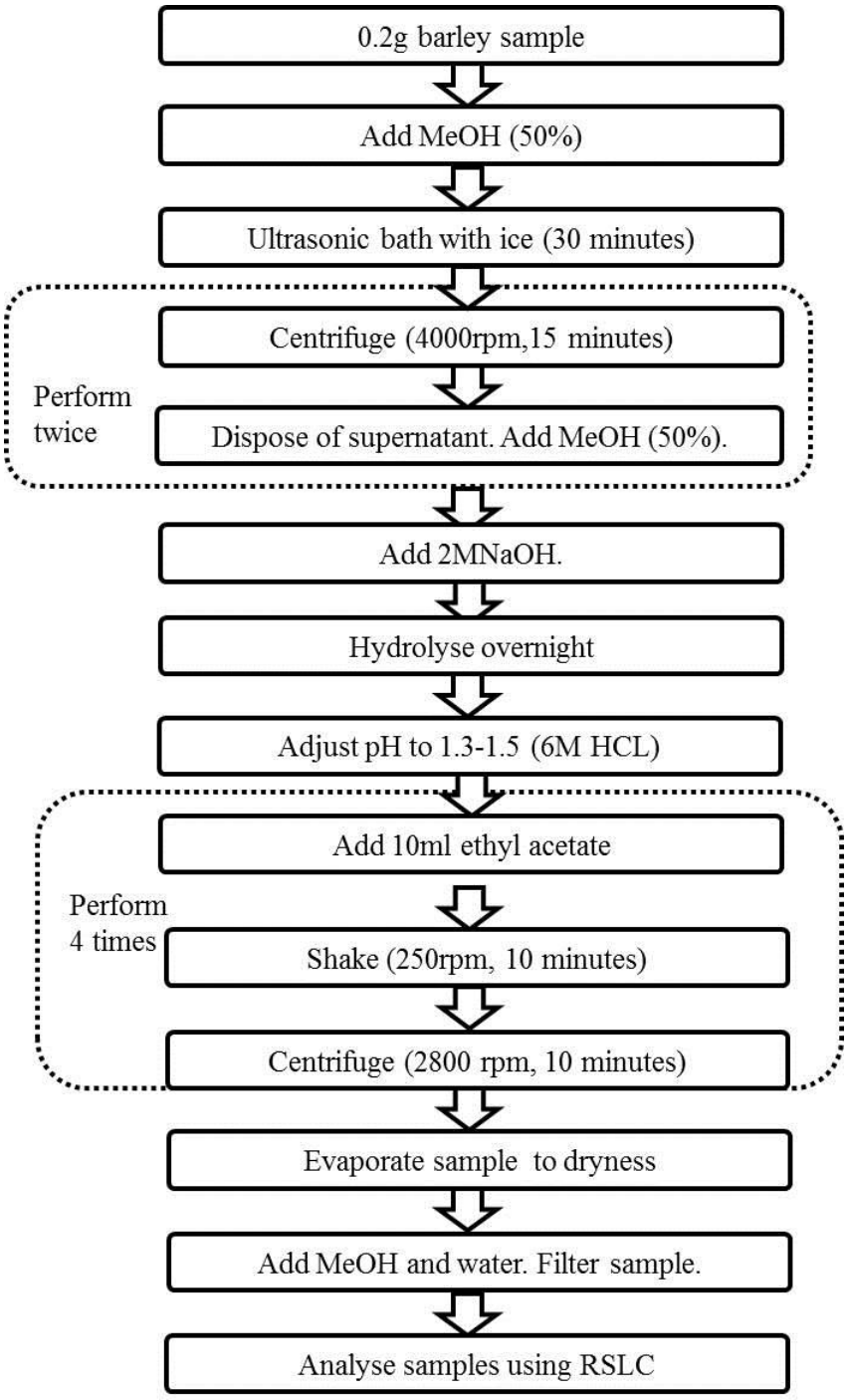

Figure 2.11: A simplified flow chart for extraction of bound phenolic acids

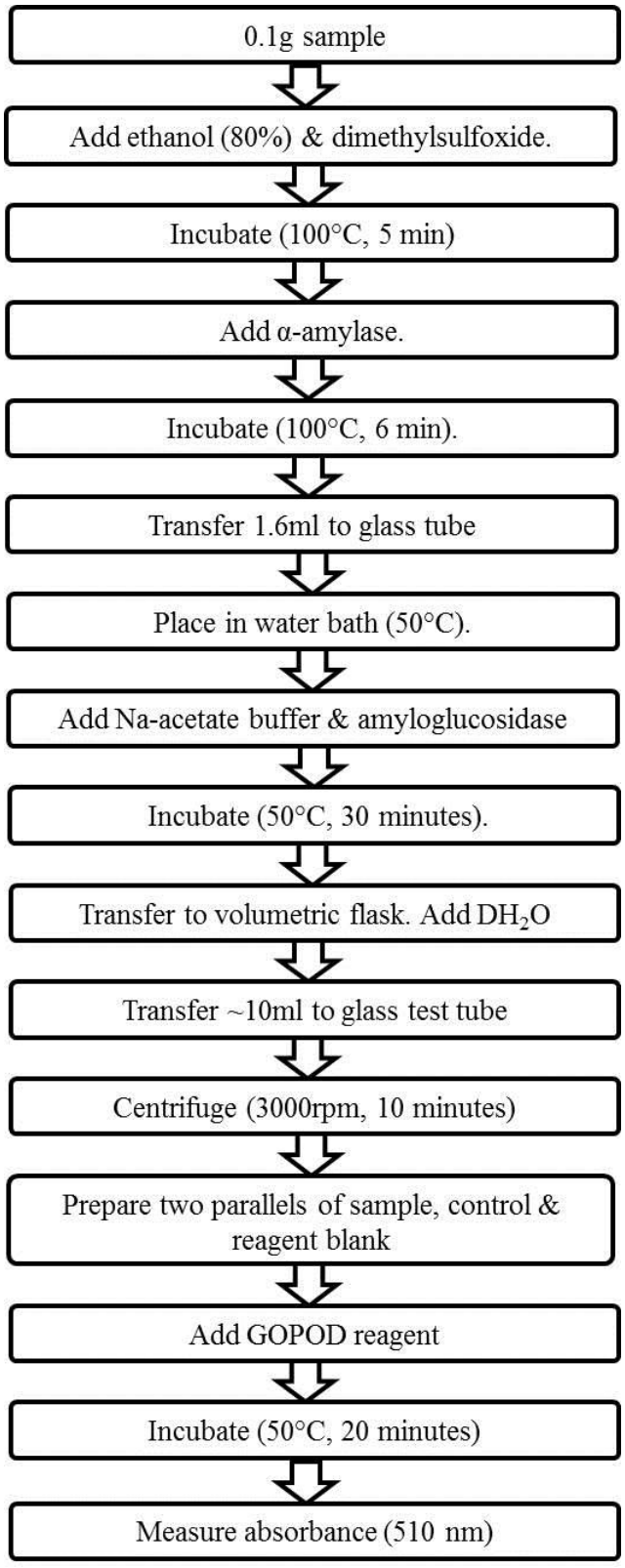

Figure 2.12: A simplified flow chart for determination of total starch

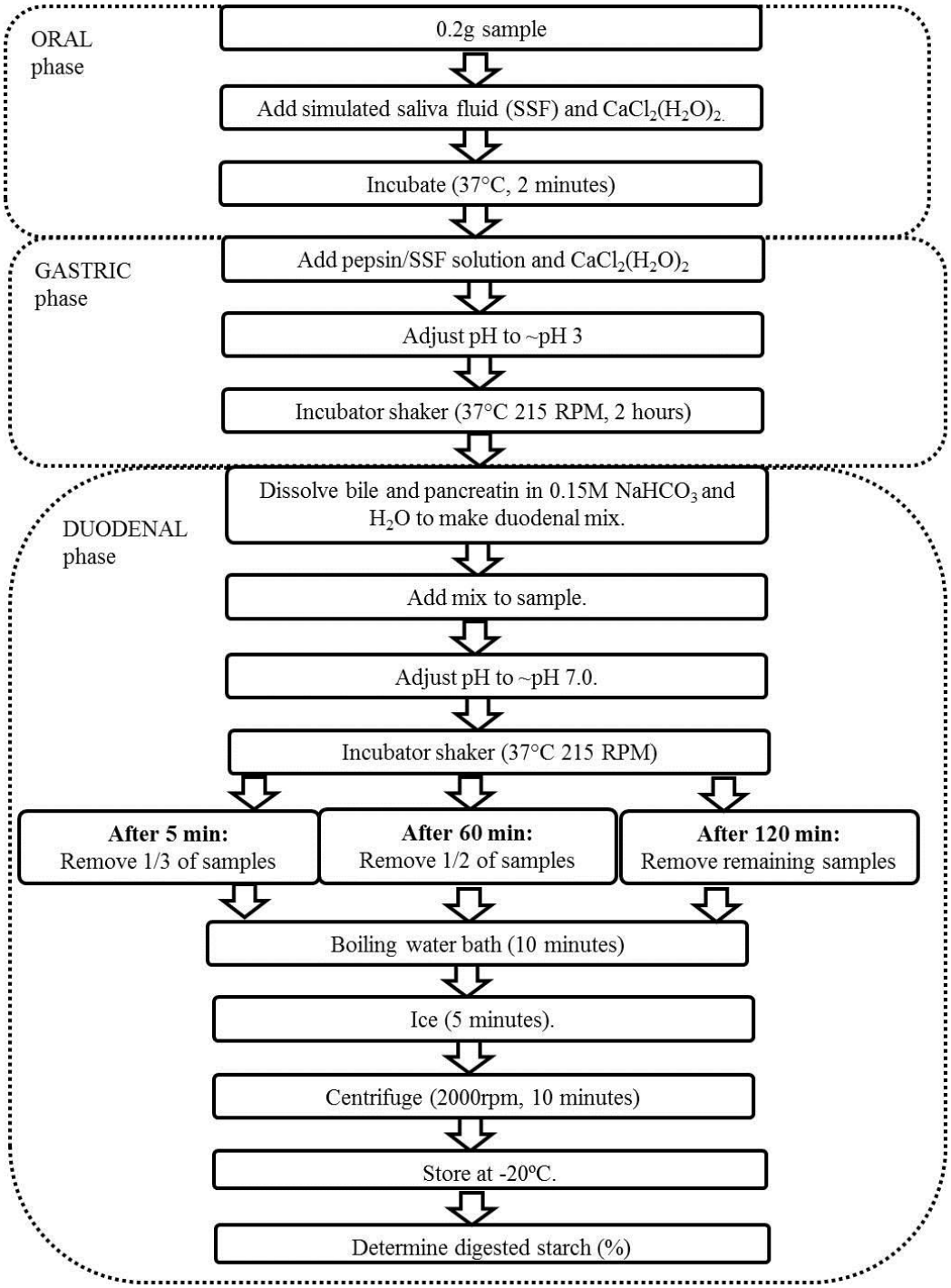

Figure 2.13: A simplified flow chart for *in vitro* digestion of starch

With respect to the determination of the amount of starch that was digested in the samples, please refer to figure 2.12. This simplified flow chart can also be used to illustrate the procedure for the determination of digested starch. However the first five steps in this flow chart can be omitted.

3 Results

3.1 Overall statistical analysis

The raw data in this thesis is analyzed using Analysis of variance (Anova) with post hoc Tukey tests. A summary of the overall statistical analysis is given in the tables below.

Table 3.1: The F-values and P-values for both the whole ANOVA model and the treatment factor. In this table the dependent variables are the four free phenolic acids; caffeic acid, p-coumaric acid, ferulic acid and sinapic acid.

Dependent variable	Model Test	Effect of treatment
Caffeic acid (Free)	$F_{(39,31)} = 746.05$, $p<0.0001$	$F_{(23,31)} = 87.45$, $p<0.0001$
p-Coumaric acid (Free)	$F_{(39,32)} = 204.98$, $p<0.0001$	$F_{(23,32)} =120.82$, $p<0.0001$
Ferulic acid (Free)	$F_{(39,32)} = 9348.63$, $p<0.0001$	$F_{(23,32)} =209.44$, $p<0.0001$
Sinapic acid (Free)	$F_{(31,23)} = 1473.06$, $p<0.0001$	$F_{(19,23)} =8.85$, $p<0.0002$
Total free phenolic acids	$F_{(39,32)} = 8463.05$, $p<0.0001$	$F_{(23,32)} =149.83$, $p<0.0001$

Table 3.2: The F-values and P-values for both the whole ANOVA model and the treatment factor. In this table the dependent variables are the four bound phenolic acids; caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid.

Dependent variable	Model Test	Effect of treatment
Caffeic acid (bound)	$F_{(1,2)} = 1.64$, $p<0.3287$	$F_{(1,2)} = 1.64$, $p<0.3287$
p-Coumaric acid (bound)	$F_{(3,4)} = 3640.42$, $p<0.0001$	$F_{(3,4)} = 3640.42$, $p<0.0001$
Ferulic acid (bound)	$F_{(3,4)} = 97.71$, $p<0.0004$	$F_{(3,4)} = 97.71$, $p<0.0004$
Sinapic acid (bound)	$F_{(3,4)} = 1.31$, $p<0.3882$	$F_{(3,4)} = 1.31$, $p<0.3882$
Total bound phenolic acids	$F_{(3,4)} = 211.32$, $p<0.0001$	$F_{(3,4)} = 211.32$, $p<0.0001$

Table 3.3: The F-values and the P-values for both the whole ANOVA model and the treatment factor. In the table the dependent variable is total starch.

Dependent variable	Model Test	Effect of treatment
Total starch	$F_{(7,8)} = 17.91$, $p<0.003$	$F_{(3,8)} = 45.03$, $p<0.0015$

Table 3.4: The F-values and the P-values for both the whole ANOVA model and the treatment factor. In the table the dependent variables are digested starch after 5, 60 and 120 minutes in the duodenum.

Dependent variable	Model Test	Effect of treatment
Digested starch 5 min	$F_{(33,170)} = 69.61$, $p<0.001$	$F_{(21,170)} = 5.19$, $p<0.0026$
Digested starch 60 min	$F_{(33,167)} = 63.53$, $p<0.001$	$F_{(21,167)} = 5.09$, $p<0.0029$
Digested starch 120 min	$F_{(33,162)} = 20.61$, $p<0.001$	$F_{(21,162)} = 6.79$, $p<0.0007$

3.2 Phenolic acid content in barley products

Covered barley had the highest level of total phenolic acids. The level of total free phenolic acids in covered barley was 4.14 $^{\mu g}/_{g\ Dry\ Matter(DM)}$. This was significantly higher than the results for all of the other barley products. The level of total bound phenolic acids in covered barley was 1052.69 $^{\mu g}/_{g\ DM}$ and was significantly higher than the values for the other barley products.

There was a significantly higher level of total free phenolic acids in barley flour than in hulled barley. In barley flour the total free phenolic acid content was 2.30 $^{\mu g}/_{g\ DM}$ while hulled barley contained 0.88 $^{\mu g}/_{g\ DM}$ total free phenolic acids.

The most abundant phenolic acid was ferulic acid (FA) while the second most abundant was *p*-Coumaric acid (*p*-CA). There was a significantly higher level of free and bound *p*-CA in covered barley than in the other three barley products. The level of free *p*-CA in covered barley was 1.43 $^{\mu g}/_{g\ DM}$ and the level of bound *p*-CA content was 344.02 $^{\mu g}/_{g\ DM.}$
A significantly higher level of bound *p*-CA was determined in barley flour in comparison to hulled barley. Barley flour contained 40.45 $^{\mu g}/_{g\ DM}$ bound *p*-CA while hulled barley contained 23.29 $^{\mu g}/_{g\ DM}$ bound *p*-CA.
The p-value for all of the results was set at $p<0.05$.

Table 3.5: gives an overview of the level of phenolic acids in various barley products. The four phenolic acids which were investigated were caffeic acid (CA), *p*-Coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA). The table shows the levels of the free and bound forms. The results are the mean of one experiment performed in duplicates ± SD and are given in µg/g dry matter (DM).

Barley product	CA Free	CA Bound	*p*-CA Free	*p*-CA Bound	FA Free	FA Bound	SA Free	SA Bound	Total Free	Total Bound
Covered barley	0.28±0.02		1.43±0.11	344.02±4.25	2.21±0.66	705.84±1.08	0.23±0.05	2.83±0.01	4.14±0.74	1052.69±5.32
Hulled barley	0.06±0.01		0.13±0.0	23.29±1.16	0.61±0.05	338.22±9.52	0.07±0.03	2.40±0.28	0.88±0.02	363.92±10.4
Barley flakes	0.07±0.01	1,90±14.63	0.18±0.0	27.23±0.06	1.09±0.02	358.43±14.72	0.08±0.02	5.38±0.28	1.43±0.05	391.04±14.5
Barley flour	0.22±0.04	5,43±20.61	0.38±0.01	40.43±5.90	1.45±0.01	376.85±48.52	0.24±0.05	3.69±3.23	2.30±0.11	420.97±57.65

3.3 Effect of boiling on the free phenolic acid content in barley products.

The results of the effect of boiling on the free phenolic acid content in hulled barley, barley flakes and barley flour are shown in figure 3.1. The levels of free caffeic acid, *p*-Coumaric acid, ferulic acid and sinapic acid in each of the boiled cereal types were compared with their corresponding un-boiled variety. There was a significantly higher level of total free phenolic acids in boiled hulled barley than in hulled barley. The total free phenolic acid content in boiled hulled barley and hulled barley was 3.33 $^{\mu g}/_{g\ DM}$ and 0.88 $^{\mu g}/_{g\ DM}$ respectively.

The total free phenolic acid content was significantly higher in boiled hulled barley than in boiled barley flakes. Total free phenolic acid content in boiled hulled barley was 3.33 $^{\mu g}/_{g\ DM}$. and the total free phenolic acid content in boiled barley flakes was 1.43 $^{\mu g}/_{g\ DM..}$

The p-value for all of the results was $p<0.05$.

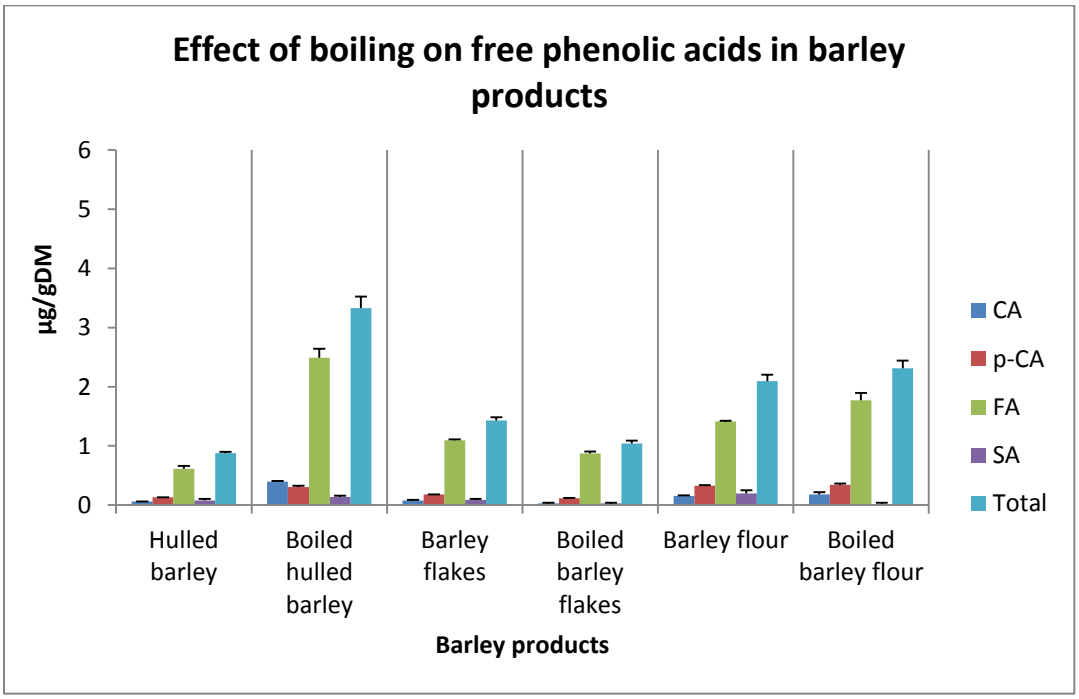

Figure 3.1 shows the effect of boiling on the content of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in hulled barley, barley flakes and barley flour. The total free phenolic acid content for each of the samples is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates ± SD.

3.4 Effect of boiling and pentopan on the free phenolic acid content in barley flour.

Figure 3.2 illustrates the joint effect of boiling and the addition of pentopan on the level of free phenolic acids in barley flour.

There was a significantly higher level of total free phenolic acids in the boiled samples containing pentopan than in the boiled barley flour samples. The total free phenolic acid content in the boiled barley flour and pentopan samples and the boiled barley flour samples was 6.04 $^{\mu g}/_{g\ DM}$ and 2.31 $^{\mu g}/_{g\ DM}$ respectively.

The barley flour, pentopan and water samples had a significantly higher level of total phenolic acids than the boiled barley flour and pentopan samples. The level of total free phenolic acids in the barley flour, pentopan and water samples was 8.05 $^{\mu g}/_{g\ DM.}$ The level of total free phenolic acids in the boiled samples and pentopan samples was 6.04 $^{\mu g}/_{g\ DM.}$
P-value was set at $p<0.05$ for all of the samples.

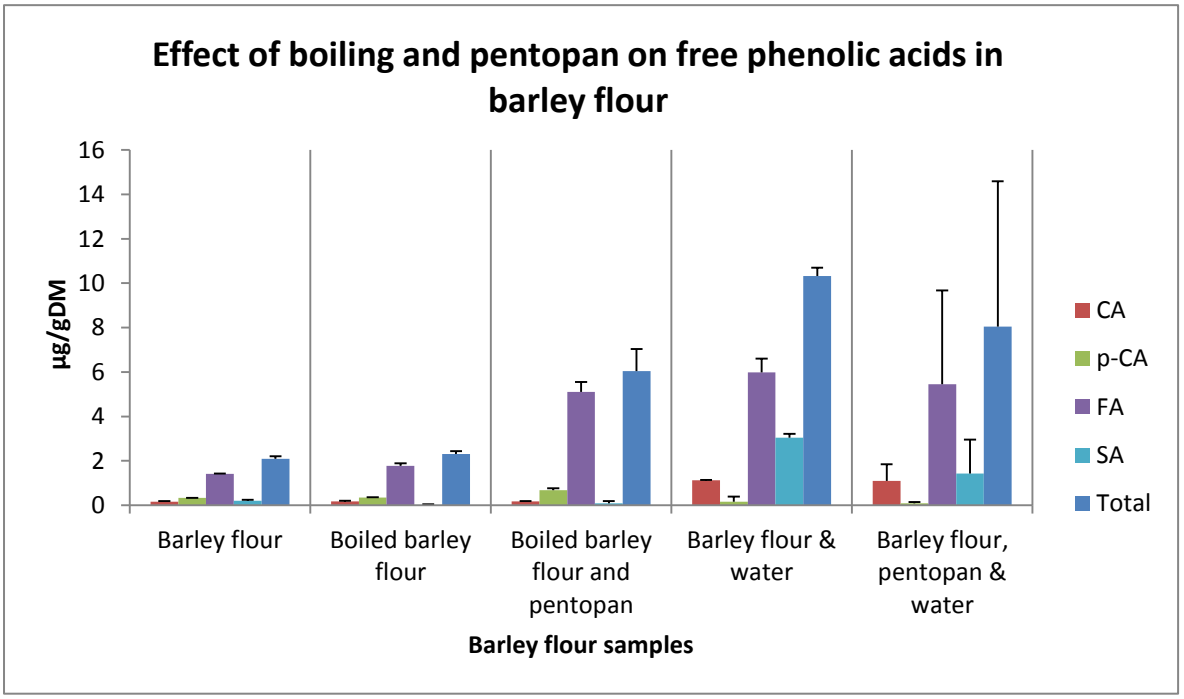

Figure 3.2 illustrates the effect of boiling and the addition of pentopan on the free phenolic acid content in barley flour. The free phenolic acids shown in the figure are caffeic acid (CA), *p*-Coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA). The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates ± SD.

3.5 Effect of water and pentopan on free phenolic content in barley flour

The effect of the addition of water on the level of free phenolic acids in barley flour is shown in figure 3.3. The p-value for all of the results was set at $p<0.05$.

Barley flour and water samples contained 10.32 $^{\mu g}/_{g\ DM}$ total free phenolic acids. This was significantly higher than barley flour, pentopan & water and barley flour which contained 8.05 $^{\mu g}/_{gDM}$ and 2.09 $^{\mu g}/_{g\ DM}$ total free phenolic acids respectively.

Figure 3.3 also illustrates the free phenolic acid content in the samples which were placed in the freezer immediately after the addition of water / water and pentopan. There was a significantly higher level of free *p*-CA in the barley flour and water samples t_0 than in the barley flour and water samples. The level of free *p*-CA was 0.78 $^{\mu g}/_{g\ DM}$ in the barley flour and water t_0 samples, while it was 0.16 $^{\mu g}/_{g\ DM}$ in the barley flour and water samples.

Total phenolic acid content in the barley flour, pentopan and water samples was 8.05 $^{\mu g}/_{g\ DM.}$ This was significantly higher than in the barley flour, pentopan and water t_0 samples which contained 3.88 $^{\mu g}/_{g\ DM}$ total free phenolic acids.
The p-value for all of the results was set at $p<0.05$.

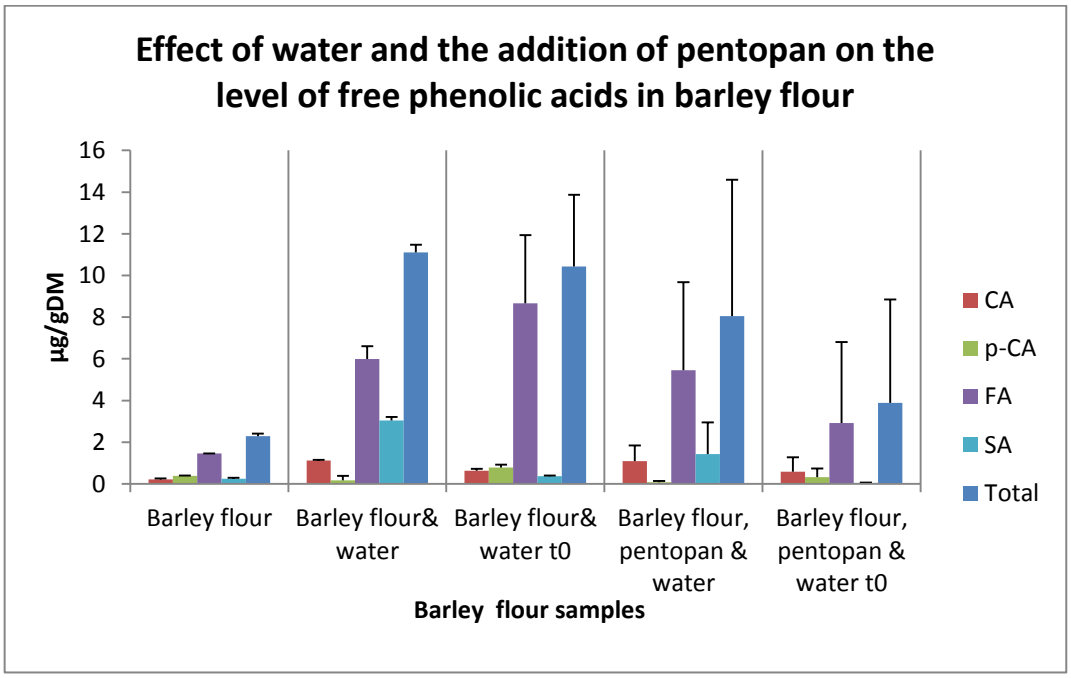

Figure 3.3 illustrates the effect of water and the addition of pentopan on the free phenolic acid content in barley flour. The free phenolic acids shown in the figure are caffeic acid (CA), *p*-Coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA). The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates ± SD.

3.6 Effect of fermentation on the free phenolic acid content in barley flour

The levels of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and total phenolic acids (Total) was significantly higher in the barley flour samples containing SD2112 and LA5 than in the barley flour and water samples.

The results are given below:

- Free CA was 5.05 $^{\mu g}/_{g\ DM}$, 4.08 $^{\mu g}/_{g\ DM}$ and 1.13 $^{\mu g}/_{g\ DM}$ in barley flour, water and SD2112 barley flour , water and LA5, and barley flour and water respectively.
- Free *p*-CA was 1.94 $^{\mu g}/_{g\ DM}$, 1.43 $^{\mu g}/_{g\ DM}$ and 0.16 $^{\mu g}/_{g\ DM}$ in barley flour, water and SD2112 barley flour, water and LA5 respectively and barley flour and water respectively.
- Free FA was 33.26 $^{\mu g}/_{g\ DM}$, 25.53 $^{\mu g}/_{g\ DM}$ and 5.99 $^{\mu g}/_{g\ DM}$ in barley flour, water and SD2112, barley flour water and LA5 and barley flour and water respectively.
- Free total was 42.48 $^{\mu g}/_{g\ DM}$, 32.82 $^{\mu g}/_{g\ DM}$ and 10.32 $^{\mu g}/_{g\ DM}$ in barley flour, water and SD2112, barley flour water and LA5 and barley flour and water respectively.

There was a significantly higher level of total free phenolic acids in the barley flour and water samples than in the barley flour samples fermented with *l.johnsonii* LA1. Barley flour and water contained 10.32 $^{\mu g}/_{g\ DM}$ total phenolic acids. The total phenolic acid content in barley flour, water and LA1 was 0.58 $^{\mu g}/_{g\ DM.}$

The p-value for all of the results was $p<0.05$.

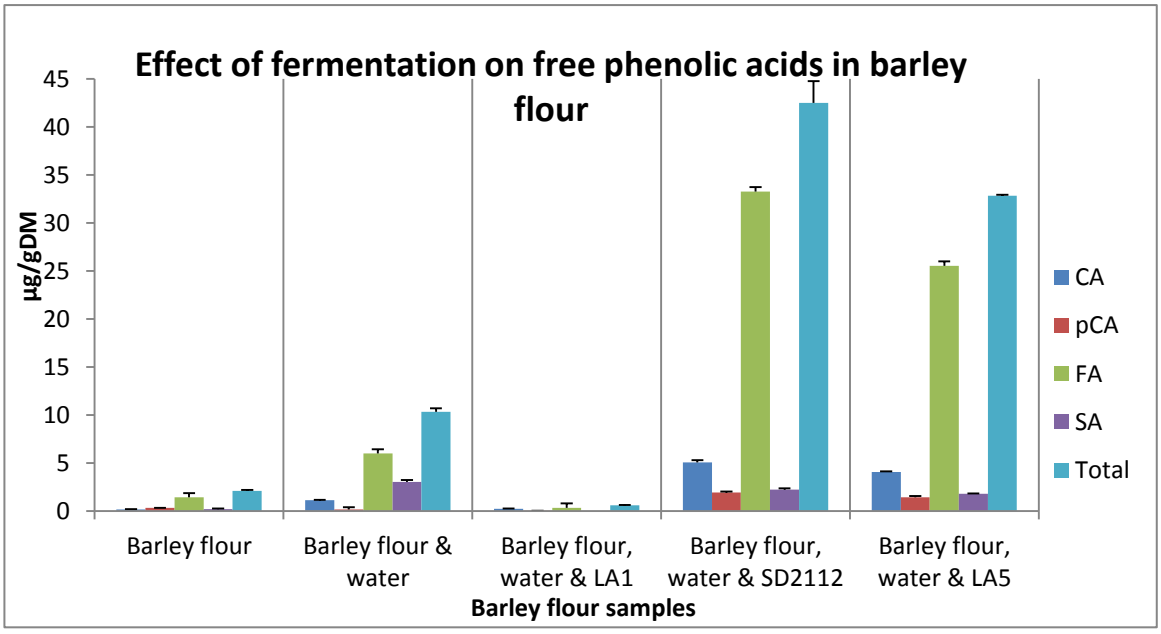

Figure 3.4 shows how the content of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in barley flour is affected by the addition of LAB. The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates ± SD.

3.7 Effect of fermentation and pentopan on free phenolic acids in barley flour

In addition to the effect of fermentation alone, the joint effect of fermentation and pentopan on the level of free phenolic acids was also investigated in this master thesis. In order to facilitate illustration of the results, three individual figures are employed. The LAB shown in figure 3.5 is *l.johnsonii* LA1. There was no significant difference in the levels of the individual free phenolic acids or total free phenolic acids following the addition of pentopan to the samples containing *l.johnsonii* LA1. Free sinapic acid (SA) was not detected in any of the samples containing *l.johnsonii* LA1.

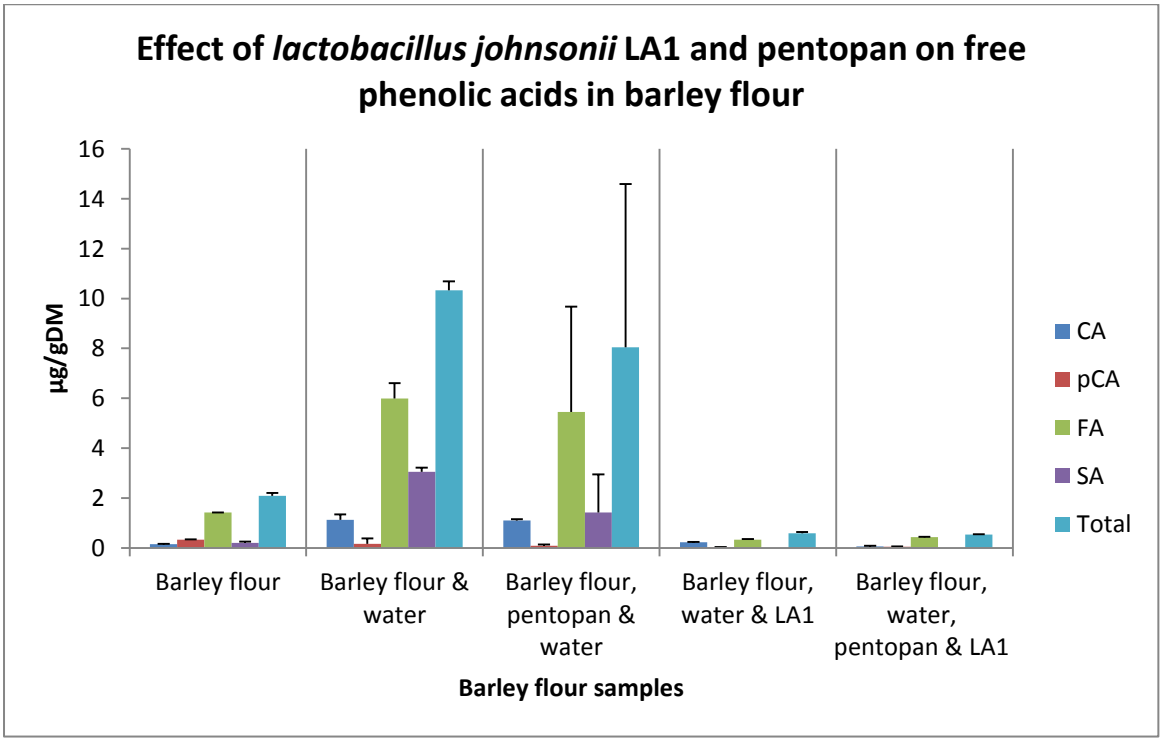

Figure 3.5 shows the effect the addition of pentopan has on the levels of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in fermented barley flour samples. The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter). In this figure each bar represents the mean of one experiment performed in duplicates ± SD.

Figure 3.6 shows the effect of a combination of *l.reuteri* SD2112 and pentopan on the level of free phenolic acids in barley flour and water samples.

There was a significantly higher level of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and total phenolic acids (Total) in the barley flour,water, pentopan & SD2112 samples than in the barley flour, water & SD2112 samples. The p-value for all of the results was set at $p<0.05$.

The results are:

- Free CA was 7.97 $^{\mu g}/_{g\ DM}$ and 5.05 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan & SD2112 and barley flour water &SD2112 respectively.
- Free *p*-CA was 2.66 $^{\mu g}/_{g\ DM}$ and 1.94 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan &SD2112 and barley flour water &SD2112 respectively.
- Free FA was 68.61 $^{\mu g}/_{g\ DM}$ and 33.26 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan &SD2112 and barley flour water and SD2112 respectively.
- Free total was 83.14 $^{\mu g}/_{g\ DM}$ and 42.48 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan and SD2112 and barley flour water and SD2112 respectively.

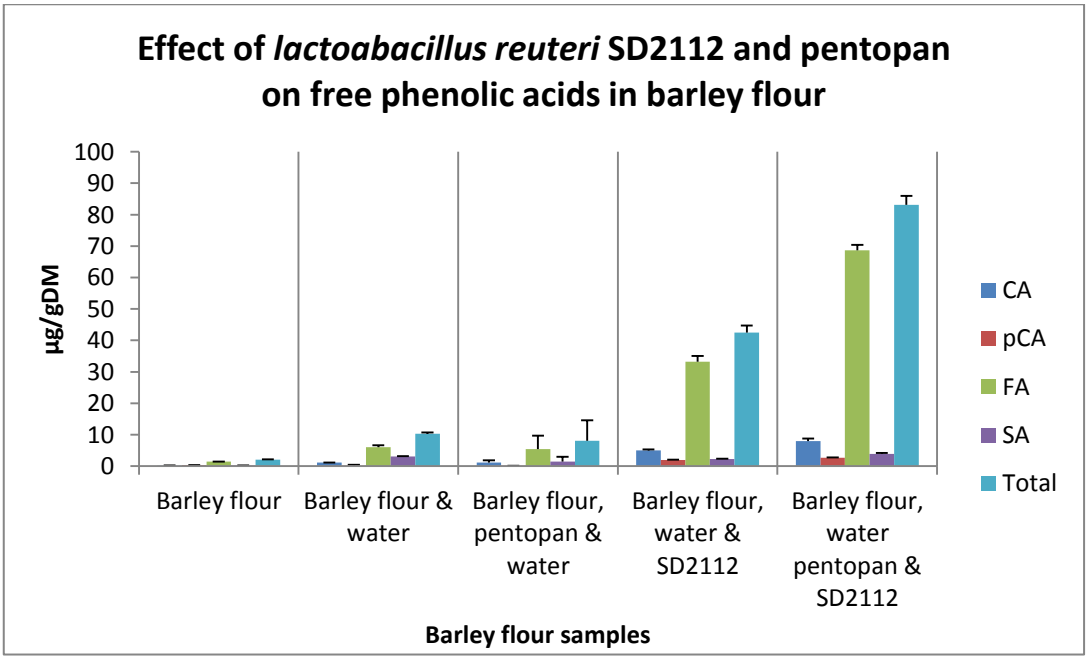

Figure 3.6: An illustration of the effect pentopan has on the levels of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in barley flour samples fermented with *lactobacillus reuteri* SD2112. The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates. ± SD

The effect of a combination of *l.acidophilus* LA-5and pentopan on the level of free phenolic acids is shown in figure 3.7. There was a significantly higher level of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and total phenolic acids in the barley flour, water, pentopan & LA5 samples than in the barley flour, water & LA5 samples. The significant results are given below. The p-value was set at $p<0.05$ for all of the results.

- Free CA was 8.33 $^{\mu g}/_{g\ DM}$ and 4.08 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan & LA5 and barley flour water &LA5 respectively.
- Free *p*-CA was 2.12 $^{\mu g}/_{g\ DM}$ and 1.43$^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan &LA5 and barley flour water &LA5 respectively.
- Free FA was 62.46 $^{\mu g}/_{g\ DM}$ and 25.53 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan &LA5 and barley flour water and LA5 respectively.
- Free total was 76.75 $^{\mu g}/_{g\ DM}$ and 32.82 $^{\mu g}/_{g\ DM}$ in barley flour, water, pentopan and LA5 and barley flour water and LA5 respectively.

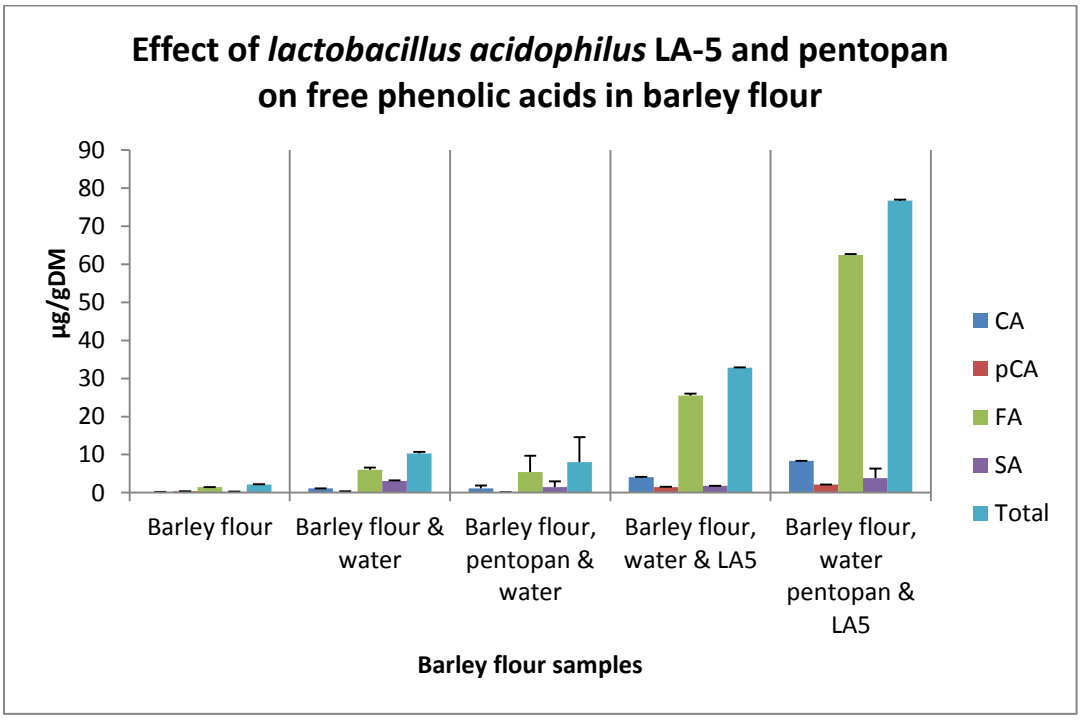

Figure 3.7: The joint effect of pentopan and *l.acidophilus* LA-5 on the levels of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in barley flour. The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates ± SD

3.8 Effect of acetic acid and pentopan on free phenolic acids in barley flour

Acetic acid was added to a number of the samples that were investigated in this thesis. These acidified samples are compared with un-acidified samples in the figure below. Acidified samples containing pentopan are also included in the figure. No significant difference in the levels of free caffeic acid (CA), *p*-Coumaric (*p*-CA) and ferulic acid (FA) was determined in the samples after the addition of acetic acid. This also applies to the samples containing pentopan. However there was a significantly higher level of total phenolic acids in the barley flour, pentopan & water samples than in the than in the barley flour, acetic acid, pentopan & water samples.

The total phenolic acid content was 8.05 $^{\mu g}/_{g\ DM}$ and 2.82 $^{\mu g}/_{g\ DM}$ in the barley flour, pentopan & water samples and barley flour, acetic acid, pentopan & water samples respectively.

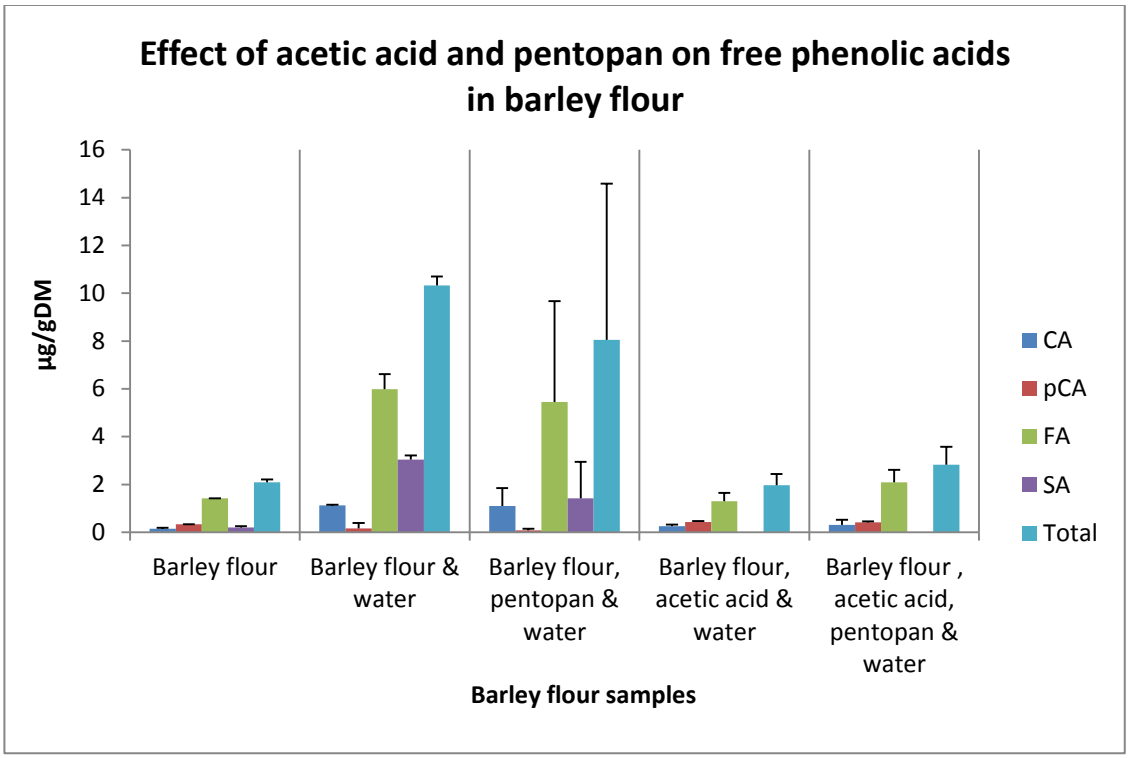

Figure 3.8 shows the effect of the addition of acetic acid and pentopan on the levels of free caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA) in barley flour. The total amount of free phenolic acids is also shown. The results are given in µg/g DM (dry matter) and each bar represents the mean of one experiment performed in duplicates. ± SD

3.9 Testing of method for extraction of free phenolic acids in barley flour

Table 3.6 shows the results of testing of the method for extraction of free phenolic acids in barley flour. The samples were prepared and analyzed as described in chapter 2 of this master thesis. No significant difference in the level of any of the individual phenolic acids was determined between the three samples. There was no significant difference in the level of total phenolic acids in the three samples.

Table 3.6: The results of testing of the method for extraction of free phenolic acids in barley flour. The free phenolic acids shown in the table are caffeic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and sinapic acid (SA). The total free phenolic acids are also shown. All the results are given in µg/g DM(dry matter). In the case of the first two samples the results are the mean of one experiment performed in duplicates ±SD. There is only one result for the third sample.

Sample ID	CA µg/gDM	p-CA µg/gDM	FA µg/gDM	SA µg/gDM	Total µg/gDM
50% original	0,09±0.03	0,36±0.05	1,51±0.40	0,08±0.00	2.06±0.48
50% speed vac only	0,14±0.00	0,29±0.01	1,45±0.03	0,30±0.03	2.18±0.04
80 % speed vac only	0,18	0,23	1,04	0,12	1.57

3.10 Determination of total starch in barley products

The effect of fermentation and boiling on *in vitro* digestion of starch was another aspect which was investigated in the course of this master thesis. In order evaluate this effect, it was first considered necessary to determine the total amount of starch in the various types of barley products. Covered barley contained 57.5% starch which was significantly less than the starch % in the other three barley products.

Barley flour contained 64.6% starch which was significantly less than the starch % in both hulled barley and barley flakes. The p-value for the results was $p<0.05$.

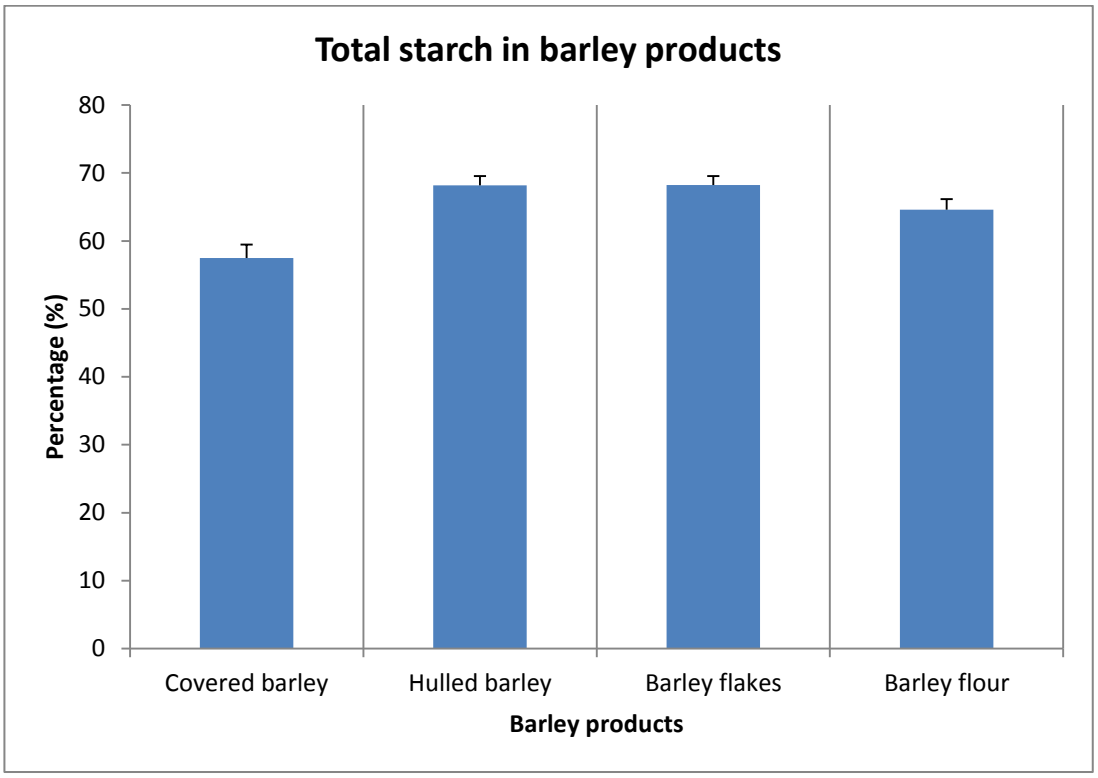

Figure 3.9 illustrates the total amount of starch determined in the various barley products utilized in this thesis. The results are given in percent and each bar represents the mean of one experiment performed in duplicates ± SD.

3.11 *In vitro* digestion of starch in barley products

Following determination of the total starch content in the four types of barley products the samples were *in vitro* digested. The amount of starch which was digested in the samples was calculated as a percentage of the total starch content (dry weight basis) in the products. There was no significant difference in the amount of starch digested between the four barley products. This applies to each of the three time points in the duodenal phase.

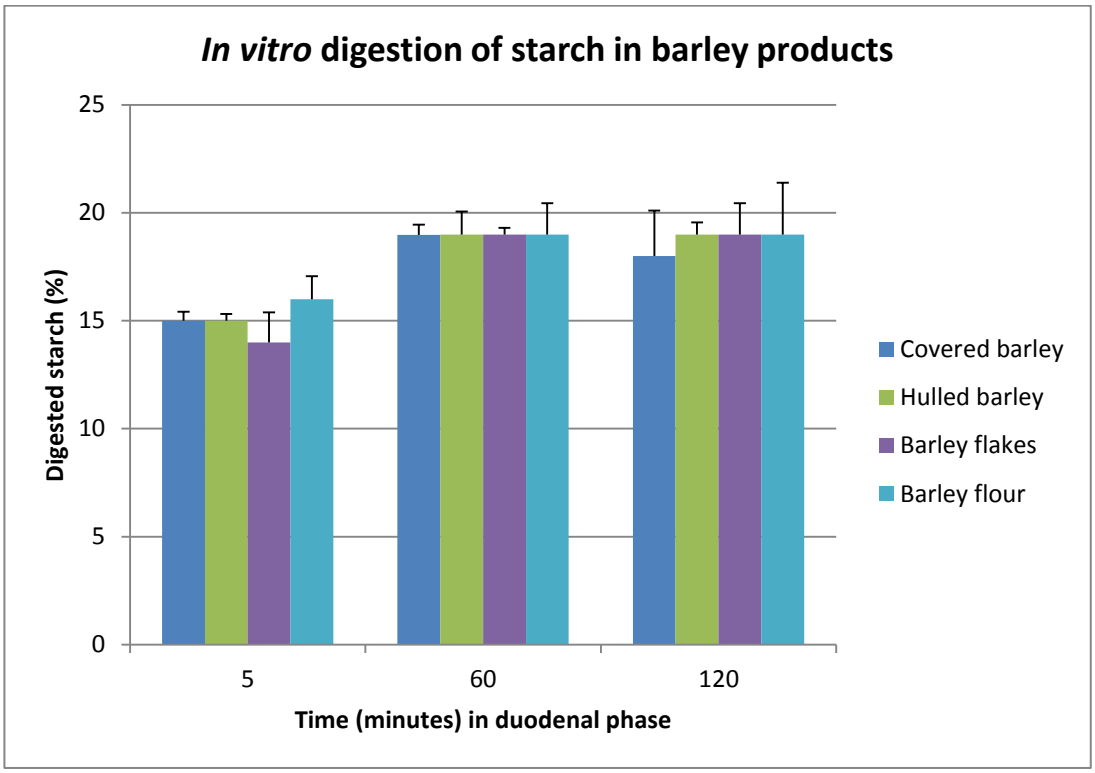

Figure 3.10 shows the amount of starch digested in covered barley, hulled barley, barley flakes and barley flour after 5, 60 and 120 minutes in the duodenal phase. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the four barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

3.12 Effect of fermentation on *in vitro* digestion of starch in barley flour

The effect of fermentation on *in vitro* digestion of starch in barley flour was one of the primary objectives of this master thesis. Post-fermentation pH was measured in all of the samples while pre-fermentation pH was measured in a selection of the samples. An overview of the pH values for the fermented samples in figure 3.13 is given in Table 3.7.

Table 3.7: An overview of the pH values for the fermented samples in figures 3.11. In the table the pH value represents the mean of two pH measurements ±SD

Sample Id	Pre-fermentation pH	Post-fermentation pH
Barley flour & water	5.87±0.04	6.20±0.12
Barley flour, water & LA1		3.73±0.01
Barley flour, water & SD2112		3.61±0.01
Barley flour, water & LA5		3.66±0.06

Figure 3.11 illustrates the effect the addition of LAB had on the digestion of starch in barley flour samples. Barley flour is included in the figure in order to illustrate the effect the addition of water can have on *in vitro* digestion of starch in barley flour. There was no significant difference between the amounts of starch digested in any of the five samples. This applies to all of the three time points in the duodenal phase.

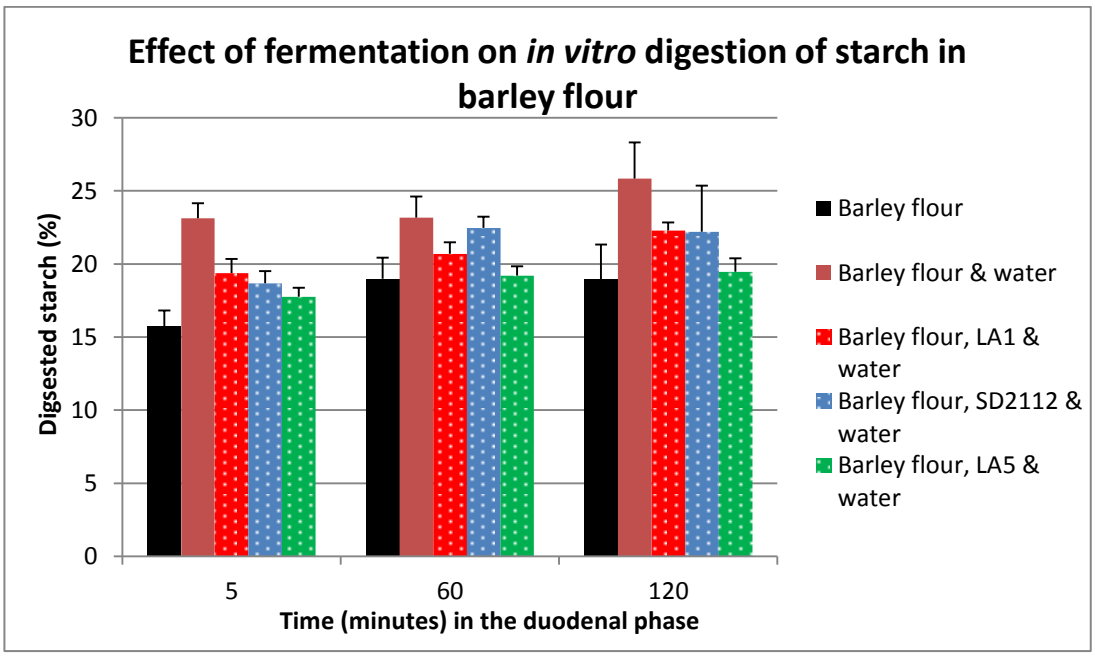

Figure 3.11 shows the effect of fermentation on *in vitro* digestion of starch in barley flour. The LAB shown in the figure above is *l.johnsonii* LA1, *l.reuteri* SD2112 and *l.acidophilus* LA-5. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the four barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

3.13 Effect of fermentation and pentopan on *in vitro* digestion of starch in barley flour

Pentopan was added to a number of the samples which were fermented with LAB. The results of the effect of the addition of pentopan to barley flour samples are shown in the figures 3.12-3-14. Tables 3.8 gives an overview of the post-fermentation pH values for the samples presented in figure 3.12. The pre-fermentation pH for barley flour and water is also included.

Table 3.8: An overview of the pH values for the fermented samples in figures 3.12. In the table the pH value represents the mean of two pH measurements ±SD

Sample Id	Pre-fermentation pH	Post-fermentation pH
Barley flour & water	5.87±0.04	6.20±0.12
Barley flour, pentopan & water		5.51±0.57
Barley flour, LA1 & water		3.73±0.01
Barley flour, LA1, pentopan & water		3.77±0.11

In order to facilitate illustration of the combined effect of fermentation and pentopan, the data is presented in three different figures. Figure 3.12 illustrates *in vitro* digestion of starch in barley flour samples containing *l.johnsonii* LA1 and pentopan. No significant difference was determined in the fermented samples after the addition of pentopan although there was a clear trend that pentopan increased starch digestion at 5 and 60 minutes in the duodenal phase.

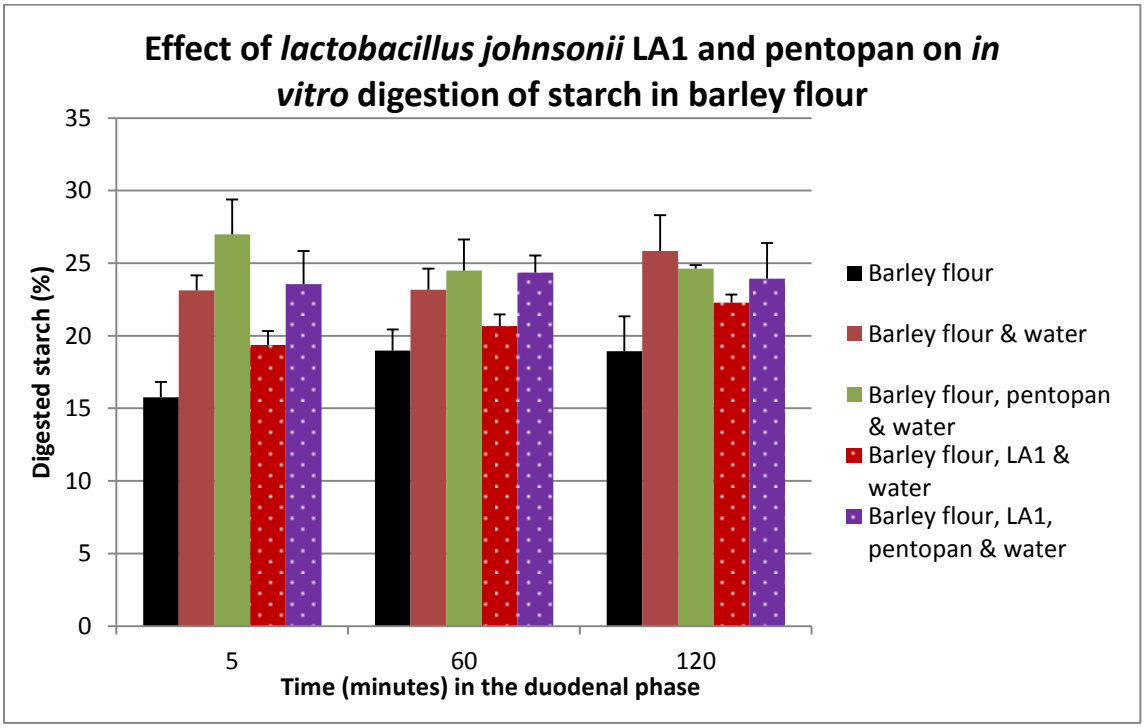

Figure 3.12 illustrates the joint effect of pentopan and *l. johnsonii* LA1 on *in vitro* digestion of starch in barley flour. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

Figure 3.13 shows the effect a combination of *l.reuteri* SD2112 and pentopan has on *in vitro* digestion of starch. The post fermentation pH values for these samples are given in the table 3.9. No significant difference in the amount of digested starch was determined in the samples containing *l.reuteri* SD2112 after the addition of pentopan.

Table 3.9: An overview of the pH values for the fermented samples in figures 3.13. In the table the pH value represents the mean of two pH measurements ±SD

Sample Id	Pre-fermentation pH	Post-fermentation pH
Barley flour & water	5.87±0.04	6.20±0.12
Barley flour, pentopan & water		5.51±0.57
Barley flour, SD2112 & water		3.61±0.01
Barley flour, SD2112, pentopan & water		3.62±0.02

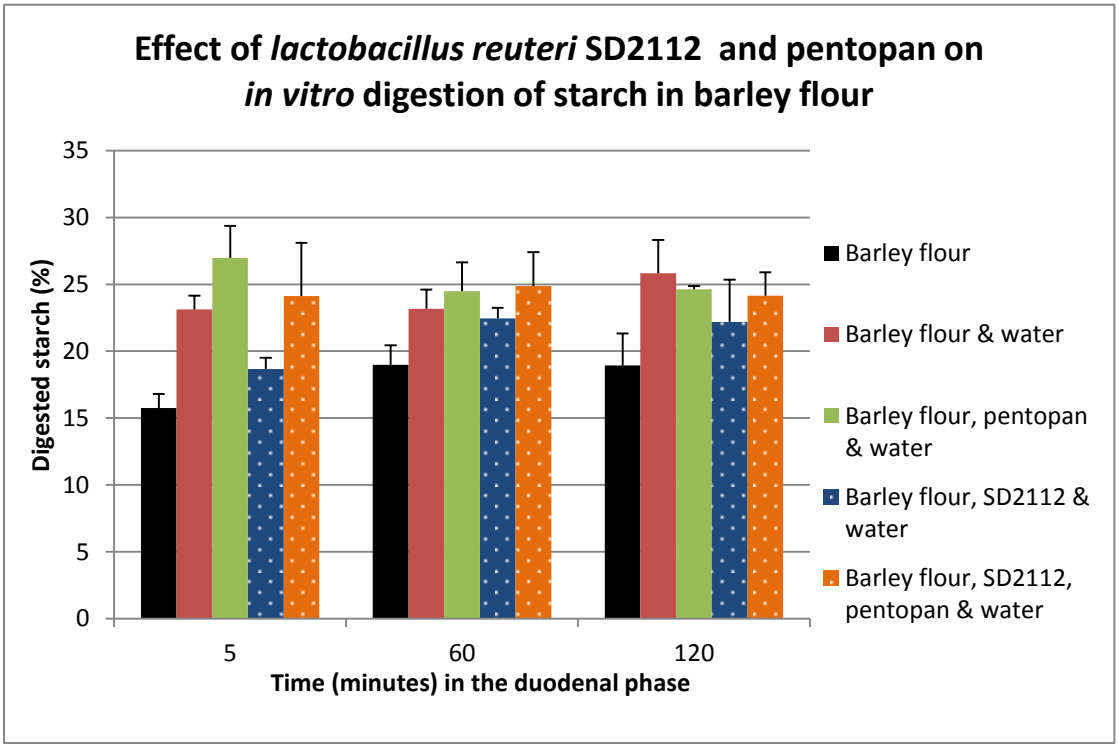

Figure 3.13 illustrates the joint effect of pentopan and *l.reuteri* SD2112 on *in vitro* digestion of starch in barley flour. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

Figure 3.14 shows the joint effect of *l.acidophilis* LA-5 and pentopan on *in vitro* digestion of starch in barley flour. The post fermentation pH values for these samples are given in the table 3.10. No significant difference in the amount of digested starch was determined in the samples containing *l.acidophilis* LA-5 after the addition of pentopan.

Table 3.10: An overview of the pH values for the fermented samples in figures 3.14. In the table the pH value represents the mean of two pH measurements ±SD.

Sample Id	Pre-fermentation pH	Post-fermentation pH
Barley flour & water	5.87±0.04	6.20±0.12
Barley flour, pentopan & water		5.51±0.57
Barley flour, LA5 & water		3.66±0.06
Barley flour, LA5, pentopan & water		3.62±0.04

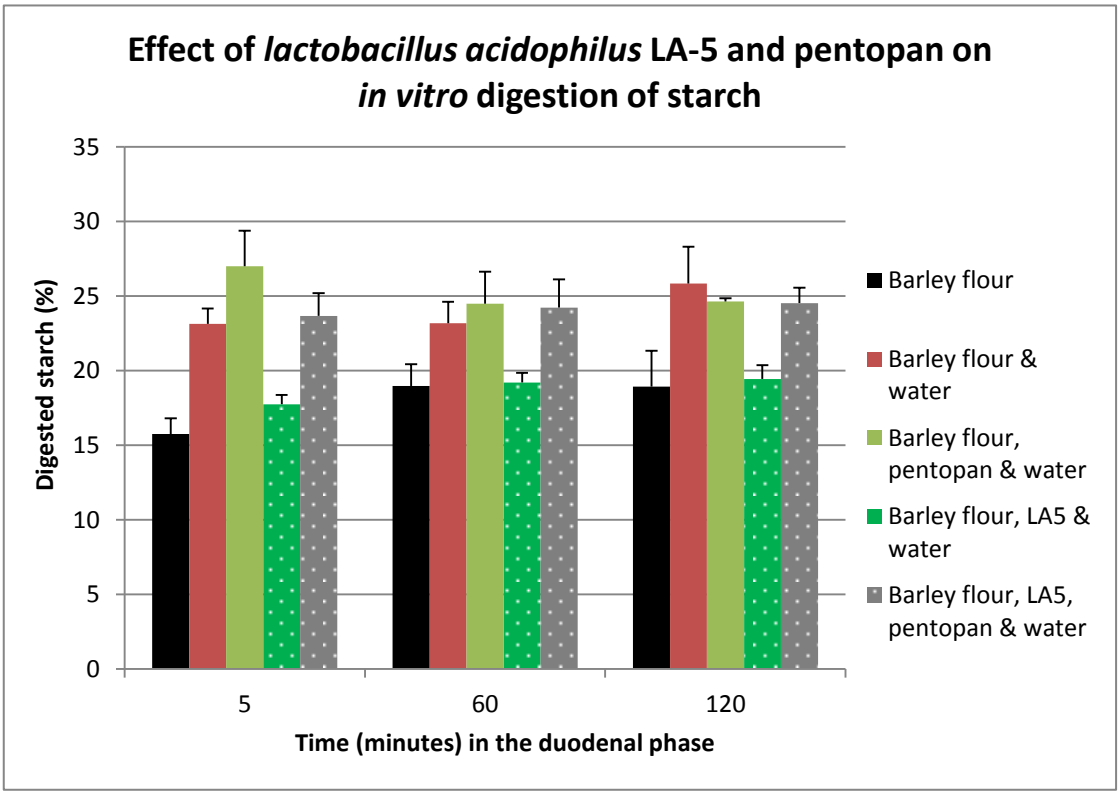

Figure 3.14 shows the combined effect of *l.acidophilus* LA-5 and pentopan on *in vitro* digestion of starch in barley flour. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

3.14 Effect of acetic acid and pentopan on *in vitro* digestion of starch in barley flour

Acetic acid was added to a number of the samples which were studied in the course of this master thesis. Both pre-fermentation and post fermentation pH values were measured in these samples. Table 3.11 gives an overview of the pH values for these samples

Table 3.11: An overview of the pH values for the fermented samples in figures 3.15. In the table the pH value represents the mean of two pH measurements ±SD

Sample Id	Pre-fermentation pH	Post-fermentation pH
Barley flour & water	5.87±0.04	6.20±0.12
Barley flour, pentopan & water		5.51±0.57
Barley flour, acetic acid & water	3.80±0.00	3.86
Barley flour, acetic acid, pentopan & H_20	3.82±0.00	3.84

The effect of the addition of acetic acid on *in vitro* digestion of starch is illustrated in figure 3.15 The combined effect of acetic acid and pentopan is also shown. No significant difference was observed in any of the samples after addition of acetic acid. This also applies to the samples containing pentopan. However there was a indication that the addition of acetic acid reduced starch digestion

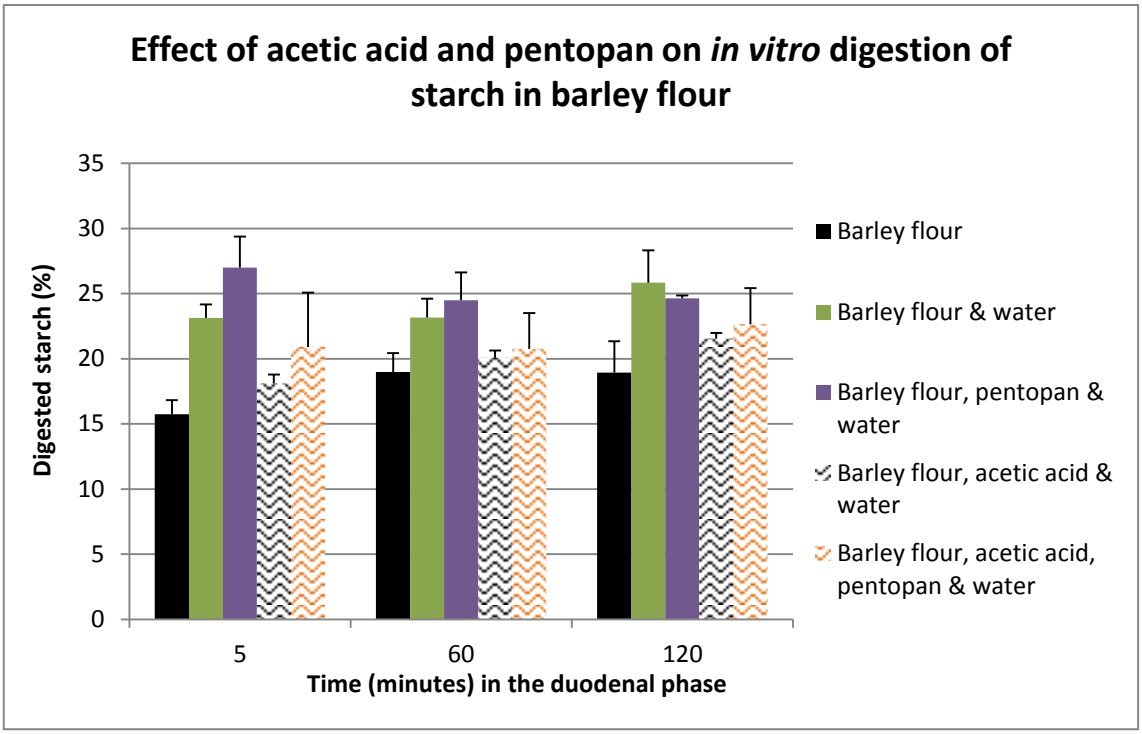

Figure 3.15 shows the effect of the addition of acetic acid on *in vitro* digestion of starch in barley flour. The combined effect of pentopan and acetic acid is also shown in this figure. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD.

3.15 Effect of boiling on *in vitro* digestion of starch in barley products

The effect of boiling on *in vitro* digestion of starch in hulled barley, barley flakes and barley flour was also investigated in this master thesis. The results are shown in figure 3.16 where un-boiled hulled barley, barley flakes and barley flour are compared with their boiled counterparts. No significant difference in the amount of i*n vitro* digested starch was determined in any of the barley products after boiling. This applies to each of the three time points in the duodenal phase.

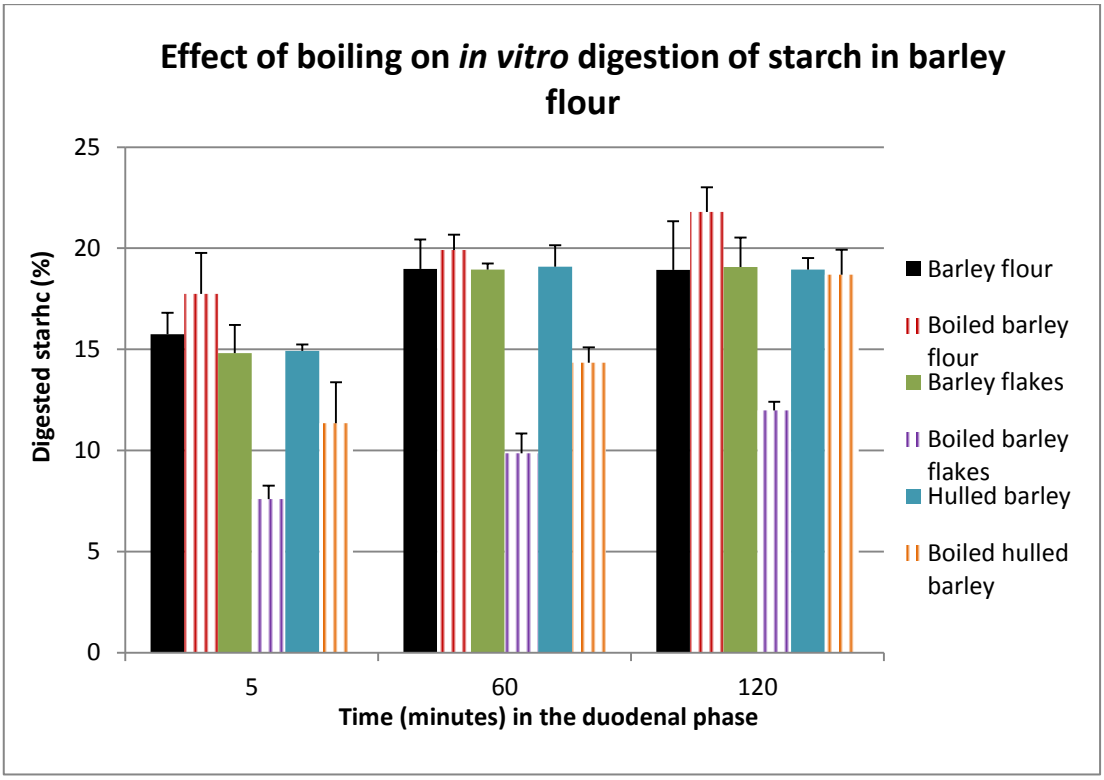

Figure 3.16 shows the effect of boiling on *in vitro* digestion of starch in the four barley products. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD

3.16 Effect of boiling and pentopan on *in vitro* digestion of starch in barley flour

The joint effect of boiling and the addition of pentopan on *in vitro* digestion of starch was another aspect which was investigated in this master thesis. Figure 3.17 illustrates *in vitro* digestion of starch in boiled barley flour containing pentopan. No significant difference in the amount of *in vitro* digested starch was determined in the boiled barley flour samples after the addition of pentopan.

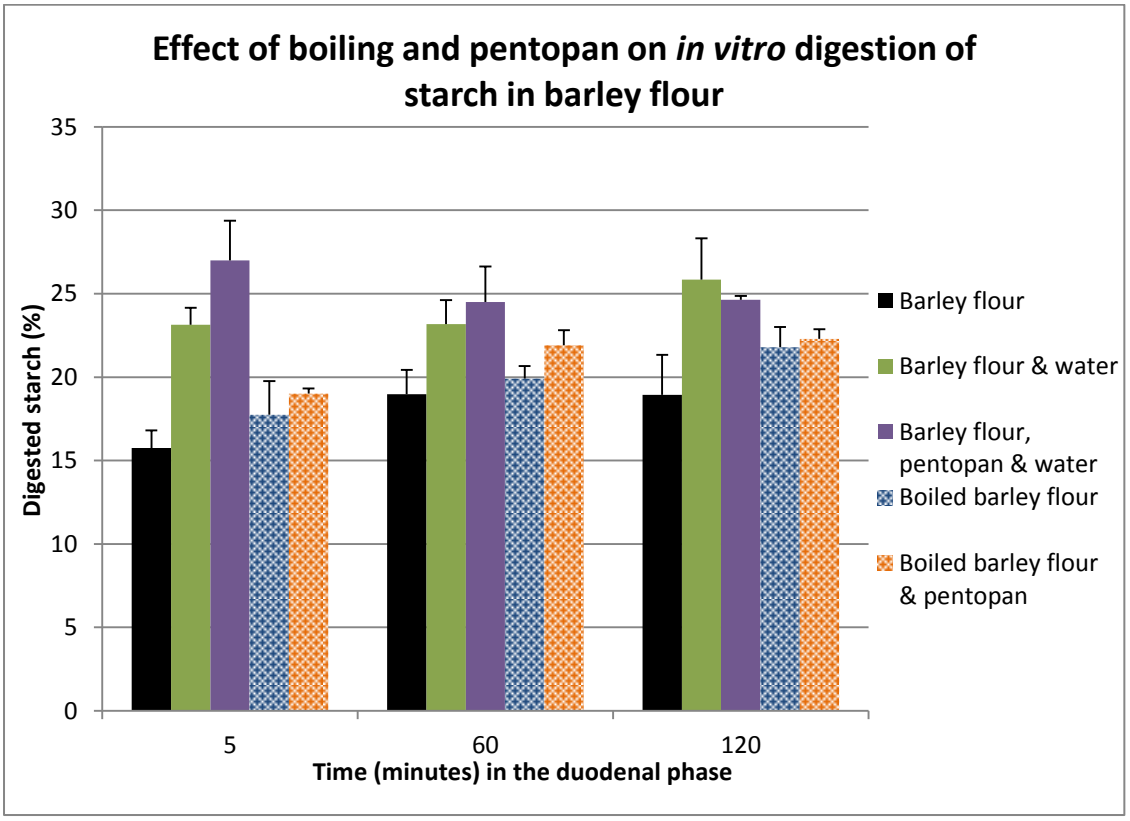

Figure 3.17 illustrates the joint effect of boiling and pentopan on *in vitro* digestion of starch in barley flour. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD.

3.17 A comparison of *in vitro* digestion of starch in barley flour and wheat flour

A comparison of *in vitro* digestion of starch in barley and wheat flour was carried out in the course of this thesis. Both boiled and un-boiled samples were compared and the results are shown in figure 3.18. No significant difference was determined in the amount of *in vitro* digested starch in the wheat flour sample after boiling. There was no significant difference between the wheat flour samples and the barley samples. This applies to both the boiled and the un-boiled samples.

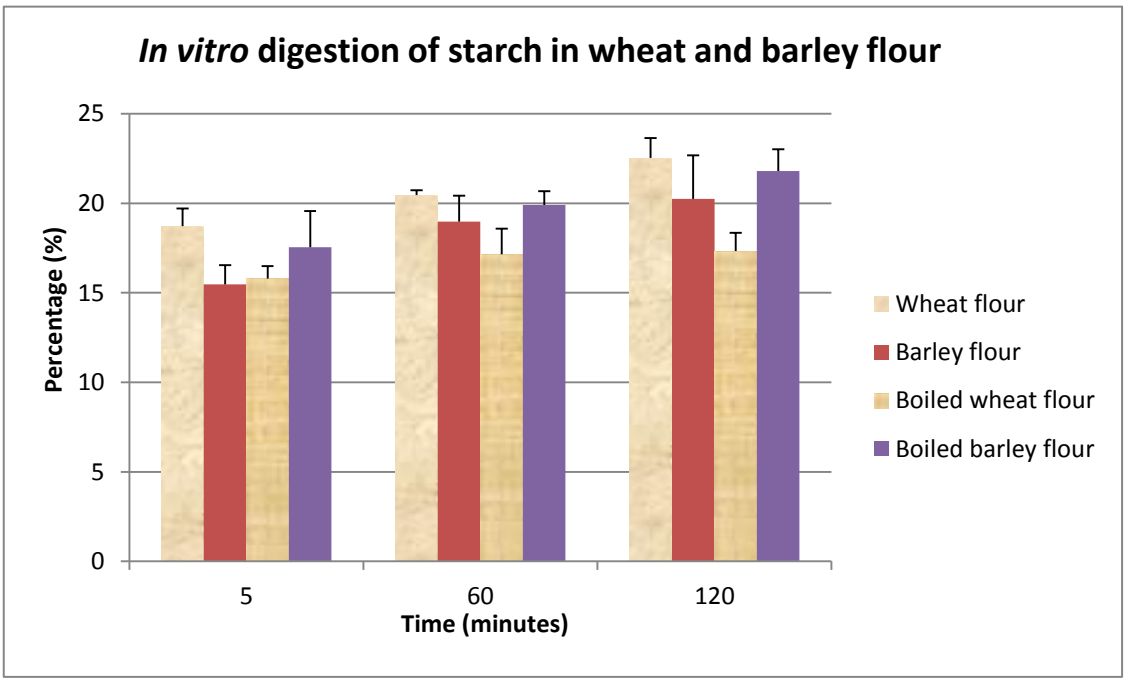

Figure 3.18 shows *in vitro* digestion of starch in wheat and barley flour. The amount of *in vitro* digested starch is calculated as a percentage of the total starch content (dry weight basis) in the barley products. In this figure each bar represents the mean of one experiment performed in triplicates ± SD.

4 Discussion

4.1 Overall statistical analysis

Any in-depth discussion of the results obtained in this master thesis needs to take into consideration the outcome of the overall statistical analysis. The raw data in this study was initially analysed using Analysis of variance (ANOVA). Given that the majority of the calculated P-values were <0.05, Tukey's post-hoc test was subsequently used to further analyse the data. The results from Tukey's test however indicated that there were few significant differences between comparable samples. The exact reason for this outcome is difficult to suggest but may be the result of a number of factors including the relatively conservative nature of Tukey's post hoc test, which is in principle a t-test, except that it corrects for the increased probability of making an error when performing multiple comparisons. Shortage of raw data or high standard deviation may also have contributed to the lack of significant results. Irrespective of the cause, it is important to bear these factors in mind when evaluating the results discussed in the following sections of this chapter (*Multiple Comparisons Among Treatment Means*)

4.2 Phenolic acid content in barley products

One of the fundamental aspects of this master thesis was to determine the effect of processing on the level of free phenolic acids in barley products. In order to achieve this objective it was first necessary to establish the free and bound phenolic acid content in these barley products. As anticipated, there was a significantly higher level of total phenolic acids in covered barley than in all of the other barley products. This applies to both the free and bound phenolic acids and may be attributed to the fact that, with the exception of covered barley, all of the other barley products have had their hull removed (*Types of Barley* 2013). Given that phenolic acids tend to accumulate in the outer layers of the *caryopsis* (Gamel & Abdel-Aal 2012; Hernanz et al. 2001), it is reasonable to assume that the removal of the outer hull would lead to a corresponding loss of phenolic acids. This assumption is also substantiated by the fact that there was approximately three times more total bound phenolic acids in covered barley than all of in the other barley products. Furthermore covered barley contained almost five times as much total free phenolic acids as hulled barley and three and two times as much as barley flakes and barley flour respectively.

Ferulic acid (FA) and *p*-coumaric acid (*p*-CA) were the two most abundant phenolic acids determined in all of the barley products, with FA being the most prominent. This is in

accordance with previous studies (Gamel & Abdel-Aal 2012; Hernanz et al. 2001). Approximately 90% of the total phenolic acid content in hulled barley, barley flakes and barley flour was made up of bound FA. The significantly higher level of free and bound *p*-CA in covered barley, in comparison with the other products, may be explained by *p*-CAs tendency to forms linkages with cell wall lignins (Higuchi et al. 1967). In general lignins are more abundant in the cell wall of the barley hull than in the other parts of the barley grain (Salomonsson et al. 1978). Covered barley is the only one of the barley products with its hull still intact. Finally with respect to the ratio of free- to bound phenolic acids, the total bound phenolic acids in all of the barley products made up 99.6% of the total phenolic acid content. This higher bound phenolic acid content has also been observed in previous studies (Bonoli et al. 2004).

4.3 Effect of boiling on the free phenolic acid content in barley products

In general whole grain cereals such as barley tend to be cooked prior to consumption. Following determination of the phenolic acid content in the untreated cereals samples, hulled barley, barley flakes and barley flour were boiled and subsequently analyzed. Given that boiling results in significant changes in the chemical composition of the grain, it was expected that both the bio-accessibility and the concentration of the phenolic acids would be effected (N'Dri et al. 2013). In the present study there was a significant increase in the level of total free phenolic acids in hulled barley after boiling. Furthermore there was an increase in all of the four individual free phenolic acids. This observation may be explained by the breakdown of cellular constituents due to thermal processing and the subsequent release of bound phenolic acids (Dewanto et al. 2002). In general caffeic acid (CA), *p*-CA and FA are found in conjugate forms and are often released by hydrolysis processes such as that induced by boiling (Clifford 1999). Hydrolysis of the conjugated acids followed by possible polymerization of the phenolic constituents (Randhir et al. 2008) may account for the significant increase in total free phenolic acids observed in boiled hulled barley. In contrast to hulled barley, barley flakes showed a decrease in the level of free phenolic acids after boiling. This observation is more difficult to explain but may be a result of poor extraction and/or degradation of phenolic acids during sample preparation. In general phenolic acids are regarded as relatively sensitive and factors such as heating or exposure to light may have contributed to their degradation (Tsao 2010).

4.4 Effect of boiling and pentopan on free phenolic acids in barley flour

Boiling alone had little effect on the level of free phenolic acids in barley flour. However the combined effect of boiling and enzymatic treatment resulted in an increase in the levels of both free FA and *p*-CA. This increase was most evident in the case of free FA and may be explained by the enzymatic actions of pentopan. Pentopan is a xylanase which cleaves the glycosidic linkages between the β-D-xylopyranosyl residues in the linear chain backbone of the arabinoxylan molecule (Selinheimo et al. 2006). These arabinoxylan chains are often cross linked by di-ferulic bridges which arise as a result of the dimerization of FA (Renger & Steinhart 2000). Cleavage of the arabinoxylan chains combined with the breakdown of cellular constituents may have attributed to the observed increase in free FA. The effect of pentopan may have been enhanced by the naturally occurring feruloyl esterases and xylanases, present in the barley flour. Both of these enzymes participate in the breakdown of arabinoxylan (Sancho et al. 1999) and may therefore have contributed to the observed effect.

4.5 Effect of water and acetic acid on the level of free phenolic acids in barley flour

In addition to the effect of boiling, the impact of fermentation on the free phenolic acid content was also investigated. In order to ensure that this impact was related to specific bacteria fermentation, barley flour samples treated with water were included in the study. In accordance with previous findings (Hole et al. 2012) there was a significant increase in the level of total free phenolic acids in barley flour treated with water. This increase may be the result of the hydrolysis and possible activation of the cereal esterases present in the barley flour (Hole et al. 2012). These esterases cleave methyl esters of cinnamic acid derivatives (Sancho et al. 1999) thereby releasing the bound cinnamic acids. Evidence of these naturally occurring cereal esterases was also seen in the water-treated samples which were immediately frozen at -20°C (t_0 samples).

The largely insignificant differences between the levels of free phenolic acids in the t_0 samples and the water-treated samples suggest that freezing does not inhibit the activity of the cereal esterases. Their activity however does seem to be impeded by the addition of acetic acid. In comparison with the water-treated samples a lower level of free phenolic acids was observed in the barley samples following the addition of acetic acid. This decrease may be a result of the fall in pH which arises as a consequence of the acidification of the samples. In this study acid-treated samples were included in order to establish if the change in phenolic

acid content was due to feruloyl esterase and xylanase or acid production. Based on the present findings it would appear that the change is primarily induced by enzymatic activity.

4.6 Effect of fermentation on the free phenolic acid content in barley flour

Barley flour fermentation with *lactobacillus johnsonii* LA1 resulted in a negative impact on the level of free phenolic acids in barley flour. This was in discordance with the findings of Hole et al. Therefore in order to ascertain the credibility of the results the samples were re-prepared and re-analysed. There was however little or no variation in the re-analysed results. An unidentified peak in the HPLC chromatogram for the *l.johnsonii* samples may suggest a possible explanation for the unexpected results. Decarboxylation of cinnamic acids is a common ability amongst a number of microorganisms including lactic acid bacteri a(van Beek & Priest 2000). This reaction may result in the formation of metabolites which are difficult to identify. The unidentified component observed in the *l.johnsonsii* samples may an example of such a case. However due to the unidentifiable nature of the component, it is difficult to reach a more definite conclusion.

With respect to the results obtained for fermentation of barley flour with *lactobacillus reuteri* SD2112 and *lactobacillus acidophilus* LA-5, these findings were in accordance with Hole et al. Fermentation of barley flour with *l.reuteri* SD2112 and *l.acidophilus* LA-5 resulted in a significant increase in a number of the free phenolic acids. This is in accordance with previous studies (Hole et al. 2012) and may be attributed to the feruloyl esterase activity of the lactic acid bacteria. Feruloyl esterases cleave the ester link joining the β-D-xylopyranosyl residues on arabinoxylan and the di-ferulic or ferulic acid (Faulds et al. 2006). This combined with the activity of the cereal enzymes is presumed to have contributed to the increase in the level of free phenolic acids.

4.7 Effect of fermentation and pentopan on free phenolic acids in barley flour

Previous studies have shown that a combination of fermentation and enzymatic treatment increases the amount of free phenolic acids in whole grain cereals (Lappi et al. 2013). This is also true for the present study. The addition of pentopan to the fermented samples resulted in a significant increase in the levels of free CA, *p*-CA FA and total phenolic acids in barley flour. This significant increase is attributed to the synergetic effects of feruloyl esterase- and xylanase activity. Both of these enzymes contribute to the degradation of arabinoxylan and the subsequent release of phenolic acids. Given that the addition of acetic acid resulted in a

decrease in the level of free phenolic acids, it is reasonable to assume that the significant increase in the levels of free phenolic acids was a result of feruloyl esterase and xylanase as opposed to acid production.

4.8 Determination of total starch in barley products

Having determined the effects of processing and enzymatic treatment on phenolic acid content, it was of interest to establish whether these effects had an impact on *in vitro* digestion of starch. Phenolic acids have been shown to inhibit the effects of pancreatic α-amylase and thereby reduce the digestion of starch (McCue et al. 2005). In order to investigate this hypothesis it was necessary to determine the total amount of starch in each of the four barley products. Covered barley contained significantly less starch than all of the other barley products while barley flour contained significantly less starch than the hulled and flaked varieties. Covered barley contains a tough outer hull which surrounds the barley grain and renders the starchy endosperm relatively inaccessible. This may account for the lower level of starch determined in the product. In the case of barley flour, the grinding or milling which flour undergoes may result in damage caused to the starch granules in the endosperm (Bettge et al. 2000). This damage may explain the lower level of starch determined in the barley flour.

4.9 *In vitro* digestion of starch in barley products

In comparison with previous studies, the amount of starch that was digested in this present study was relatively low. *Anker-Nilssen et al*. reported levels of up to 80% digestion of starch after 120 minutes incubation time (Anker-Nilssen et al. 2006). In this present study starch digestion never reached more than 25% for the same incubation period. Several factors may have played a role in this result including the raw barley materials themselves. In contrast to *Anker-Nilssen et al,* the commercial barley products employed in this thesis were a mixture of various barley types. The exact composition of their starch content was therefore unknown and this combined with other factors may have contributed to the low level of starch digestion in this present study. Growth temperature is another element which may have influenced the level of starch digestion and is particularly pertinent given that summer 2013 was a relatively warm summer in Norway. According to *Anker-Nilssen et al*., there is a negative correlation between growth temperature and starch digestion. A number of factors may be responsible for this. In general a high growth temperatures results in an increase in the level of soluble β-glucans in the barley grain. An increase in soluble β-glucans could result in a corresponding increase in viscosity and thereby account for the low level of starch digestion. A second

possible explanation could be related to the protein content of the barley grain. Protein content increases in accordance with growth temperature. A high growth temperature would therefore increase the protein content in the matrix of the endosperm and possible impede the digestion of the endospermic starch (Anker-Nilssen et al. 2006).

4.10 *In vitro* digestion of starch in boiled barley products

Following the determination of starch digestion in the untreated barley products, a similar study was carried out on the processed barley samples. Furthermore the possible link between a change in the level of free phenolic acids and starch digestion was also investigated. As mentioned previously, boiling resulted in only a slight increase in the level of free phenolic acids in barley flour. It was therefore deemed unlikely that there was a link between this increase and the increase in the amount of digested starch in barley flour after boiling. Presumably there are other factors which have contributed to this phenomenon. Boiling, in general, results in an increase in the level of starch digestion. This is due to the fact that boiling induces gelatinization of starch which renders the starch granule more susceptible to enzymatic hydrolysis (Aura et al. 1999). Furthermore boiling results in the denaturation of protein, which renders the starchy endosperm more susceptibility to pancreatic α-amylase digestion. Degradation of protein is further heightened by the employment of pepsin which breaks down the protein matrix surrounding the endospermic starch granules. The addition of bile, which acts as an emulsifying agent, enhances the interaction between the starch granules and pancreatic α-amylase (Aura et al. 1999). Thus both bile and pepsin play a vital role in the digestion of starch.

In contrast to barley flour, a decrease in starch digestion was observed in hulled barley, barley flakes and wheat flour after boiling. This decrease was unexpected particularly in the case of wheat flour which has experienced similar processing to barley flour. One possible hypothesis is the formation of a gluten network which may have arisen as a result of stirring the flour during boiling. This gluten network could serve as an obstacle to the digestive actions of the hydrolytic enzymes, thereby rendering the starch in the endosperm relatively inaccessible. As previously mentioned, an increase in the level of free phenolic acids was observed in hulled barley after boiling. However there is no evidence to suggest that this increase is connected to the decrease in digested starch observed in boiled hulled barley. This is due to the fact that a similar decrease in starch digestion was observed in barley flakes despite the fact that boiling resulted in a decrease in the phenolic acid content

One of the possible reasons for the observed decrease however is the inaccessibility of the starch granules. Boiling leads to the breakdown of cellular constituents which can result in the release of soluble β-glucans. These soluble β-glucans are capable of forming a gel which can act as a physical barrier to the digestive actions of the hydrolytic enzymes. This protective action of the gel combined with the inaccessibility of the starch granules may therefore account for the decrease in starch digestion observed in hulled barley and barley flakes after boiling. Similar to hulled barley, barley flakes have a tightly packed physical form and therefore a relatively small surface area available for enzymatic attack. This small surface area combined with the barrier effect of the gel may have contributed to the decrease in starch digestion (Snow & O'Dea 1981).

With respect to all of three of the aforementioned products, starch retrogradation can provide an explanation for the decrease in starch digestion. Starch retrogradation occurs when gelatinized starch molecules begin to re-associate and develop a more ordered structure (Atwell et al. 1988). Amylose in particular can retrograde to a form that is highly resistant to pancreatic amylase hydrolysis (Muir & O'Dea 1992). Retrogradation can occur during cooling of starch rendering the starch resistant to enzymatic digestion.(Eerlingen et al. 1994).

4.11 *In vitro* digestion of starch in fermented barley flour

With respect to fermentation, all of the bacteria fermented samples showed a lower level of starch digestion than the samples which were treated with water alone. Once again this phenomenon cannot be directly attributed to an increase in free phenolic acids. This is due to the fact that the lower level of starch digestion was observed in all of the bacteria fermented samples including those fermented with *l.johnsonii* LA1. As mentioned earlier fermentation with *l.johnsonii* LA1 resulted in a decrease in the level of free phenolic acids. It is therefore reasonable to reject the possibility of a link between an increase in free phenolic acids and a decrease in starch digestion.

The possibility that pH played a role in the reduction of starch digestion, observed in the fermented and acid treated samples was considered. Post fermentation pH measured in theses samples was between pH 3.7-3.8. Given that pancreatic α-amylase functions best at pH 6.9 (Rowe et al. 1968), it was not unreasonable to assume that the low pH in the samples may have impeded the digestive actions of the enzyme. However this hypothesis was rejected due to the fact that prior to incubation in the duodenal phase, pH in the samples was adjusted to

pH 7.0. Rejection of this theory presented the possibility of a second hypothesis that the presence of lactic acid or acetic acid in the samples may have contributed to the decrease in starch digestion. It would appear that the acids exerted an inhibitory effect on starch digestion. However the exact mechanisms of this hypothesis are not known.

4.12 Effect of pentopan on *In vitro* digestion of starch in barley flour

The addition of pentopan to the bacteria fermented samples resulted in a slight increase in the level of starch digestion. This in comparison to the bacteria fermented samples. Again this increase was observed in all of the bacteria fermented samples and is therefore not connected to an increase in the level of free phenolic acids. One of the factors which may have contributed to the increase in starch digestion is the enzymatic breakdown of arabinoxylan. Approximately 20% of the cell wall of the barley endosperm is composed of arabinoxylan (Delcour & Hoseney 2010). Enzymatic degradation of arabinoxylan would presumably render the starchy endosperm more accessible to the digestive actions of pancreatic α-amylase. This could possibly account for the observed increase in the level of starch digestion.

A similar observation of the effect of pentopan was seen in the boiled, water-treated and acid-treated samples. In each of these cases, the addition of pentopan resulted in an increase in the level of starch degradation. The reasons behind this increase are presumably the same as those discussed in the previous paragraph and are therefore not considered to be connected to an increase in the level of free phenolic acids.

5 Conclusion

Based on the findings of the present studies, fermentation of barley flour with *L.acidophilus* LA-5 and *L.reuteri SD2112* appears to have positive effects in terms of an increase of phenolic acids. The level of total free phenolic acids was 3 and 4 times higher in the samples fermented with *L.acidophilus* LA-5 and *L.reuteri* SD2112 respectively. This is in comparison with the water-treated samples. These effects were further enhanced by the commercial xylanase, pentopan. In the case of *L.acidophilus* LA-5, the addition of pentopan resulted in a twofold increase in the level of free FA and total free phenolic acids. This was also the case for *L.reuteri SD2112.* With respect to boiling, there was almost four times as much total free phenolic acids observed in hulled barley after boiling.

However despite these significant increases in the level of free phenolic acids, no link between these acids and the inhibition of pancreatic α-amylase is supported by this study. The results show however that starch digestion does appear to be impeded by the presence of lactic acid or acetic acid. This is most likely due to the fact that these acids inhibit the digestive actions of pancreatic α-amylase. However while the exact mechanism of this inhibition is not known, it appears to be counteracted by the addition of commercial xylanase pentopan.

In addition to the inhibitory effects of acid, starch digestion in also appears to be hindered in by thermal processing such as boiling. This is the case for hulled barley and barley flakes and may be caused by retrogradation, gel formation or the formation of a gluten network. All of these phenomena could hinder the digestive actions of enzymes, thereby providing plausible explanations for the observed decrease.

An evaluation of these findings should simultaneously consider the shortcomings of this study including the conservative post hoc statistical tests and the static nature of the *in vitro* model. In order to obtain a more realistic picture, further research is therefore required.

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Appendix 1: Materials

Chemicals

Wet chemicals

Acetic acid glacial 100% (Merck)

Methanol gradient grade for liquid chromatography (Merck)

Hydrochloric acid fuming 37% for analysis (Merck)

Ethyl acetate for analysis (Merck)

Acetonitrile isocratic grade for HPLC (VWR)

Rektifisert sprit (Kemetyl)

Dimethyl sulfoxide (Aldrich)

Glycerol 85% (Merck)

pH calibration buffer solutions 4 & 7 (Radiometer Analytical)

Dry chemicals

Pentopan 500 BG (Novo Nordisk A/S, Denmark)

Caffeic acid (Fluka Chemika)

p-Coumaric acid (4-Hydroxycinnamic acid) (Aldrich)

Ferulic acid (4-Hydroxy-3-methoxycinnamic) (Aldrich)

Sinapic acid (Fluka Chemika)

Potassium chloride pro analysi (Merck)

Potassium phosphate monobasic ACS reagent, ≥99.0% (Aldrich)

Sodium azide ReagentPlus, ≥99.5% (Aldrich)

Sodium bicarbonate pro analysi (Merck)

Sodium hydroxide pellets for analysis (Merck)

Sodium chloride for analysis (Merck)

MOPS sodium salt ≥99.5% (Aldrich)

Magnesium chloride hexahydrate (Aldrich)

Calcium chloride dehydrate pro-analysi (Merck)

Pepsin from porcine gastric mucosa (Aldrich)

Bile from bovine and ovine bile acid mixture (Aldrich)

Pancreatin from porcine pancreas (Aldrich)

Megazyme Total Starch Assay Procedure Kit

Thermostable α-amylase

Amyloglucosidase

GOPOD Reagent Buffer

GOPOD Reagent Enzymes

D-Glucose standard solution

Equipment

Isolation in Pure Strain

Lactobacillus johnsonii LA1

Lactobacillus reuteri SD2112

Lactobacillus acidophilus LA-5

MRS Broth for microbiology (Aldrich)

Inoculating loop (VWR)

Agar plates (Aldrich)

Glass tubes 25ml (Duran)

Bunsen burner (VWR)

Matches (Nitedals)

Cryotube vials (VWR)

Moisture determination in barley products

Thermo Control YTC 01 L Infrared Dryer (Sartorius)

YDP03-OCE Data Printer (Sartorius)

Aluminum Weighing Dish (Aldrich)

Metal spatula (VWR)

Fermentation of barley flour

5ml volumetric flask (Duran)

X5105 Dual Range (Mettler Toledo)

Graduated laboratory bottle capacity 1000 mL, clear glass (Duran)

Waterbath 1086 (GFL Gesellschaft für Labortechnik mbH)

50ml conical polypropylene centrifuge tube (VWR)

Multifuge 4KR (Heraeus)

Multipipette plus (Eppendorf)

Combitips advanced 50ml (Eppendorf)
MS2 Minishaker (Ishikawa et al.)
100-1000ul mLINE Pipette (Biohit)
100-1000ul mLINE Pipettetips (Biohit)
1.5ml tubes (Eppendorf)
Balance basic (Sartørius)
100ml polypropylene disposable cups (Sarstedt)
Metal spatula (VWR)
Disposable Pasteur Pipets (VWR)
Pasteur pipette rubber bulbs (Aldrich)
PHM210 standard pH meter (Meter Lab Radiometer)
20-200µl mLINE Pipette (Biohit)
20-200µl mLINE Pipettetips (Biohit)
Freezer (Bosch)
Incubator (Termaks)

Freeze drying

Balance basic (Sartørius)
100ml polypropylene disposable cups (Sarstedt)
Metal spatula (VWR)
Freezer (Bosch)
Freeze Dryer alpha 1-2/ LD plus (Christ)
Balance basic (Sartørius)

Boiling of barley products

Balance basic (Sartørius)
100ml polypropylene disposable cups (Sarstedt)
Metal spatula (VWR)
Measuring cylinder Class A 500 ml (Duran)
Erlenmeyer flask 500ml (Pyrex)
PARAFILM® M-forseglingsfilm (Brand)
Oven plate (Electrolux)
Saucepan (Tefal)
Wooden spoon

Freezer (Bosch)

X5105 Dual Range (Mettler Toledo)

8ml Hexagonal Antistatic Polystyrene Weighing Dishes (VWR)

Stopwatch timer (VWR**)**

Grinding of barley products

Balance basic (Sartørius)

100ml polypropylene disposable cups (Sarstedt)

Metal spatula (VWR)

ZM 200 Ultra Centrifugal Mill (Retsch)

Extraction phenolic acids in barley flour

Balance Basic (Sartørius)

50ml conical polypropylene centrifuge tube (VWR)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

MS2 Minishaker (Ishikawa et al.)

Ultrasonic Clear (VWR)

Stopwatch timer (VWR)

Multifuge 4KR (Heraeus)

100ml glass flask (Duran)

El 131 Rotavapor and water bath (Buchi)

PHM210 standard pH meter (Meter Lab Radiometer)

Multishaker PSU 20 (Bisan)

SPD131DDA SpeedVac Concentrator plus Universal Vacuum System UVS 800- DDA (Thermo Electron Savant)

100-1000ul mLINE Pipette (Biohit)

100-1000ul mLINE Pipettetips (Biohit)

1.5 ml glass crimp neck vial (VWR)

0.2ml flat bottom clear glass micro inserts (VWR)

Aluminum crimp caps with central hole, 5.5 mm and septum (VWR)

Millex-HV Filter, 0.45 µm, PVDF, 33 mm, gamma sterilized (Millipore)

3 ml SOFT-JECT® – 3-part disposable syringes (Henke Sass Wolf)

Preparation of stock solutions and working solutions

X5105 Dual Range (Mettler Toledo)

8ml Hexagonal Antistatic Polystyrene Weighing Dish (VWR)

5ml 10ml 20ml, volumetric flask (Duran)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

1.5 ml glass crimp neck vial (VWR)

0.2ml flat bottom clear glass micro inserts (VWR)

Aluminum crimp caps with central hole, 5.5 mm and septum (VWR)

Millex-HV Filter, 0.45 µm, PVDF, 33 mm, gamma sterilized (Millipore)

3 ml SOFT-JECT® – 3-part disposable syringes (Henke Sass Wolf)

Analysis of phenolic acids in barley flour using Rapid Separation Liquid Chromatography (RSLC)

Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) System (Thermo Scientific)

Dionex™ UltiMate™ 3000 Rapid Separation Diode Array Detector (Thermo Scientific)

Dionex™ Ultimate™ 3000 Binary Rapid Separation (RS) Pump (Thermo Scientific)

Dionex™ Ultimate™ 3000 Rapid Separation Autosampler (Thermo Scientific)

Dionex™ Ultimate™ 3000 Rapid Separation Thermostatted Column Compartment (Thermo Scientific)

Acquity UPLC BEH C8 1.7μm 2.1 x 150 mm column (Waters)

Determination of total starch in barley products

Balance Basic (Sartørius)

Metal spatula (VWR)

8ml Hexagonal Antistatic Polystyrene Weighing Dish (VWR)

100ml, 1000ml volumetric flask (Duran)

Measuring cylinder Class A 20ml, 50ml, 100 ml (Duran)

PHM210 standard pH meter (Meter Lab Radiometer)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

100-1000ul mLINE Pipette (Biohit)

100-1000ul mLINE Pipettetips (Biohit)

15ml conical polypropylene centrifuge tube (VWR)

Refrigerator, Freezer (Bosch)

Aluminum foil

ZM 200 Ultra Centrifugal Mill (Retsch)

Glass test tubes round bottomed 16 x 120 mm (VWR)

MS2 Minishaker (Ishikawa et al.)

Stopwatch timer (VWR)

Multipipette plus (Eppendorf)

Combitips advanced 50ml (Eppendorf)

Waterbath 1086 (GFL Gesellschaft für Labortechnik mbH)

Multifuge 4KR (Heraeus)

UVmini-1240 Spectrophotometer (Shimadzu)

Microline 280 9 pin printer (Oki)

***In vitro* digestion of starch - preparation of buffers and digestive juices**

Balance Basic (Sartørius)

Metal spatula (VWR)

8ml Hexagonal Antistatic Polystyrene Weighing Dish (VWR)

500ml, 1000ml volumetric flask (Duran)

Measuring cylinder Class A 100 ml (Duran)

PHM210 standard pH meter (Meter Lab Radiometer)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

Oral phase

Balance Basic (Sartørius)

Metal spatula (VWR)

Glass conical centrifuge tube, 35ml (Kimax)

2-20ul mLINE Pipette (Biohit)

2-20ul mLINE Pipettetips (Biohit)

100-1000ul mLINE Pipette (Biohit)

100-1000ul mLINE Pipettetips (Biohit)

MS2 Minishaker (Ishikawa et al.)

Waterbath 1086 (GFL Gesellschaft für Labortechnik mbH)

Stopwatch timer (VWR)

Gastric phase

X5105 Dual Range (Mettler Toledo)

8ml Hexagonal Antistatic Polystyrene Weighing Dishes (VWR)

Measuring cylinder Class A 100 ml (Duran)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

2-20ul mLINE Pipette (Biohit)

2-20ul mLINE Pipettetips (Biohit)

MS2 Minishaker (Ishikawa et al.)

20-200µl mLINE Pipette (Biohit)

20-200µl mLINE Pipettetips (Biohit)

PHM210 standard pH meter (Meter Lab Radiometer)

Innova 40 benchtop incubator shaker (New Brunswick Scientific)

Stopwatch timer (VWR)

Duodenal phase

X5105 Dual Range (Mettler Toledo)

Balance Basic (Sartørius)

8ml Hexagonal Antistatic Polystyrene Weighing Dishes (VWR)

Metal spatula (VWR)

MS2 Minishaker (Ishikawa et al.)

Measuring cylinder Class A 100 ml (Duran)

500-5000ul mLINE Pipette (Biohit)

500-5000ul mLINE Pipettetips (Biohit)

PHM210 standard pH meter (Meter Lab Radiometer)

20-200µl mLINE Pipette (Biohit)

20-200µl mLINE Pipettetips (Biohit)

Innova 40 benchtop incubator shaker (New Brunswick Scientific)

Waterbath 1086 (GFL Gesellschaft für Labortechnik mbH)

Stopwatch timer (VWR)

Glass conical centrifuge tube, 35ml (Kimax)

Freezer (Bosch)

Appendix 2: Flow charts

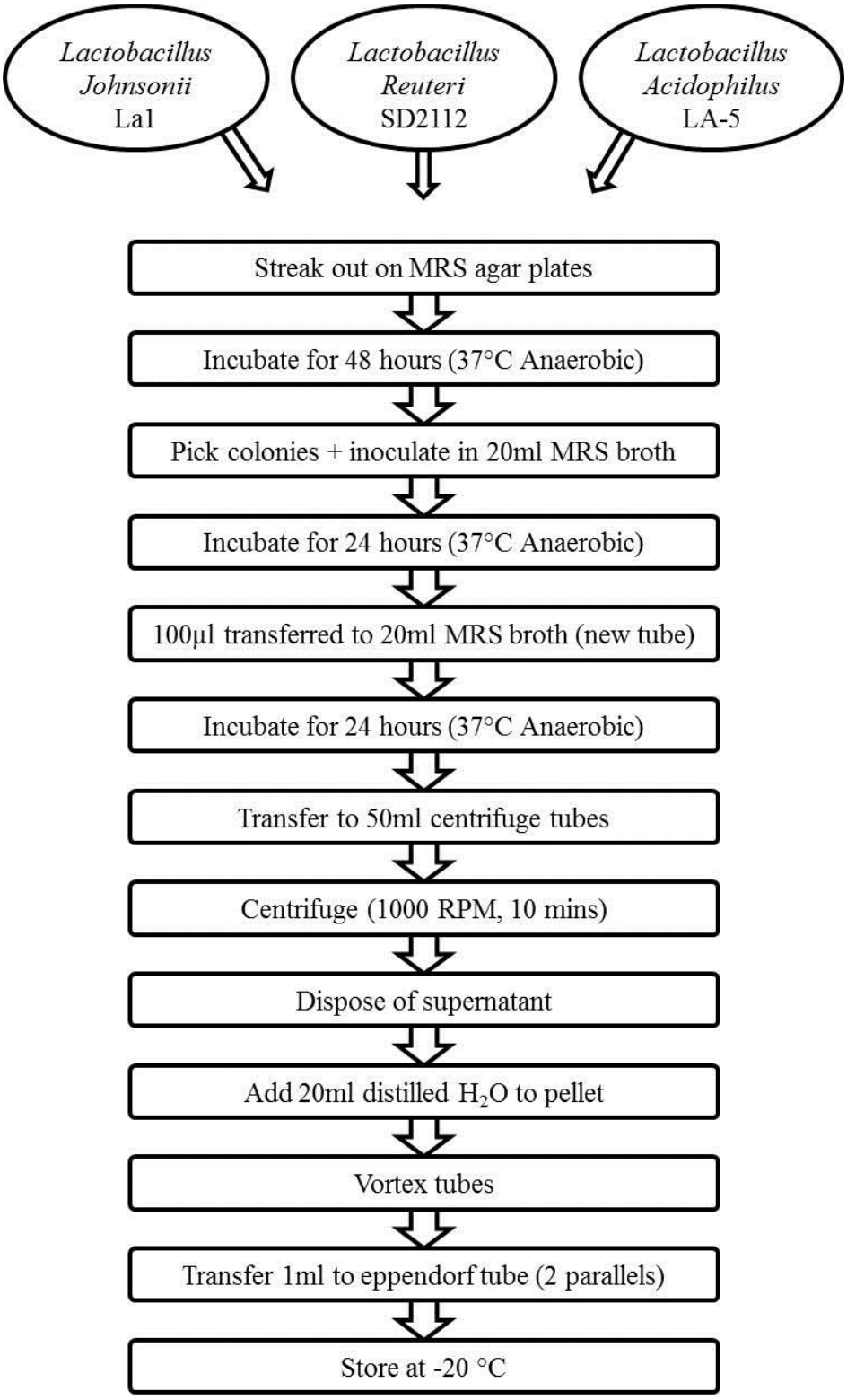

Figure 1: Flow diagram for isolation in pure strains.

Fermentation of barley flour samples

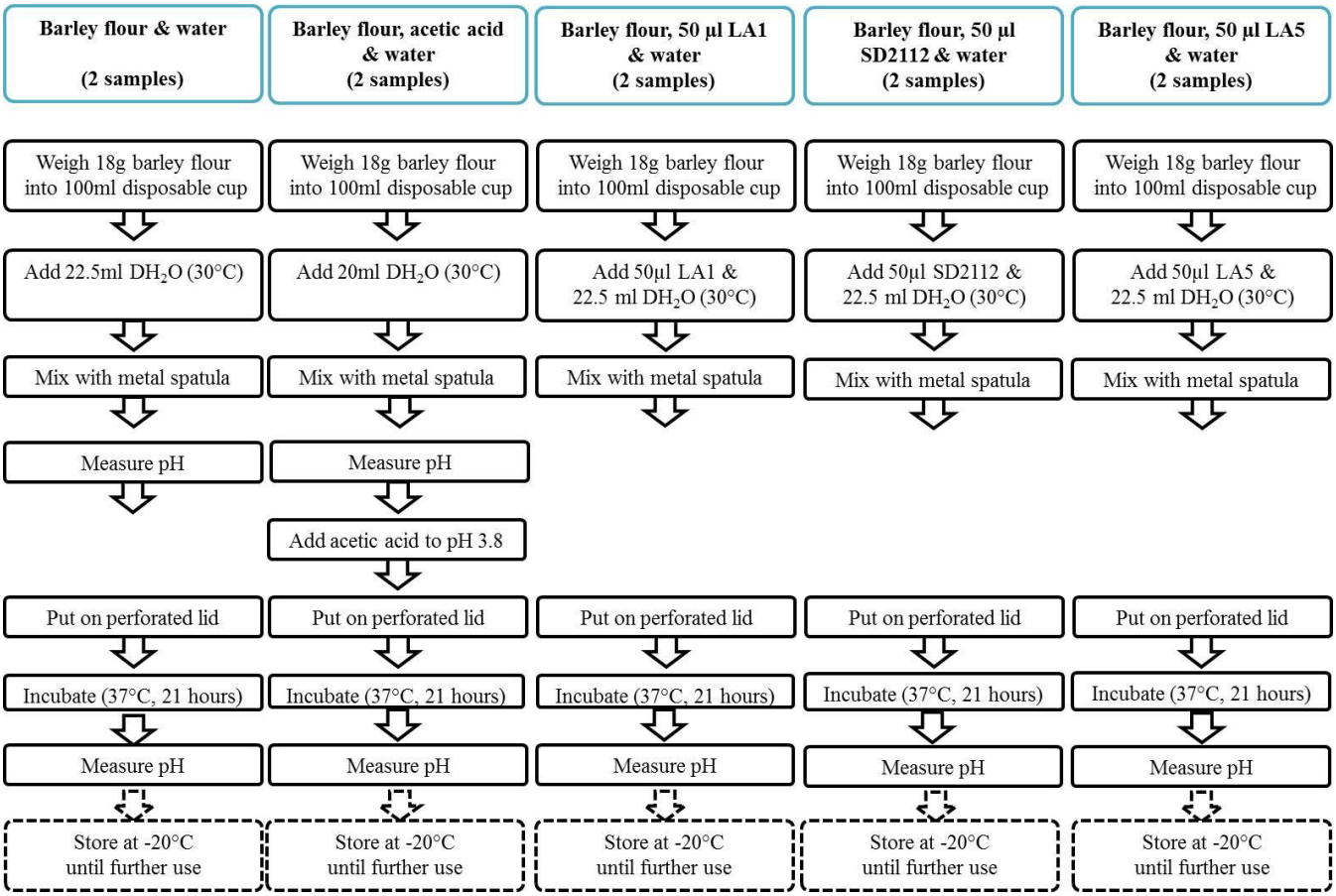

Figure 2a: Flow diagram for fermentation of barley flour samples. The first ten samples are shown in the chart.

Fermentation of barley flour samples containing pentopan

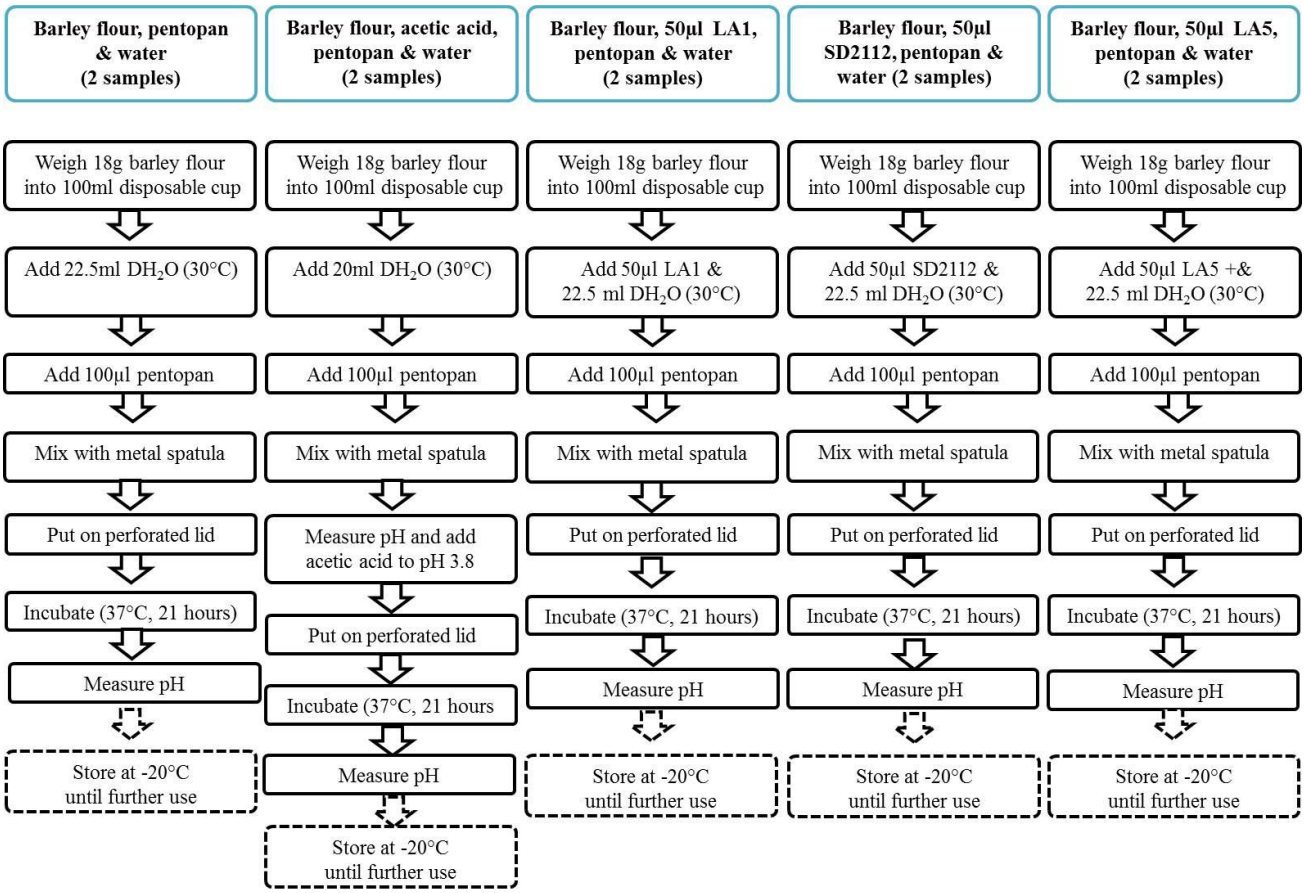

Figure 2b: Flow diagram for sample preparation of the fermented barley flour samples containing pentopan.

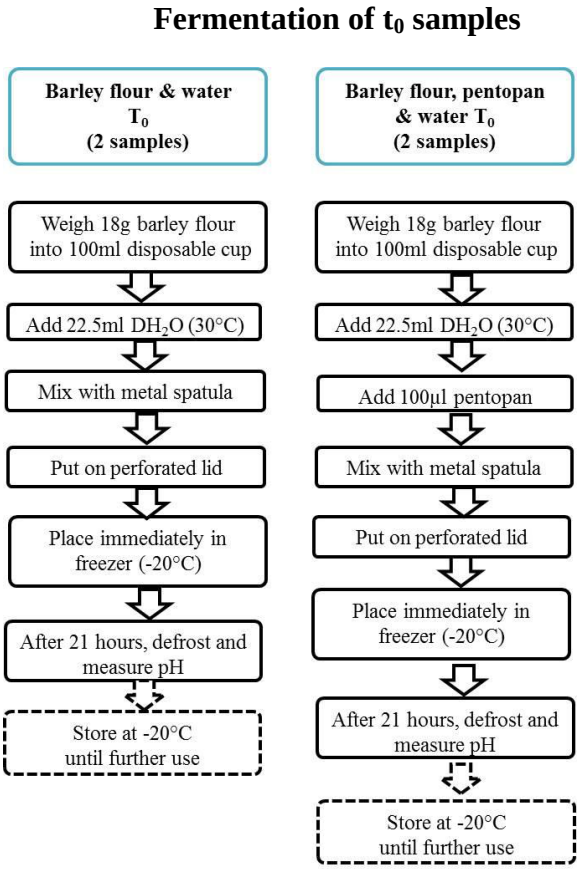

Figure 2c: The fermented samples which were placed directly in the freezer are shown in the flow chart above.

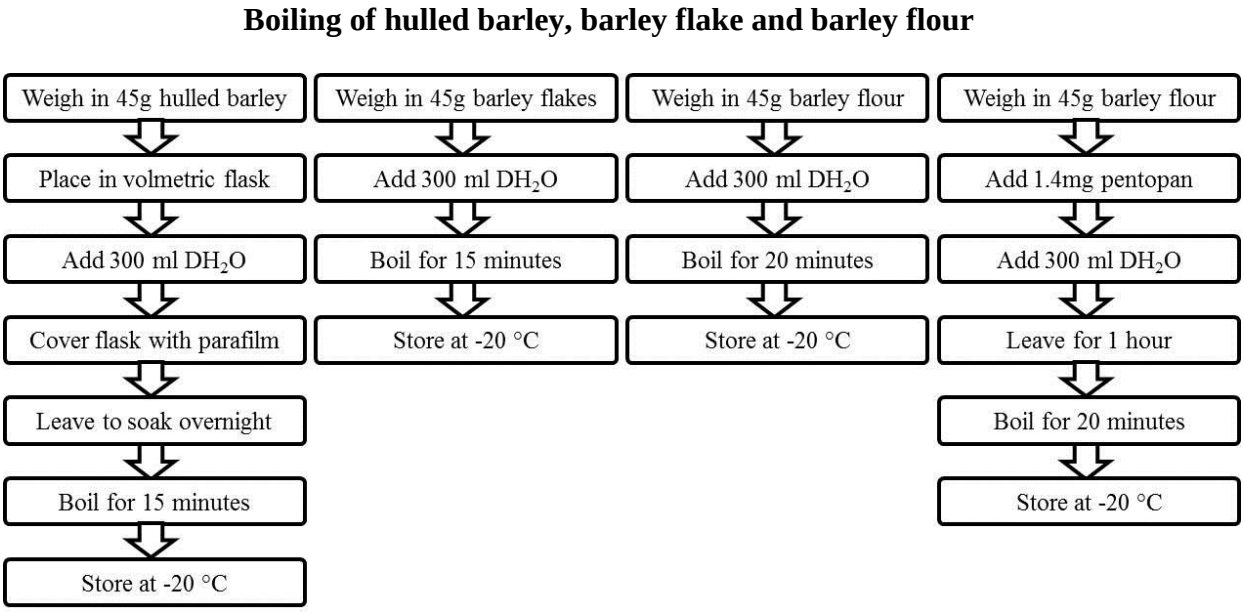

Figure 3: Flow diagram for boiling of hulled barley, barley flake and barley flour samples.

Sample preparation of free phenolic acids in barley samples

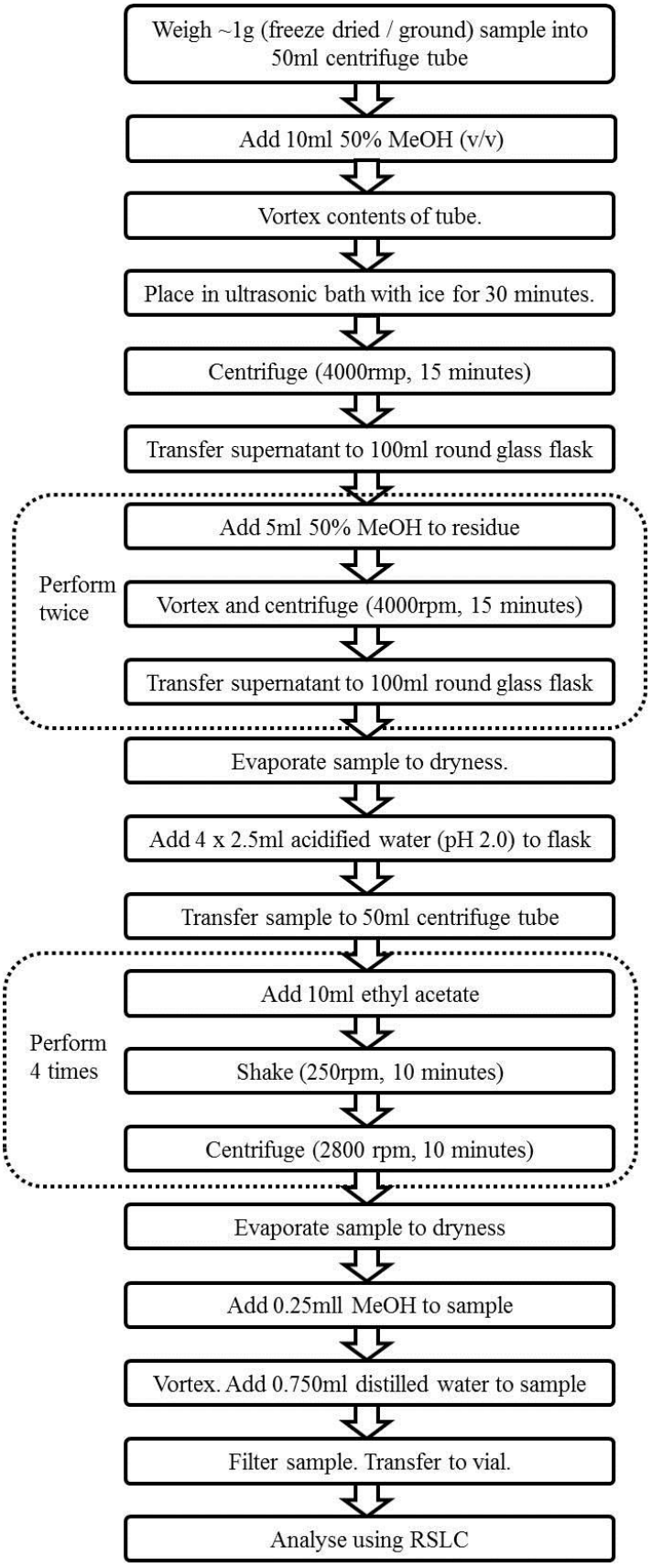

Figure 4: Flow chart for sample preparation of free phenolic acids in barley.

Sample preparation of bound phenolic acids in barley products

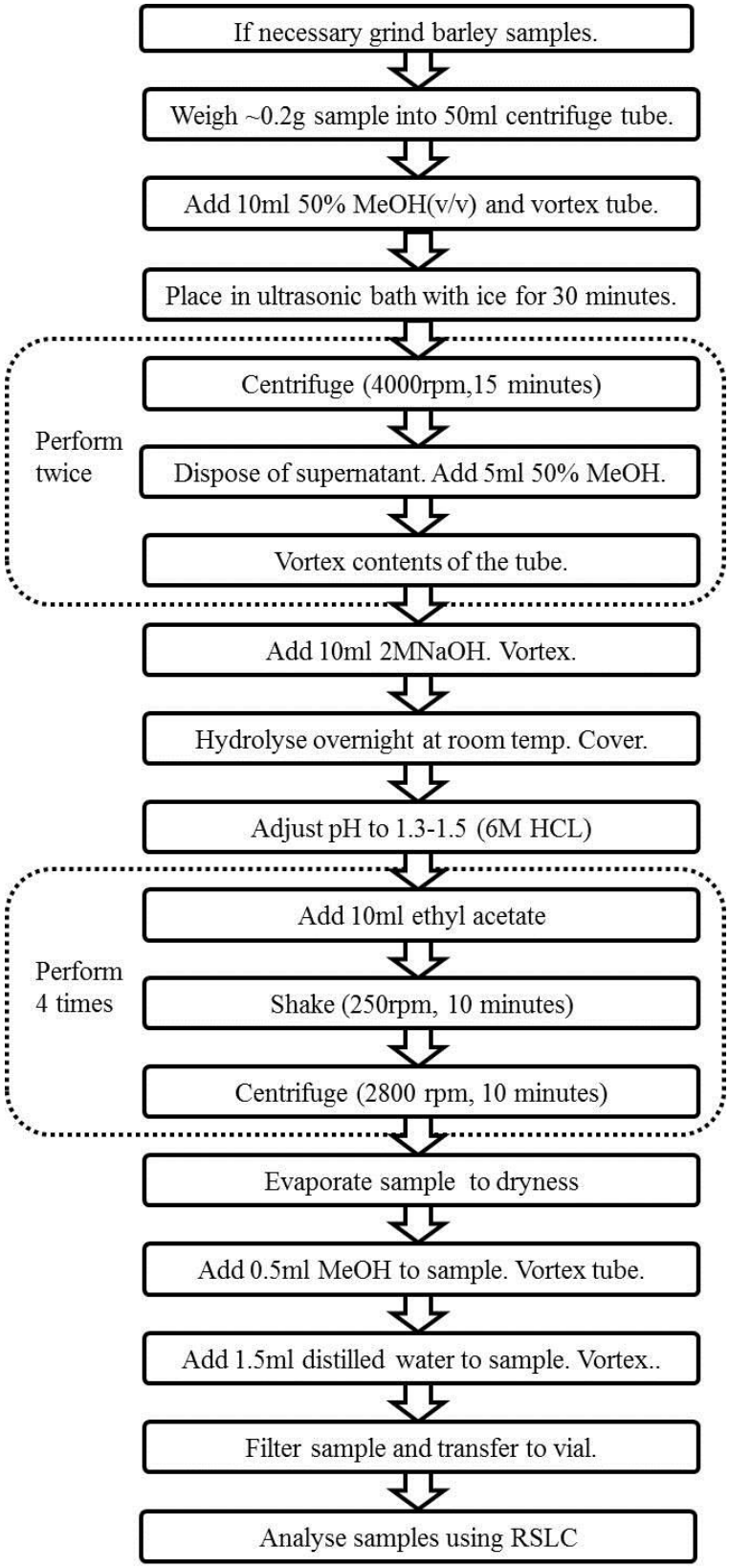

Figure 5: Flow chart for the sample preparation of bound phenolic acids in barley products

Determination of total starch in barley samples

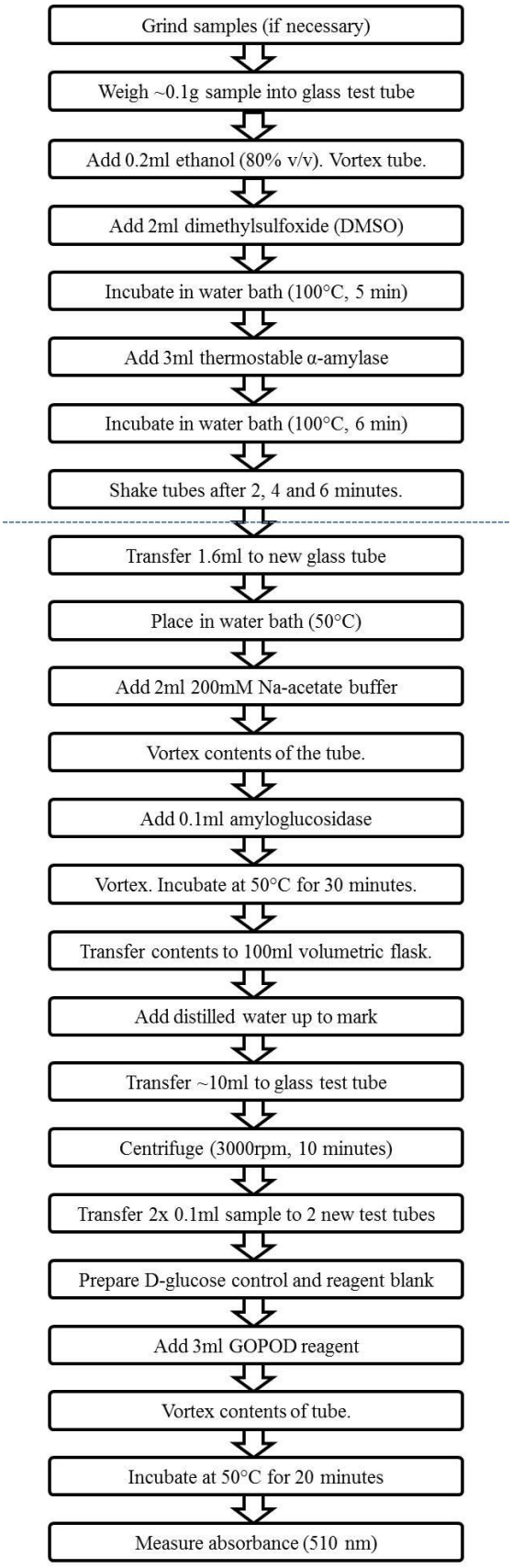

Figure 6: Flow chart for determination of total starch in barley products

in vitro digestion of starch in barley

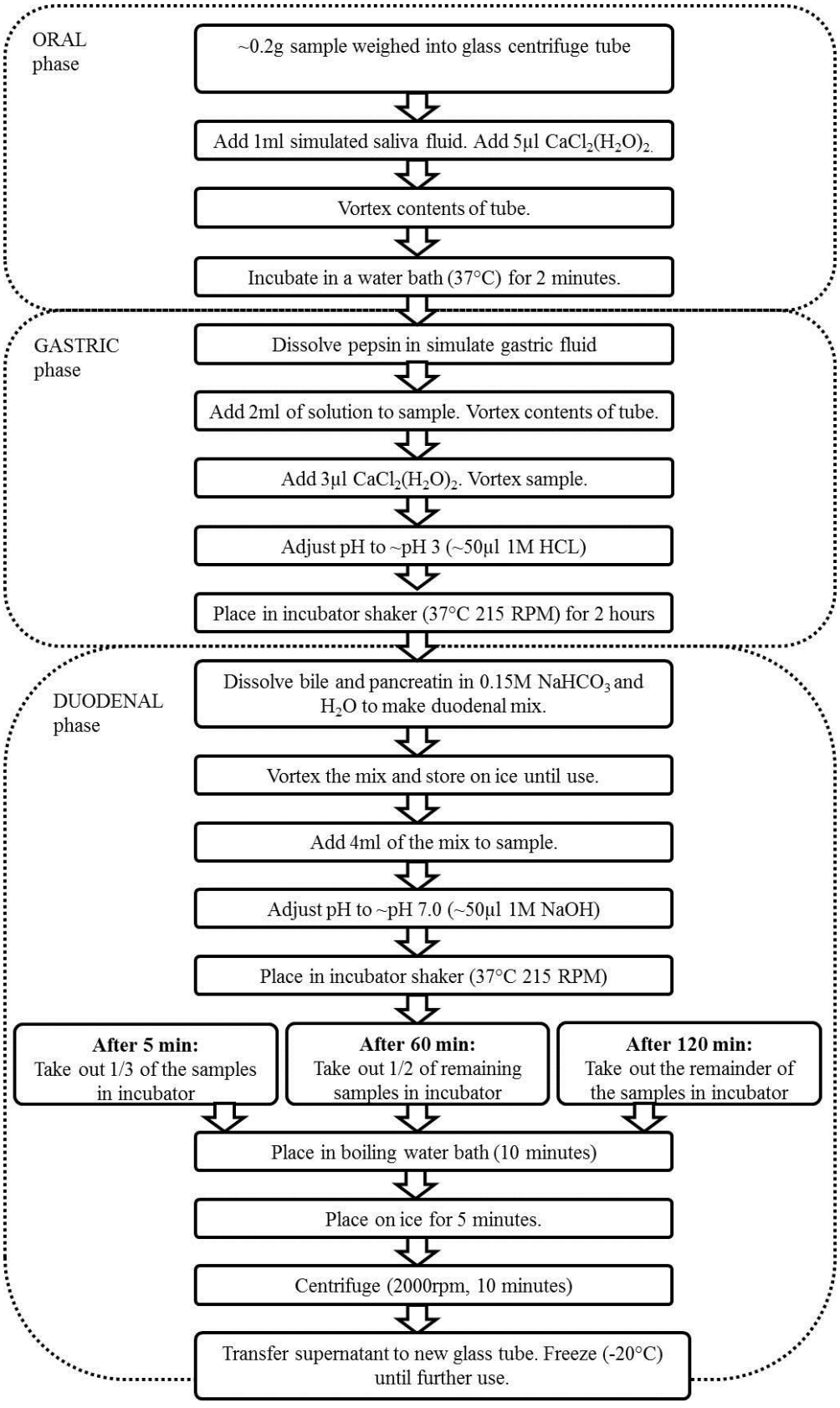

Figure 7: Flow chart for *in vitro* digestion of starch in barley samples

Determination of starch in in vitro digested barley samples

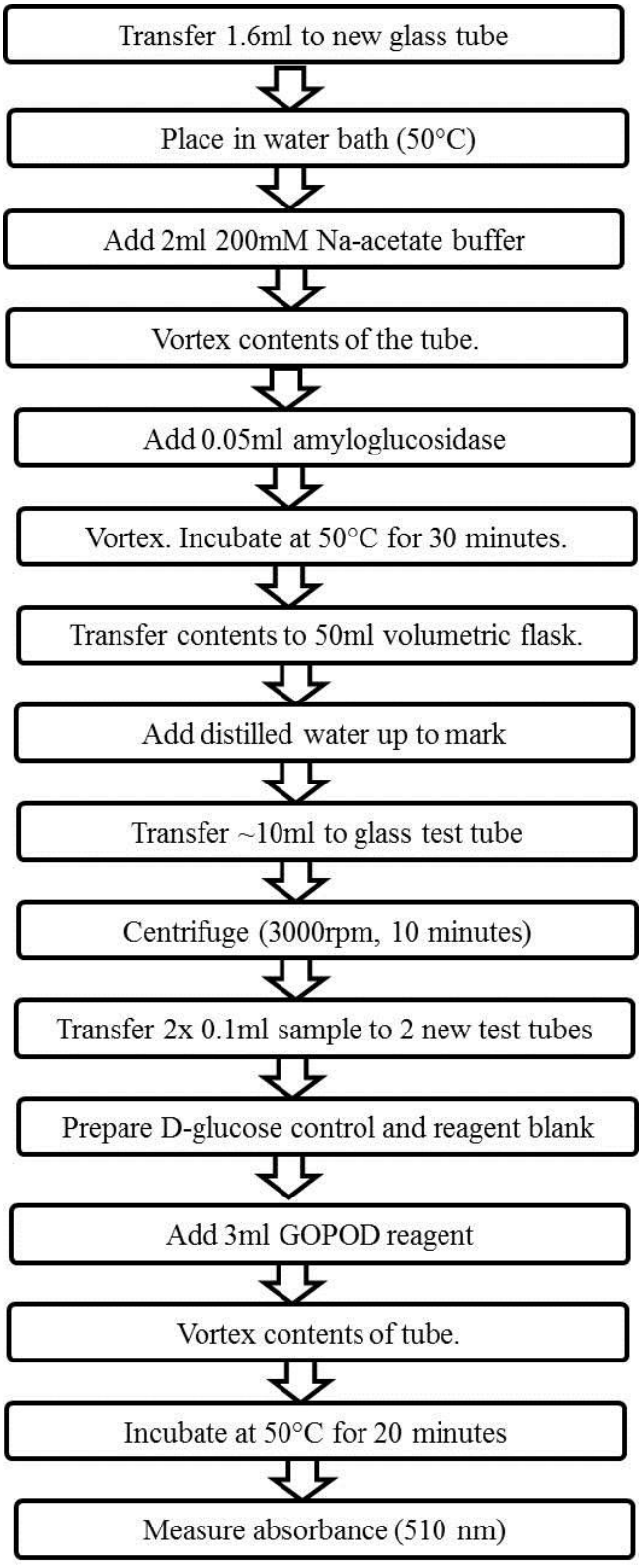

Figure 8: Flow chart for *in vitro* digestion of starch in barley samples

Appendix 3: Buffer preparation

6M HCL: 50ml 37% HCL was added to 50ml Milli-Q water in a 100ml Class A volumetric flask. The flask was shaken and marked with the contents, date and students initials.

2M NaOH: 16g sodium hydroxide pellets was weighed into a weighing dish using an analytical balance. The NaOH transferred to a laboratory glass bottle and dissolved in 200ml Milli-Q water.

1M HCL was made by adding 82.0 mL hydrochloric acid (37%) to approximately 600 mL distilled water in a 1 liter volumetric flask. The mixture was diluted up to the graduation mark with distilled water and subsequently stored at room temperature.

1M NaOH was made by dissolving 40.0 g sodium hydroxide pellets in 1L distilled water. The pellets were weighed into a weighing dish and subsequently transferred to a 1000ml volumetric flask. The flask was filled up to the mark with distilled water and stored at room temperature.

50mM MOPS buffer: 11.55g of MOPS sodium salt was weighed into a weighing dish and subsequently transferred to a 1000ml volumetric flask. 900ml distilled water was added to the flask and the pH in the solution adjusted to pH 7 by the addition of ~17ml 1M HCl. 0.74g calcium chloride dehydrate was added to the solution followed by 0.2g sodium azide. The solution was vigorously shaken and filled up to the graduation mark with distilled water. The buffer was stored at 4°C.

200mM Na-acetat buffer: 11.6ml glacial acetic acid was added to 900ml distilled water in a 1000ml volumetric flask. The pH in the solution was adjusted to pH 4.5 by the addition of ~60ml 1M NaOH. 0.2g sodium azide was added to the solution and the volume in the flask was adjusted to 1000ml. The flask was vigorously shaken and stored at 4°C until further use.

GOPOD Reagent Buffer (Bottle 3, Megazyme kit) was diluted up to the graduation mark with distilled water in a 1000ml Class A volumetric flask.

0.15M $NaHCO_3$ Buffer: 12.6 g $NaHCO_3$ was weighed into a weighing dish using an analytical balance and subsequently transferred to a 1000ml volumetric flask. The flask was filled up to the graduation mark with distilled water, marked with the date and placed in the refrigerator until further use.

Appendix 4: SSF and SGF

Preparation of the solutions required to make simulated saliva fluid (SSF) and simulated gastric fluid (SGF) used in the *in vitro* digestion of starch in barley flour

Potassium chloride (KCl) solution: 2.340g KCl was weighed into a weighing dish using an analytic balance (Sartørius Basic). The KCl was transferred to a 50 ml class A volumetric flask and the weighing dish was rinsed several times with distilled water. The distilled water was transferred to the flask which was subsequently filled up to the graduation mark with distilled water. The contents of the flask were thoroughly mixed until the KCl was completely dissolved. The final concentration in the solution was 0.0468 $^{g}/_{ml}$.

Monopotassium phosphate (KH_2PO_4) solution: 3.400g KH_2PO_4 was weighed into a weighing dish and subsequently transferred to a 50 ml class A volumetric flask. The weighing dish was rinsed with distilled water which was transferred to the flask. The flask was filled up to the mark with distilled water and shaken vigorously until the KH_2PO_4 was dissolved. The concentration in the solution was 0.068 $^{g}/_{ml}$.

Sodium bicarbonate ($NaHCO_3$) solution: 2.100g $NaHCO_3$ was weighed into a weighing dish and then transferred to a 25 ml class A volumetric flask. The flask was filled up to the graduation mark with distilled water and shaken until the $NaHCO_3$ was dissolved. Following addition of water the concentration of $NaHCO_3$ in the solution was 0.084 $^{g}/_{ml.}$

Sodium chloride (NaCl) solution: 6.000g NaCl was dissolved in 50ml distilled water in a 50 ml class A volumetric flask. In order to dissolve the NaCl, the flask was shaken and turned upside down several times. The final concentration of NaCl in the flask was 0.12 $^{g}/_{ml.}$

Magnesium chloride hexahydrate ($MgCl_2(H_20)_6$) solution: 0.305 g $MgCl_2(H_20)_6$ was weighed into weighing dish and subsequently transferred to a 10 ml class A volumetric flask. The weighing dish was rinsed with distilled water which was transferred to the flask. The flask was filled up to the mark with distilled water and shaken vigorously. The final concentration of $MgCl_2(H_20)_6$ in the flask was 0.0305 $^{g}/_{ml.}$

Calcium chloride ($CaCl_2(H_20)_2$) solution: 0.588 g $CaCl_2(H_20)_2$ was dissolved in 10 ml distilled water in a 10 ml class A volumetric flask. The final concentration in the solution was 0.0588 $^{g}/_{ml}$**.**

Appendix 5: Calculations

Example of a calculation of the amount of <u>free</u> caffeic acid in barley flour

Ethyl acetate was used to extract free phenolic acids in ~1g of the sample **barley flour A**. The sample was evaporated and the residue dissolved in 1ml MeOH. A calibration curve comprising of four calibration standards was used to calculate the amount of caffeic acid in the sample. The result was 0.0919 $^{\mu g}/_{ml.}$

The following formula was used to calculate the correct concentration of caffeic acid in the sample: $\mathbf{c_1.v_1 = c_2.v_2}$

where: $c_1 = 0.0919^{\mu g}/_{ml}$

$v_1 = 1ml$

$c_2 = X$

$v_2 = 1.006g$

$$\frac{0.0919\ ^{\mu g}/_{ml} \times 1ml}{1.006g} = 0.0914\ ^{\mu g}/_{g}$$

Moisture content was determined in the barley flour sample as described in section 3.2.1 of this thesis. The dry matter (DM) content was determined by subtracting the moisture content from the total content (100%).

Moisture content = 12.19%

Dry matter content = 100% - 12.19% = **<u>87.81%</u>**

The answer, 0.0914 $^{\mu g}/_{g,}$ was multiplied by 100 and divided by 87.81.

This gave a final answer of **<u>0.1040 $^{\mu g}/_{gDM}$</u>** caffeic acid in the barley flour sample.

Example of a calculation of <u>bound</u> caffeic acid in barley flour

Ethyl acetate was used to extract ~0.2g of the sample **barley flour A**. The sample was evaporated and subsequently dissolved in 2ml MeOH. A calibration curve, comprising of four calibration standards was used to calculate the amount of caffeic acid in the sample. The result was $0.2351^{\mu g}/_{ml.}$

The following formula was used to calculate the correct concentration of caffeic acid in the sample: $\mathbf{c_1.v_1 = c_2.v_2}$

where: $c_1 = 0.2351\ ^{\mu g}/_{ml}$

$v_1 = 2ml$

$c_2 = X$

$v_2 = 0.200g$

$$\frac{0.2351\ ^{\mu g}/_{ml} \times 2ml}{0.200g} = 2.351\ ^{\mu g}/_{g}$$

The dry matter content in the barley flour used in this thesis is 87.81%. The answer $2.351\ ^{\mu g}/_{g}$ is multiplied by 100 and divided by 87.81 giving a final answer of $2.678\ ^{\mu g}/_{gDM}$ caffeic acid in the barley flour sample.

Example of a calculation of the total amount of starch in a barley flour sample

Starch % w/w (as is) was calculated using the formula below:

Starch % w/w (as is) = $^{\Delta}$ A x F x (FV/0.l) x (1/1000) x (100/W) x (162/180)

Where:

$^{\Delta}$ A = Absorbance read against the reagent blank

F = 100 (µg of D-glucose)
Absorbance for 100 µg of glucose

FV = Final volume (i.e. 100ml)

0.1 = volume of sample analyzed

1/1000 = conversion from µg to mg

100/W = Factor to express "starch" as a percentage of flour weight

W = weight in milligrams ("as is" basis) of the flour analyzed

162/180 = conversion from free D-glucose to anhydro D-glucose (as occurs in starch)

If we use the following details for a barley flour sample:

Sample Absorbance at 510nm = 0.683

Reagent blank Absorbance at 510nm = 0.022

$^{\Delta}$ A = 0.661

D-glucose standard Absorbance = 1.0805

F = (100µg/1.0805) = 92.6µg

FV/0.1 = 100ml/0.1ml = 1000ml

1/1000 = 0.001mg

Sample weight = 100.1mg

100/W = 100/100.1 = 0.999mg

162/182 = 0.9

If we put the highlighted figures into the formula above we get the following answer:

Starch % w/w (as is) = (0.661) x (92.6µg) x (1000ml) x (0.001mg) x (0.999mg) x (0.9) = 55.03

Starch % w/w (dry wt.basis): = Starch % w/w (as is) x (100 / (100 – moisture content (%)))

The moisture content in the barley flour used in this thesis is **12.19%.**

Starch % w/w (dry wt.basis) = 55.03 x (100 / (100 -12.19)

= 55.03 x (100/87.81)

= 55.03 x 1.138

= 62.63%

Example of a calculation of the total amount of starch *in vitro* digested in a barley flour sample.

The formula used is similar to the formula above. However the final volume is 50ml as opposed to 100ml. An example of a calculation of the amount of starch *in vitro* digested in barley flour after 60 minutes is given below.

Starch % w/w (as is) = Δ A x F x (FV/0.l) x (1/1000) x (100/W) x (162/180)

Where:

Δ A = Absorbance read against the reagent blank

F = 100 (µg of D-glucose)/ (Absorbance for 100 µg of glucose)

FV = Final volume (i.e. 50ml)

0.1 = volume of sample analyzed

1/1000 = conversion from µg to mg

100/W = Factor to express "starch" as a percentage of flour weight

W = weight in milligrams ("as is" basis) of the flour analyzed

162/180 = conversion from free D-glucose to anhydro D-glucose (as occurs in starch)

If we use the following details for a barley flour sample:

Sample Absorbance at 510nm = 0.627

Reagent blank Absorbance at 510nm = 0.047

Δ **A = 0.580**

D-glucose standard Absorbance = 1.074

F = (100µg/1.074) = **93.1µg**

FV/0.1 = 50ml/0.1ml = **500ml**

1/1000 = **0.001mg**

Sample weight = 203mg

100/W = 100/203 = **~0.49mg**

162/182 = **0.9**

If we put the highlighted figures into the formula above we get the following answer:

Starch % w/w as is = 0.580 x 93.1 x 500 x 0.001 x 0.49 x 0.9 = 11.91

Starch % w/w (dry wt.basis): = Starch % w/w (as is) x (100 / (100 – moisture content (%)))

The moisture content in the barley flour used in this thesis is 12.19%.

Starch % w/w (dry wt.basis): = 11.91 x (100/ (100 – 12.19))

= 11.91 x (100 / 87.81)

= 11.91 x (1.1388)

= 13.56

13.56 is expressed as a fraction of total starch previously determined in barley flour i.e.

(13.56/62.63) x 100 = **21.65%**

Answer: The amount of starch which has been *in vitro* digested in the above flour sample after 60 minutes is **21.65%.**

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