Evaluation of seed hardness and malting characteristics of barley grain for use in ruminant diets

by

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ABSTRACT

In western Canada, over 70% of the barley grain grown for malt is sold as livestock feed. The main objective of this thesis was to investigate the relationship between ruminal dry matter digestibility (DMD) and seed hardness (SH) and malting characteristics (MC) of barley grain by measuring *in situ* DMD, and changes in the nutrient composition as predicted by Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (ATR-FTIR) before and after *in situ* rumen incubation. Results demonstrated that diastatic power and SH were associated with an improved *in situ* rumen digestion at early incubation time, but level of SH and MC had no impact on rumen digestion in relation to nutrient molecular structures using ATR-FTIR spectra. However, ATR-FTIR identified the differences of molecular structures in barley grain as affected by rumen digestion, indicating a potential of ATR-FTIR for use to predict nutrient digestion of barley grain in the rumen.

PREFACE

This thesis is an original work by Shuai Ding. The first study of the project using rumen cannulated animals received research ethics approval from the Animal Care Committee, Agriculture and Agri-Food Canada (AAFC), under protocol number of ACC1309.

The research conducted for this thesis was a collaborative project led by Drs. W.Z. Yang and T.A. McAllister at the Lethbridge research center (LRC) of AAFC, Dr. M.L. Swift at the Alberta Agriculture and Rural Development, and Dr. M. Oba at the University of Alberta. Barley grain used in the project was part of a large scale project by Dr. J.T. O'Donovan at the Lacombe research center of AAFC. The results of analysis of malting quality attributes were obtained from Dr. M. Edney at Canadian Grain Commission. The experimental design and data analysis in Chapter 2 and 3 were conducted by Shuai Ding, with the assistance and contribution of Drs. W.Z. Yang, M.L. Swift, M. Oba and T.A. McAllister.

Chapter 2 of this thesis has been submitted to Canadian Journal of Animal Science as a Short Communication manuscript. Shuai Ding was responsible for the manuscript composition, and Drs. W.Z. Yang, M.L. Swift, M. Oba and T.A. McAllister contributed to manuscript edits.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ASBC	American Society of Brewing Chemists
ATR	Attenuated total reflectance
ATR-FTIR	Fourier transform infrared with attenuated total reflectance
CBMBTG	Canadian Barley Malting and Brewing Technical Guide
CP	Crude protein
DDGS	Dried distillers grains with solubles
DM	Dry matter
DMD	Dry matter disappearance
DMI	Dry matter intake
DP	Diastatic power
DRIFT	Fourier transform infrared with diffuse reflectance
FIR	Far infrared
FRI	Friability
FTIR	Fourier transform infrared
FT-MIR	Fourier transform mid infrared
FT-NIR	Fourier transform near infrared
IR	Infrared
MC	Malting characteristics
MIR	Mid infrared
NDF	Neutral detergent fiber
NIR	Near infrared
OM	Organic matter
PC	Principal component
PCA	Principal component analysis
SH	Seed hardness
SR-FTIR	Fourier transform infrared with advanced synchrotron
VFA	Volatile fatty acids
WBG	Wort beta-glucan

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

1.1.1 Production and history of barley grain

Barley (*Hordeum vulgare*) is the fourth largest cereal crop in the world, behind maize, wheat and rice (FAO, 2012). Its wide distribution is a consequence of strong adaptability and wide genetic variation within the crop, with specific cultivars adapted to specific growing environments (Nilan and Ullrich, 1993). In 2012, global production of barley was 134 MMT with the top barley producing countries being Germany, France, Ukraine and Russia (FAO, 2012). Canada was the sixth largest producer of barley in the world, producing 8.1 MMT in 2011-2012 (FAO, 2012). More than 90% of Canadian barley is grown in the prairie provinces of Alberta, Saskatchewan and Manitoba (Camm, 2008).

1.1.2 Uses: food, malt, and feed

Barley was historically used as an important food source for humans worldwide (Baik and Ullrich, 2008). However, due in part to the rise in prominence of wheat and rice, barley grain has gradually evolved to be primarily used for feed, and brewing (Baik and Ullrich, 2008). In North America, barley is mainly grown for the value-added malting market and is used as a substrate for the production of beer (Canadian Barley Malting and Brewing Technical Guide [CBMBTG], 2012). However, to ensure brewing efficiency, barley for malting is subject to a number of assessments based on physical, chemical and malting characteristics (MC) such as, crude protein (CP) content, malt extract, diastatic power (DP), friability (FRI) and wort beta-glucan (WBG; CBMBTG, 2012; Fox et al., 2009). Barley that fails to meet malt grade is mainly used as animal feed. Currently about 70% of the barley produced is used for feed, 20% for malting, 5% for food, and 5% for undefined uses (Wang, 2005).

1.2 Quality characteristics of barley grain

1.2.1 Physical characteristics of barley grain

Kernel size

Kernel size of barley is usually characterized by measuring plumpness, which is the fraction retained on a 6/64" slotted sieve (CBMBTG, 2012; Fox, 2008). Plumpness reflects well-filled kernels that are seen as higher quality than thin kernels (Meredith et al., 1962). Growing environment, agronomic practices and barley genetics influence kernel size by affecting starch synthesis in the kernel during the grain filling phase of barley development (Coventry et al., 2003). Plump barley was found to produce adequately modified malt with high levels of malt extract under standard processing conditions (Agu et al., 2007; Edney et al., 2012). Besides plumpness, uniformity of kernel size is critical for both feed and malt barley. Excessively large grains decrease malting quality by reducing the efficiency of the chemical conversion in endosperm of barley during the malting process, which decreases the conversion of starch to fermentable sugars during the brewing process (Fox, 2008). Kernel uniformity supports consistent germination and leads to higher quality malt (Edney et al., 2005; O'Donovan et al., 2011). Kernel size also plays a role in the quality of barley as feed. In particular, the uniformity of kernel size influences processing quality for dry rolling barley grain, as small kernels may be under processed whereas large kernels may be over processed (Yang et al., 2013). Under processing of barley grain may reduce starch availability for fermentation by rumen microbial populations, whereas over processing may reduce feed intake and increase the risk of digestive disorders in ruminants (Ahmad et al., 2010).

Thousand kernel weight

Thousand kernel weight is another indicator of grain quality in breeding, and is simply measured as the weight of 1000 kernels by a seed counting device (Hori et al., 2003). A high

thousand kernel weight is associated with a high degree of kernel plumpness (Edney et al., 2005). Thus, factors affecting kernel size will impact thousand kernel weight. For malting purposes, thousand kernel weight only gives an indication of potential malt extract in plump barley, but not in thin barley (Edney et al., 2005). In ruminants, thousand kernel weight reflects both highly soluble and highly degradable dry matter (DM; Khorasani et al., 1995). In ruminants, barley grain with higher thousand kernel weight may be more digestible than barley with a lower thousand kernel weight, but the high thousand kernel weight may also be associated with a rapid rate of fermentation and a higher risk of digestive upsets.

Seed hardness

Seed hardness (SH) defined as the resistance of the kernel to fracture, is a critical factor affecting grain processing and product quality for feeding or malting purposes (Camm, 2008). Malting varieties are usually softer, while feed varieties are usually harder (Allison et al., 1979). Barley SH is increasingly important in quality evaluation, whilst the wheat industry has applied this trait for decades to differentiate grain quality and market classes (Camm, 2008). Seed hardness can be measured as a hardness index using a Single Kernel Characterisation System (SKCS; Martin et al., 1993), or by milling energy using a 'Comparamill' (Allison et al., 1979). Seed hardness is associated with starch, protein and beta-glucan content, and malting quality parameters (Fox, 2008). Seed hardness had positive relationship with barley protein and grain beta-glucan, but negative relationship with malt extract and the chemical processes involved in germination that result in endosperm modification (Allison et al., 1979; Henry and Cowe, 1990).

1.2.2 Chemical composition of barley grain

Chemical composition of barley grain consists of carbohydrates, fats, protein, vitamins, minerals and moisture content. The content of these constituents in barley grain vary

considerably depending primarily on genetics, agronomic practices, and growing environment (Nilan and Ullrich, 1993).

Starch

Starch content of barley ranges from 45.9-62.8% (Ovenell-Roy et al., 1998), and barley starch normally consists of amylose and amylopectin in an approximate ratio of 3:1 (Palmer, 1983). Amylose is composed of alpha-(1, 4)-glycosidic linkages, whereas amylopectin has a higher molecular weight with alpha -(1, 4)-glycosidic and alpha -(1, 6)-glycosidic linkages (Pomeranz, 1985). Differences in amylose:amylopectin ratio affects the degradation properties of barley starch during malting and ruminal fermentation. In amylose, the glucose chains that make up amylose are linear as compared with branching chains within amylopectin. This enable the chains in amylose to tightly bind together through hydrogen bonding, increasing the resistance of starch to enzymatic hydrolysis during malting and in the digestive tract, particularly in monogastrics (MacGregor and Bhatty, 1993). In comparison, amylopectin is a highly branched chain polymer of glucose molecules, which has a large surface area that is favorable for amylase degradation (Foley et al., 2006; Fox, 2010; Yang, 2013). Barley grain high (up to 100%) in amylopectin is known as "waxy" barley (Pomeranz, 1985). Fox (2010) suggested that high amylopectin reduces the malting quality of barley, as it is negatively related to malt extract and cell wall modification, and positively associated with grain beta-glucan content. Cell wall modification of barley grain refers to the breakdown of beta-glucan and arabinoxylan content in endosperm cell wall by hydrolytic enzymes during the malting process of barley grain. Thus, the impact of starch type on malting quality of barley grain depends on both starch structures and other factors, such as malt extract, cell wall modification and grain beta-glucan content. Feeding waxy barley to ruminants has been observed to reduce ruminal starch digestibility as compared

to feeding “normal” barley grain due to a positive relationship of amylopectin content with protein, grain beta-glucan, neutral detergent fiber (NDF) content, and extent of crystallinity in waxy barley grain (Foley et al., 2006). A high degree of crystallinity associated with high amylopectin content in waxy barley has been suggested to have low susceptibility to enzyme hydrolysis (Foley et al., 2006). Thus, the effect of amylose: amylopectin ratio on starch digestion of barley grain is not only determined by starch structure itself, but also by the differences in protein and fiber content of barley grain (Foley et al., 2006; Yang, 2013).

Protein

Barley protein content ranges from 10-17%, and has complex interactions with other quality attributes (Baik and Ullrich, 2008; Fox, 2010). High protein content is undesirable for malting purposes due to negative impacts on malt extract and starch content, and an increase in the cloudiness of beer (Fox, 2008). Also, increasing protein content generally decreases yield of barley grain, thus, it is desirable to select for low protein content if the objective is to increase grain yield (Emebiri et al., 2004; Fox, 2010). Extremely low protein content may also compromise brewing performance and the feed value of barley grain (Fox, 2010). In terms of amino acid composition, barley protein has a moderate nutritional quality due to its low lysine content (Ullrich et al., 1986).

Neutral detergent fiber

Barley NDF content ranges from 11 to 34%, and is attributed to the hull, pericarp and aleurone layer in the physical structure, among which the hull is the major contributor (Baik and Ullrich, 2008; Bell et al., 1983). The NDF content of barley is normally higher than corn and is responsible for its comparable lower digestible energy content as compared to corn (Baik and Ullrich, 2008; Boyles, 2001). Increasing the NDF content of barley grain above 18% DM has

been shown to reduce digestibility in swine (Bell et al., 1983; Jha, et al., 2010). However, in ruminants, the fibrous hull is not a factor that limits the ruminal fermentation of starch if it is adequately fractured.

Beta-glucan

Beta- glucan accounts for about 75% of the barley endosperm cell wall, and 2-9% DM of the whole grain (Baik and Ullrich, 2008; Newman and Newman, 1992). The relationship between beta-glucan content and quality of food, feed and malt have been widely discussed. Beta-glucan benefits human health by lowering blood cholesterol and glycemic index (Baik and Ullrich, 2008). However, high beta-glucan levels in barley grain increase the viscosity of malt and act as a barrier to the hydrolytic enzymes that degrade starch and protein within endosperm cells (Fox, 2008). In terms of feed value, increasing beta-glucan reduces digestion in monogastric animals (Campbell and Bedford, 1992). In poultry, beta-glucan reduces productivity and increases viscosity of the digesta which decreases the digestion and absorption of nutrients (McNab and Smithard, 1992). The effects of barley beta-glucan content on rumen digestion and ruminant productivity has been inconsistent (Engstrom et al., 1992; Foley et al., 2006) with these inconsistencies possibly being e associated with the method of processing. Beta-glucan disappearance of barley grain at 8 h *in situ* rumen incubation was higher when barley grain was dry-rolled as compared to when it was steam-rolled due to more small particles being produced from dry-rolling (Engstrom et al., 1992). However, it has been suggested that rumen microorganisms produce sufficient beta-glucanase to efficiently degrade beta-glucan in barley grain (Hristov et al., 2000).

1.2.3 Malting characteristics of barley grain

The malting process of barley grain consists of three stages: steeping, germination and kilning. Steeping is the process of immersing grain in water to increase the moisture content levels to 40-46% under low temperature (15-18 ˚C). This process enables hydrolytic enzymes to move throughout the endosperm, and activate the desired biochemical reactions that facilitate the germination process. Endosperm modification enables various enzymes to breakdown protein and cell wall components to release the starch granules for brewing, and yield soluble substances including sugars, amino acids, and peptides. It is important to note that in the brewing process, the released starch granules are extracted into liquid fraction and converted to smaller molecules or simple sugars, known as "wort" after a processing step called "mashing" (MacGregor and Bhatty, 1993). The kilning process is initiated when sufficient malt has been formed. In kilning process, dry air is introduced at increasing temperatures up to approximately 80˚C through the malt to halt enzyme activity and reduce its moisture content to a stable stage. Several important malting characteristics (MC) including DP, FRI, WBG can indicate the efficiency and quality of malting process. Diastatic power and FRI are measured in the malt of barley grain after the kilning process, whereas WBG is measured in the wort. These MC are affected by genetics, growing environment and agronomic practices (Edney et al., 2012; Molina-Cano et al., 1995).

Diastatic power

Diastatic power is a critical quality factor which represents the activity of total starch-degrading enzymes in the malt and quantifies the ability of malt to convert starch to fermentable sugars (°L, CBMBTG, 2012; Fox, 2008) in the brewing process. The key enzymes involved in this conversion include alpha-amylase, beta-amylase, and limit dextrinase, among which beta-amylase hydrolyses nearly 70% of the amylose and 50% of the amylopectin in starch (Fox, 2008). Diastatic power is determined with a segmented flow analyzer, using an automated

neocuproin assay for reducing sugars calibrated with malt standards analyzed using the official ferricyanide reducing sugar method [American Society of Brewing Chemists (ASBC), 2009]. Diastatic power has been found to be positively correlated with protein content and negatively correlated with malt extract and barley grain yield (Arends, 1995; Fox, 2008). Greater DP is desirable for increased malting quality of barley grain. However, selection of barley grain with high DP requires caution as too high of protein can adversely impact malting quality and grain yield.

Friability

Friability is the measure of readiness of malt to crumble when subjected to crushing (Fox et al., 2008). It is an estimation of the physical modification of the malt grains, and a reliable indicator of the ability of barley to produce soft and friable malt (Edney and Mather, 2004; Fox et al., 2008). Thus, higher FRI values are more desirable for malting as measured using a friabilimeter, which presses malt samples against a rotating sieve (2.2 mm) using a rubber roller with the percentage of malt by weight which passes through the sieve being a measurement of FRI (CBMBTG, 2012; Edney and Mather, 2004). Thus, high FRI values indicate that the malt is more easily fractured during milling process with this value being positively associated with malt extract and germination, and negatively associated with protein content, DP and increased kernel size (Edney and Mather, 2004; Edney et al., 1999).

Wort beta-glucan

Beta-glucan content of the wort indicates the extent of beta-glucan breakdown in barley cell wall by beta-glucanase, thus both beta-glucan content in the cell wall and the level of active beta-glucanase determines WBG (Edney et al., 2007; Fox, 2008). High content of WBG indicates insufficient breakdown of grain beta-glucan during the malting process, which is undesirable for brewing efficiency and quality as an increase in wort viscosity and slows beer filtration rates

prior to packaging (MacGregor and Fincher, 1993). Edney et al. (2007) also suggested that the high content of WBG reduced fermentability of malt, by restricting enzyme activity during mashing, and delaying the release of starch from under-modified endosperm. The WBG is determined by segmented flow analyzer using the calcofluor fluorescence method (ASBC, 2009), in which diluted wort is combined with the calcofluor solution in order to bind with the calcofluor. The resulting fluorescence is measured and reported as ppm of beta-glucan content in wort. A WBG content of less than 100 ppm has been recommended to improve malting quality and efficiency (Edney et al., 2007).

1.3 Digestion and utilization of barley grain in the rumen

1.3.1 Microbial digestion

Rumen microorganisms consist of 10^9-10^{10} bacteria, 10^5-10^6 protozoa and 10^3-10^4 fungi per milliliter of rumen fluid, with bacteria being responsible for the majority of feed digestion in the rumen (Cheng et al., 1991; McAllister and Cheng, 1996). Ruminal bacteria are principally responsible for starch digestion with less participation of protozoa and fungi (McAllister et al., 1993; 1994). The bacterial digestion of chemical components including starch, NDF and protein is initiated by microbial attachment (McAllister et al., 1994). Starch degradation requires that bacteria form a complex microbial digestive consortia on the surface of cereal grains to hydrolyze the alpha-(1, 4) and/or alpha-(1, 6) bonds of amylose and amylopectin (McAllister et al., 1993; 1994). The sequential process establishes a primary microcolony of amylolytic bacteria which adheres to the surface of starch granules (McAllister et al., 1993; 1994), and further attracts secondary colonizers, resulting in an establishment of a climax microbial population capable of digesting the majority of the barley kernel (McAllister and Cheng, 1996).

Microbial digestion of fiber involves bacteria which produce cellulolytic enzymes on their surfaces that possess cellulose binding domains (McAllister and Cheng, 1996). The dominant roles of these cellulolytic bacteria in attachment to fiber have been reviewed (McAllister et al., 1994). In addition, starch granules are embedded in a protein matrix within the endosperm of barley. In barley, starch granules are loosely associated with the protein matrix, whereas in corn they are tightly embedded within the protein matrix of the horny endosperm (McAllister et al, 1993; 1994). Barley endosperm is more rapidly fermented by ruminal microorganisms than that of corn, but breaking down its fibrous husk is a prerequisite for efficient utilization of barley starch due to the low ability of microorganisms to attach and degrade this structure (McAllister et al., 1993; 1994). Therefore, barley starch digestion is not limited by a protein matrix but by the fibrous hull and pericarp, which requires processing of barley grain to increase microbial accessibility and digestion of barley grain (McAllister et al., 1993).

1.3.2 Factors affecting ruminal digestion of barley grain

Effect of chemical composition

Barley grain ranks third in rate of ruminal digestion only behind that of oats and wheat if whole grain is fed to ruminants, but after processing, barley grain has been shown to have faster digestion rate than oats in the rumen (Campling, 1991; Nikkhah, 2012). Generally, increasing starch content increases DMD and the production of volatile fatty acids (VFA) from barley grain in the rumen (Yang et al., 2001). The composition of starch also affects barley digestion in the rumen. Barley grain with a high content of amylose may be more resistant to hydrolysis by rumen microbial enzymes due to its tight bond structure and reduced surface area for amylase digestion as compared to barley with a high amylopectin content (Yang, 2013). Waxy barley has

also been reported to reduce ruminal starch digestibility due to the higher crystallinity of starch granules as compared to normal type of barley, as crystalline regions generally have low susceptibility to enzyme hydrolysis (Foley et al., 2006). The decreased rumen digestion rate and digestibility of DM in waxy barley grain could potentially be associated with increased fiber and decreased starch content as amylopectin content was greater in waxy barley grain, compared to those in normal barley grain (Foley et al., 2006). In addition, increased fiber content of barley grain, such as increase in hull content may also cause a lower DMD, and consequently, lower its nutritive value (Hoover, 1986). Among the components of fiber, lignin is the most recalcitrant fiber component limiting digestibility of barley grain in the rumen (Du, 2008). Lignin negatively affects hemicellulose digestion in the rumen by acting as a physical barrier to restrict the access of rumen microbes and enzymes to digestible polysaccharides (Moore and Jung, 2001). Protein in barley is highly degradable in the rumen. However, high protein content may be indirectly associated with reduced starch content, reducing the digestible energy and feed value of barley grain (Damiran and Yu, 2010).

Effect of barley grain processing

The digestibility of whole barley in cattle is much lower, because the hull and intact pericarp of whole barley grain are very resistant to bacterial attachment and digestion in the rumen (McAllister et al., 1994). Hence, barley grain needs to be processed prior to feeding to make its endosperm, encased within indigestible pericarp and hull layers, accessible to the microbial population in the rumen. Methods used for processing and extent of processing are the primary factors affecting the feeding value of barley grain.

Processing methods

Dry processing: Dry processing by grinding using a hammer mill or rolling with a roller mill cracks or crushes the fibrous hull and pericarp to provide rumen microorganisms and enzymes with access to the internal endosperm (Dehghan-banadaky et al., 2007; McAllister et al., 1994). Fine grinding can produce particles that increase the risk of rumen acidosis and reduce DMI. Dry-rolling is commonly used in feedlots to process barley grain because it is cheaper as compared to temper or steam-rolling. However, the quality of barley produced by dry rolling is affected by kernel hardness and uniformity (Swan et al., 2006).

Temper rolling: In temper rolling, water is added to whole barley to increase moisture to 18 to 20%, and the mixture is left for 12 to 24 h before rolling (Dehghan-banadaky et al., 2007). Temper rolling increases the uniformity of the particles produced during processing, decreasing shattering and the production of fines, which decreases the rate of starch digestion as compared to aggressive dry processing (Dehghan-banadaky et al., 2007). Yang et al. (1996) and Wang et al. (2003) reported a reduction of 34% and 45%, respectively, in rate of ruminal DMD with temper-rolled versus dry rolled barley. Christen et al. (1996) suggested that temper rolling is advantageous over dry rolling when the original grain is extensively rolled, has a moisture level of less than 10%varies in kernel size and is fed with a limited amount of forage.

Steam processing: Steam rolling or flaking involves application of steam at either low or high pressure to increase grain moisture and temperature (Dehghan-banadaky et al., 2007). The combination of heat and moisture with rolling gelatinizes starch, which increases swelling of starch granules due to absorption of water, breaking down their crystalline structure (Ratnayake and Jackson, 2009). Gelatinization of starch may reduce the rate of starch degradation (Zinn et al., 1993) if retrogradation occurs. Similar to tempering, steam processing tends to reduce production of fines, and increase the uniformity of particle sizes as compared to dry processing,

but it requires additional investment in equipment and energy, increasing processing cost (Dehghan-banadaky et al., 2007). The effect of steam processing on the ruminal digestion of barley grain has been inconsistent (Plascencia et al., 1998; Malcom and Kiesling, 1993). Steam flaking has more consistently increased ruminal degradability of corn than barley (Fiems et al., 1990), an outcome that likely reflects the destruction of the protein matrix in corn, a structure that is less of a barrier to microbial starch digestion in barley.

Extent of Processing

Increasing the severity of processing increases rate and extent of digestion of barley in the rumen, but also the risk of acidosis (Beauchemin et al. 2001; Yang et al., 2000). Therefore, processing methods needs to be optimized so that they maximize rumen digestion without causing digestive and metabolic disorders. Processing index (PI) is widely used by feed industry as a means of describing the extent of processing. The PI is the test weight (TW) of the grain after processing expressed as a percentage of the TW before processing. The optimum PI varies with type of ruminant production system, with a PI of 65% being optimal for lactating dairy cows fed steam rolled barley (Yang et al., 2000) and 75% for finishing beef cattle fed temper rolled barley (Beauchemin et al., 2001). The PI varies with the method selected for processing as well as the flatness and particle size distribution of processed barley grain differs among processing methods. The optimum extent of processing also depends on the amount of forage, and the quality of barley such as kernel uniformity and NDF content in the diet (Bengochea et al., 2005; Hironaka et al., 1992).

Precision processing

Precision processing can simply be defined as processing barley according to kernel size of grain (Yang et al., 2013). Kernel size varies substantially (Yang et al., 2013) and this variability

is further magnified if loads of heavy and light barley are blended, which is common practise at grain terminals. The substantial variation in kernel size within a lot makes it difficult to achieve optimal processing with a single roller setting, especially when the barley is dry-rolled prior to feeding. Precision processing of grain provides alternatives to ensure uniform cracking of all kernels as it separates grain kernels to achieve a uniform kernel size within a lot, with each lot then being processed with an optimum roller setting specific to the kernel size (Ahmad et al. 2010; Yang et al., 2013).

Ahmad et al. (2010) suggested that adjusting roller settings according to specific kernel sizes could increase the starch utilization of barley grain by ruminants. Yang et al. (2013) showed that precision processing resulted in more uniformly processed grains with fewer whole kernels and less fines compared to conventional processing.

Forage to concentrate ratio

The maximum utilization of barley grain also depends on the amount of forage in the diet of ruminants. To maximize the energy intake of high-producing ruminants, grain needs to be included in the diet at high levels. However, feeding a high grain diet increases the risk of digestive disorders, an outcome that can decrease feed conversion efficiency. Thus, minimizing the amount of forage in the diet without causing digestive upset is of interest, but a challenge. Manipulating grain processing can potentially avoid any negative impacts that arise from the reduced feeding of forage. Koenig et al. (2003) studied the interaction between PI of barley grain and proportion of silage, and suggested that barley grain had an optimal PI of 86% when 5 % barley silage was in the diet but when this level was increased to 20% barley silage, the grain could be more extensively processed to a PI of 61%. Although increasing the fineness of particles in the diet may cause digestive upsets, with sufficient forage included in the diet, fine

particles could be beneficial for nutrient utilization due to improved digestion. Sadri et al. (2007) and Soltani et al. (2009) showed that finely ground barley and steam-rolled barley had a similar effect on rumen digestion and productivity of dairy cows if barley was included in the diet at less than 30% of diet DM.

1.3.3 Digestive disorder from feeding barley grain

Feeding barley grain to high-producing beef cattle or dairy cows can be associated with several nutritional disorders, such as rumen acidosis and bloat. These nutritional disorders may reduce weight gain, milk production, feed efficiency or cause displaced abomasum, liver abscesses, lameness and other health related issues (Oetzel, 2007). The general risk factors for digestive disorders include extensive grain processing, a low proportion of forage in the diet, and abrupt changes in the amount of grain supplied (Beauchemin and McAllister, 2008). Thus, one of the approaches to minimize acidosis is to enable rumen microbes to adapt to dietary changes. A desirable result is a balance between the production and the neutralization or removal of VFA to achieve a stabilized rumen pH above 5.6 (Beauchemin and McAllister, 2008). In addition, balancing starch and effective fiber, and using proper processing methods can prevent the rapid production of VFA in the rumen, and reduce risk of digestive disorders (Beauchemin and McAllister, 2008). Including forage in the diet at adequate levels and with longer particle lengths can stimulate rumination and the production of saliva which can buffer against acid production in the rumen (Beauchemin and McAllister, 2008).

1.3.4 Assessment of ruminal digestion

Information on rates and extent of grain digestion in the rumen is critical in diet formulation to optimize rumen microbial growth and ruminant production. *In vivo* measurement of grain

digestion in the rumen is expensive, labour-intensive, time-consuming, and confounded by inherent animal variation (Stern et al., 1997). To overcome the drawbacks of *in vivo* measurements, various *in vitro* and *in situ* methods are frequently used to characterize ruminal grain digestion.

In vitro digestibility estimation

Two common *in vitro* techniques for this purpose include the Tilley and Terry method and the gas production technique. The two-stage system developed by Tilly and Terry (1963), involves a 48 h digestion period with rumen fluid to simulate rumen digestion followed by a second 48 h digestion period using pepsin and weak acid to simulate post-ruminal digestion. It is used widely due to its convenience, especially when large numbers of feedstuffs are being characterized (Getachew et al., 1998). However, the original procedure may not create conditions that are optimal for digestion kinetics, resulting in lack of detection of differences among varying substrates (Stern et al., 1997; Walker, 2007). Therefore, the procedure has been modified by considering factors such as ratio of buffer to ruminal inoculum, type of buffer, particle size of the substrate, substrate processing, and type of diet fed to cattle that serve as a source of rumen fluid (Stern et al., 1997; Walker, 2007). The gas production technique is another widely used method to model grain digestion in the rumen assuming the gas production during grain fermentation is proportional to the amount of carbohydrate fermented. However, it is important to note that if gas pressure accumulates in the fermentation vessels above 48 kPa, it may reduce microbial growth and cause gas to dissolve in the medium, which reduces the amount of gas measured (Rymer et al., 2005).

In situ digestibility estimation

The *in situ* or nylon bag technique has been widely used to measure ruminal digestion of feeds (Ørskov and McDonald, 1979). The technique involves suspending nylon bags containing feedstuffs in the rumen and measuring degradation characteristics of DM, CP, starch and NDF by analyzing the residues in bags after a series of time intervals of incubation in the rumen (Stern et al., 1997). The advantages and disadvantages of the technique are well documented. A number of factors may cause variation in the estimation of nutrient digestion, which requires control and standardization to obtain consistent data (Stern et al., 1997). These factors include bag porosity, feed particle size, sample size to bag surface ratio, variation in rumen function among animals and the type of diet fed (Stern et al., 1997). A potential problem with this technique could be over or under estimating the actual digestibility of feed due to loss through bags or the fact that the feed within the bag is not subject to break down by chewing (Ørskov et al., 1980).

1.3.5 Effect of site of digestion on animal performance

Maximizing animal production for cattle fed barley-based diets requires an understanding of interactions between the site of digestion of barley grain and its utilization (Owens and Soderlund, 2012). Extensive ruminal carbohydrate fermentation may be undesirable due to the energy loss through methane production and excessive acid production resulting in reduced fiber digestion and increased risk of bloat and acidosis (Hunt, 1996; Rowe et al., 1999). However, digestion of carbohydrates in the rumen is distinctively advantageous compared with intestinal digestion in two ways (Hunt, 1996). Firstly, ruminal fermentation of carbohydrates may increase protein synthesis by ruminal microorganisms. The greater microbial protein production from increased energy availability appears to compensate for gaseous related energy losses associated with the fermentation of starch in the rumen (McLeod et al., 2006). Second, total tract starch

digestibility is greater due to a larger proportion of starch degradation in the rumen, rather than in the small intestine, mainly due to the limitations of intestinal starch digestion in ruminants (Hunt, 1996). It is a general practice that the digestion of barley grain in the rumen should be maximized to the point that it does not cause an increase in incidence of digestive upsets. .

1.4 Infrared Spectroscopy

1.4.1 The fundamentals of Infrared Spectroscopy

Infrared (IR) spectroscopy provides information about the molecular structure of a compound (Griffiths, 2002a). The fundamental principle behind the technique is on the basis of vibrational motions of atoms and chemical bonds within molecules. When a beam of IR radiation is passed through a sample, the IR energy, i.e. photon, is absorbed by atoms and bonds, and transformed into molecular vibrations at specific frequency and mode (Griffiths, 2002a).

Electromagnetic radiation: Infrared electromagnetic radiation includes visible light, X-rays, ultraviolet radiation, microwaves, and radio frequency waves (Loudon, 2002a). These waves differ from each other in the length (Loudon, 2002a). Wavelength is the length of one complete wave cycle, which is inversely related to wavenumber (Loudon, 2002a). The IR region consists of three areas including near IR (NIR), mid IR (MIR) and far IR (FIR), which are located in the region of 13,000-4,000, 4,000-200 and 200-10 cm^{-1}, respectively with NIR and MIR being the most commonly used in the prediction of chemical components and identification of functional groups in biological samples (Griffiths, 2002a). The MIR is commonly reported in wavenumber (cm^{-1}), whereas NIR is preferably reported in wavelength expressed in unit of nanometer.

Vibrational theory: Two major vibrational modes of a compound are stretching and bending (Loudon, 2002a). For the stretching vibration, it includes symmetrical and asymmetrical

stretching vibration (Figure 1-1). For bending vibration, it includes: 1) in-plane, which can be separated to scissoring and rocking vibration, and 2) out-of-plane bending vibration, which can be divided into wagging and twisting vibration (Figure 1-1; Griffiths, 2002a; Stuart, 2005). Molecules vibrate at various frequencies with the minimum energy at room temperature when no IR radiation excites the molecules (Loudon, 2002a; Stuart, 2005). However, when a frequency of IR radiation matches the frequency of a vibrating bond, energy from the IR radiation is absorbed and the intensity of the bond vibration increases (Stuart, 2005). This absorption gives rise to the peak in the IR spectrum. Therefore, it is possible to identify unknown chemical bonds predicted by FTIR, and relate them to organic compounds and determine the composition of a mixture according to the frequencies, intensities and patterns of the characteristic peaks (Stuart, 2005).

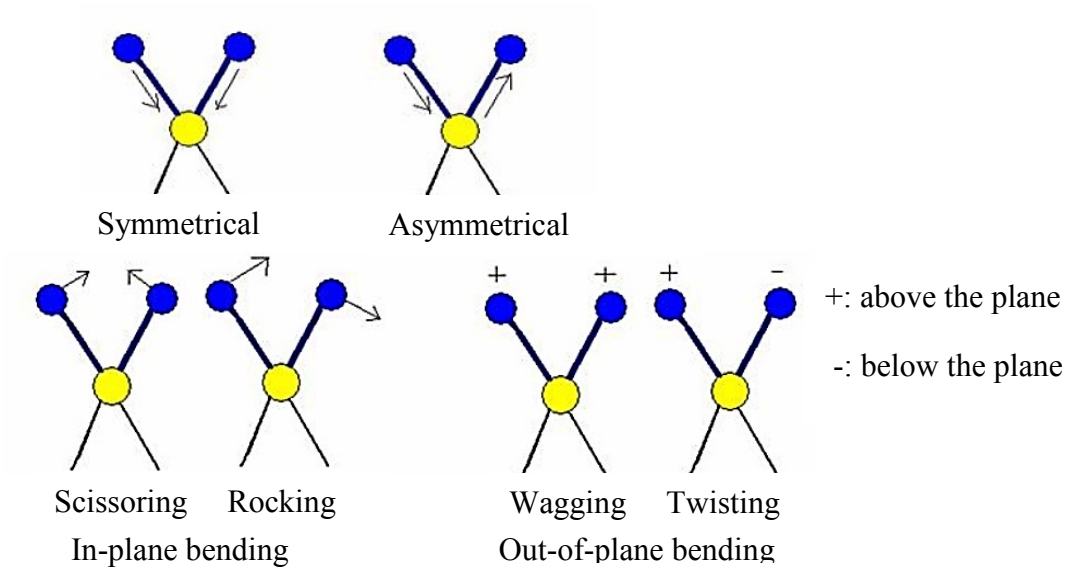

Figure 1–5. Stretching and bending of a methylene group (-CH_2-). Source: http://www.pharmatutor.org/pharma-analysis/analytical-aspects-of-infra-red-spectroscopy-ir/types-of-vibrations.

Beer-Lambert law: The Beer-Lambert law (commonly known as Beer's law) is the fundamental law of quantitative absorption spectroscopy, which indicates a linear relationship between the absorbance and the concentration of target molecules in the sample of interest (Griffiths, 2002b; Stuart, 2005). The formula is $A=\varepsilon cl$, where A is the absorbance of the sample in IR spectrum, c *is* the concentration of the analyte, and l *is* the path length of the radiation, ε is the molar absorptivity (Stuart, 2005). For IR spectroscopy, the path length and molar absorptivity are constants (Stuart, 2005). Although absorbance is directly proportional to the concentration of the analyte, some factors may affect the nature of this proportional relationship (Griffiths, 2002b). Firstly, the sample must be homogeneous to avoid scattering of the incident radiation, and deviations from Beer's law. As Beer's law assumes the absorbing medium is homogeneous and does not scatter the radiation, scattering of the radiation causes the effective path length to vary significantly, resulting in deviations from Beer's law (Griffiths, 2002b; Wang, 2014). In addition, errors in sample placement in relation to the IR source should be avoided (Griffiths, 2002b) as a constant angle is necessary to avoid small wavenumber shifts, and an alternation of measured absorbance (Griffiths, 2002b).

Comparisons between NIR, MIR, and FIR spectroscopy: The main difference between ranges of MIR and NIR is that absorption of MIR range represents fundamental bands of molecular vibrations, but absorptions in NIR range are associated with overtones and combinations of these fundamental bands (Stuart, 2005). An MIR spectrum consists of fundamental vibrations (4000-1500 cm^{-1}) and fingerprint regions (1500-600 cm^{-1}), which have clear and strong signals in many cases (Subramanian and Rodriguez-Saona, 2009). The fingerprint region is useful in identifying or discriminating sample conformation and molecular structural conformations as compared to functional groups. Different molecules may have

specific fingerprint areas, but possess the same functional group region if they have similar functional groups (Stuart, 2005). However, caution in interpreting functional groups in the fingerprint region is still required for biological samples, as biological samples usually contain complex mixtures of compounds which overlap within the same fingerprint region (Subramanian and Rodriguez-Saona, 2009). Unlike MIR spectra, NIR spectra are comprised of overtones and combination absorptions with weak and overlapping signals (Stuart, 2005). Overtones are bands that can be produced at frequencies 2 to 3 times the fundamental frequency, whereas combination absorption is a combination of two or more vibrations arising through addition and subtraction of energies to produce a single band (Stuart, 2005; Subramanian and Rodriguez-Saona, 2009). The broad overlapping peaks and large variation in baseline make interpretation of NIR spectrum difficult for qualitative analysis (Subramanian and Rodriguez-Saona, 2009). However, NIR spectroscopy is advantageous and has been more widely used in quantitative analysis and requires minimal or no sample preparation unlike MIR spectroscopy (Subramanian and Rodriguez-Saona, 2009). To enhance spectral characteristics, spectra are often mathematically processed using derivatization. Predictive models (calibrations) are derived using multivariate regression statistics, where absorption is the independent variable, and the factor to be predicted is the dependent variable. Compared to NIR and MIR spectroscopy, FIR spectroscopy is not commonly used in quantitative and qualitative analysis, due to the low efficiency of FIR beam-splitters, low signal-to-noise ratio and strong water vapor absorption which causes interference with spectral features. It also requires more specialized sampling techniques than NIR or MIR spectroscopy (Ferraro and Rein, 1985).

1.4.2 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a technique of IR spectroscopy. The term "Fourier Transform" refers to the collection and conversion of data from an interference pattern (interferogram) to an IR spectrum (Griffiths, 2002a). In a traditional dispersive technique, an IR beam is dispersed by a monochromator into individual frequency, which is then directed to the compound, and received by the detector to generate an electrical signal (Griffiths, 2002a). A dispersive IR spectrometer shows each resulting frequency sequentially, but FTIR spectroscopy using both NIR and MIR region examines all frequencies simultaneously, which extends the capabilities of IR spectroscopy into areas of analysis that are limited to dispersive instruments (Stuart, 2005). Additionally, FTIR has been suggested to have the advantages of speed, sensitivity, accuracy, high throughput, constant resolution and a signal to noise ratio as compared by dispersive IR spectrometry (McCluskey, 2000). However, Armstrong et al. (2006) indicated that advantages of FTIR vs. dispersive IR spectroscopy are in the MIR region, but not the NIR region, as the low signal-to-noise ratio in the detector-noise limited instruments of traditional MIR spectroscopy limits their application. However, NIR spectroscopy does not use detector-noise limited instrumentation, and as biological materials absorb over broad regions in NIR spectroscopy, they may not be easily related to the measurement of specific wavelengths. Thus, the advantages of the Fourier transform method are less prominent in the NIR as compared to MIR region.

Configurations of FTIR spectroscopy*:* Common configurations that have their own strengths and weaknesses are transmittance and reflectance (Stuart, 2005). Transmittance is the simplest configuration as the IR radiation is directly passed through the sample and detected on the other side (Stuart, 2005). This technique is cost effective, possesses a high signal to noise ratio in solid,

liquid and gaseous samples. However, the need for thin samples for transmittance makes sample preparation technically challenging and time consuming, limiting application (Stuart, 2005).

Reflectance is the reverse of transmittance as it collects IR light reflected back from the sample (Griffiths, 2002a; Stuart, 2005). Compared to transmittance, reflectance is simpler, faster and employs less sample preparation as thin samples are not a requirement (Stuart, 2005). Although the signal to noise ratio of reflectance method is typically lower than that of transmittance, the speed and simplicity of the reflectance technique makes it the preferred method (Stuart, 2005; Subramanian and Rodriguez-Saona, 2009). Common reflectance methods include attenuated total reflectance (ATR), diffuse reflectance and specular reflectance (Stuart, 2005).

1.4.3 Interpretation of spectra: major absorption groups

Interpretation of comprehensive data within spectra requires an understanding of the spectroscopic properties of the individual elements of the sample tissue (Stuart, 2005). In MIR spectrum, major absorption bands in starch, fiber, protein and fat are particularly relevant and are discussed below.

The structure of starch can be revealed by IR spectroscopy, although the precise assignment of absorptions associated with this polysaccharide is still difficult due to the complexity of overlapping bands associated with starch, fiber and non-starch polysaccharides at 1300 – 800 cm^{-1}. This region is dominated by ring vibrations overlapped with stretching vibrations of (C-OH) side groups and (C-O-C) glycosidic bonds (Kačuráková et al., 2000). Specifically, an important band near 930 cm^{-1} is solely attributed to alpha-(1, 4)-glycosidic linkages in the molecular structure of starch (Nurrulhidayah et al., 2012). Amylose and amylopectin can be characterized using the ratio of 1018 and 999 cm^{-1} (Kuhnen et al., 2010).

Fiber t is composed of cellulose, hemicellulose and lignin and can been identified by associating peaks formed at certain wavelengths. Several studies have identified absorption peaks associated with cellulosic material at approximately 1400 cm^{-1} and 1246 cm^{-1} (Peitrzak and Miller, 2005). Himmelsbach et al. (1998) determined differences in distribution of cellulose and hemicellulosic material at 1335 cm^{-1} and 1250^{-1} cm within the stems of two varieties of flax. The absorption bands of lignin have been widely studied (Olga Derkacheva, 2008). An aromatic compound in lignin shows two major bands at 1600 and 1510 cm^{-1} (Yu, 2004).

Protein is characterized by the presence of N and peptide bonds, and absorption bands of protein structures including Amide I, II, alpha-helix and beta-sheets within the MIR region (Carbonaro and Nucara, 2009). Molecules that contain a significant number of C=O bonds, such as the amide groups within proteins exhibit an absorption band at 1650 cm^{-1}, a result of stretching vibrations of C=O bonds (Stuart, 2005). The amide I bands between 1700 to 1600 cm^{-1} are strongly correlated to the levels of alpha-helix (1648-1640 cm^{-1}), beta-sheet (1635-1628, 1525-1521 cm^{-1}) and unordered structures in proteins (Carbonaro and Nucara, 2009). Amide II has 60% N–H bending and 40% C–N stretching at 1575-1510 cm^{-1} (Xin et al., 2013).

Functional groups of fat include methyl, methylene, and backbone of long carbon chain with double or single bonds. The absorption bands of these functional groups are well-characterized in the MIR region. Absorption bands of lipid can be determined from the symmetric and asymmetric stretching vibrations of CH_2 at 2922 and 2852 cm^{-1}, and CH_3 at 2956 and 2874 cm^{-1} within acyl chains (Stuart, 2005). Another strong absorption band used to indicate lipids is at approximately 1779-1725 cm^{-1}, which arises from the stretching vibration of the C=O ester groups (Stuart, 2005).

1.4.4 Spectra analysis methods

Multivariate analysis offers convenient alternatives to distinguish differences of chemical-structural conformation between samples using entire spectra (Yang, 2013). Due to the large data sets in common FTIR data and problem of selectivity, multivariate data analysis extracts the maximum useful information from the spectra and presents the results as easily interpretable plots.

The objective of principle component analysis (PCA) is to decompose the original data set and concentrate the source of variability to a new set of uncorrelated variables called principal components (PC, Hori and Sugiyama, 2003). The first PC (PC-1) generally describes the maximum variation or spread in the samples, and the second PC (PC-2) describes the second component responsible for the most variation in the data set eigenvector (Dunteman, 1989), and so on. Thus, the first few PC typically accounts for the most variance. Each sample is assigned a score on each PC eigenvector, and the scores plot of PCA shows the distribution of the data, and summarizes the relationship between samples. The loadings plot of PCA is a representation of the wavenumber(s) associated with each PC (Bonnier and Byrne, 2012). The intensity of the bands in the loading plot of each PC explains the intensity of the band's influence on the variations of groupings in the scores of PCA. The stronger the band intensity, the greater the band contributes to the variation or groupings in the spectral data. Therefore, PCA can not only help to identify variances but also provide information on which factors affect the grouping. In feed analysis, Yu et al. (2011) used PCA to reveal structural changes in carbohydrates as result of its use in bioethanol production. PCA analysis could fully distinguish differences in protein amide I region of Valier and Harington barley varieties using FTIR (Yu et al., 2008). Zhang and Yu (2012 a; 2012 b) used PCA to reveal differences in the protein and carbohydrate structural

conformation of barley blended with different levels of dried distillers grains with solubles (DDGS).

1.4.5 Use of IR spectroscopy in feed analysis

Optimizing feed utilization of livestock genuinely requires simple, fast and accurate techniques to estimate the chemical composition of feedstuffs and characterize nutrient degradation in the rumen (Yu, 2004). The metabolic characteristics of feed nutrients can be determined by their related molecular structures and biopolymer conformation (Yu, 2004; 2005). Vibrational spectroscopic techniques can identify chemical and molecular structural composition, with little or no sample preparation, no need for reagents with high-throughput and cost-effectiveness (Yu, 2004; 2005).

Use of NIR spectroscopy *in feed analysis:* NIR spectroscopy can estimate a wide range of plant components including NDF, protein and starch as well as the nutrient digestibility of feeds by measuring undigested residues arising after *in vitro*, *in situ* or *in vivo* digestion (Corson et al., 1999; Swift, 2003). For instance, NIR spectroscopy has widespread application in forage characterization, including prediction of composition and *in vivo* digestibility (Baker and Barnes, 1990; Givens and Deaville, 1999). In addition, Zijlstra et al. (2011) showed that NIR spectroscopy could predict the digestible energy content of barley grain for swine. Applications of NIR spectroscopy are limited by the quality of the calibration data that is derived from wet chemistry. If accurate and robust calibration curves are employed, NIR spectroscopy is a rapid and cost effective method for screening large numbers of samples for nutrient quality (Zijlstra et al., 2011).

Use of MIR spectroscopy *in feed analysis:* In the late 1960s, most MIR instruments started to employ FTIR spectroscopy, using interferometers instead of monochromators in traditional dispersive spectroscopy, due to their advantages for speed of analysis, high sensitivity, cost and precision and higher signal-to-noise ratio (Subramanian and Rodriguez-Saona, 2009).

Use of FT-NIR spectroscopy *in feed analysis:* Use of FT-NIR spectroscopy in feed analysis is not as common as its use has been in food analysis. However, Chen et al. (2014) used FT-NIR spectroscopy to analyze protein and total fat of corn with dual-component analysis being a more reliable estimation strategy as compared to partial least square models. The FT-NIR has been used to evaluate the glucan, xylan, galactan, arabinan, lignin and ash content in corn stover and switchgrass, and predict the chemical composition of wheat straw (Liu et al., 2010). Sohn et al. (2007) used both FT-NIR and NIR spectroscopy to determine the chemical composition of a number of barley grain varieties to assess their potential as a feedstock for fuel ethanol production. They suggested that FT-NIR had no predictive advantage over a dispersive system for most of the components in barley grain. Further, similar to the limitation of NIR, FT-NIR also relies on accurate calibrations models to provide reliable predictions (Stuart, 2005).

Use of FT-MIR spectroscopy *in feed analysis:* FT-MIR spectroscopy has increasingly become a powerful tool for investigating the molecular spectral characteristics of animal feeds (Yu, 2005; Yu, 2012). FT-MIR spectroscopy has been used to detect structural and non-structural carbohydrate and protein secondary structural characteristics in carinata and canola meal, and in mixtures of co-products such as DDGS with barley grain and among different varieties of barley and corn (Duodu et al., 2002; Gholizadeh et al., 2014; Yu et al., 2009; 2011; Xin and Yu, 2013).

In particular, the spectra profile of protein structures has been widely studied in animal feeds. Zhang and Yu (2012a) reported the relationship between the spectral profile of the protein molecular structure and protein availability in blends of DDGS and barley grain. In addition, FT-MIR can detect processing and heat-induced changes in protein secondary structure (Gamage et al., 2012). Furthermore, FTIR techniques have been successfully used in predicting protein digestibility from undigested residues of barley grain in swine (Wang et al., 2012a). Peng et al. (2014) used FT-MIR to determine the relationship between the intrinsic molecular structures of protein feeds and protein solubility, *in situ* rumen degradability and intestinal digestibility. The feeds investigated were barley, corn, oat, wheat, lentil, peas, canola meal, expeller meal (extruded canola meal), soybean meal, mill feeds (pelleted by-products from cereal grains), sugar beet pulp, blood meal and meat meal. They concluded that the protein amide II area and beta-sheet height could be used to predict the contents of intestinally digestible rumen undegradable protein ($r^2 = 0.8$) and total digestible CP ($r^2 = 0.8$) of these feeds for dairy cattle.

1.5 Conclusion, hypothesis and objectives

Barley is primarily grown for the value-added malting and brewing market in North America. However, to ensure brewing efficiency and achievement of malt, barley is subject to a number of strict malting quality specifications which considering its physical and chemical characteristics. Therefore, over 75% of malting varieties of barley grain are marketed down as feed, primarily for ruminants. Although a number of physical and chemical factors that affect the digestion of barley grain by ruminants are well documented, the relationship between SH and MC such as DP, FRI and WBG, and feed value are largely unknown. Studying the effect of parameters including SH, DP, FRI and WBG on rumen digestion may provide alternative

approaches to evaluate the feed value of barley grain for ruminants. Thus, my first hypothesis is that SH and MC of barley grain (DP, FRI, WBG) can be used to predict *in situ* rumen digestibility of barley grain.

The alternation of barley grain digestion in the rumen as affected by SH and MC may be associated with its inherent molecular structures. The ATR-FTIR spectroscopy is widely applied in analysis of the chemical and molecular composition of plants as it has the advantages of rapid analysis with simple operation, and can simultaneously measure multiple nutrients. The MIR region between 4000-400 cm^{-1} is the most widely used region for characterization and identification of bonds and functional groups in chemical compounds, due to their unique absorption patterns in this region. From now on, I will use FTIR to specifically refer to Fourier transform technology used in MIR region. In combination with PCA, ATR-FTIR spectroscopy is powerful tool to reveal information on chemical structural conformations of grain components of interest. Thus, my second hypothesis is that ATR-FTIR spectroscopy can be used as an analytical tool to study structural characteristics of barley grain associated with nutrient digestion in ruminants, and the effect of SH and MC on the rumen digestion of barley grain.

The project consisted of two studies, with the objective of the first study being to evaluate the relationship between *in situ* DMD of barley grain, and the SH and MC (DP, FRI, WBG). The second study used the same barley grain and residues from 4 and 12 h rumen incubation to investigate the relationship between barley grain digestion, and SH and MC using ATR-FTIR spectroscopy, and to characterize the ruminal digestion of barley grain overtime using ATR-FTIR spectroscopy.

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CHAPTER 2 THE EFFECT OF SEED HARDNESS AND MALTING CHARACTERISTICS ON *IN SITU* DRY MATTER DIGESTIBILITY OF BARLEY GRAIN IN BEEF HEIFERS

2.1 Introduction

In Canada, barley is primarily grown for the value-added malt market where it is used as a substrate for the production of beer [Canadian Barley Malting and Brewing Technical Guide (CBMBTG), 2012]. To ensure brewing efficiency, it is assessed for a number of strict quality specifications which it must possess to be approved for the malting process (Fox et al., 2009). Barley that fails to meet the malt grade is mainly used as animal feed and priced on the basis of bushel weight (CBMBTG, 2012), a parameter that is poorly related to its ruminal digestibility (Mathison et al., 1992). As such, it is of interest to determine if malt quality traits are also indicators of the feed value of barley. If such a relationship exists, barley could be simultaneously assessed for its value for malt or as a feed for ruminant livestock.

A number of characteristics that used to evaluate malt quality including seed hardness (SH) index, and malting characteristics (MC) such as diastatic power (DP), friability (FIR), and wort beta-glucan (WBG) concentration could be related to the feed value of barley grain (Fox et al., 2009). For example, the hardness of the grain affects the extent to which it shatters during processing, and the degree to which starch in the endosperm is digested by microbial and host enzymes. Diastatic power represents the activity of total starch degrading enzymes in the malt, which will be used to convert starch to fermentable sugars in the fermentation of brewing process. Wort beta-glucan indicates the extent of beta-glucan breakdown in endosperm cell wall by beta-glucanase, thus both beta-glucan content in the cell wall and the level of active beta-glucanase determine WBG (Edney et al., 2007; Fox, 2008). Wort beta-glucan content is

positively associated with grain beta-glucan content (Wang et al., 2004). In the brewing industry, a high content of grain beta-glucan in barley may restrict the degradation of endosperm cell walls and limit the access of amylases to starch granules, if the level of beta-glucanase in the rumen is low. Thus, high content of WBG is undesirable for malting and brewing efficiency and quality (MacGregor and Fincher, 1993). A recent study by Dr. Yu at the University of Saskatchewan showed that barley with higher grain beta-glucan content exhibited a reduced extent of degradation in the rumen (personal communication). However, no research has studied the effect of WBG on rumen digestion of barley grain. Friability is an indicator of the readiness of malt to crumble when subjected to crushing. However, possible relationships between these malting quality attributes and the feed value of barley for ruminants have not been well investigated. I hypothesize that SH and the MC of barley grain could be used to predict rumen digestibility of barley. Therefore, the objective of this study was to evaluate the relationship between *in situ* dry matter (DM) digestibility (DMD) of barley grain and SH, DP, FRI or WBG.

2.2 Materials and methods

Barley grain samples were collected from five locations across western Canada during 2010, 2011 and 2012 (as part of a larger agronomic study by Drs. O'Donovan and Edney). For each year, barley samples were selected for high and low values of SH, DP, FRI, and WBG. A total of 72 samples (3 years × 4 attributes × 2 levels × 3 samples) were evaluated in this study. Three samples represent three varieties including AC Metcalfe, Bentley, and Merit 57. The SH and MC were determined at the Grain Research Laboratory of the Canadian Grain Commission, Winnipeg, Canada. Protein content of barley grain was predicted using near-infrared spectroscopy. Wort beta-glucan (in volume of 400 mL) was measured using the calcofluor

fluorescence method, and reported as ppm [American Society of Brewing Chemists (ASBC), 2009]. Diastatic power was analyzed using a segmented flow analyzer, using an automated neocuproin assay for reducing sugars, which was calibrated with malt standards analyzed using the official ferricyanide reducing sugar method (ASBC, 2009). To analyze FRI, malt samples in steel mesh drum, were pressed against the rotating sieve by means of the pressure force of a rubber roller. Friability was reported as the percentage of malt by weight which passed through the sieve. Seed hardness was determined by measuring crush force, using the Perten Single Kernel Characterization System (Martin et al., 1993), which is expressed as an index ranged from 0 to 100 (0 = softest and 100 = hardest). Selected samples ranged from 47.8 to 68.4 in SH index, from 123 to 226°L in DP, and from 36.9 to 92.3% in FRI, and from 11 to 434 ppm in WBG content (Table 2-1).

Barley samples were ground through a 6-mm sieve using a hammer mill. Particle size distribution of ground samples selected for SH and FRI was measured using a series of sieves at 3.35, 2.36, 1.18, 0.85 mm, and a pan in a Ro-Tap machine (RX-29, W. S. Tyler, Mentor, OH). The selection of the SH and FRI for measuring the particle size distribution was based on the suggestion that these two malt characteristics were likely related to the particle size distribution generated during grinding.

The *in situ* rumen DMD of barley was measured using three ruminally cannulated beef heifers at the Agriculture and Agri-Food Canada, Lethbridge Research Center Metabolism barn. Heifers were fed a total mixed ration containing 65% barley silage, 30% barley grain, and 5% mineral and vitamin supplement (DM basis) ad libitum throughout the entire trial. Heifers had free access to drinking water, and were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009). Barley (5 g) was placed into a bag (10 × 20 cm) made

of monofilament PeCAP polyester (pore size, 51 ± 2 μm; B. & S. H. Thompson, Ville Mont-Royal, QC, Canada), heat sealed, and placed in a polyester mesh bag (20 × 30 cm) anchored to the rumen cannula by a 90-cm rope. Duplicate bags for each incubation time and each sample were placed in the rumen of each heifer for 4, 12, and 48 h, in the reverse order of incubation time so that all bags were removed simultaneously. Upon removal, bags were rinsed under running cold tap water until the water became clear. The bags containing fermentation residues were dried in a forced air oven at 55^{o}C for 48 h to estimate DMD.

Data were analyzed using the MIXED procedure of SAS, with the model including the fixed effect of malting attribute level, and the random effects of year, heifer and samples. Effects of the fixed factor were declared significant at $P < 0.05$, and trends were discussed at $P < 0.10$. Pearson correlation coefficients between SH, MC, protein content and *in situ* DMD were estimated using the CORR procedure of SAS.

2.3 Results and discussion

Considerable variation among samples was observed in SH, DP, FRI and WBG (Table 2-1). Wort beta-glucan content varied the most with coefficient of variation (CV) of 76.4%, followed by FRI with CV of 31.4%, DP with CV of 18.4%, and SH with CV of 10.3%. The particles retained on 3.35, 2.36 or 1.18-mm sieve did not differ between the high and low SH samples (Table 2-2). However, the materials retained or that passed through the 0.85-mm sieve were greater ($P < 0.01$) for high than for low SH samples. The particle size distribution did not differ between the low and high FRI samples.

Barley grains that possessed high and low WBG content or degree of FRI in malt did not differ in *in situ* DMD (Table 2-3). However, the *in situ* DMD of barley grains with low SH was

lower ($P = 0.02$) compared to the barley with high SH after 4 h of ruminal incubation. In addition, barley grain with low DP had greater ($P = 0.02$) DMD after 4 h of incubation or tended ($P = 0.07$) to have greater *in situ* DMD after 12 h of incubation compared to barley with a high DP.

The greater *in situ* DMD of barley grain with high SH index is not consistent with a previous report (Ramsey et al., 2001), which showed no relationship between SH and rate of *in situ* DMD for barley grain ground through a 3-mm screen. Increased kernel hardness is expected to result in a slower rate of rumen digestion due to the increased difficulty for microbes to access starch in the endosperm. As SH is defined as the resistance of the kernel to fracture (Anjum and Walker, 1991), it is logical to expect that harder grains have stronger resistance to microbial and enzymatic degradation. However, the current study showed that 4-h *in situ* DMD was greater for barley grains with high seed hardness index. The difference observed in the current study and that of Ramsey et al. (2001) may arise due to the fact that the grains were processed through different screen sized between the two studies (6 mm and 3 mm, respectively). In addition, the proportion of fine particles that passed through 1.18-mm sieve was greater for high SH than low SH samples (13.9 vs.11.7%) in the current study. These results indicated that grinding barley samples through a 6-mm screen promoted shattering and produced fine particles to a greater extent for harder barley compared to softer barley, resulting in higher *in situ* DMD. In wheat, ground samples with greater SH had a higher rate of DM digestion as compared to samples that were softer, suggesting that grinding generated more fine particles from harder kernels (Swan et al., 2006). The higher DMD at 4 h may be indicative of an increased risk of rumen acidosis following feed ingestion. However, the greater DMD at 4 h of incubation did not appear to impact DMD at 12 or 48 h of incubation. Therefore, the overall relationship between SH and the

extent of ruminal digestion of barley grain DM would be minimal as the actual rumen retention time of processed grain is approximately 12 h (De Boer et al., 1987).

Diastatic power represents the activity of total starch-degrading enzymes including alpha-amylase, beta-amylase, limit dextrinase, and alpha-glucosidase, which are responsible for the break down starch to fermentable sugar during malting (CBMBTG, 2012). Therefore, barley grain with high DP was expected to have higher *in situ* DMD as it reflects an increase in the activity of starch degrading enzymes. However, in the current study, barley with high DP actually exhibited a lower *in situ* DMD. The unexpected lower *in situ* DMD of barley samples with high DP is unclear. However, DP was positively correlated with protein content (Table 2-4) for barley samples evaluated in the current study, suggesting that DP is also potentially related to the density of the protein matrix. Although starch granules are loosely associated with protein matrix in barley, we speculate that an increase in protein content may still indicate greater embedment of starch granules within a protein matrix, a factor that could decrease the access of the microbes to starch granules and the degradation of starch at early incubation times.

The undesirable effect of WBG on malting quality of barley has been well documented (Edney et al., 2007), whereas, its effect on ruminal digestion of barley has been not been evaluated. However, WBG is positively associated with the beta-glucan content of grain (Wang et al., 2004). The impacts of grain beta-glucan content of barley on *in situ* DMD have been inconsistent. Engstrom et al. (1992) reported that the *in situ* rumen degradation of barley grain varying in beta-glucan concentration from 3.5 to 4.8% did not differ. In contrast, Foley et al. (2006) found that the higher beta-glucan content of waxy barley decreased the rate of *in situ* degradation as compared to barley with a normal starch genotype. In fact beta-glucan is the major structural component of aleurone cell walls in the endosperm, accounting for 75% of

the endosperm cell walls in barley grain (Fincher, 1975). Greater structural components with high beta-glucan content may require longer for microbial attachment and degradation to occur. However, the lack of difference in *in situ* DMD of barley with high and low beta-glucan content suggests that the beta-glucanase activity associated with rumen microorganisms was sufficiently high to degrade the endosperm cell walls within barley grain.

The lack of difference in *in situ* DMD between high and low FRI barley samples is in agreement with the results of Fox et al. (2009) as FRI had no relationship with 3-h *in situ* DMD of forty barley cultivars they examined. It is expected that barley grain with higher FRI would have higher microbial degradation, hence higher *in situ* DMD. The FRI of malt is calculated as the percentage of the friable malt, by weight, which passes through a sieve (2.2 mm) after processing (CBMBTG, 2012). Higher values of FRI indicate a high percentage of grain that is easily fractured during processing. The higher percentage of broken kernels could potentially lead to greater surface area after processing, which would favor rumen microbial colonization and a more rapid rumen digestion (McAllister et al., 1994). However, the particle distribution of ground samples did not vary with the level of FRI, which is consistent with the lack of differences in *in situ* DMD. In addition, FRI was negatively related to protein content of barley grain (Table 2-4), indicating that barley grain with greater FRI likely has a less developed protein matrix. Current results suggest that the difference in FRI of barley grain may not affect microbial attachment or the enzymatic degradation of barley in the rumen.

Barley grains that exhibited high or low WBG content or wide differences in FRI did not differ in *in situ* DMD. However, after 4 h of incubation, barley grain with greater SH or low DP had higher *in situ* DMD compared to those with low SH or high DP. Considering that *in situ* DMD of barley with high and low SH or DP differed only at early incubation times, these malt

traits may not be suitable predictors of extent of barley grain digestion in the rumen since the average retention time of barley grain in the rumen is likely longer than 4 h.

Table 2–1. Variation range of seed hardness and individual malting characteristic of barley grain

Item	Mean	SD[z]	Minimum	Maximum	CV[y]
Seed hardness (Index)	64.8	6.7	47.8	68.4	10.3
Beta-glucan (ppm)	203	155	11	434	76.4
Friability (%)	63.4	19.9	36.9	92.3	31.4
Diastatic power(°L)	174	32	123	226	18.4

[Z] SD = standard deviation.
[Y] CV = coefficient of variation.

Table 2–2. Effect of seed hardness and friability of barley grain (n = 18 for each attribute) on particle size distribution

	Seed hardness Index			Friability		
Item	Low	High	SEM[z]	Low	high	SEM[z]
Particle size on sieve (%)						
3.35 mm	4.4	4.2	0.07	4.2	4.1	0.06
2.36 mm	31.1	29.8	0.81	30.6	30.5	0.43
1.18 mm	52.8	52.1	0.88	52.9	52.0	0.61
0.85 mm	4.2[b]	4.9[a]	0.04	4.9	5.0	0.08
< 0.85 mm	7.5[b]	9.0[a]	0.17	8.6	8.5	0.27

[z] Standard error of the mean.
Means with different letters in the same row are significantly different ($P < 0.05$).

Table 2–3. Effects of seed hardness and malting attributes of barley grain on *in situ* dry matter disappearance (%).

	Low	High	SEM[z]	*P* <
Seed hardness (index)	53.4±3.1	64.8±2.0		
4h	33.6	36.0	0.59	0.02
12h	68.0	67.7	1.23	0.86
48h	83.6	82.5	1.00	0.15
Beta-glucan (ppm)	122±111	316±118		
4h	36.7	36.3	2.8	0.69
12h	67.7	66.7	1.32	0.41
48h	83.2	83.5	0.47	0.68
Diastatic power (°L)	146±11	203±17		
4h	38.1	35.7	2.89	0.02
12h	67.6	65.3	1.47	0.07
48h	84.1	83	0.59	0.18
Friability (%)	46.0±7.5	80.7±10.3		
4h	36.4	37.3	2.73	0.43
12h	66.6	68.4	1.56	0.21
48h	82.7	84.1	0.87	0.18

[z] Standard error of the mean.

Table 2–4. Correlation coefficients between seed hardness, malting characteristics (MC) and protein content or *in situ* dry matter disappearance (DMD) of barley grain (n = 18 for each MC)

			DMD (%)					
	Protein (% DM)		4 h		12 h		48 h	
	r	*P*<	r	*P*<	r	*P*<	r	*P*<
Seed hardness (Index)	0.15	0.56	0.04	0.84	-0.03	0.89	-0.24	0.33
Beta-glucan (ppm)	0.45	0.06	-0.62	0.01	-0.32	0.20	0.39	0.10
Diastatic power (°L)	0.79	0.01	-0.15	0.55	-0.41	0.08	-0.28	0.27
Friability (%)	-0.80	0.01	0.29	0.23	0.12	0.63	0.36	0.14

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CHAPTER 3. ASSESSING RELATIONSHIP BETWEEN SEED HARDNESS, MALTING CHARACTERISTICS AND RUMEN DIGESTION OF BARLEY GRAIN IN RELATION TO MOLECULAR STRUCTURE USING ATR-FTIR

3.1 Introduction

Chapter 2 has demonstrated that studying the effect of physical and malting characteristics (MC) that used to evaluate malt quality, such as seed hardness (SH), diastatic power (DP), friability (FRI) and wort beta-glucan (WBG) on rumen digestion of barley grain, may provide alternatives to evaluate feed quality for optimal utilization of barley grain in ruminants.

Theoretically, determining the distribution of components, such as neutral detergent fiber (NDF), protein and starch in grain could reveal important information regarding its biochemistry and fermentability (Yu, 2004). Differences in SH and MC among barley samples arise from differences in growing environment, variety and physical structure, and are potentially associated with the chemical composition of a kernel and structure of chemical components. Molecular structures of chemical components include functional groups that represent specific groups of atoms and chemical bonds of molecules responsible for the characteristic chemical properties of feed (Loudon, 2002b). Traditional wet chemistry is unable to detect the differences in the functional groups (Yu, 2004). Digestion of barley grain in the rumen may be affected by malting quality characteristics as a result of their inherent molecular structures.

Previous work (Chapter 2) indicated that DP and SH may impact the digestion of barley grain in early incubation time, but not the extent of barley grain digestion in the rumen. The results showed that barley samples with low DP had higher *in situ* dry matter digestion (DMD) compared to those with high DP after 4 h, a trend which continued after 12 h of rumen

incubation. In addition, barley grain with low SH had lower *in situ* DMD than those with high SH after 4 h of incubation. Further study on the relationship of molecular structural features of barley grain in relation to spectral characteristics using Fourier transform infrared (FTIR) spectroscopy may enhance our understanding of the mechanisms by which *in situ* DMD varied with levels of DP or SH. This could enable barley grain to be selected based on certain SH or MC that reflects its degree of feed value for ruminants.

Fourier transform infrared spectroscopy has been suggested as a rapid, convenient and economical tool to be able to detect and define functional groups of biological samples. The attenuated total reflectance (ATR) is one of the most widely used techniques for FTIR in grain-based samples (Kim et al., 2007) due to its rapid analysis, ease of operation, and simultaneous determination of multi-nutrient composition. For instance, FTIR equipped with ATR (ATR-FTIR) has been used to detect differences in structural makeup of proteins within different varieties of barley and protein sources (Zhang and Yu, 2012a). This information was used to predict the digestibility of wheat protein in swine (Wang et al., 2012a); and to evaluate *in vitro* digestibility of canola co-products (Wang et al., 2012b). However, to date, little research has been published using ATR – FTIR spectroscopy to study changes in the molecular structure of protein and carbohydrates during *in situ* incubation, and if these changes are affected by SH, DP, friability (FRI), or wort beta-glucan (WBG). Additionally, principle component analysis (PCA) is able to reveal differences in the structural makeup in the Amide I and II regions of raw vs autoclaved canola seeds (Theodoridou and Yu, 2013), and in the protein and carbohydrate structural makeup of a barley grain diet mixed with varying levels of DDGS (Zhang and Yu, 2012a; 2012b). Therefore, in combination with PCA, ATR-FTIR may be a powerful tool for characterizing nutrient digestion of barley grain in the rumen.

Thus, the hypothesis for this study was that ATR- FTIR spectroscopy can be used as an analytical tool to evaluate the chemical and structural fractions (e.g. Amide I and II in protein, aromatic skeletal ring in lignin) of barley grain and barley residues following ruminal digestion, in order to better understand factors that dictate the nature of barley grain digestion. The variation of *in situ* incubation time, SH, MC, variety and growing environment of barley grain may alter the chemical and structural composition of the *in situ* residues. Therefore, the objectives of this research were to characterize the rumen residues of barley grain using ATR-FTIR spectra in order to investigate the relationship between MC and *in situ* rumen digestion.

3.2 Materials and Methods

3.2.1 Sample Selection and Preparation

Samples from the previous *in situ* study (Chapter 2) with high and low levels of SH, DP, FRI and WBG listed previously (Table 2-1) were used for this study. The samples varied by variety (AC Metcalfe, Bentley and Merit57), harvest years (2010, 2011, 2012), and location (Brandon, Scott and Lacombe, Lethbridge and Indian Head). For each year, barley samples were selected for high and low values of SH, DP, FRI, and WBG, ranging from 47.8 to 68.4 in SH index, 123 to 226°L in DP, and 36.9 to 92.3% in FRI, and 11 to 434 ppm in WBG content , respectively (Table 2-1). Barley samples and residues of 4 and 12 h of *in situ* incubation were selected from the study conducted in Chapter 2, with 4 h representing the time point when differences of *in situ* DMD were observed in barley grain with high and low DP and SH, and 12 h representing the potential extent of rumen digestion of barley grain (De Boer et al., 1987). In the current study, a total of 72 samples (4 attributes × 2 levels for each attribute × 3 varieties ×3 years) after 0, 4, and 12 h of rumen incubation were used. The techniques for measurement of *in*

situ DMD are described in Chapter 2 and the results are reported in Table 2-3 (Chapter 2). Prior to scanning in an FTIR, barley samples (approximately 1-5 g) incubated in the rumen for 0, 4 and 12 h were ground through 1-mm sieve (standard model 4, Arthur Thomas Co., Philadelphia, PA), followed by grinding using a TissueLyser II system (Qiagen, Valencia, CA) for 15 min at 30Hz/s.

3.2.2 Spectra collection

Each sample was sub-sampled three times and each sub-sample (approximately 10 mg) was scanned on a FTIR spectrometer (MB 3000, ABB, Montréal, QC, Canada) equipped with a deuterated triglycine sulfate detector and a single-bounce ATR with a diamond plate (MIRacle ATR, PIKE Technologies, Madison, WI). The spectrum of each sub-sample were obtained by averaging 32 scans using Horizon MB FTIR software (ABB) in the range of 4000-600 cm^{-1} with a resolution setting of 4 cm^{-1} (Wang et al., 2013a). The diamond plate of ATR was cleaned carefully with lint-free tissue after each scan to avoid cross contamination. Constant pressure was applied to ensure consistent contact of the sample with the diamond plate. Prior to acquisition of each sub-sample spectrum, a background spectrum containing no sample was scanned and subtracted from spectrum of the sample before conversion to absorbance units. Spectra of three sub-samples were averaged for each sample.

3.2.3 Spectral and multivariate data analysis

Wavenumbers of 3050-700 cm^{-1} were selected from the total wavenumbers (4000-600 cm^{-1}) due to the clear presence of peaks associated with structural and chemical features of barley in the selected region. Then, the selected ATR- FTIR spectra was divided into five regions (region

1: 3050-2812 cm^{-1}; region 2: 2626-1471 cm^{-1}; region 3: 1469-1207 cm^{-1}; region 4: 1205-941 cm^{-1}; region 5: 937-650 cm^{-1}, Figure 3-1 a) to facilitate characterization of spectra.

Software Unscrambler 10.3 (CAMO, AS Oslo Norway) was used for preprocessing and PCA. Raw spectra were preprocessed using the Standard Normal Variate procedure to reduce scattering of raw data (Barnes et al., 1989), followed by second- order derivative transformation using the method proposed by Savitzky and Golay with smoothing side points of 9. Savitzky-Golay Second-order derivative using smoothing algorithm can correct baseline, which is a low frequency signal that contains noise and needs to be eliminated from raw spectra to improve the signal-to-noise ratio of raw spectra. Preprocessed spectra were analyzed according to incubation time and level of SH, DP, FRI and WBG (high versus low) in order to determine their effects on chemical composition of barley grain and residues as indicated by molecular structures. Principal component analysis was performed to identify the greatest sources of variation in ATR-FTIR spectra (selected region and five sub-regions) and identify clear patterns in the spectra according to grouping by factors such as incubation time, level of SH, DP, FRI and WBG, harvesting site and variety. Principle component analysis decomposes the original data set and concentrates the source of variability to a new set of uncorrelated variables called principal components (PC, Hori and Sugiyama, 2003). The first PC (PC-1) describes the maximum variation or spread in the samples, and the second PC (PC-2) represents the next largest source of variation in the data set (Dunteman, 1989).Thus, the first few components typically account for the most variance. In addition, each sample (each spectrum in this case) is assigned a score, and the score plots of PCA visually show the distribution and grouping of the data, and summarize the relationship between samples. The loadings of PCA represent the origin of spectral variation based on recorded wavenumbers (Bonnier and Byrne, 2012). The intensity of the bands in the loading value of each

PC explains the intensity of the band's influence on the variations of groupings. The stronger the band intensity, the greater the band contributes to the variation of groupings in the spectral data.

3.3 Results

Results of PCA indicate the only grouping along axis was by incubation time, and there was no grouping along PC axis when sample scores were grouped by level of SH and MC values, harvest location, or variety for the four characteristics in spectra. Grouping by incubation time and the lack of grouping by level of DP and SH are shown. In addition, there was no impact of FRI or WBG on rumen digestion in the first study (Chapter 2), and spectra patterns for FRI and WBG were similar to those selected for SH and DP. Figures associated with FRI and WBG are shown in the Appendix (Appendix-1, 2, 3, 4, 5 and 6).

3.3.1 Selected ATR- FTIR spectra of barley grain after *in situ* rumen incubation

Overall, the selected spectra showed significant peaks at 2925, 2854, 1762, 1743, 1481, 1460, 1153, 1080, 1062, 992, 982 and 930 cm^{-1}, and broad peaks at 1650 - 1627 and 1608-1571 cm^{-1}, which are potentially associated with molecular structures of lipid, lignin, protein, cellulose, hemicellulose, and starch, respectively (Figure 3-1 a). Total spectra had similar pattern between SH and DP (Figure 3-1 a, 3-2 a).

The PCA scores showed groupings by incubation time along the PC-1 (Figure 3-1 b) which uses samples selected for DP. However, spectra were not grouped by level of DP (Figure 3-1 c). The grouping by incubation time was due to differences at certain wavenumbers in loadings plot for PC-1 (Figure 3-1 d). The PC-1 that explained 53% variation in spectra pattern was mainly attributed to large variations at 2923, 2852, 1762, 1743, 1481, 1460, 1062, 982, 964 and 930cm^{-1}

(Figure 3-1 b, d), which confirms significant peaks observed in spectra. The PCA of samples selected for SH had similar patterns in that sample scores tended to group by incubation time but not by level of SH (Figure 3-2 a, b, c, d).

3.3.2 Five sub- regions of ATR-FTIR spectra of barley residues

Samples selected for DP showed larger peaks at 2923, 2854 cm^{-1} at 12 h compared to those at 0 h and 4 h (Figure 3-3 a) which corresponds to peaks for lipids or potentially lignin or carbohydrates. This was confirmed by results of PCA where groupings are clearly seen according to time points (Figure 3-5 a) but not by level of DP (Figure 3-5 b). Peaks were also seen at 2945 and 2837 cm^{-1} (Figure 3-3 a); however, variation at time points were smaller. The intensity of these bands at 2923, 2845, 2945 and 2837 cm^{-1} increased as incubation time increased, except the broad band at 2995-2974 cm^{-1}, which showed a slightly higher intensity at 12 h compared to 4 h residues (Figure 3-3 a). In the scores plot of PCA (Figure 3-5 a), incubation time was grouped along the PC-1 axis, accounting for 99% of the spectra variation. The loadings plot (Figure 3-5 c), showed that the variation represented by PC-1 is mainly attributed to significant bands at 2923, 2854 cm^{-1}, and weaker bands at 2945 and 2837 cm^{-1}. Samples selected for SH showed similar results (Figure 3-4 a; Figure 3-6 a, b, c) in region 1 to those selected for DP where large peaks were seen at 2923 and 2854 cm^{-1}.

Samples selected for DP had higher peaks at 1762, 1743 and 1726 cm^{-1} (region 2) at 12 h in the spectra compared to those at 0 and 4 h (Figure 3-3 b), which mainly correspond to lipids components, although carbohydrates and lignins may have a contribution at similar wavenumbers. There were also clear differences according to incubation time at these wavenumbers (Figure 3-3 b). The peak at 1743 cm^{-1} showed greater differences between 0 and

12 h than other peaks (Figure 3-3 b). A strong peak was also seen at 1481 cm^{-1}, which likely refers to lignin components, but variation at time points were smaller (Figure 3-3 b). There was no difference between incubation times in spectra at broad peaks of 1654-1627 and 1608-1527 cm^{-1}, which correspond to protein molecular structures such as amide I and II (Figure 3-3 b). In addition, the scores plot of PCA (Figure 3-4 d) showed distinct grouping of sample scores along PC-1 between 0 and 12 h, or 4 and 12 h, but not between low and high levels of DP (Figure 3-4 e). The plot also showed a greater similarity of spectra between samples at 0 and 4 h as compared to samples at 12 h of incubation for both DP and SH (Figure 3-4 d, 3-6 d). The loadings plot of PCA showed that these groupings were mainly associated with bands at 1762, 1743, 1726, 1627 and 1481 cm^{-1} (Figure 3-4 f), which confirms the groupings by incubation time observed in the spectra (Figure 3-3 b). Samples selected for SH showed similar results to DP in region 2 (Figure 3-5 b; Figure 3-6 d, e, f).

In region 3, samples selected for DP had higher peaks at 1460, 1394, 1375 and 1234 cm^{-1} at 12 h as compared to those at 4 h, which potentially corresponds to lignin, cellulose and hemicellulose related structures (Figure 3-3 c). However, spectra showed lower peaks at 1332, and 1317 cm^{-1} at 12 h as compared to those at 4 h (Figure 3-3 c), which are assigned to carbohydrates. At peaks of 1332 and 1317 cm^{-1}, the difference between 4 and 12 h of incubation were more visible (Figure 3-3 c). Samples selected for SH showed a similar pattern of differences in spectra (Figure 3-5 c). The scores plot of PCA showed clear grouping by incubation time for both DP and SH (Figure 3-4 g, h; 3-6 g, h). The loadings plot of PCA (Figure 3-4 i; 3-6 i) showed that the presence of clear grouping by incubation time along PC-1 was mainly due to a significant band from 1460 cm^{-1}, with less influence at 1394, 1375, and 1234 cm^{-}

[1], which partially confirms the differentiation of spectra according to incubation time for samples selected for both DP and SH (Figure 3-3 c; 3-5 c).

Weaker peaks were observed at 1080, 1062, 992, 982 cm^{-1} at 12 h as compared to those at 4h in samples selected for DP (Figure 3-3 d), which are mainly associated with carbohydrate molecular structures, and more likely starch related structures. The scores plot of PCA (Figure 3-4 j) showed a separation between 0 h and 4 and 12 h along both PC-1 axis, with overlaps between 4 and 12 h. Spectra of barley sample were not grouped by level of DP (Figure 3-4 k). This separation was mainly attributed to variations at 1080, 1062, 982 and 964 cm^{-1} in PC-1 (Figure 3-4 l). Samples selected for SH showed a similar pattern of spectra separation and PCA grouping by incubation time but not by level of SH in region 4 (Figure 3-5 d; Figure 3-6 j, k).

In region 5, samples selected for DP had weaker peaks at 930, 862, 763 cm^{-1} at 12 h, compared to those at 4 h (Figure 3-3 e), which are mainly due to non-structural carbohydrates with a small contribution from structural carbohydrates. The presence of an absorption band near 930 cm^{-1} is solely attributed from alpha-(1, 4)-glycosidic linkages in starch. The score plots of PCA (Figure 3-4 m) showed that samples at 12 h of incubation grouped separately from those at 0 or 4 h along the PC-1 axis, whereas samples at 0 and 4 h of incubation were distinguishable from each other along PC-2 axis, with some overlap. However, spectra were not grouped by level of DP (Figure 3-4 n). Samples selected for SH showed a similar pattern of spectra and PCA in region 5 (Figure 3-5 e; Figure 3-6 m, n). Grouping by incubation time for both samples selected for DP and SH was attributed to variations at 930, 862 and 763 cm^{-1} for PC-1, and at 862, 844 and 822 cm^{-1} for PC-2 (Figure 3-4 o, p; 3-6 o,p) likely corresponding to non-structural carbohydrates.

3.4 Discussion

Effect of malting quality characteristics on molecular structure of barley grain as indicated by FTIR and PCA

In the first study (Chapter 2), barley grain with low DP and high SH had higher DMD at 4 h of incubation compared with barley grain with high DP and low SH, respectively, with FRI and WBG having no impact on DMD. Based on these results, we assumed that ATR-FTIR would not be able to detect differences in molecular structure of barley grain with high vs low levels of FRI and WBG across time points. However, due to the observed difference in 4 h DMD in the previous study, as affected by level of DP or SH, the molecular structures in barley residues from rumen incubation may have been altered, and thus detectable by ATR-FTIR. The lack of difference detected in molecular structures of barley grain and residues as affected by high and low FRI and WBG are in agreement with our assumption for FRI and WBG. However, in contrast to our hypothesis, the lack of grouping by level of SH and DP in PCA scores at any region of spectra suggested that level of each attribute had no impact on molecular structures of barley grain as a result of rumen digestion. This may be due to the small magnitude of difference in *in situ* DMD (2.3-2.4%) of barley grain with high and low DP or SH.

Effect of *in situ* rumen digestion on molecular structure of barley grain as indicated by ATR-FTIR spectra and PCA

The clear grouping by incubation time in spectra and PCA analysis showed that rumen digestion impacts molecular structures in grain residues. Overall, carbohydrates, lipids and lignins appeared to be the main components that contribute to the differences of spectra, as shown in the PCA analysis of the total spectra.

Effect of Starch and NDF digestion: Although the precise assignment of absorptions associated with polysaccharides is still difficult due to the complexity of overlapping bands associated with starch, fiber and non-starch polysaccharides at 1300 – 800 cm^{-1} (Stuart, 2005), structures of starch, cellulose and hemicellulose were still revealed by ATR-FTIR. The results showed a relatively higher peak absorption of wavenumbers potentially associated with NDF structures (1460, 1375, 1394, 1481 and 1234 cm^{-1}) in spectra of 12 h residues, and higher peak absorption of wavenumbers associated with starch molecules (1062, 1080, 992, 982 and 930 cm^{-1}) in spectra of 4 h residues. In particular, the presence of an absorption band near 930 cm^{-1} is solely attributed to the alpha-(1, 4)-glycosidic linkages in starch (Kizil et al., 2002; Nurrulhidayah et al., 2012). The bands at 992 and 982 cm^{-1} are likely associated with amylose, although they could be affected by cellulose at 993 and 983cm^{-1}, respectively (Nikonenko et al., 2000; Smits et al, 1998). Absorption bands at 1080 and 1062 cm^{-1} could correspond to amorphous region in starch structures (Krim and Liang, 1956). Absorption bands at 1460, 1375 and 1394cm^{-1} are potentially associated with cellulose and hemicellulose (Brewer and Wetzel, 2010; Pandey and Pitman, 2003; Sun et al., 2004). Bands at 1481 and 1234 cm^{-1} can be more confidently assigned to aromatic skeletal and phenolic ring structures in lignin (Pandey and Theagarajan, 1997; Liu and Yu, 2011).These results indicated that as rumen incubation progressed from 0 to 12 h, NDF concentration increased, and overall starch concentration decreased in barley residues. This could potentially be attributed to the faster digestion of starch than NDF in the rumen (Theurer, 1986). In addition, starch molecules, likely amylose structures (Nikonenko et al., 2000; Smits et al, 1998), contributed to the greatest differences between spectra as affected by *in situ* rumen digestion, however, this may be attributed to an increasing concentrations of other components that are not digested at the same rate. For example, the

variation in NDF related components including hemicellulose, cellulose and lignin could be attributed to the preferential digestion of starch, resulting in their concentrations increasing in the residue. These results were expected as hulled barley grain consists of approximately 46 to 63% starch including amylose and amylopectin, commonly in a 3:1 ratio, and 17 to 32% NDF (Baik and Ullrich, 2008; Overnell-Roy et al., 1998). Barley starch is readily digestible in the rumen once the fibrous hull is broken (Dehghan-Banadaky et al., 2007; McAllister et al., 1994), and up to 80% of starch disappears after 12 h of *in situ* rumen incubation (Ahmad et al., 2010). However, the fibrous hull, pericarp and aleurone layer that contains large amounts of cellulose, hemicellulose and lignin are slowly degradable or completely indigestible (Du et al., 2009). This results in a meaningful drop in starch concentration with a relative increase in NDF concentration in barley residues as observed in the current results. In addition, the clear grouping of 0 and 12 h samples in PCA scores at region 4 and region 5 due to C-O-C stretch and alpha- (1, 4)-glycosidic linkages reflects the degradation of starch after 12 h of ruminal incubation. More specifically, the significant decrease of concentration in glycosidic linkage after 12 h of incubation as compared to 0 and 4 h is a sign of starch digestion by the rumen microbial population (French, 1973).

Effect of Protein digestion: Protein is characterized by the presence of N and peptide bonds, and absorption bands of protein structures including Amide I (at 1710-1580 cm^{-1}), II (at 1575-1510 cm-1), alpha-helix (1648-1640 cm^{-1}) and beta-sheets (1635-1628, 1525-1521 cm^{-1}) within the MIR region (Pelton and McLean, 2000; Mantsch and Chapman, 1996). In the current study, the weak contribution of ATR-FTIR absorption bands associated with these protein molecular structures and PCA of total spectra and region 2 indicate this technique was not sensitive enough to detect changes in the molecular structures associated with protein. This is likely due to a lower

concentration of barley protein (9-14% DM), as compared to starch and NDF concentration (52 to 66% and 16-25% DM, respectively, predicted using near-infrared spectroscopy), however, as protein is important in ruminant digestion, this is an area that needs to be addressed further. In addition, chemical components such as starch could cause a confounding effect on detection of protein-associated molecular structures due to similar spectral bands by ATR-FTIR. Yu (2006) and Wetzel et al. (2003) suggested that if studying the molecular structure of proteins is the main interest of using FTIR spectroscopy, relatively "pure" protein or protein-dominant tissues should be used to reduce potential effects of other biological components such as starch on derived measurements. Due to the presence of many large starch granules in the protein matrix of barley grain, the contribution of starch in samples is disproportionally high, even after grinding and mixing of samples (Wetzel et al., 2003). This would distort the ability to discern the details of the protein molecular structures in FTIR spectra (Wetzel et al., 2003). Use of FTIR microspectroscopy with advanced synchrotron technology (SR-FTIR) may help to localize relatively pure protein in barley kernels, and specifically study protein-associated molecular structures (Wetzel et al., 2003). The structural features of relatively pure protein in a barley kernel can be revealed using the microscopic capabilities of SR-FTIR, whereas conventional FTIR spectroscopy lacks this capability. Previous research using ATR-FTIR to study barley grain protein digestion in the rumen has focused on predicting feed crude protein concentration and rumen degradability using 1450-600 cm^{-1} in the MIR region (Belanche et al., 2013), and distinguishing differences in protein structure between different varieties of barley (Damiran and Yu, 2011; Du et al., 2009; Liu and Yu, 2010), and mixtures of barley with DDGS (Zhang and Yu, 2012 a; 2012 b). However, none of the studies focused on characterizing barley nutrient digestion using rumen residues of barley grain in a visual manner using ATR-FTIR and PCA.

Lipid digestion: In the current study, functional groups of fat include CH_2 at 2922 and 2852 cm^{-1}, CH_3 at 2956 and 2874 cm^{-1} and C=O ester groups at approximately 1779-1725 cm^{-1} (Boeriu et al., 2004; Tipson, 1968). The higher peak associated with lipid molecular structures and its major contribution to spectra variation was not expected. The small amount of lipid (approximately 3% of DM, Baik and Ullrich, 2008; Overnell-Roy et al., 1998) in barley was expected to be digested rapidly, and the amount of lipid present in the residue was expected to decrease as incubation time increased (Jenkins, 1993). One speculation of the cause of increased lipid concentration during rumen incubation could be associated with lipid synthesis by de novo rumen microbes from carbohydrate precursors (Demeyer et al., 1978). Also, free fatty acids can be utilized by rumen microbes for the synthesis of phospholipids, and are important for building cell membranes (Bauman and Lock, 2006). Thus, lipids in the rumen consist of fatty acids of both dietary and microbial origin (Jenkins, 1993). Total lipid content of bacterial dry mass in the rumen ranges from 10 to 15%, and proportions are higher in solid-associated bacteria than in liquid-associated bacteria (Bauchart et al, 1990; Jenkins, 1993). Thus, in the current study, the significant contribution of lipid-related molecules to grouping of incubation time in PCA could be due to an increasing amount of microbial lipid synthesized as a result of colonization of the grain by rumen microbes as ruminal digestion proceeded. There might have been an increasing amount of lipid synthesized de novo by microbes from carbohydrate precursors, and possibly a small contribution from phospholipids in the microbial dry mass attached to solid feed. However, the unchanged protein concentration as incubation progressed does not support this speculation, as the increased microbial mass on the feed should have also increased protein concentration in ATR-FTIR spectra. Therefore, significant increase of CH_2, CH_3, and C=O concentration in spectra could be associated with increased concentration of lignins and structural carbohydrates

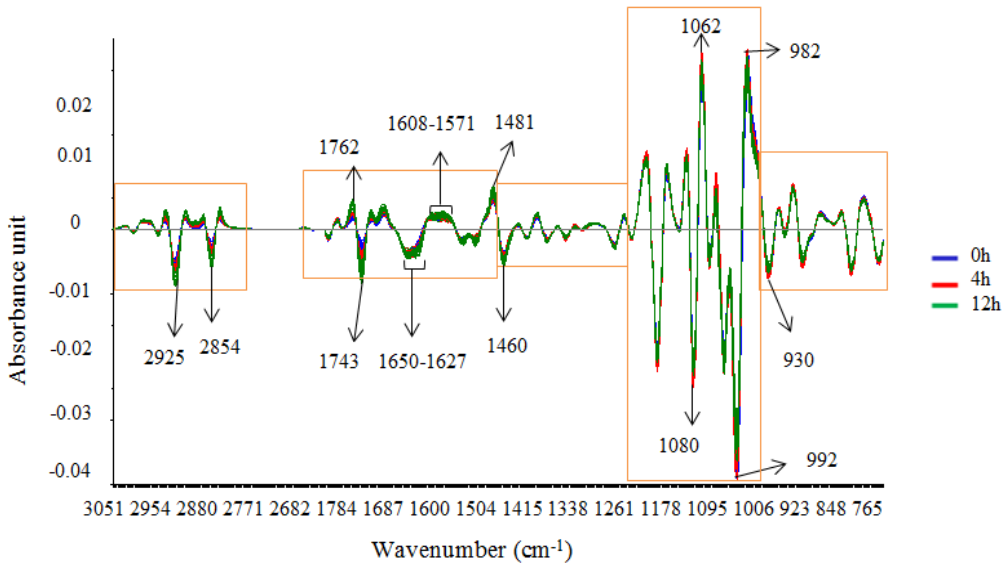
as rumen incubation prolonged, because these chemical components also contain CH_2, CH_3, and C=O in their molecular structures (Nadji et al., 2009; Popescu et al., 2011).

Effect of harvesting site and variety on molecular structure of barley grain

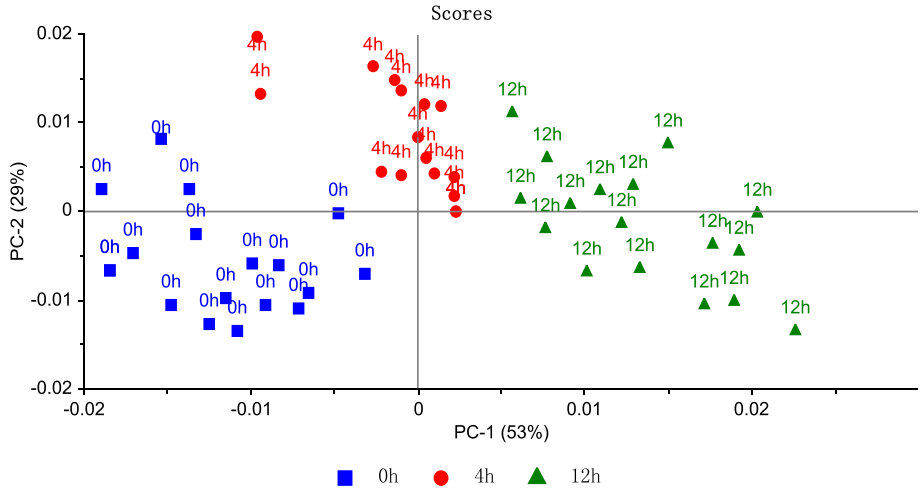
The lack of grouping by variety and harvesting site in PCA sample scores (data not shown) indicated that these factors did not explain the variation of the spectra, and had no impact on rumen digestion over time. Genotype of barley grain and growing condition were expected to impact the chemical composition and molecular structure of barley grain, which might also affect rumen digestion and be reflected in differences detected by ATR-FTIR (Damiran and Yu, 2011; Liu and Yu, 2010; O'Donovan et al., 2011). Liu and Yu (2010; 2011) used ATR-FTIR with multivariate analysis to successfully distinguish molecular structural differences at regions associated with carbohydrate and protein among barley varieties including CDC Helgason, AC Metcalfe, McLeod and CDC Cowboy. Damiran and Yu (2011) using Fourier transform infrared with diffuse reflectance (DRIFT) also observed differences in molecular structural makeup of protein Amide I and II among different varieties of barley grain (CDC Fibar, CDC Rattan, HB08302 conventional and CDC McGwire) but no difference was observed in carbohydrate related regions. However, Yu et al. (2004c) identified no statistical difference in seed structure (protein and starch molecules) between a feed type (Valier) and malting type (Harrington) barley grain based on SR –FTIR spectra. Previous results show that successfully distinguishing molecular structural differences among varieties of barley depends on the variety selected. Thus, the current lack of difference between varieties and locations of barley could be due to a lack of difference in structural composition.

3.5 Conclusion

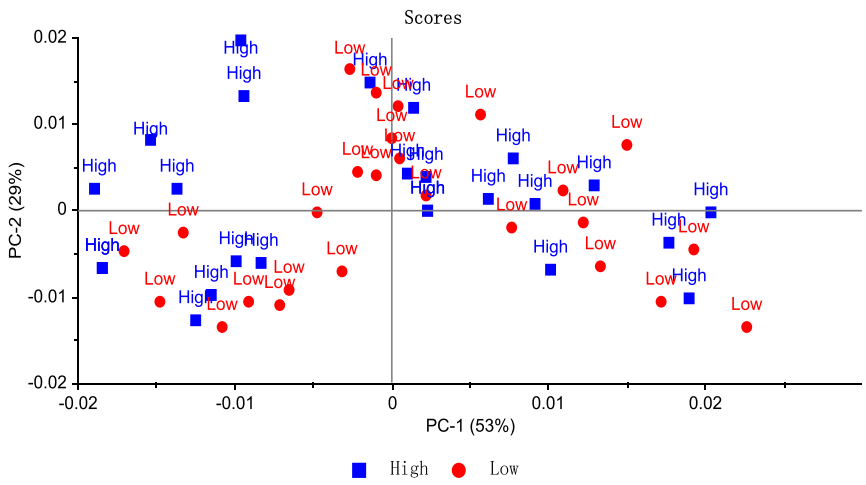
ATR – FTIR spectra varied according to *in situ* incubation time in barley samples selected for SH and DP; however, no effect of WBG and FRI was observed across time points. This indicates a lack of sensitivity in ATR–FTIR required to detect changes in the chemical composition of barley grain during digestion, as observed in the results of Chapter 2. Although changes in molecular structures were observed, the bands potentially represent multiple molecular structures making interpretation of specific functional groups difficult. Carbohydrate components, likely starch-associated structures appeared to differ in ATR-FTIR spectra as a result of *in situ* ruminal digestion according to our expectations. These results indicated that ATR-FTIR was not able to detect differences of barley samples according to SH, DP, FRI or WBG before and after rumen digestion, but it may have potential to identify changes in molecular structures of barley grain as result of *in situ* digestion.


Absorbance unit
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
1062
982
1608-1571
1481
1762
2925
2854
1743
1650-1627
1460
930
1080
992
3051 2954 2880 2771 2682 1784 1687 1600 1504 1415 1338 1261 1178 1095 1006 923 848 765
Wavenumber (cm-1)
0h
4h
12h

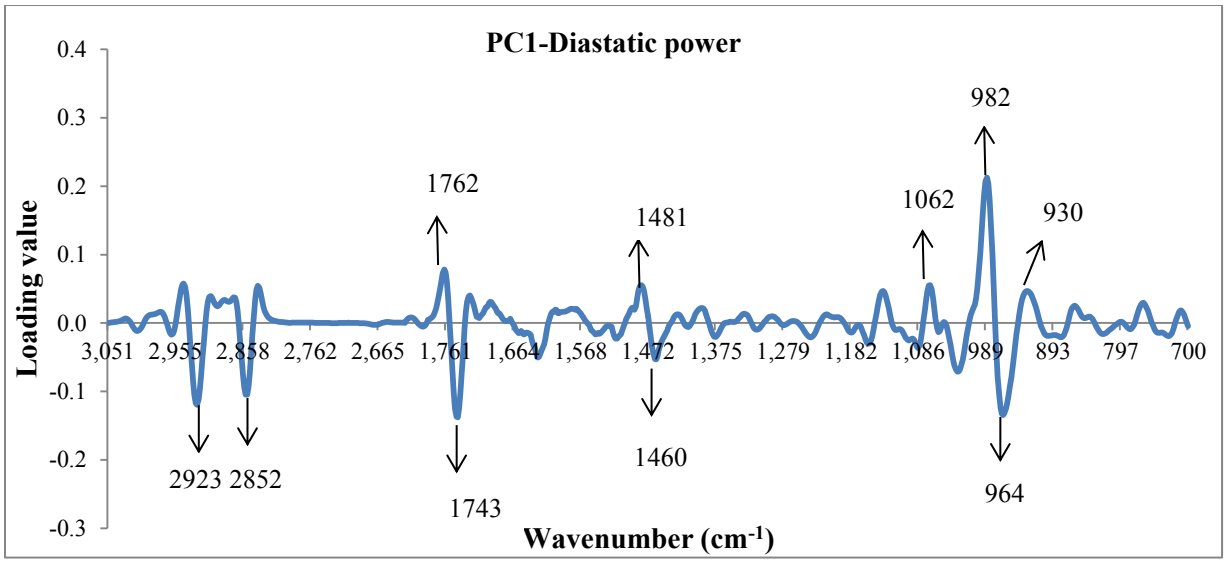
(a)


Scores
PC-2 (29%)
PC-1 (53%)
0.02
0.01
0
-0.01
-0.02
0h
4h
12h

(b)

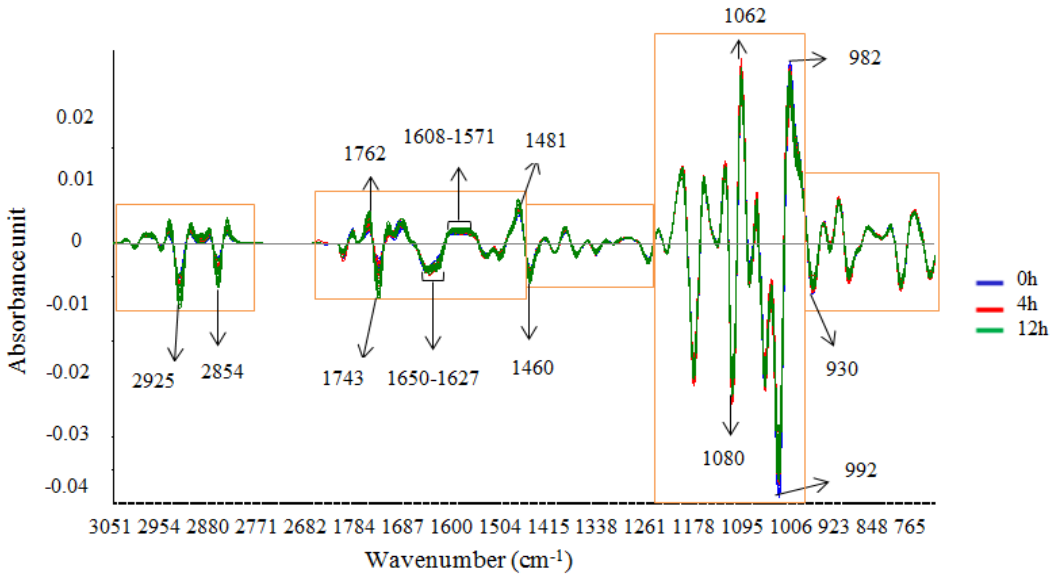

Scores
PC-2 (29%)
PC-1 (53%)
0.02
0.01
0
-0.01
-0.02
-0.02
-0.01
0
0.01
0.02
High
Low

(c)

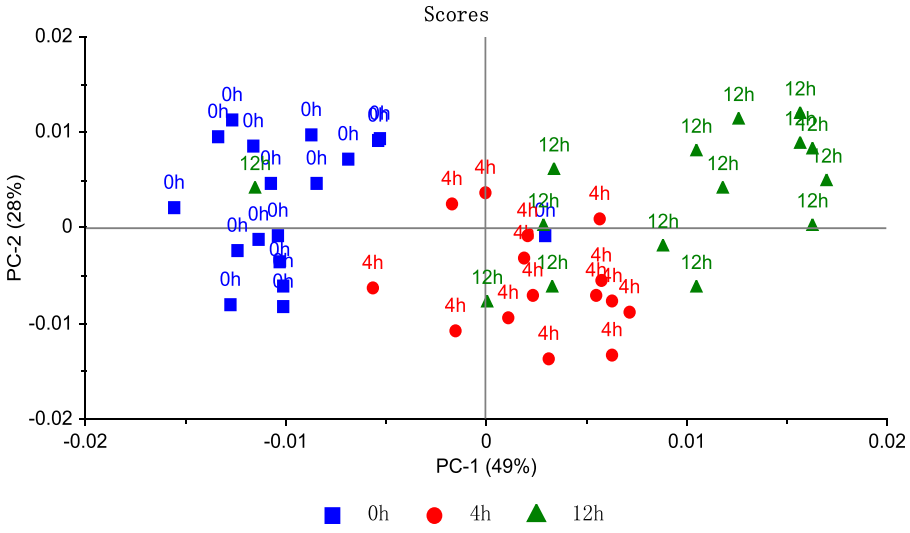

PC1-Diastatic power
Loading value
Wavenumber (cm-1)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3,051 2,955 2,858 2,762 2,665 1,761 1,664 1,568 1,472 1,375 1,279 1,182 1,086 989 893 797 700
1762
1481
1062
982
930
2923 2852
1743
1460
964

(d)

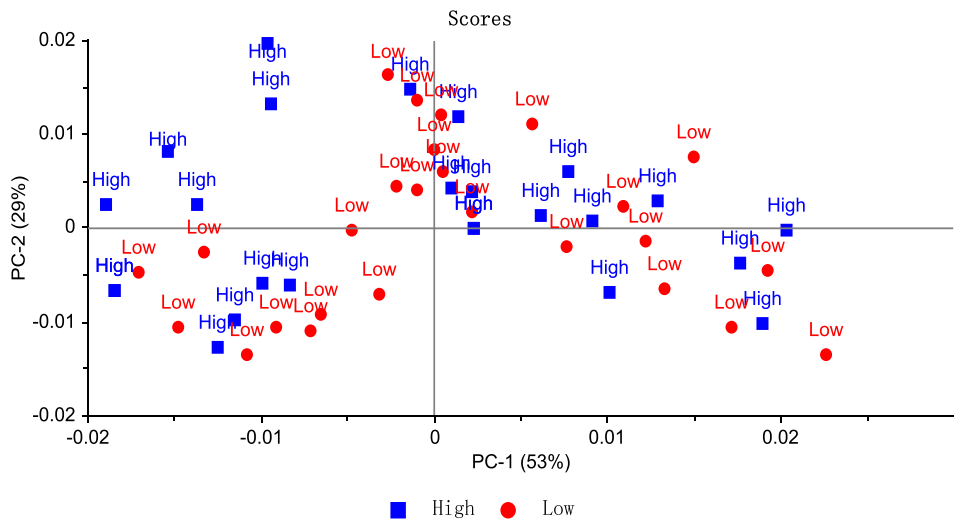
Figure 3–1. Spectra analysis and principle component analysis (PCA) of selected ATR-FTIR spectra (3050-700 cm^{-1}) of barley samples selected for high and low values of diastatic power (DP) at 0 (original barley grain), 4 and 16h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Spectra analysis-separation of spectra according to incubation time point; (b) Scores plot of PCA-grouping of spectra according to incubation time point; (c) Scores plot of PCA-lack of grouping of spectra according to level of DP; (d) Loadings plot of PCA-the origin of variations in ATR-FTIR spectra based on recorded wavenumbers. Each spectrum (line) represents an individual sample. Orange boxes indicate the areas of five sub-divided regions.


1062
982
0.02
0.01
1762
1608-1571
1481
0
-0.01
-0.02
-0.03
-0.04
Absorbance unit
2925
2854
1743
1650-1627
1460
930
1080
992
0h
4h
12h
3051 2954 2880 2771 2682 1784 1687 1600 1504 1415 1338 1261 1178 1095 1006 923 848 765
Wavenumber (cm-1)

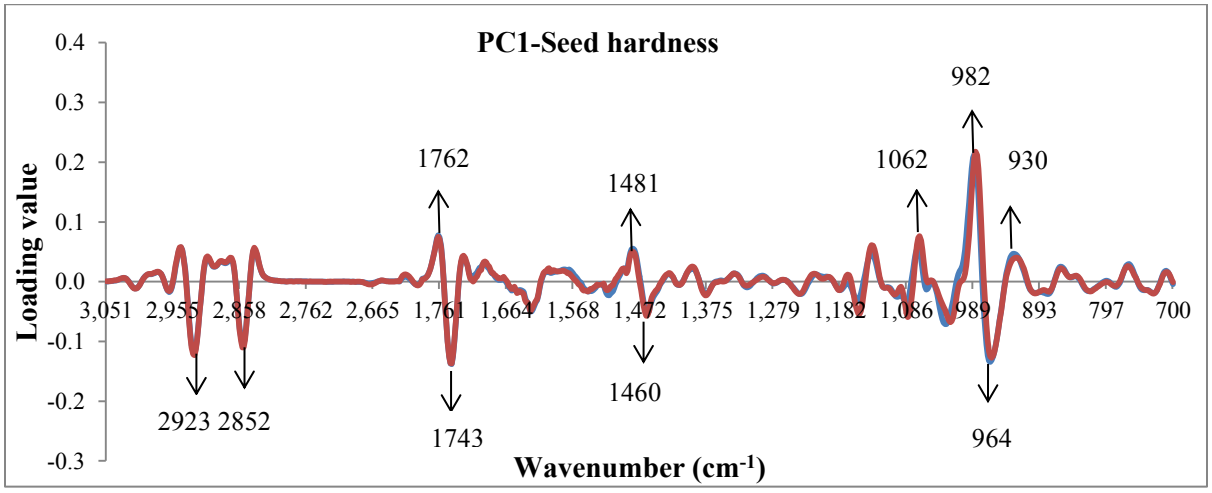
(a)


Scores
0.02
0.01
0
-0.01
-0.02
PC-2 (28%)
-0.02
-0.01
0
0.01
0.02
PC-1 (49%)
0h
4h
12h

(b)

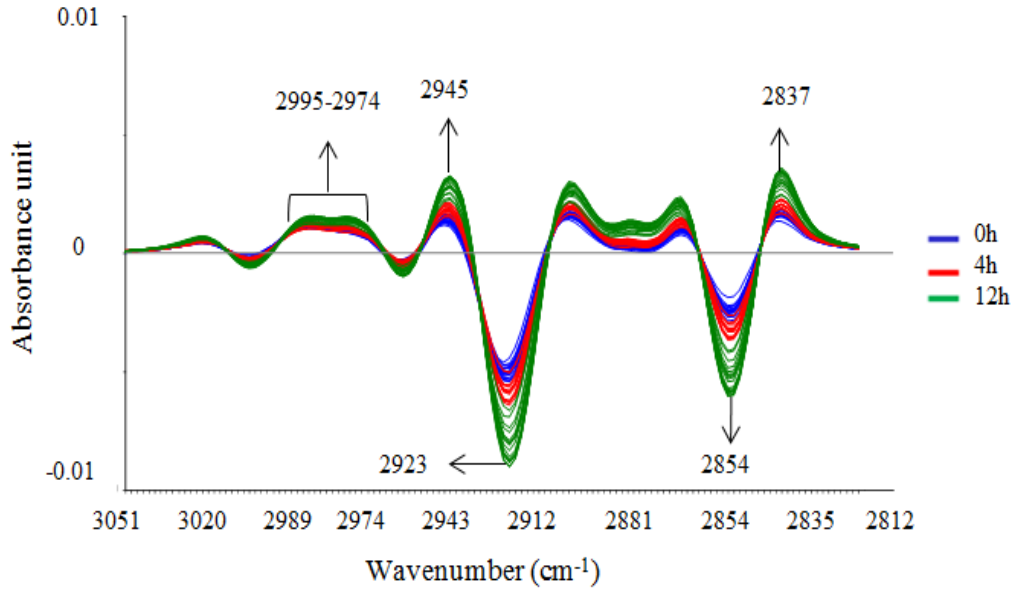

Scores
PC-2 (29%)
PC-1 (53%)
0.02
0.01
0
-0.01
-0.02
-0.02
-0.01
0
0.01
0.02
High
Low

(c)

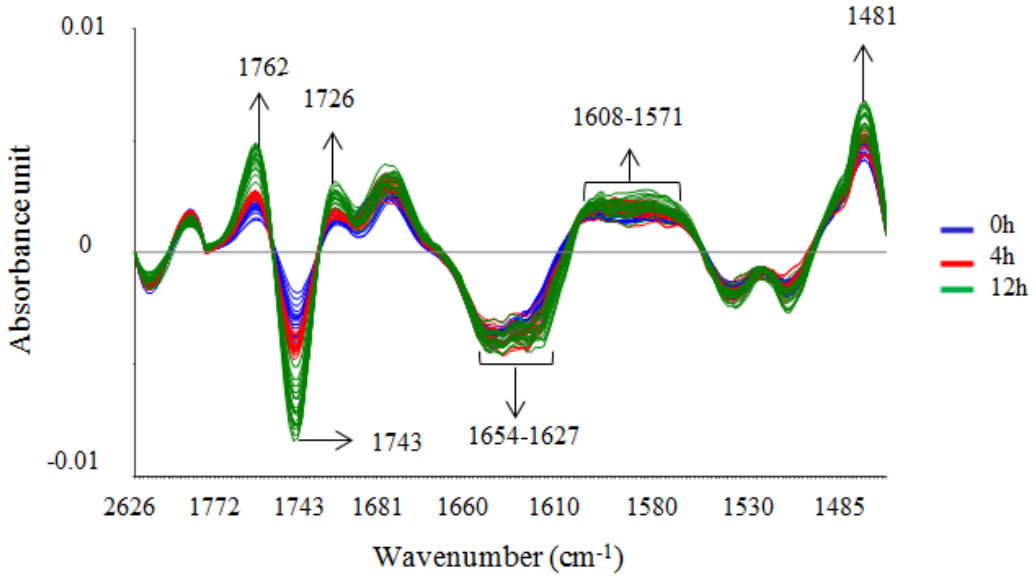

PC1-Seed hardness
Loading value
Wavenumber (cm-1)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3,051 2,955 2,858 2,762 2,665 1,761 1,664 1,568 1,472 1,375 1,279 1,182 1,086 989 893 797 700
2923
2852
1762
1743
1481
1460
1062
982
964
930

(d)

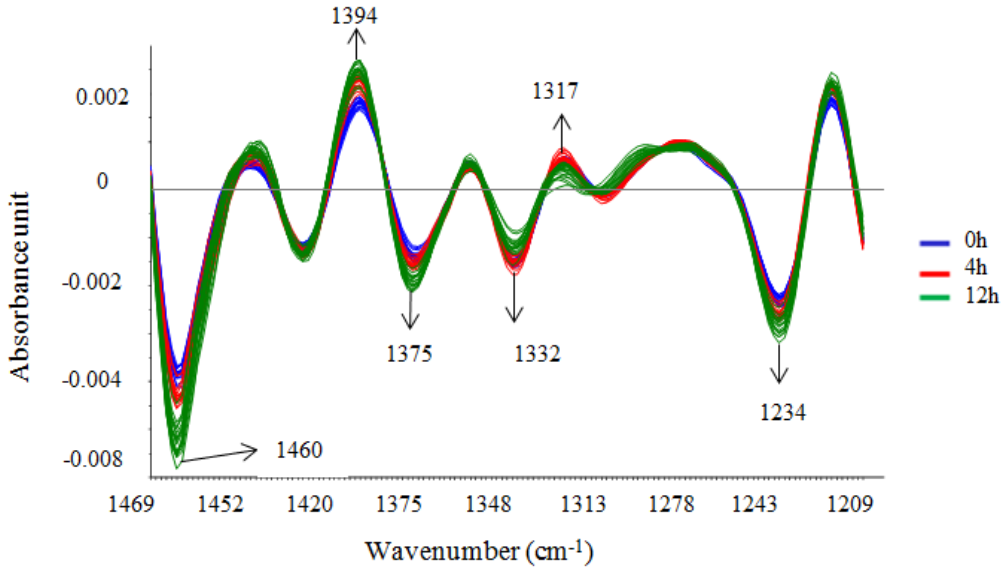
Figure 7–2. Spectra analysis and principle component analysis (PCA) of selected ATR-FTIR spectra (3050-700 cm^{-1}) of barley samples selected for high and low values of seed hardness (SH) at 0 (original barley grain), 4 and 18h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Spectra analysis-separation of spectra according to incubation time point; (b) Scores plot of PCA-grouping of spectra according to incubation time point; (c) Scores plot of PCA-lack of grouping of spectra according to level of SH; (d) Loadings plot of PCA-the origin of variations in ATR-FTIR spectra based on recorded wavenumbers. Each spectrum (line) represents an individual sample. Orange boxes indicate the areas of five sub-divided regions.


0.01
0
-0.01
Absorbance unit
2995-2974
2945
2837
2923
2854
0h
4h
12h
3051
3020
2989
2974
2943
2912
2881
2854
2835
2812
Wavenumber (cm-1)

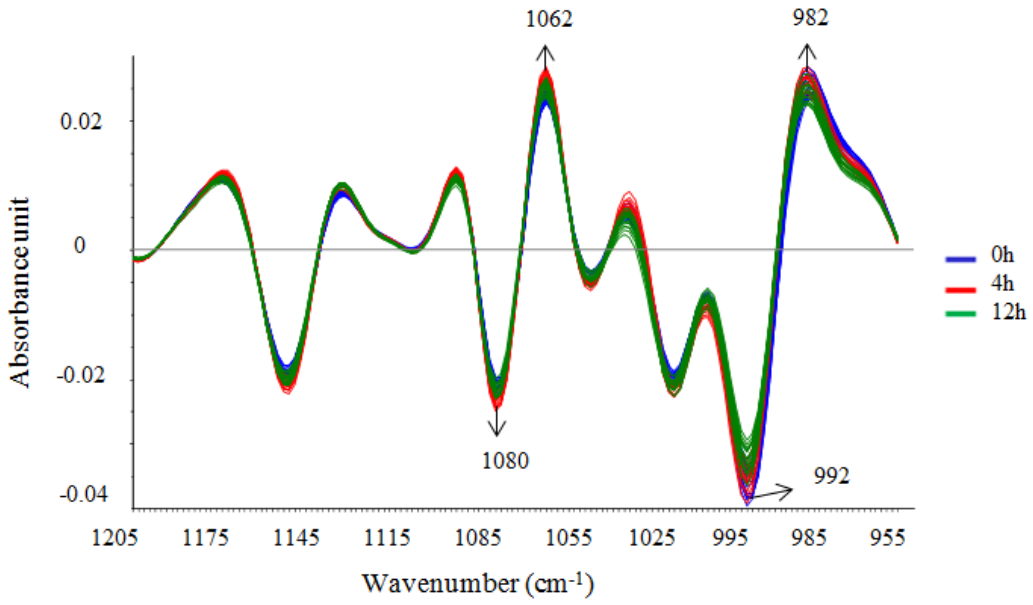
(a)


0.01
0
-0.01
Absorbance unit
1762
1726
1608-1571
1481
1743
1654-1627
0h
4h
12h
2626
1772
1743
1681
1660
1610
1580
1530
1485
Wavenumber (cm-1)

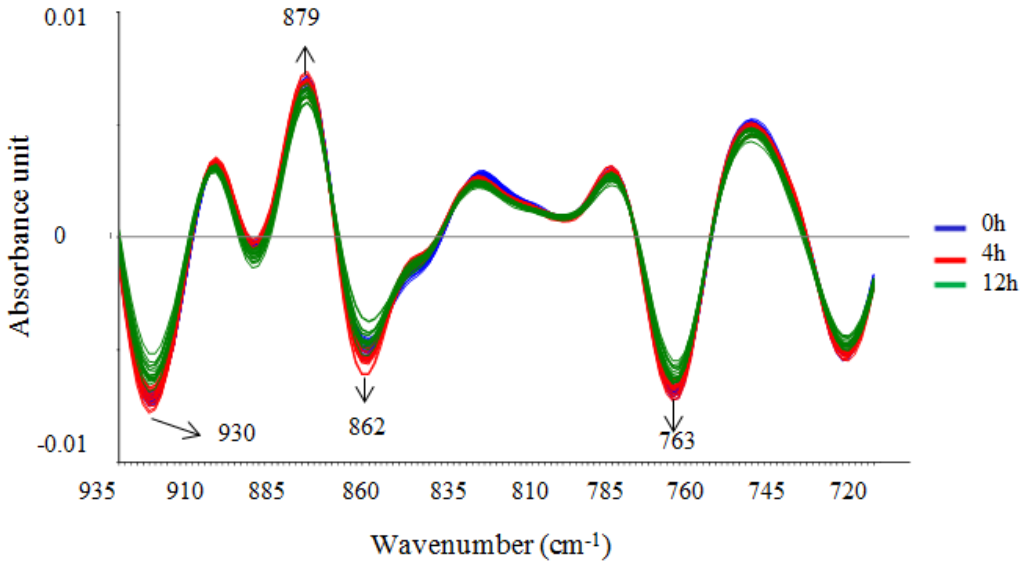
(b)


1394
1317
0.002
0
-0.002
-0.004
-0.008
Absorbance unit
1375
1332
1234
1460
1469
1452
1420
1375
1348
1313
1278
1243
1209
Wavenumber (cm-1)
0h
4h
12h

(c)


1062
982
0.02
0
-0.02
-0.04
Absorbance unit
1080
992
1205
1175
1145
1115
1085
1055
1025
995
985
955
Wavenumber (cm-1)
0h
4h
12h

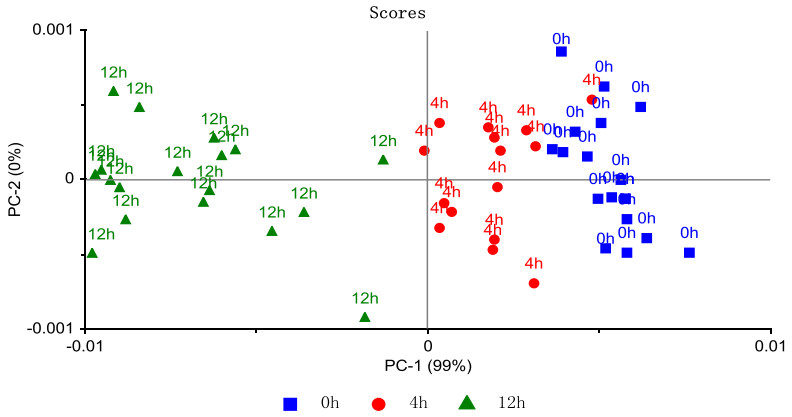
(d)



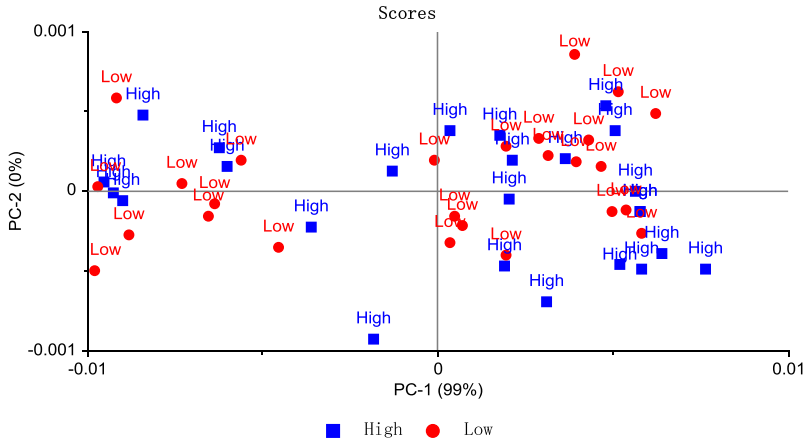
(e)

Figure 3–3 Spectra analysis of five sub-divided regions of ATR-FTIR spectra of barley samples selected for high and low values of diastatic power at 0 (original barley grain), 4 and 12h of *in situ* rumen incubation, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Region 1: 3050-2812 cm^{-1}; (b) Region 2: 2626-1471 cm^{-1}; (c) Region 3: 1469-1207 cm^{-1}; (d) Region 4:1205-941 cm^{-1}; (e) Region 5: 937-650 cm^{-1}. Spectra of each region were separated according to incubation time point (0, 4 and 12h) of *in situ* rumen digestion at certain wavenumbers. Each spectrum (line) represents a sample.

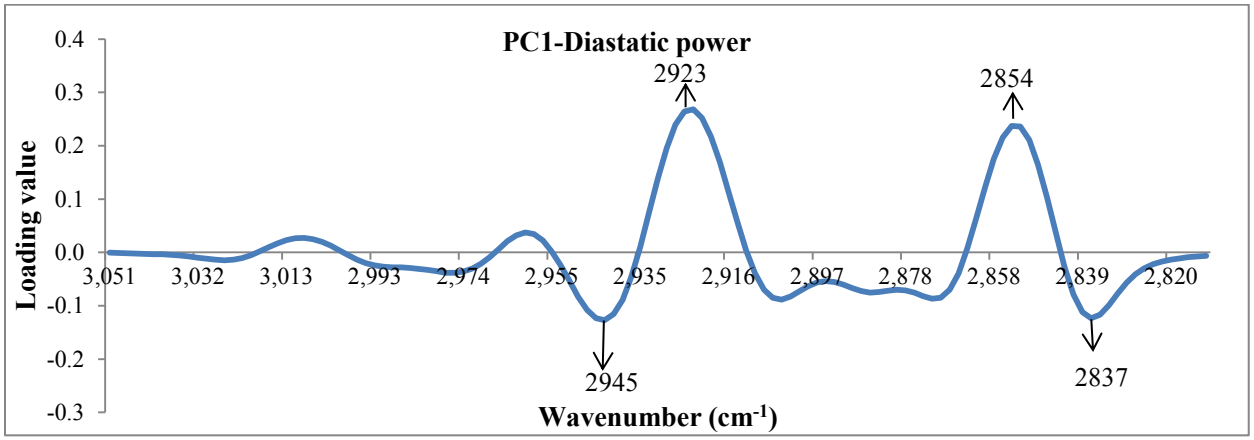
Region 1


Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.01
0
0.01
0h
4h
12h

(a)

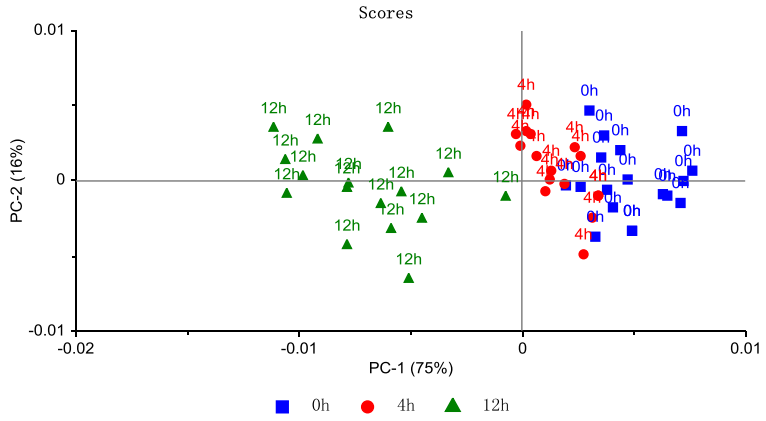

Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.01
0
0.01
High
Low

(b)

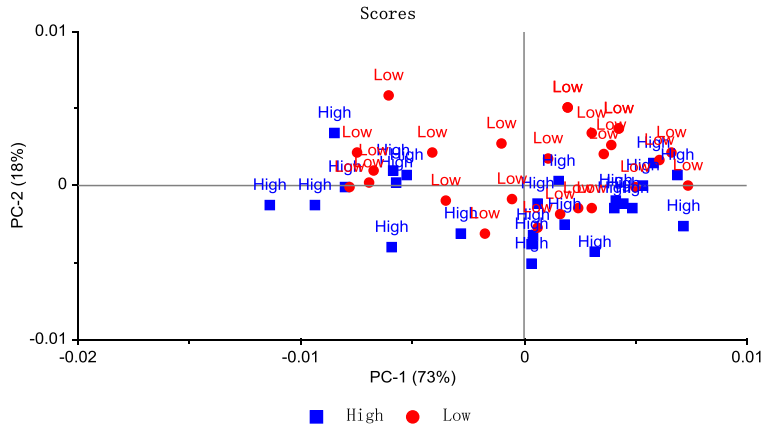

PC1-Diastatic power
Loading value
Wavenumber (cm-1)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3,051
3,032
3,013
2,993
2,974
2,955
2,935
2,916
2,897
2,878
2,858
2,839
2,820
2923
2854
2945
2837

(c)

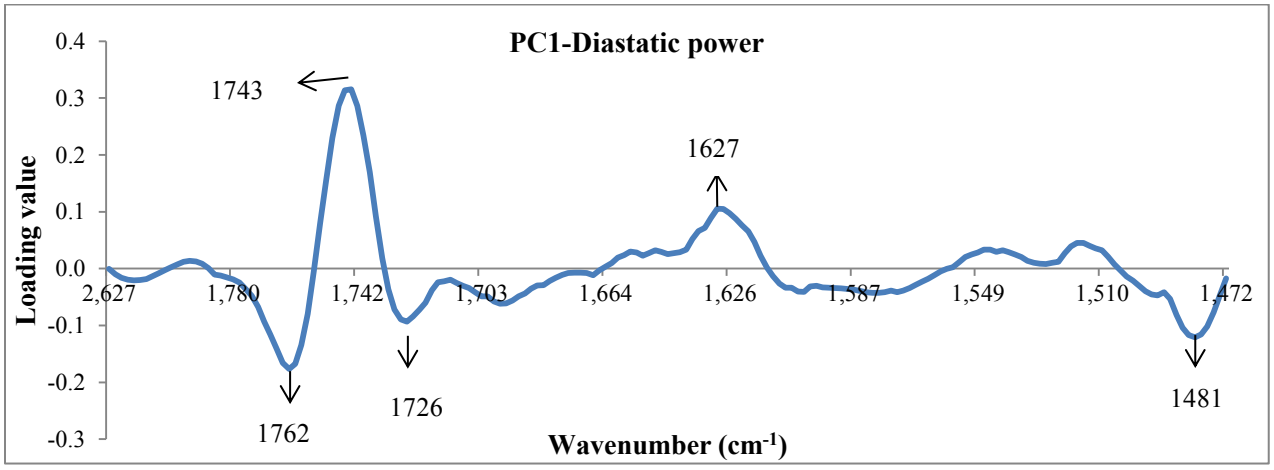
Region 2


Scores
PC-2 (16%)
PC-1 (75%)
0h
4h
12h

(d)

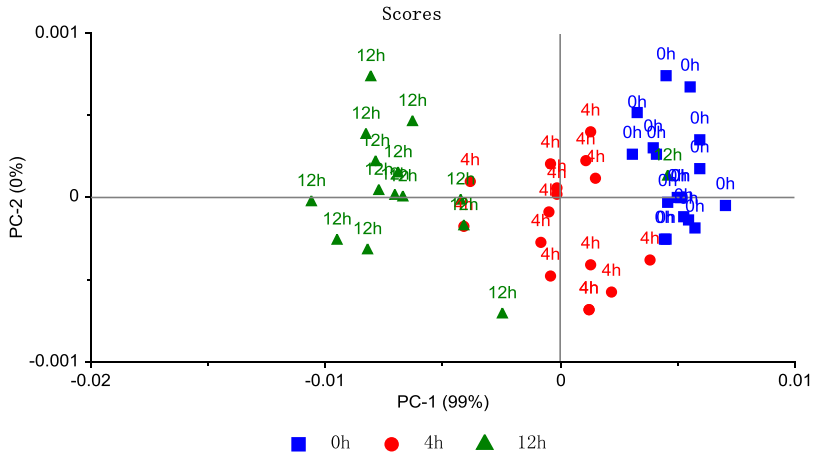

Scores
PC-2 (18%)
PC-1 (73%)
High
Low

(e)

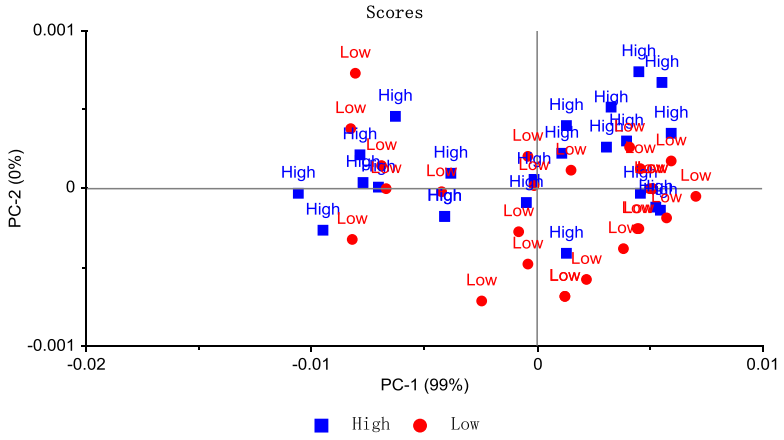

PC1-Diastatic power
Loading value
Wavenumber (cm-1)
1743
1762
1726
1627
1481
2,627
1,780
1,742
1,703
1,664
1,626
1,587
1,549
1,510
1,472

(f)

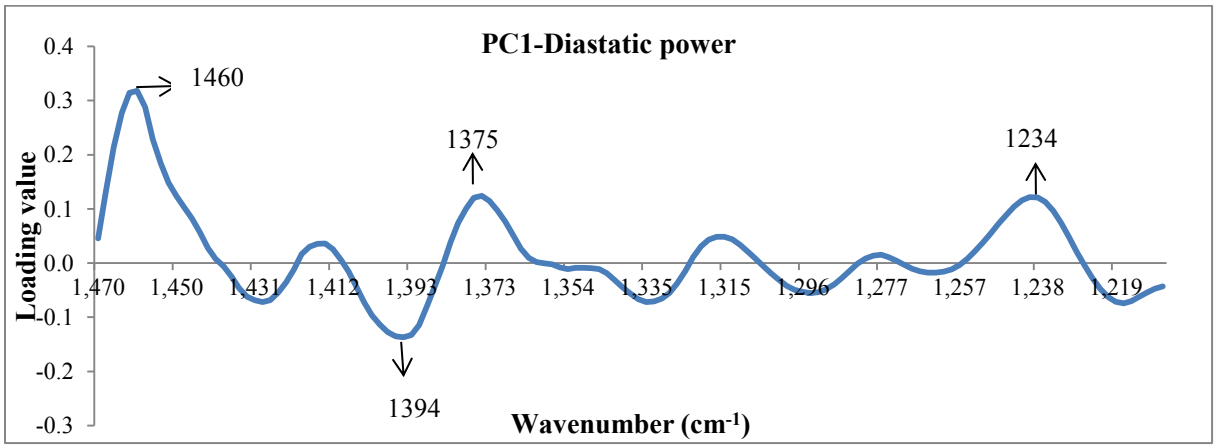
Region 3


Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.02
-0.01
0
0.01
0h
4h
12h

(g)

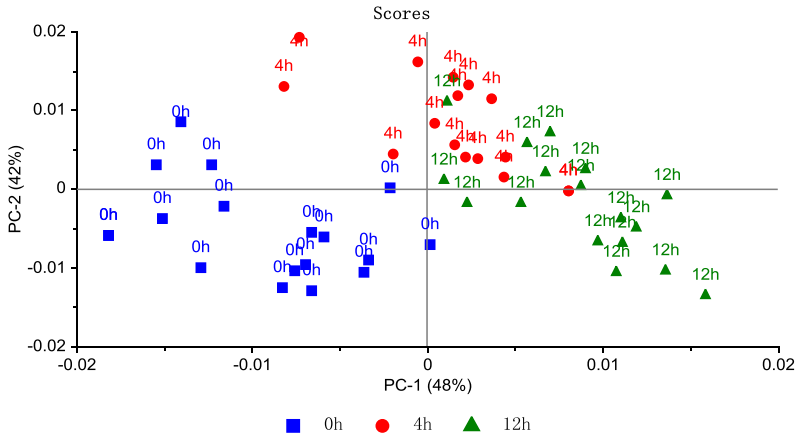

Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.02
-0.01
0
0.01
High
Low

(h)

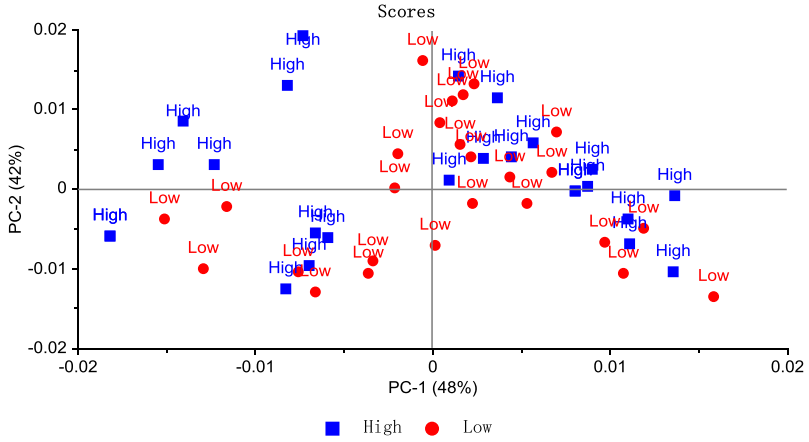

PC1-Diastatic power
Loading value
Wavenumber (cm-1)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
1460
1375
1234
1394
1,470
1,450
1,431
1,412
1,393
1,373
1,354
1,335
1,315
1,296
1,277
1,257
1,238
1,219

(i)

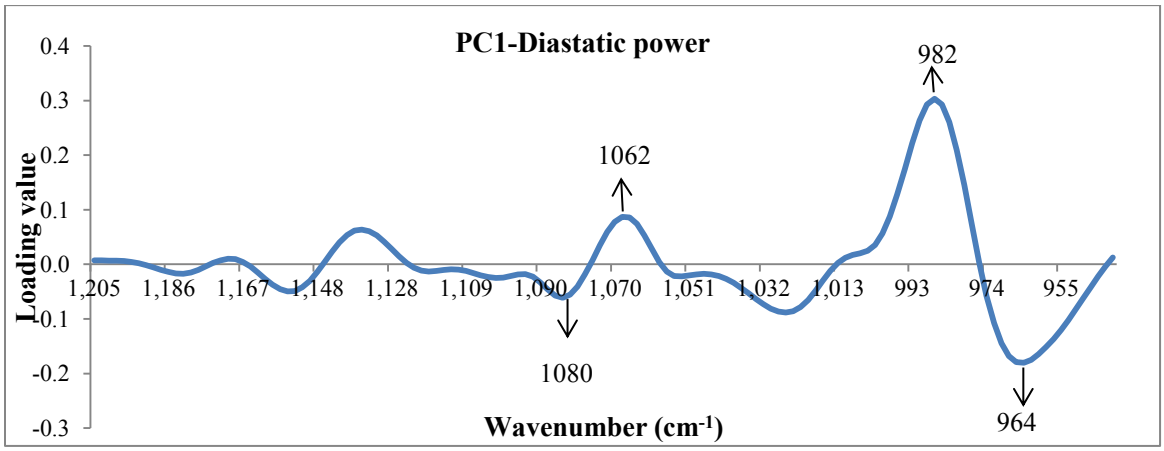
Region 4


Scores
PC-2 (42%)
PC-1 (48%)
0h
4h
12h

(j)

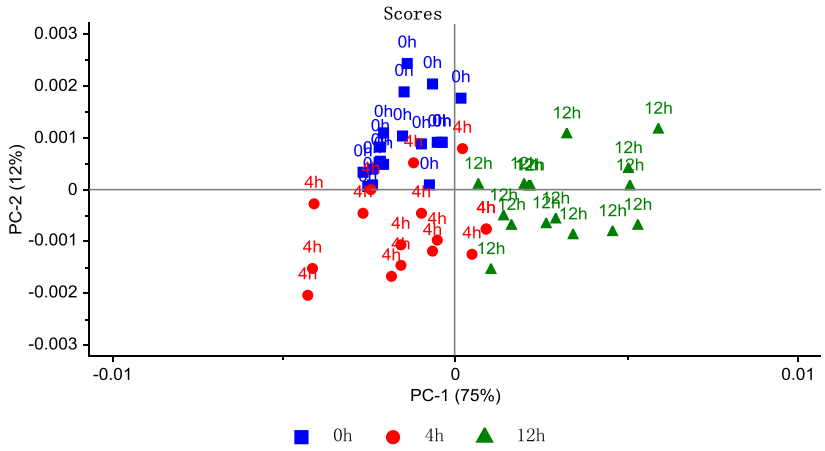

Scores
PC-2 (42%)
PC-1 (48%)
High
Low

(k)

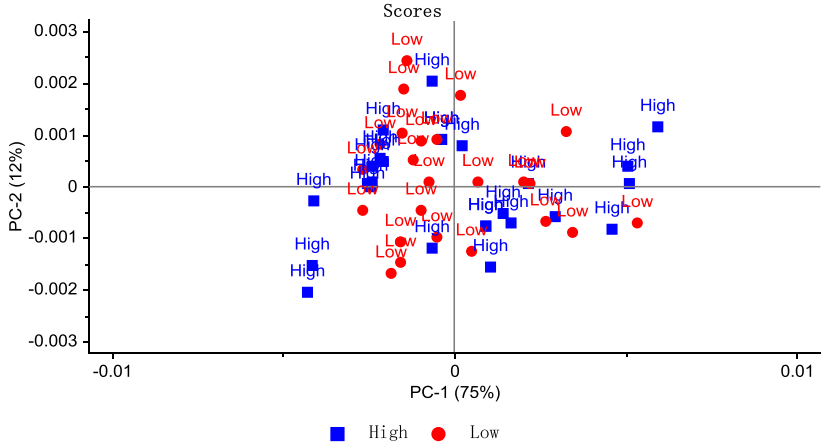

PC1-Diastatic power
Loading value
Wavenumber (cm-1)
982
1062
1080
964

(l)

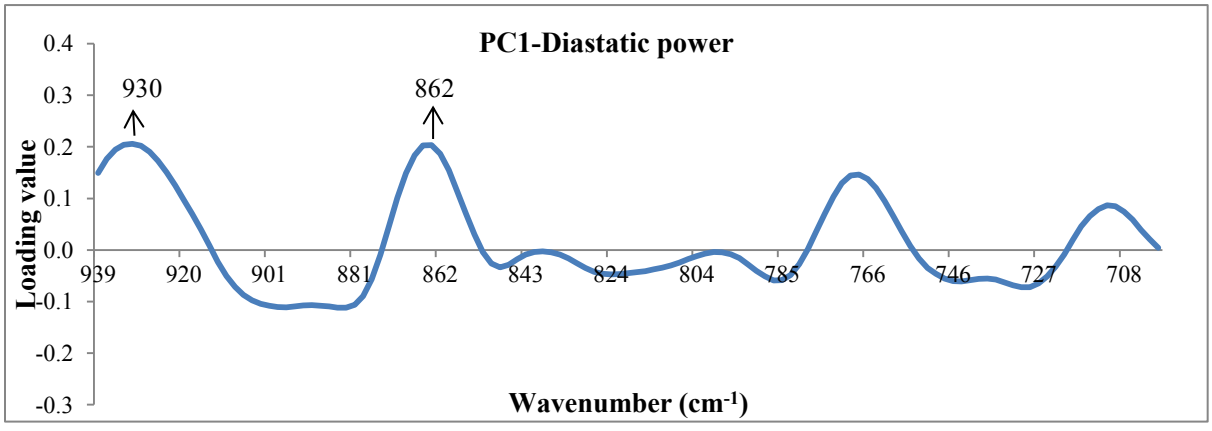
Region 5


Scores
PC-2 (12%)
PC-1 (75%)
0h
4h
12h

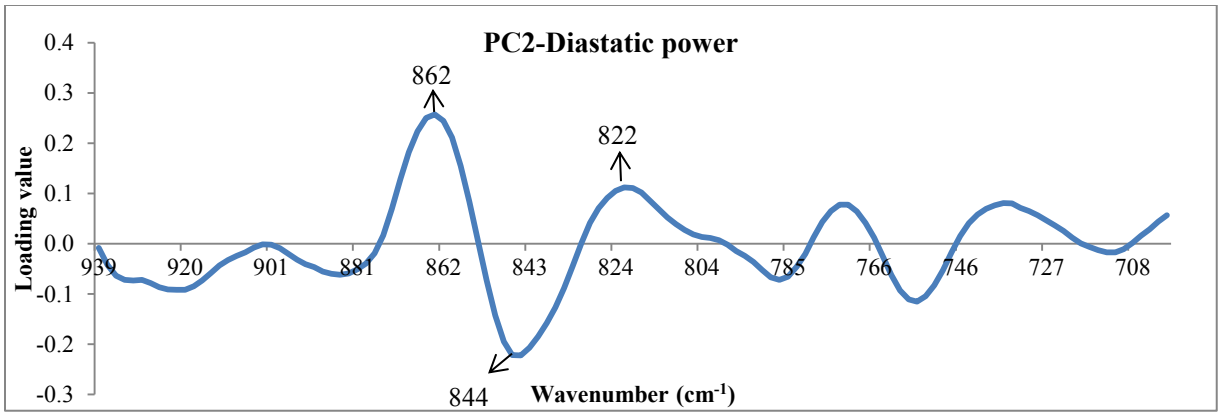
(m)


Scores
PC-2 (12%)
PC-1 (75%)
High
Low

(n)


PC1-Diastatic power
930
862
Loading value
Wavenumber (cm-1)

(o)



(p)

Figure 3–4. Scores and loadings plots of principle component analysis (PCA) based on five sub-divided regions of ATR-FTIR spectra at 0 (original barley grain), 4 and 12 h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative, using samples selected for high and low diastatic power (DP).

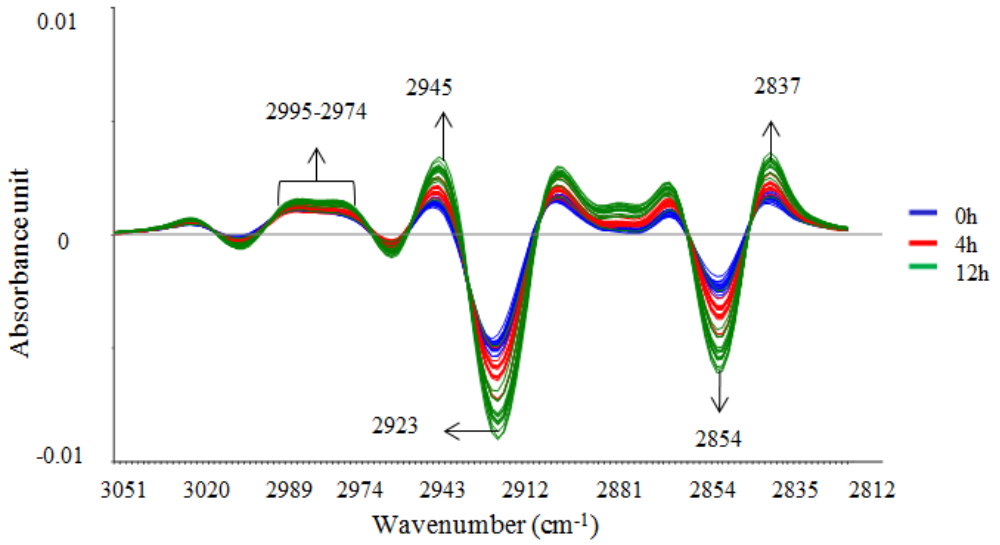
Region 1 3050-2812 cm^{-1}: a) Scores plot of PCA-spectra grouped by incubation time point; b) Scores plot of PCA-lack of spectra grouping by level of DP; C) PC-1 loadings plot of PCA-origin of variation in the spectra in region 1.

Region 2 2626-1471 cm^{-1}: d) Scores plot of PCA-spectra grouped by incubation time point; e) Scores plot of PCA-lack of spectra grouping by level of DP; f) PC-1 loadings plot of PCA-origin of variation in the spectra in region 2.

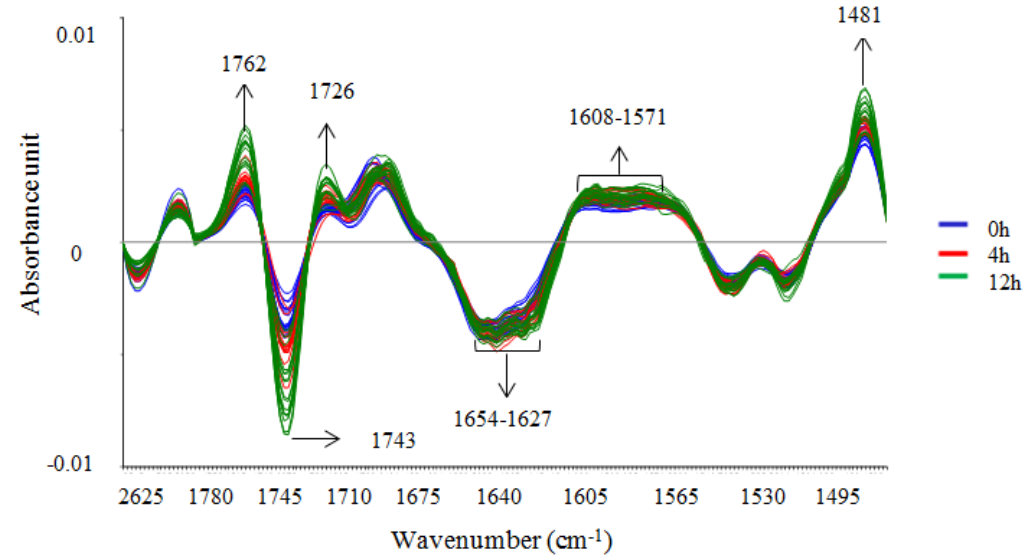
Region 3 1469-1207 cm^{-1}: g) Scores plot of PCA-spectra grouped by incubation time point-; h) Scores plot of PCA-lack of grouping by level of DP; i) PC-1 loadings plot of PCA-origin of variation in the spectra in region 3.

Region 4 1205-941 cm^{-1}: j) Scores plot of PCA-spectra grouped by incubation time point-DP; k) Scores plot of PCA-lack of spectra grouping by level of DP; l) PC-1 loadings plot of PCA-origin of variation in the spectra in region 4.

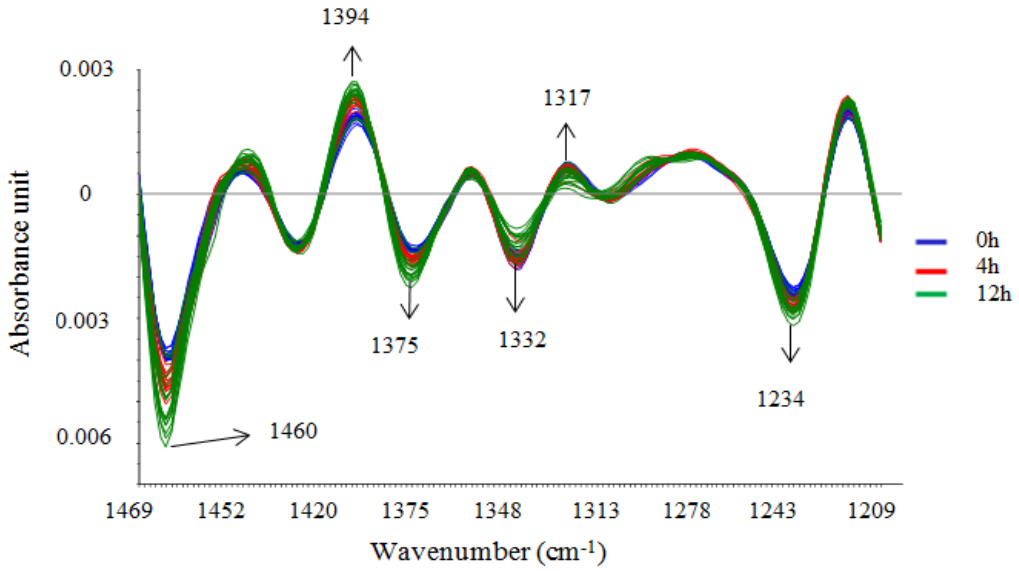
Region 5 937-650 cm^{-1}: m) Scores plot of PCA- spectra grouped by incubation time point-DP; n) Scores plot of PCA-lack of spectra grouping by level of DP; o) PC-1 loadings plot of PCA-origin of variation in the spectra in region 5. p) PC-2 loadings plot of PCA-origin of variation in the spectra in region 5.


0.01
0
-0.01
Absorbance unit
2995-2974
2945
2837
2923
2854
3051
3020
2989
2974
2943
2912
2881
2854
2835
2812
Wavenumber (cm-1)
0h
4h
12h

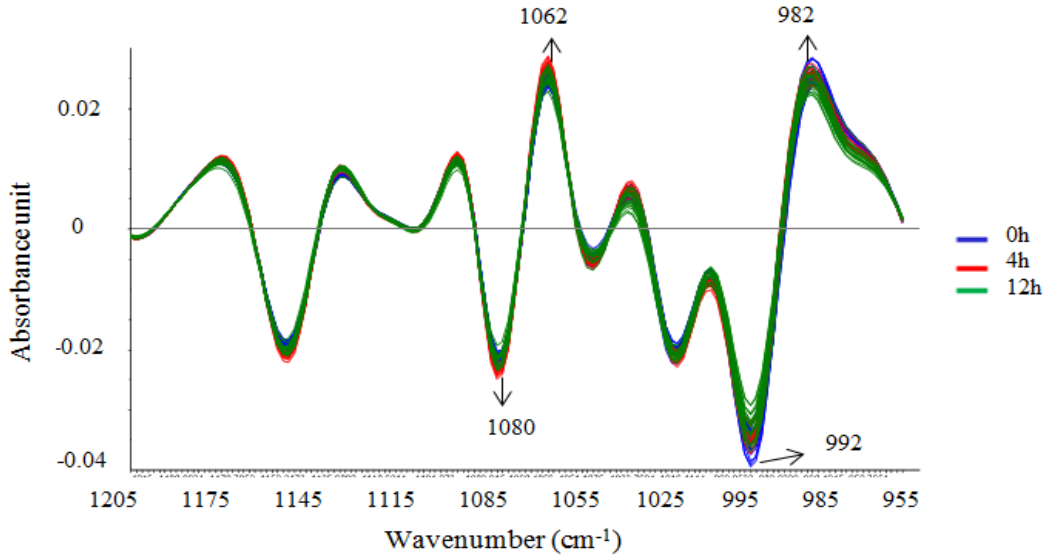
(a)


0.01
0
-0.01
Absorbance unit
1762
1726
1608-1571
1481
1654-1627
1743
2625
1780
1745
1710
1675
1640
1605
1565
1530
1495
Wavenumber (cm-1)
0h
4h
12h

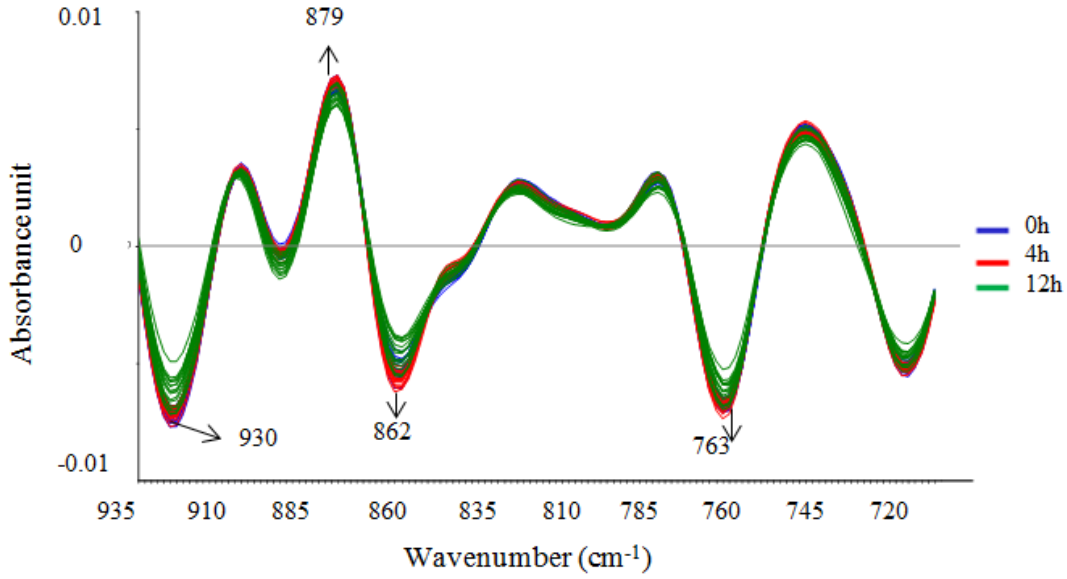
(b)


1394
1317
0.003
0
0.003
0.006
Absorbance unit
1375
1332
1234
1460
0h
4h
12h
1469
1452
1420
1375
1348
1313
1278
1243
1209
Wavenumber (cm-1)

(c)


1062
982
0.02
0
-0.02
-0.04
Absorbance unit
1080
992
0h
4h
12h
1205
1175
1145
1115
1085
1055
1025
995
985
955
Wavenumber (cm-1)

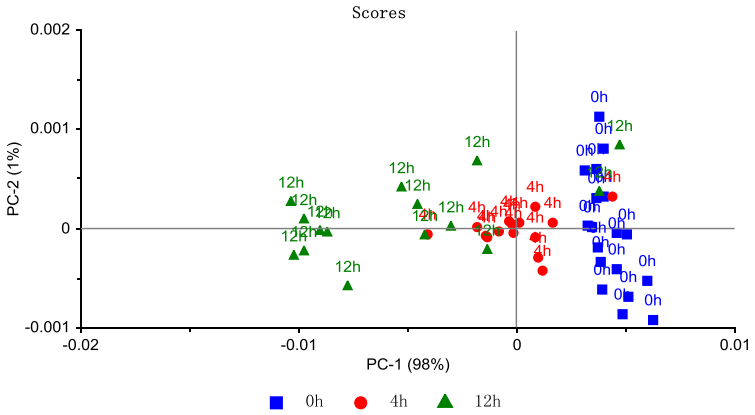
(d)



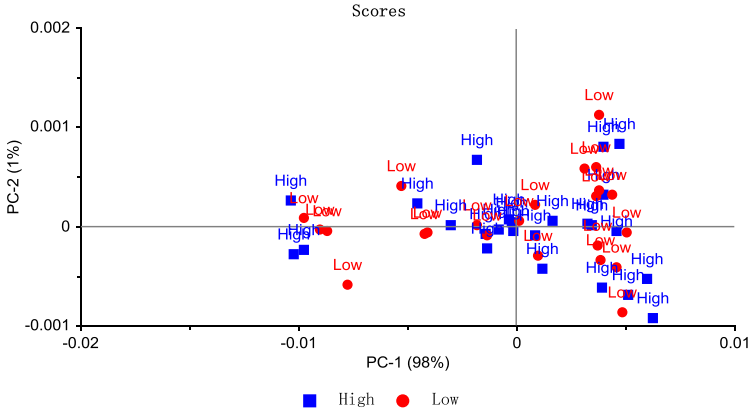
(e)

Figure 3–5. Spectra analysis of five sub-divided regions of ATR-FTIR spectra of barley samples selected for high and low values of seed hardness at 0 (original barley grain), 4 and 12h of *in situ* rumen incubation, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Region 1: 3050-2812 cm^{-1}; (b) Region 2: 2626-1471 cm^{-1}; (c) Region 3: 1469-1207 cm^{-1}; (d) Region 4:1205-941 cm^{-1}; (e) Region 5: 937-650 cm^{-1}. Spectra of each region were separated according to incubation time point (0, 4 and 12h) of *in situ* rumen digestion at certain wavenumbers. Each spectrum (line) represents a sample.

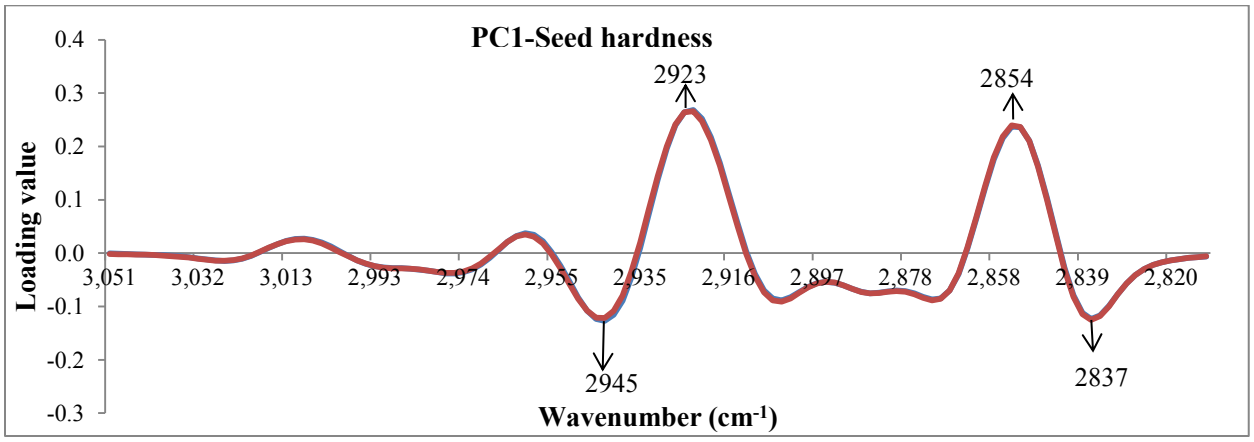
Region 1


Scores
PC-2 (1%)
PC-1 (98%)
0.002
0.001
0
-0.001
-0.02
-0.01
0
0.01
0h
4h
12h

(a)

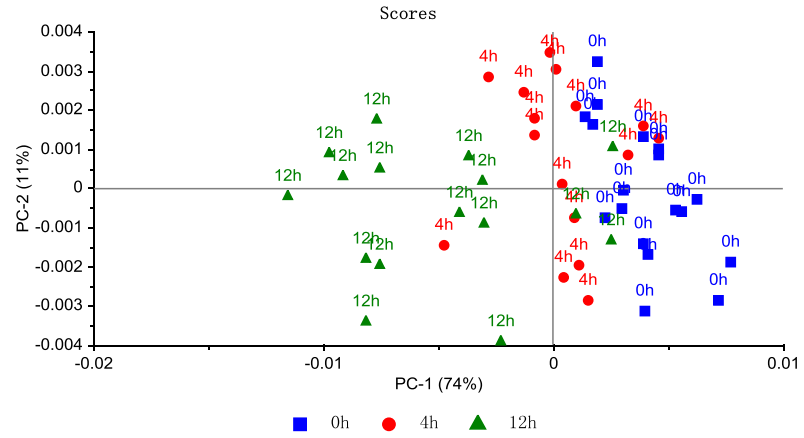

Scores
PC-2 (1%)
PC-1 (98%)
0.002
0.001
0
-0.001
-0.02
-0.01
0
0.01
High
Low

(b)

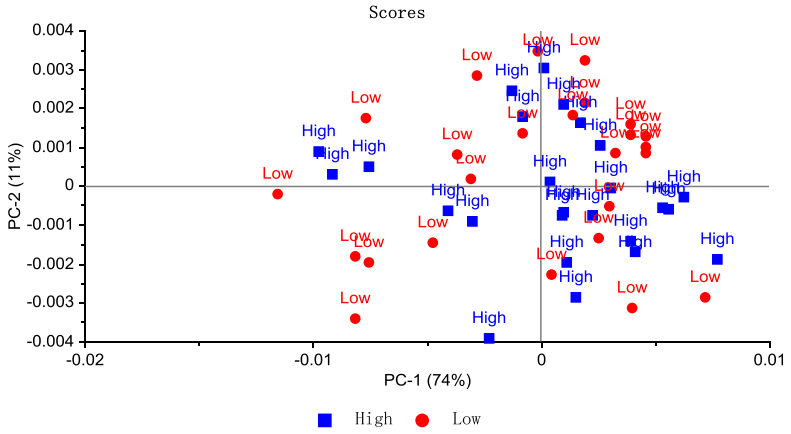

PC1-Seed hardness
Loading value
Wavenumber (cm-1)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3,051
3,032
3,013
2,993
2,974
2,955
2,935
2,916
2,897
2,878
2,858
2,839
2,820
2923
2854
2945
2837

(c)

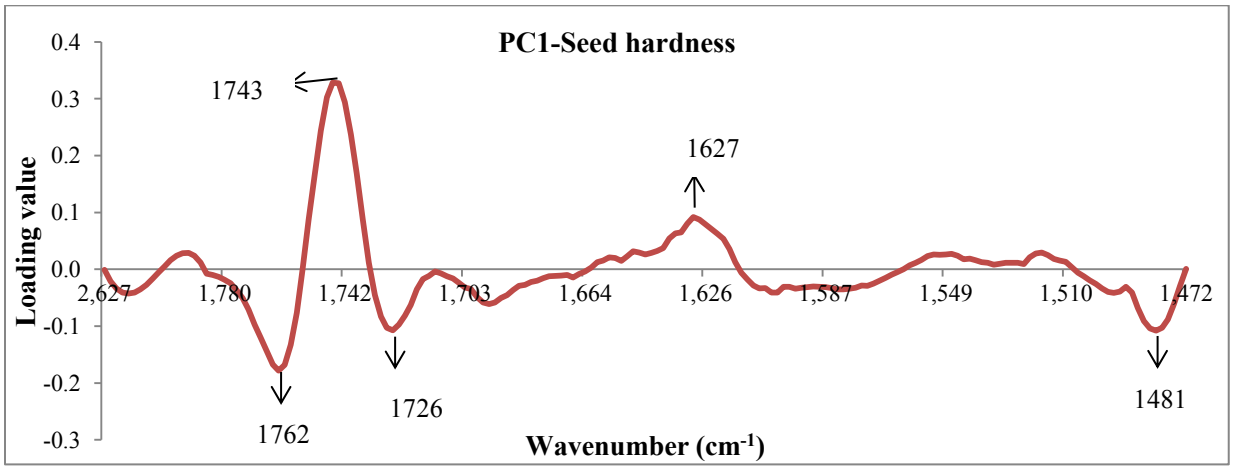
Region 2


Scores
PC-2 (11%)
PC-1 (74%)
0h
4h
12h

(d)

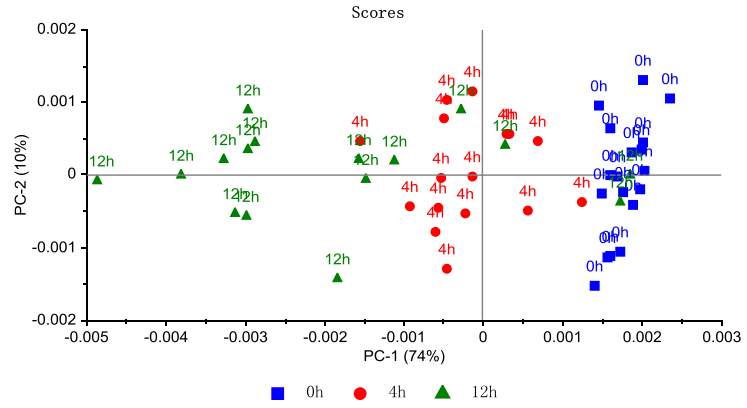

Scores
PC-2 (11%)
PC-1 (74%)
High
Low

(e)

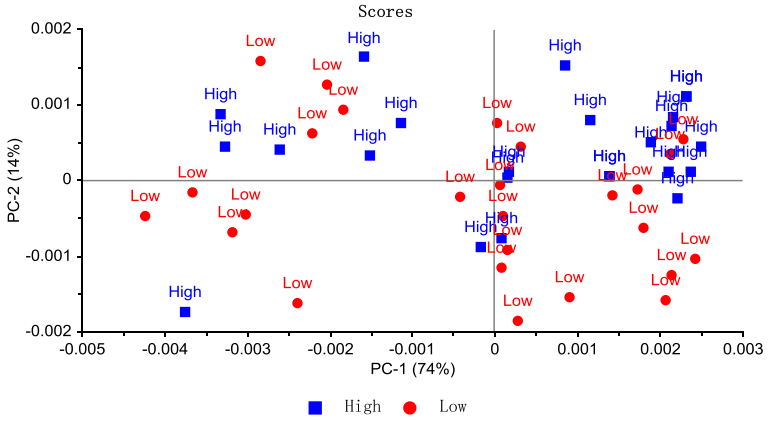

PC1-Seed hardness
Loading value
Wavenumber (cm-1)
1743
1762
1726
1627
1481

(f)

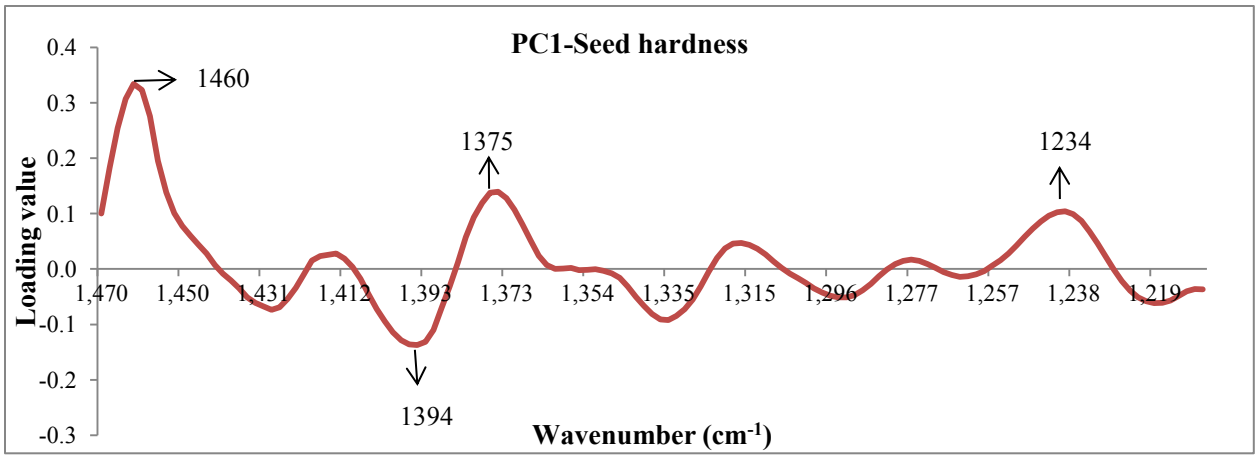
Region 3


Scores
PC-2 (10%)
PC-1 (74%)
0h
4h
12h

(g)

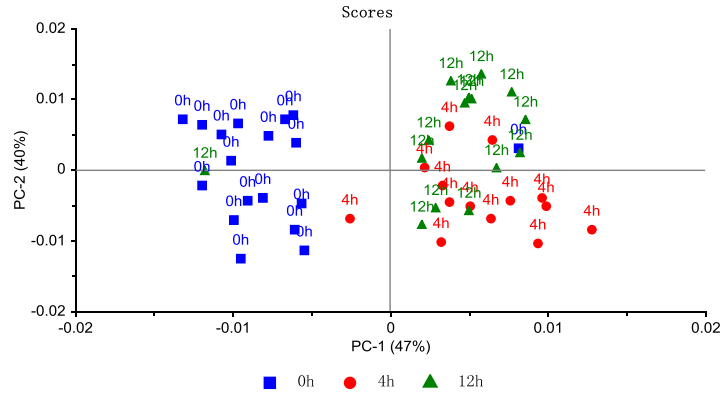

Scores
PC-2 (14%)
PC-1 (74%)
High
Low

(h)

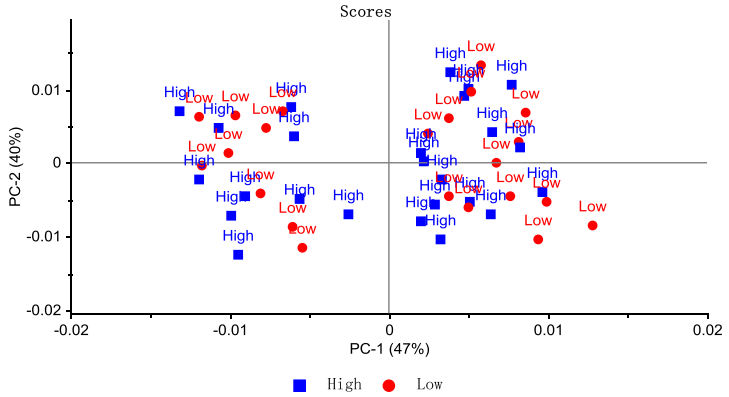

PC1-Seed hardness
Loading value
Wavenumber (cm-1)
1460
1375
1234
1394

(i)

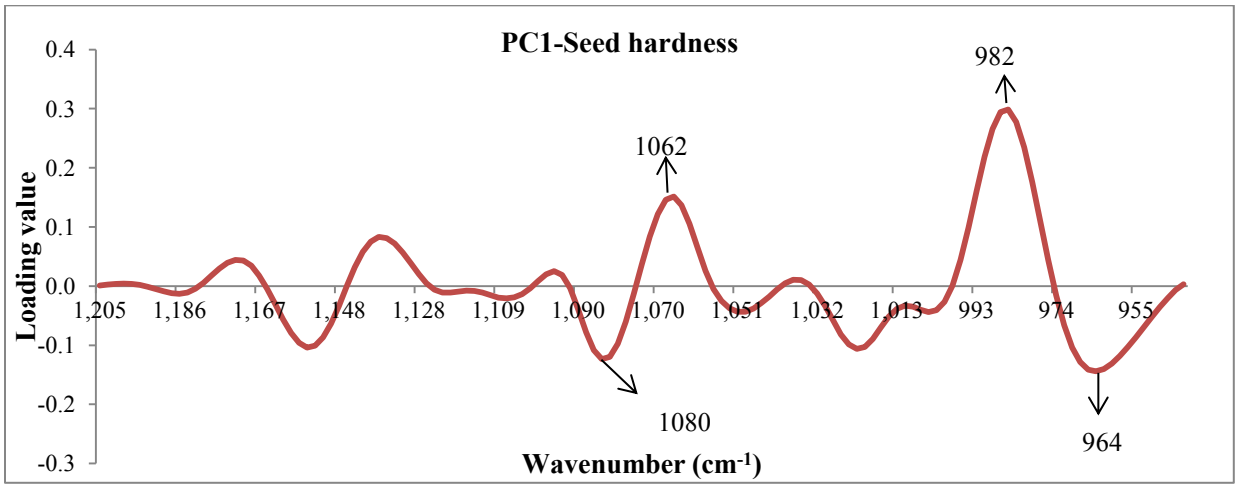
Region 4


Scores
PC-2 (40%)
PC-1 (47%)
0h
4h
12h

(j)

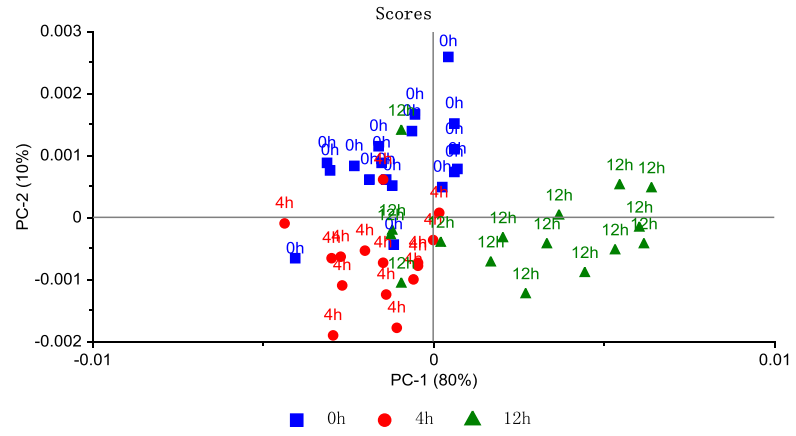

Scores
PC-2 (40%)
PC-1 (47%)
High
Low

(k)

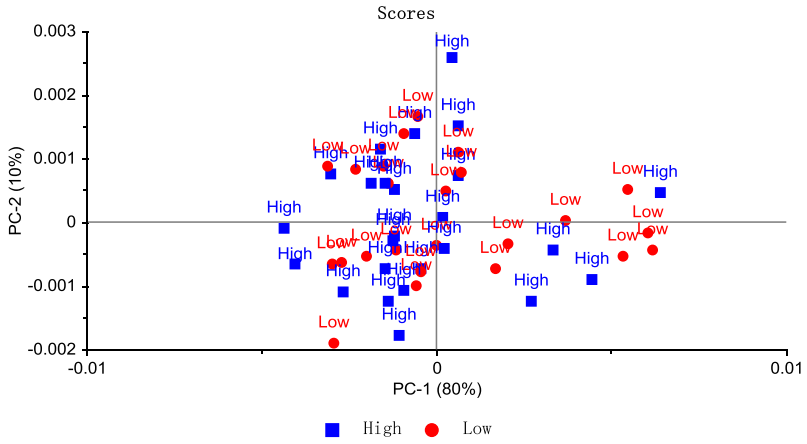

PC1-Seed hardness
Loading value
Wavenumber (cm-1)
982
1062
1080
964

(l)

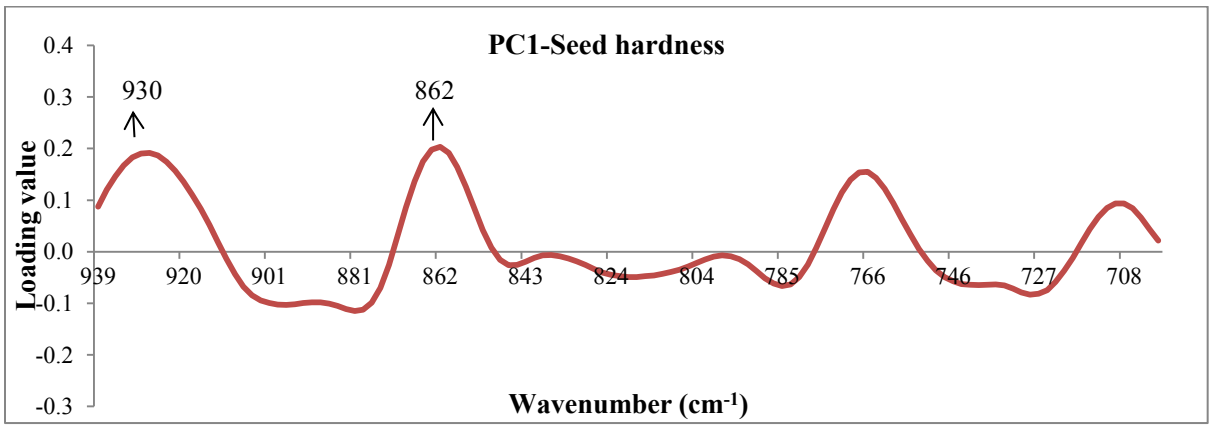
Region 5


Scores
PC-2 (10%)
PC-1 (80%)
0.003
0.002
0.001
0
-0.001
-0.002
-0.01
0
0.01
0h
4h
12h

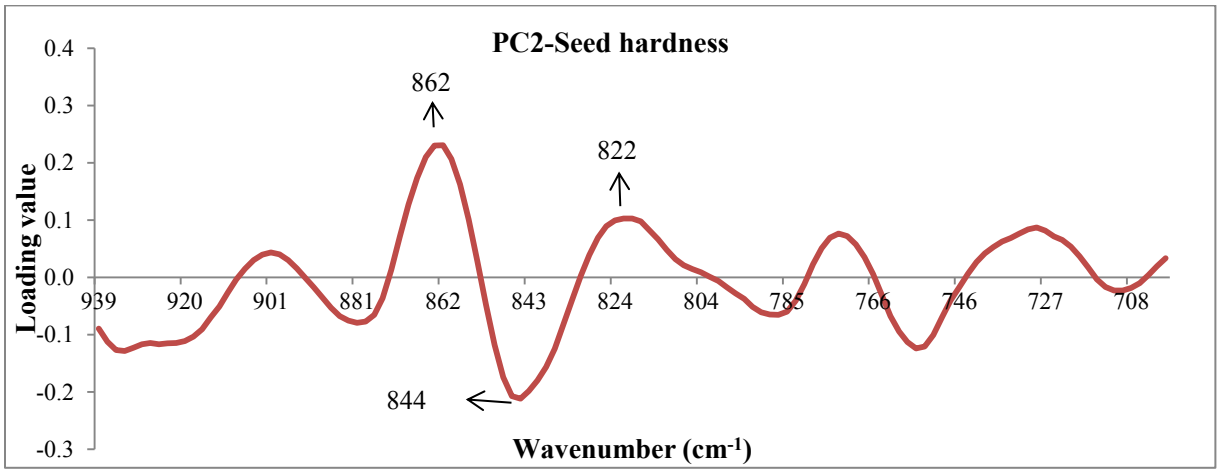
(m)


Scores
PC-2 (10%)
PC-1 (80%)
0.003
0.002
0.001
0
-0.001
-0.002
-0.01
0
0.01
High
Low

(n)


PC1-Seed hardness
Loading value
Wavenumber (cm-1)
930
862
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
939
920
901
881
862
843
824
804
785
766
746
727
708

(o)



(p)

Figure 3–6. Scores and loadings plots of principle component analysis (PCA) based on five sub-divided regions of ATR-FTIR spectra at 0 (original barley grain), 4 and 12 h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative, using samples selected for high and low seed hardness (SH).

Region 1 3050-2812 cm^{-1}: a) Scores plot of PCA-spectra grouped by incubation time point; b) Scores plot of PCA-lack of spectra grouping by level of SH; C) PC-1 loadings plot of PCA-origin of variation in the spectra in region 1.

Region 2 2626-1471 cm^{-1}: d) Scores plot of PCA-spectra grouped by incubation time point; e) Scores plot of PCA-lack of spectra grouping by level of SH; f) PC-1 loadings plot of PCA-origin of variation in the spectra in region 2.

Region 3 1469-1207 cm^{-1}: g) Scores plot of PCA-spectra grouped by incubation time point-; h) Scores plot of PCA-lack of grouping by level of SH; i) PC-1 loadings plot of PCA-origin of variation in the spectra in region 3.

Region 4 1205-941 cm^{-1}: j) Scores plot of PCA-spectra grouped by incubation time point-DP; k) Scores plot of PCA-lack of spectra grouping by level of SH; l) PC-1 loadings plot of PCA-origin of variation in the spectra in region 4.

Region 5 937-650 cm^{-1}: m) Scores plot of PCA- spectra grouped by incubation time point-DP; n) Scores plot of PCA-lack of spectra grouping by level of SH; o) PC-1 loadings plot of PCA-origin of variation in the spectra in region 5; p) PC-2 loadings plot of PCA-origin of variation in the spectra in region 5.

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CHAPTER 4 GENERAL DISCUSSION

4.1 Summary of findings and discussions

Overview

Our first study aimed to evaluate the relationship between ruminal dry matter digestibility (DMD) and seed hardness (SH) and malting characteristics (MC) of barley grain. *In situ* DMD did not vary with wort beta-glucan (WBG) content or friability (FRI). However, after 4 h of incubation, barley grain with low SH and high diastatic power (DP) had lower *in situ* DMD than those with high SH and low DP. The second study was built upon the findings from the first study, in which the same barley grain and residues from 4 and 12 h incubation were scanned using a Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (ATR-FTIR) instrument. The four attributes exhibited similar PCA grouping in which scatter plots tended to be clustered by incubation time, rather than by level of SH, DP, FRI and WBG. The grouping by incubation time could be explained by differences in certain wavenumbers: 2923, 2852, 1743, 1481, 1062, 992 and 982 cm^{-1} in PCA. These bands primarily correspond to carbohydrate components, likely amylose-associated structures in starch as shown by peaks at 992 and 982 cm^{-1}, and to a lesser extent, lignin- and lipid-related molecular structures. However, changes in protein molecular structures were not detected in ATR-FTIR spectra.

These results suggested that FRI and WBG are not considered suitable predictors of barley grain digestion in ruminants. Additionally, although DP and SH are potentially associated with improved 4 h *in situ* rumen DM digestion, neither DP nor SH affected molecular-structural conformation in barley samples before and after rumen digestion as detected by ATR-FTIR. However, the results showed an association between incubation times and alteration of carbohydrate molecular structures. The alteration of carbohydrates molecular structures as

affected by incubation times suggests the breakdown of nutrients to be available for the animals, although greater changes of carbohydrate molecular structures of barley grain may suggest a higher risk of rumen acidosis in ruminants

Seed hardness

Grains with harder kernels normally have stronger resistance to microbial and enzymatic degradation, resulting in slower digestion compared to softer grains (Anjum and Walker, 1991). However, the greater *in situ* DMD of harder barley samples at 4 h of incubation appeared to contradict this expectation, a response that could be related to the effects of physical processing. The particle size distribution of ground barley (6-mm screen) differed between high and low SH barley, such that the proportion of fine particles that passed through 1.18-mm sieve was greater for high versus low hardness samples (13.9 vs.11.7%). These results indicated that grinding barley promoted shattering and produced finer particles to a greater extent for harder than softer barley. Lack of difference in molecular-structural conformation of original barley grain with high and low SH was in agreement with this assumption that the differences in *in situ* DMD was potentially due to effect of processing. However, the lack of effect of high and low SH on molecular structures of residues after 4-h digestion contradicted differences observed in *in situ* DMD (36.0 vs. 33.6% for harder and softer grain, respectively). It is important to mention that ATR-FTIR might not have been sensitive enough to detect the difference in molecular structures, as affected by *in situ* DMD.

Diastatic power

We expected that barley samples with high DP had higher *in situ* DMD, as high level of DP represent a higher activity of total starch-degrading enzymes which break down starch to fermentable sugar during malting (Canadian Barley Malting and Brewing Technical Guide,

2012). However, the lower *in situ* DMD of barley grain with high DP at 4 h of incubation contradicted to this expectation. The fact that the starch-degrading enzymes are only active during germination indicates that these enzymes have limited contribution to starch digestion of barley grain in the rumen. In the first study, we speculated that a positive correlation of DP and protein content of barley samples might have contributed to the differences in *in situ* DMD at 4 h digestion between high and low DP grains. In fact, the increased protein content may indicate greater embedding of starch granules within the protein matrix, a factor that could decrease the access of microbes to starch granules and the degradation of starch during early incubation times. We expected ATR-FTIR could detect this difference in chemical components and corresponding molecular structures, particularly protein-related structures, of barley samples with high and low DP before and after 4 h *in situ* incubation. In contrast, such difference in ATR-FTIR spectra of barley samples with high and low DP was not detected, suggesting that the observed difference in 4 h *in situ* DMD (2.3%) of barley grain was relatively small for detection.

The inability of ATR-FTIR to detect differences in total protein concentrations of high and low DP barley after *in situ* rumen degradation may not adequately reflect differences in the composition of protein fractions, for example hordein. Previous research suggested that hordein is associated with beta-amylase content-the major enzyme in DP (Fox, 2010). In particular, B hordein group may play a role in regulating DP instead of A, C and D groups (Peltonen et al., 1994). The classification of hordein into A, B, C, D is according to their increasing apparent molecular weights (Peltonen et al., 1994). In addition, the lack of detectable differences in molecular structures in barley residues with high and low DP after rumen incubation may be due to confounding effects of other chemical components. Yu (2006) and Wetzel et al. (2003) suggested that if studying protein molecular structures is the main interest using Fourier

transform infrared (FTIR) spectroscopy, use of relatively "pure" protein or protein-dominant tissues is recommended to reduce potential effects of other biological components, such as starch. Due to the presence of starch granules in the protein matrix of barley grain, the contribution of starch to this tissue would be disproportionally high as compared to protein, even after grinding and mixing of samples (Wetzel et al., 2003). This would cause scattering and disturbance to the signals associated with protein molecular structures in FTIR spectra (Wetzel et al., 2003). Use of FTIR microspectroscopy with advanced synchrotron technology (SR-FTIR) may help to localize relatively pure protein in barley kernel, and specifically study protein-associated molecular structures (Wetzel et al., 2003). The structural features of relatively pure protein in a barley kernel can be revealed in the microscopy of SR-FTIR, whereas a conventional FTIR spectroscopy does not have this capability.

Wort beta-glucan

This was the first study to examine the effect of WBG on ruminal digestion of barley. However, WBG indicates the amount of grain beta-glucan that is not degraded by beta-glucanases during malting. It has been suggested to be positively associated with grain beta-glucan content (Wang et al., 2004). In the first study, the lack of difference in DMD of barley with high and low beta-glucan content suggests that the ruminal beta-glucanase activity was sufficiently high to degrade the endosperm cell walls within barley grain in both high and low beta-glucan barley. As a result of similar *in situ* DMD it was expected that no differences would be observed by ATR-FTIR and PCA, as confirmed by our results.

Friability

It was expected that barley grain with higher FRI would have greater *in situ* DMD, as higher FRI indicates a high percentage of grain that is easily fractured during physical processing. The

result was in agreement with Fox et al. (2009) that FRI had no relationship with 3 h *in situ* DMD for forty barley cultivars examined. This was also consistent with lack of impact of FRI on molecular structures of barley grain before and after *in situ* incubation.

Use of PCA with ATR-FTIR spectra

Our results showed that the PCA analysis was able to identify differences in ATR-FTIR spectra as a result of incubation times. More importantly, the scores and loadings of PCA have shown differences that the spectra analysis was unable to identify. The scores of PCA visually distinguished the differences of treatment or variables in ATR-FTIR spectra by grouping. For instance, at region 4, there was a lack of differentiation between 0 and 4 h (or 12 h) at bands of 992 cm^{-1} in ATR-FTIR spectra (Figure 3-3 g), but the PCA analysis showed distinction between 0 and 4 h (or 12 h) along PC-1 axis (Figure 3-4 l). Together with loadings of PCA, maximum sources of variation in samples selected from different time points were shown at bands of 992 cm^{-1} (using samples selected for DP as an example). This indicates that using PCA analysis provides further insight into the sources of spectral variability in a visual manner. The current results clearly indicated that use of PCA provides visual identification of the maximum sources of variation in the spectra, and characterization of rumen residues to its molecular structures.

4.2 Implications of current research

This project evaluated the use of SH and MC of barley grain as indicators of feed value in ruminants. The correlation of lower DMD of barley grain with low level of SH or high level of DP appeared to slow the rate of barley digestion, this could potentially avoid the rapid drop of ruminal pH and reduce the risk of rumen acidosis associated with feeding high-barley grain diets to ruminants. When barley grain for malting production is downgraded for feed use, ruminant

nutritionists or producers need to predict its feed value for ruminants. The current results suggest that ATR-FTIR was not able to detect differences in molecular structures of SH or each of MC as a result of rumen digestion; however, ATR-FTIR detected changes in molecular structures of barley grain due to rumen digestion. Additionally, as a result of no difference observed in DMD at 12 h between barley selected for high or low SH, and MC, it can be concluded that perhaps SH and each MC are not ideal predictors of digestion in ruminants.

Use of ATR-FTIR spectroscopy can provide an alternative for nutritionists, cereal breeders, food and feed chemists, grain processors and scientists to qualitatively analyze chemical composition and molecular structure makeups of barley grain for various purposes. The requirement of rapid analysis of feed composition, and feed quality evaluation in livestock drives a need to develop tools, such as FTIR spectroscopy for feed analysis. However, the current project demonstrated that ATR-FTIR shows promise in the detection of differences in molecular structures of barley grain across incubation time points, in particular starch structures.

4.3 Future directions

The current results indicated that the ruminal DMD may be influenced by SH and grain processing. Future research should study the effects of processing methods and extent on *in situ* barley grain digestion and animal performance varying with SH. Information derived from such studies may help to achieve optimal rumen digestion and minimize rumen acidosis. In addition, the correlation of rumen DMD of barley with DP also needs to be confirmed using *in vivo* studies.

Future research investigating the mechanism by which DP affects rumen digestion may consider the following aspects. First, previous research suggested that specific protein fraction-

hordein, in particular, B hordein group is potentially associated with DP in barley grain. Thus, the study on relationship between DP and hordein fraction, in particular B hordein group of barley grain, or on the effect of hordein content and composition on rumen digestion of barley grain would be of interest. In addition, to better understand the effect of DP on protein secondary structure of barley grain, the potential of SR-FTIR microspectroscopy could be explored to particularly localize relatively pure protein region in barley grain to reduce possible disturbance of other chemical components such as starch on protein structure detection. Previous research (Yu, 2006; Wetzel et al., 2003) indicated that SR-FTIR microspectroscopy with high ultra-spatial resolution allows the determination of spectral data with structural, chemical and spatial information of the sample tissues in microscopic regions.

As interpreting FTIR spectra always requires references from previous literature, some assignments of bands to corresponding molecular structures are subjective. Purifying starch, protein, fiber and lipid of barley grain, and scanning these chemical components separately help to validate the band assignment, and provide accurate reference for qualitative and quantitative analysis using FTIR spectroscopy. However, developing robust models for quantitative prediction of FTIR spectroscopy is fundamental and in demand. Future work can focus on the study of mathematical methods for FTIR analysis, and the standardization of models for accurate prediction of rumen digestion of dry matter and specific nutrient in barley grain. Further, although the potential of using near-infrared (NIR) spectroscopy to predict rumen digestion of feeds has been widely studied, no research has compared the accuracy and efficiency of FTIR and NIR spectroscopy to predict rumen digestion of barley grain and other feeds. Thus, future research is warranted to not only ascertain the usefulness of FTIR spectroscopy in predicting rumen digestion, but also to compare the accuracy and efficiency of using FTIR spectroscopy

and NIR to predict rumen digestion of barley grain, in order to optimize prediction of nutrient digestion using the most appropriate tool.

The current project observed a clear discrimination of spectra by incubation time in lipid-associated areas. We speculated that it could be due to microbial synthesis as rumen digestion progressed. However, the lack of impact of rumen digestion on protein molecular structures contradicts this speculation. It is possible that ATR- FTIR spectroscopy might have had limitations in detecting differences in molecular structures associated with microbial protein due to disturbance of other chemical components. Alternatively, the accumulation of other lipid sources such as bacterial lipids, as rumen incubation proceeded might have contributed to the discrimination of spectra by incubation time in lipid-associated areas. However, protein accumulation as a result of increasing microbial attachment of barley grain was not detected by ATR-FTIR due to rumen digestion. Further research may separate residue and microbial pellet to distinguish if the lipids originate from microbial biomass. The increased concentration of lipids in corresponding wavenumbers of ATR- FTIR spectroscopy may be a more reliable indicator of increased microbial biomass as rumen digestion proceeded compared with alteration of protein content in its associated regions.

4.4 General conclusions

Overall, although diastatic power and seed hardness are associated with improved *in situ* rumen digestion at 4 h of incubation, the lack of difference at 12 h of barley grain with high and low diastatic power and seed hardness suggested that they are not suitable predictors of barley grain digestion in the rumen. Seed hardness of barley grain may potentially be associated with processing, but the mechanism of how diastatic power affects rumen digestion during early

digestion warrants future research. The current project showed that ATR-FTIR spectroscopy did not detect changes in molecular structures of barley grain selected for SH and MC as a result of *in situ* rumen digestion. However, ATR-FTIR spectroscopy distinguished some differences between molecular structural conformations as affected by rumen digestion. Principle component analysis identified the major factors that contribute to the differences in spectra. These factors are mainly attributed to carbohydrate-related components, and to a lesser extent, lignin- and lipid-related molecular structures. These results indicate that ATR-FTIR may have potential to predict changes in molecular structures of barley grain as a result of rumen digestion.

4.5 References

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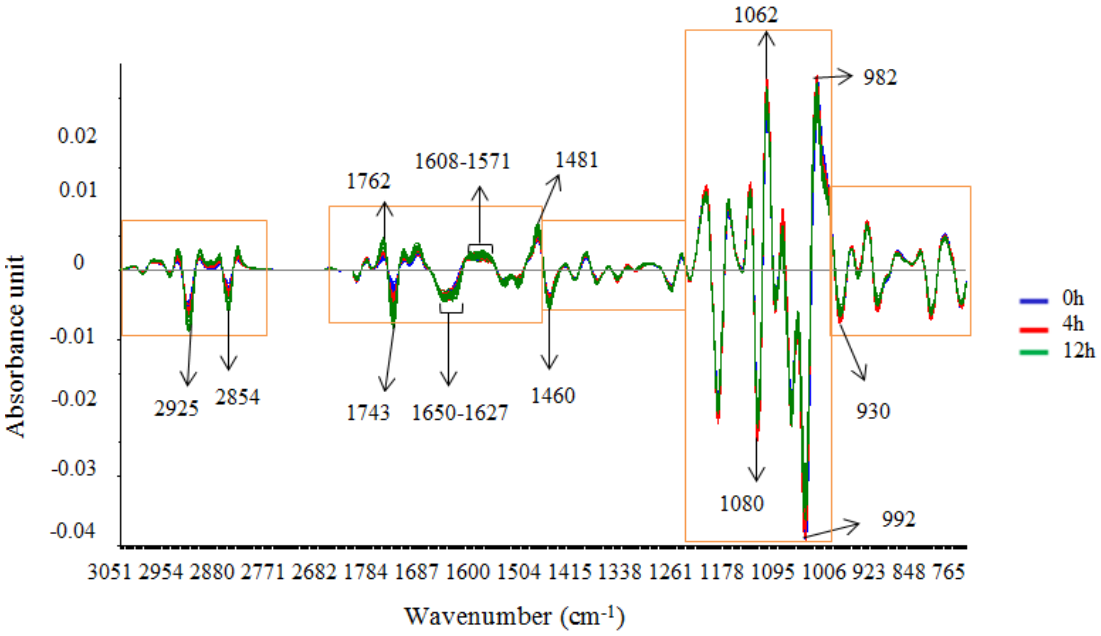
APPENDIX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1481
1762
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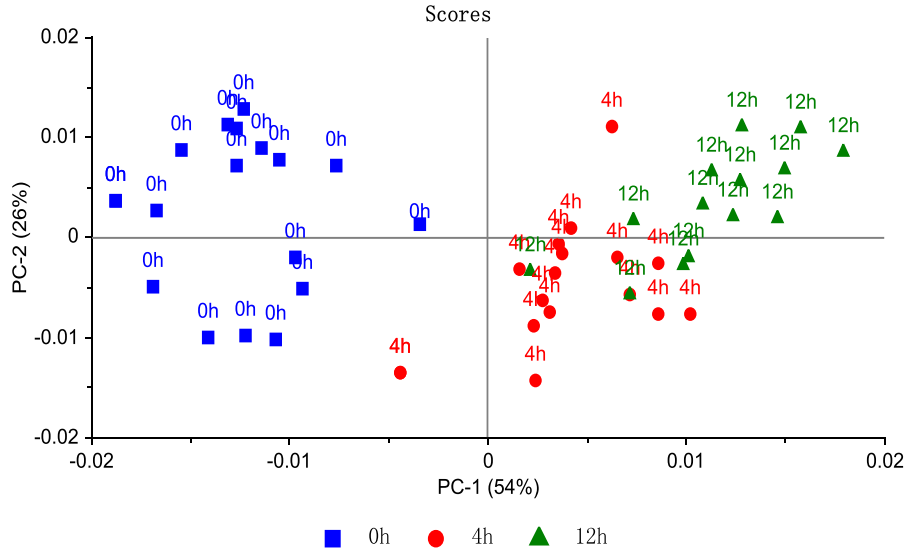
0.01
0
-0.01
-0.02
-0.03
-0.04
Absorbance unit
2925
2854
1743
1650-1627
1460
930
1080
992
3051 2954 2880 2771 2682 1784 1687 1600 1504 1415 1338 1261 1178 1095 1006 923 848 765
Wavenumber (cm-1)
0h
4h
12h

(a)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PC-1 (54%)
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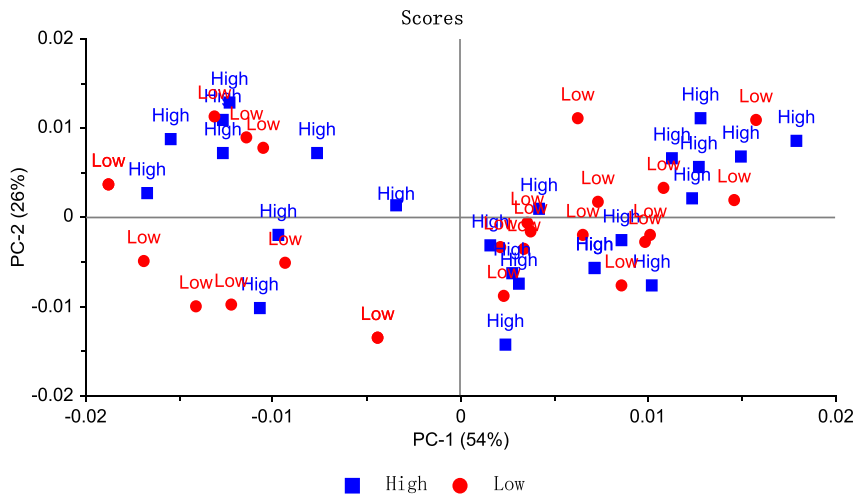
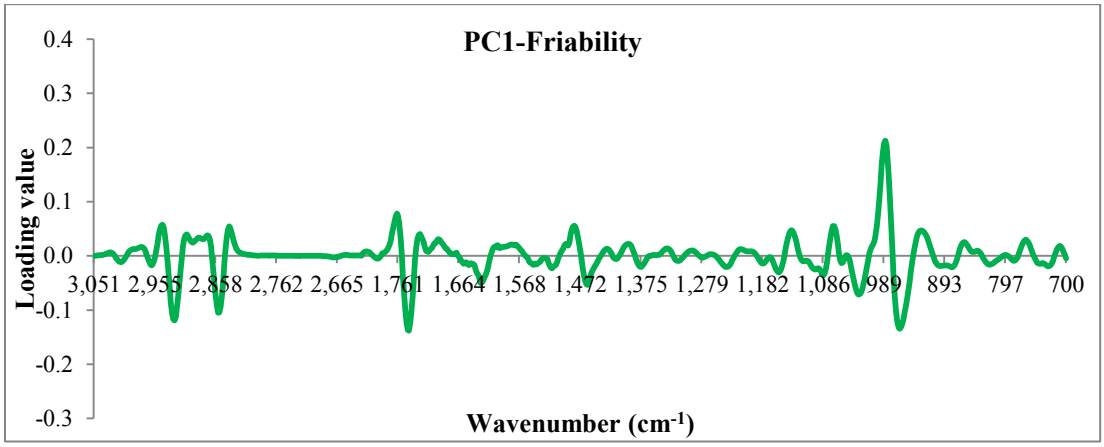
0
-0.01
-0.02
0h
4h
12h

(b)

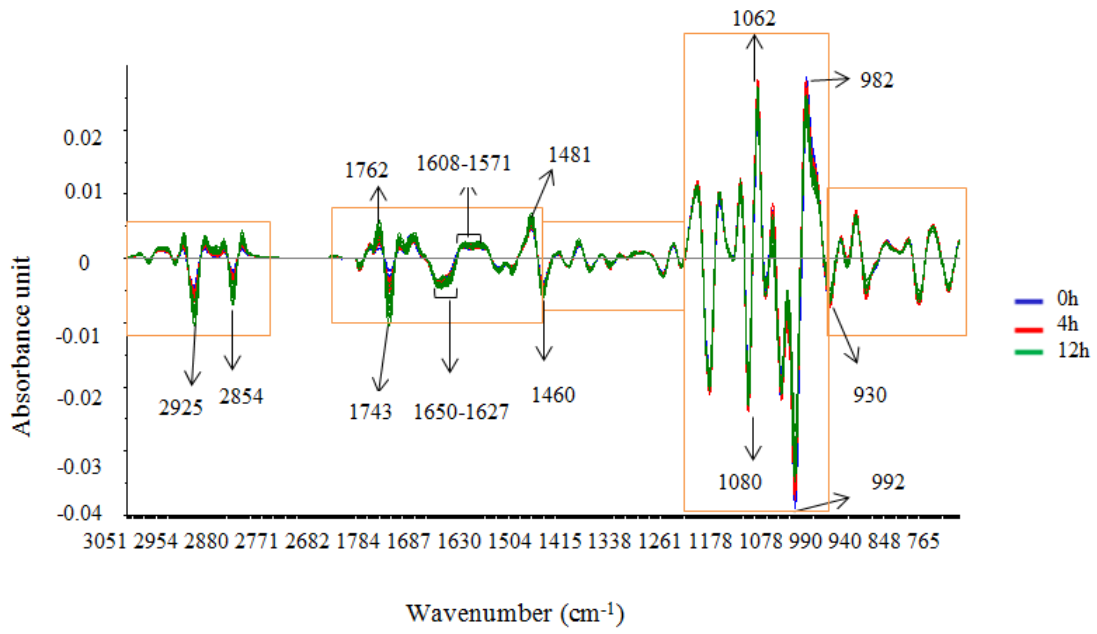


(c)



(d)

Appendix-1 Spectra analysis and principle component analysis (PCA) of selected ATR-FTIR spectra (3050-700 cm^{-1}) of barley samples selected for high and low values of friability (FRI) at 0 (original barley grain), 4 and 19h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Spectra analysis-separation of spectra according to incubation time point; (b) Scores plot of PCA-grouping of spectra according to incubation time point; (c) Scores plot of PCA-lack of grouping of spectra according to level of FRI; (d) Loadings plot of PCA-the origin of variations in ATR-FTIR spectra based on recorded wavenumbers. Each spectrum (line) represents an individual sample. Orange boxes indicate the areas of five sub-divided regions.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0
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-0.02
-0.03
-0.04
1062
982
1762
1608-1571
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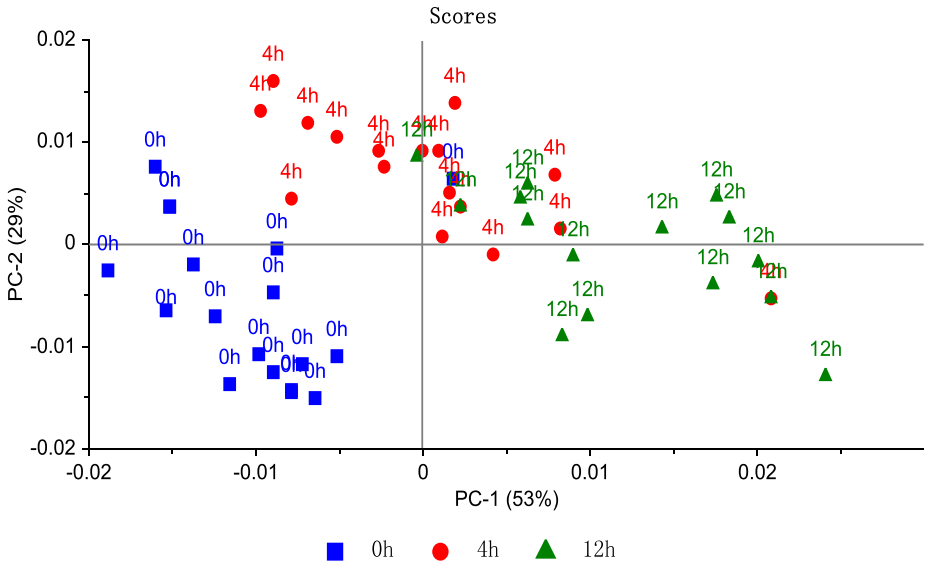
2925
2854
1743
1650-1627
1460
930
1080
992
3051 2954 2880 2771 2682 1784 1687 1630 1504 1415 1338 1261 1178 1078 990 940 848 765
Wavenumber (cm-1)
0h
4h
12h

(a)


Scores
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PC-1 (53%)
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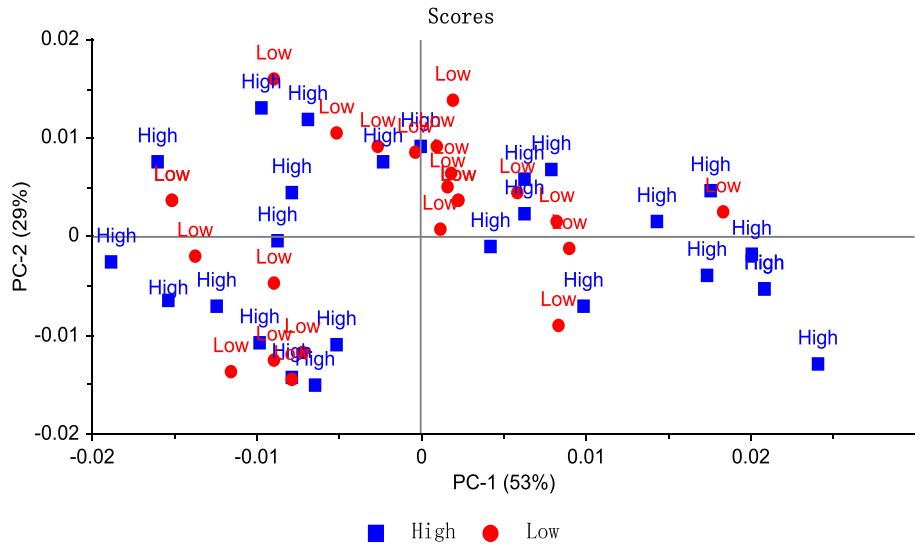
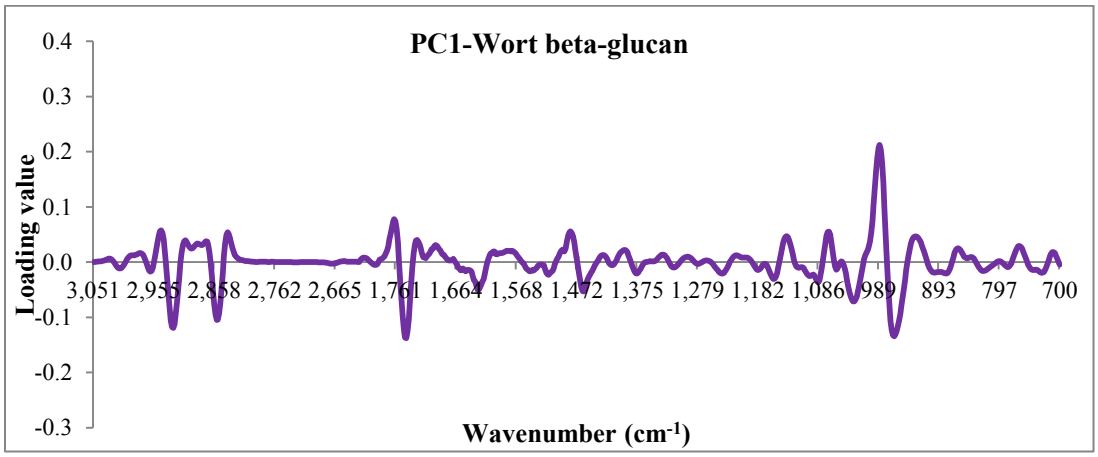
0.01
0
-0.01
-0.02
0h
4h
12h

(b)

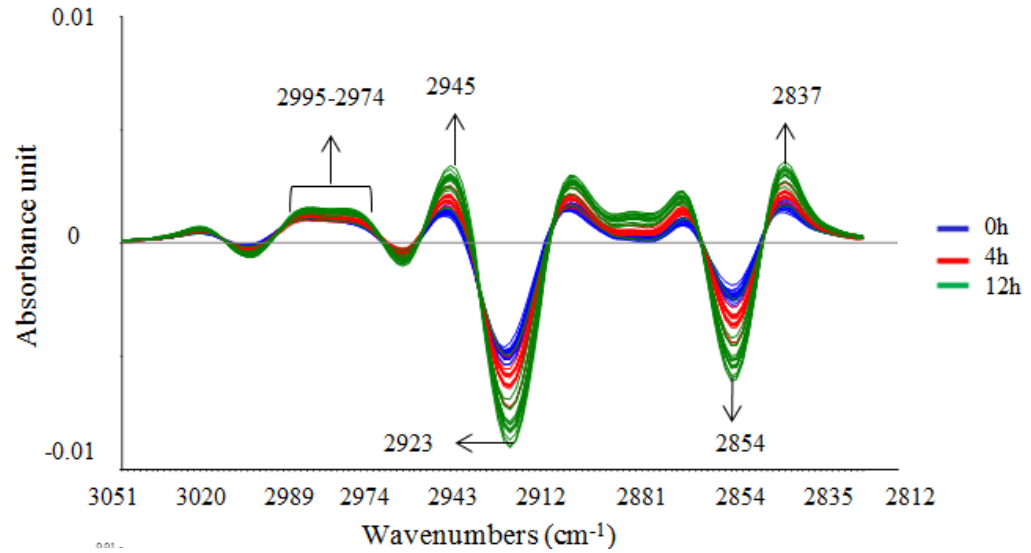


(c)



(d)

Appendix-2 Spectra analysis and principle component analysis (PCA) of selected ATR-FTIR spectra (3050-700 cm^{-1}) of barley samples selected for high and low values of wort beta-glucan (WBG) at 0 (original barley grain), 4 and 110h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Spectra analysis-separation of spectra according to incubation time point; (b) Scores plot of PCA-grouping of spectra according to incubation time point; (c) Scores plot of PCA-lack of grouping of spectra according to level of WBG; (d) Loadings plot of PCA-the origin of variations in ATR-FTIR spectra based on recorded wavenumbers. Each spectrum (line) represents an individual sample. Orange boxes indicate the areas of five sub-divided regions.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-0.01
Absorbance unit
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2923
2854
0h
4h
12h
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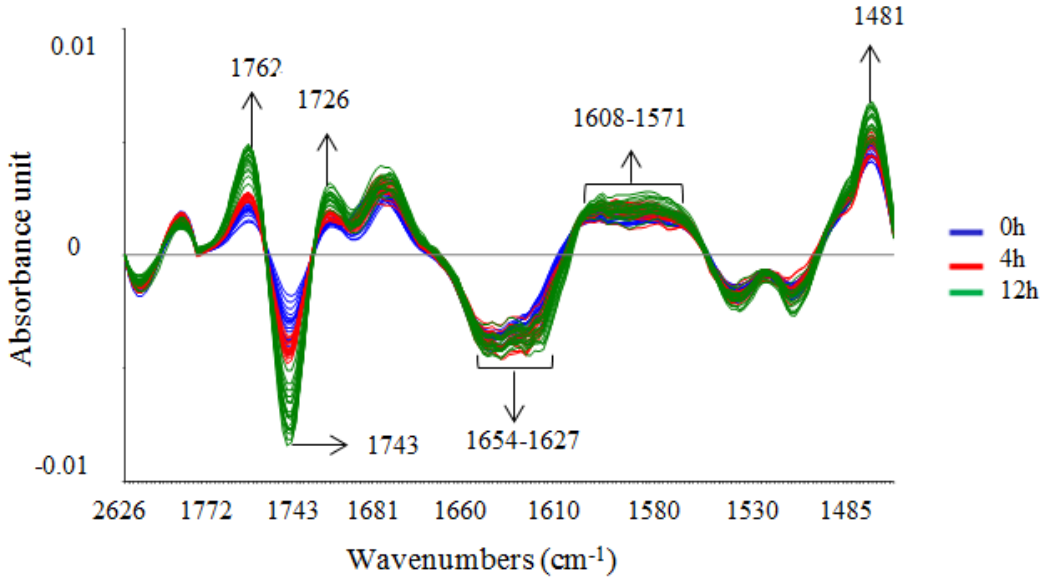
2989
2974
2943
2912
2881
2854
2835
2812
Wavenumbers (cm-1)

(a)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Absorbance unit
1762
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1481
1743
1654-1627
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4h
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1681
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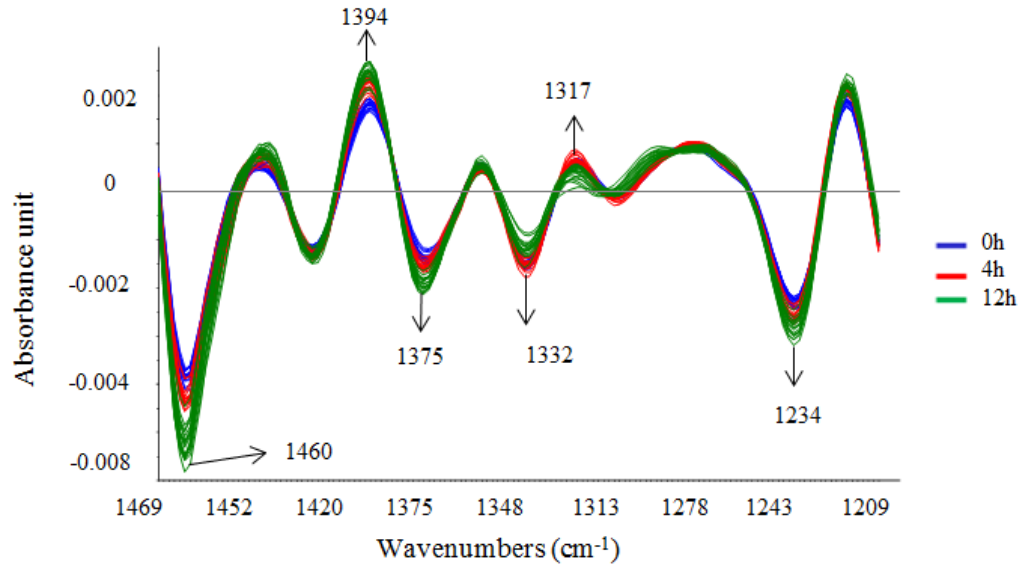
1580
1530
1485
Wavenumbers (cm-1)

(b)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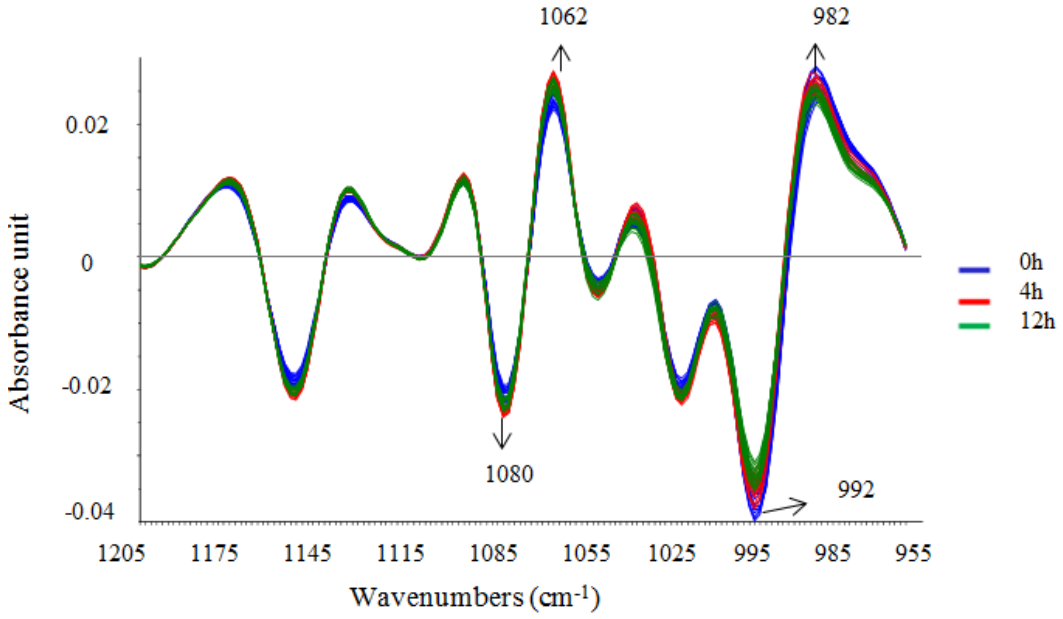
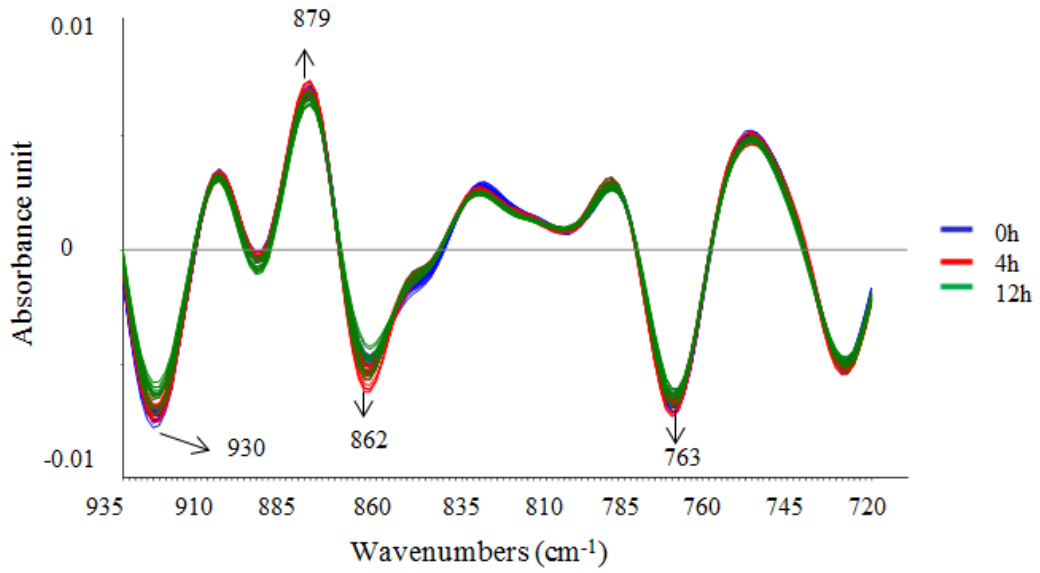
1375
1332
1234
1460
1469
1452
1420
1375
1348
1313
1278
1243
1209
Wavenumbers (cm-1)
0h
4h
12h

(c)

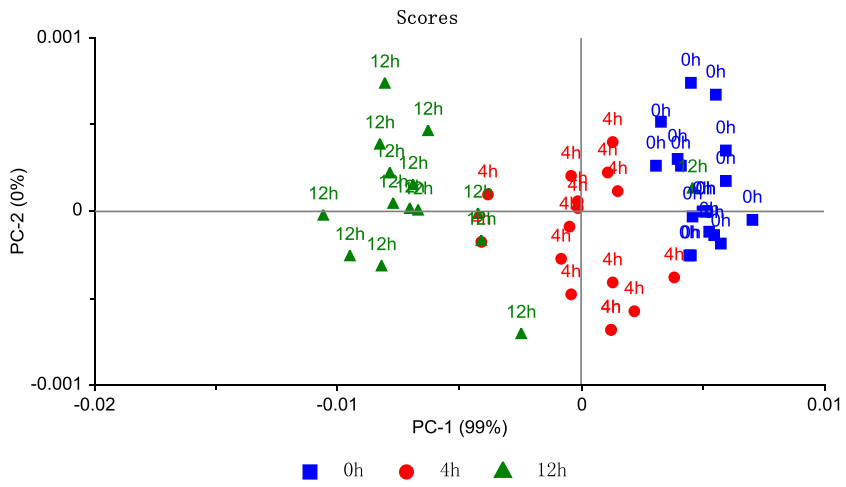

1062
982
0.02
0
-0.02
-0.04
Absorbance unit
1080
992
1205
1175
1145
1115
1085
1055
1025
995
985
955
Wavenumbers (cm-1)
0h
4h
12h

(d)

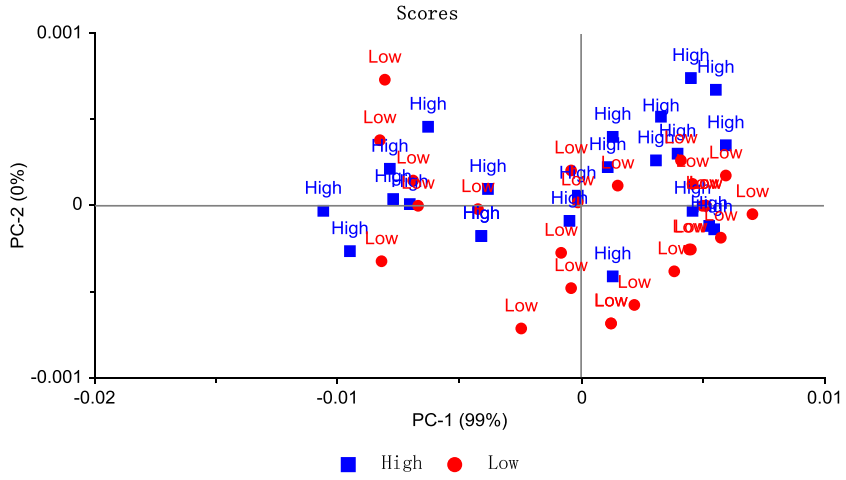


(e)

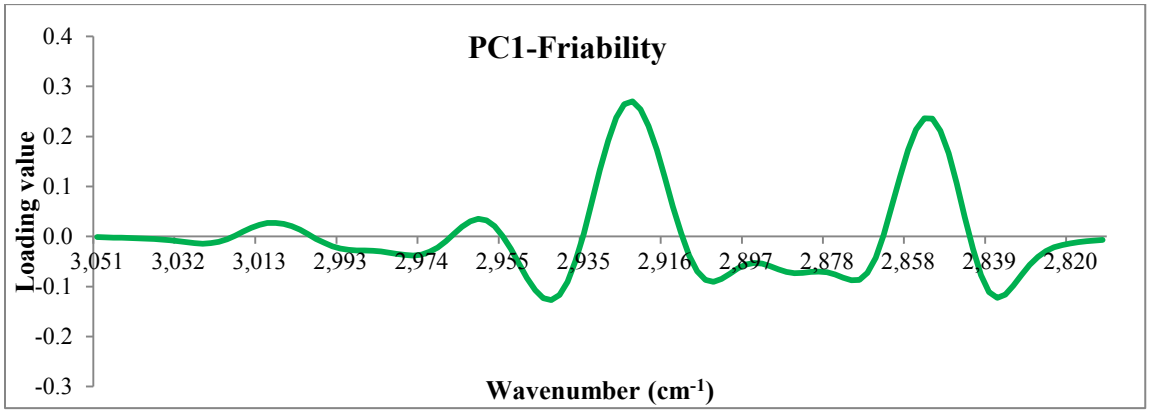
Appendix-3 Five sub-divided regions of ATR-FTIR spectra of barley samples selected for high and low values of friability transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Region 1: 3050-2812 cm^{-1}; (b) Region 2: 2626-1471 cm^{-1}; (c) Region 3: 1469-1207 cm^{-1}; (d) Region 4:1205-941 cm^{-1}; (e) Region 5: 937-650 cm^{-1}. Spectra of each region were separated according to incubation time point (0, 4 and 12h) of in situ rumen digestion at certain wavenumbers. Each spectrum (line) represents a sample.


Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.02
-0.01
0
0.01
0h
4h
12h

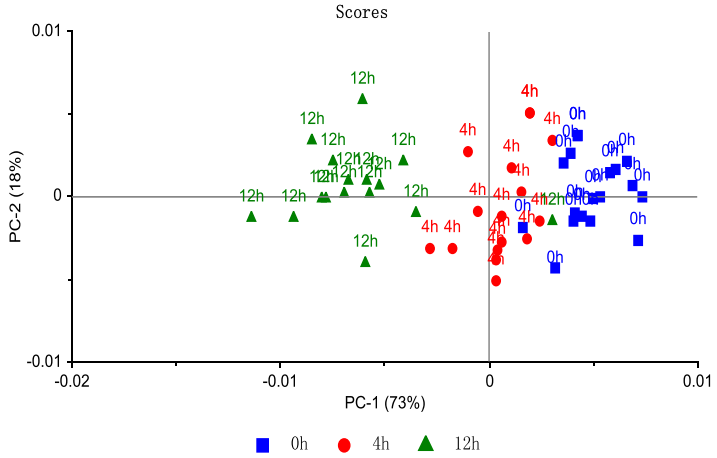
(a)


Scores
PC-2 (0%)
PC-1 (99%)
0.001
0
-0.001
-0.02
-0.01
0
0.01
High
Low

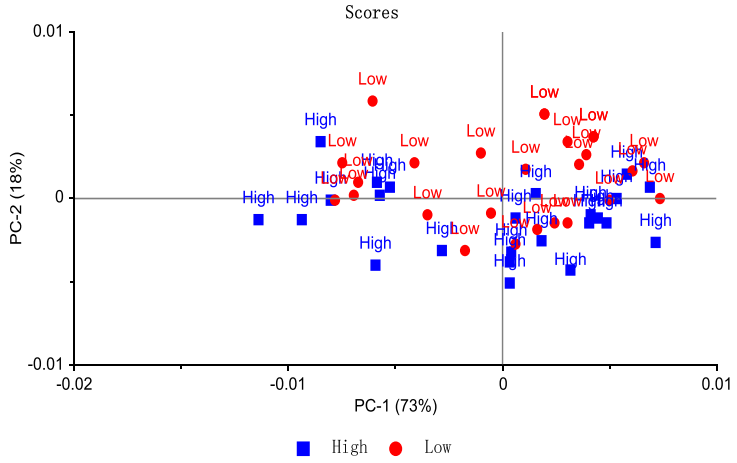
(b)


PC1-Friability
Loading value
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
3,051
3,032
3,013
2,993
2,974
2,955
2,935
2,916
2,897
2,878
2,858
2,839
2,820
Wavenumber (cm-1)

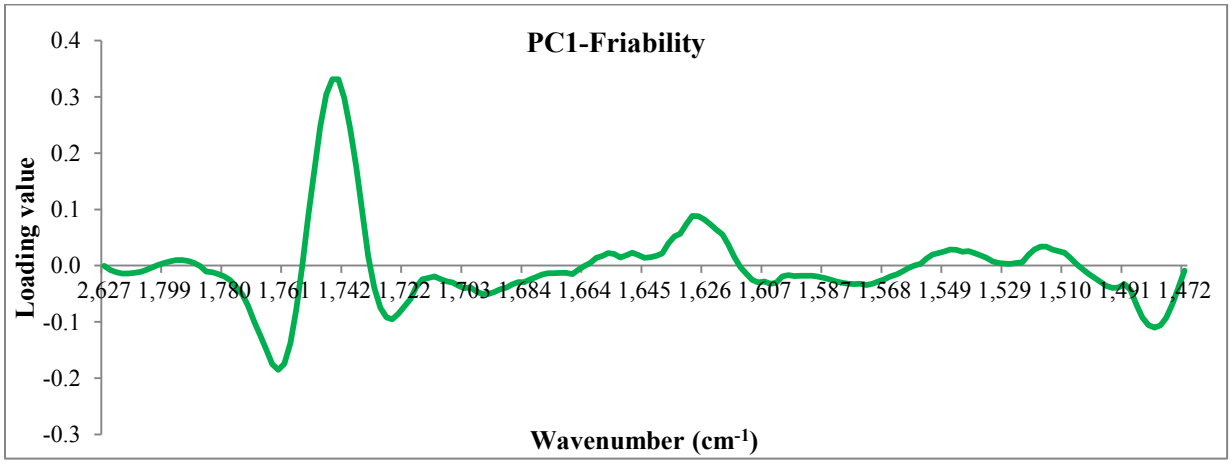
(c)


Scores
PC-2 (18%)
PC-1 (73%)
0.01
0
-0.01
-0.02
-0.01
0
0.01
0h
4h
12h

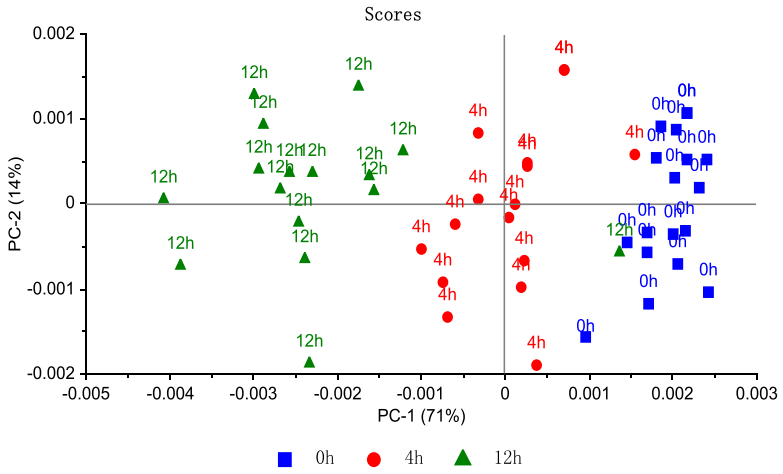
(d)


Scores
PC-2 (18%)
PC-1 (73%)
0.01
0
-0.01
-0.02
-0.01
0
0.01
High
Low

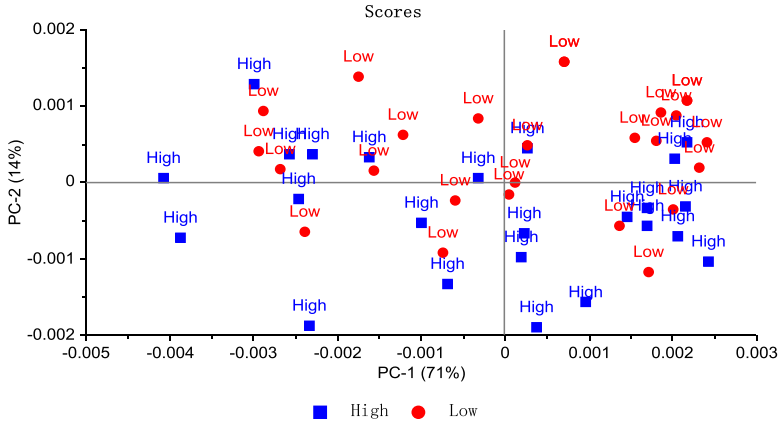
(e)


PC1-Friability
Loading value
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
2,627 1,799 1,780 1,761 1,742 1,722 1,703 1,684 1,664 1,645 1,626 1,607 1,587 1,568 1,549 1,529 1,510 1,491 1,472
Wavenumber (cm-1)

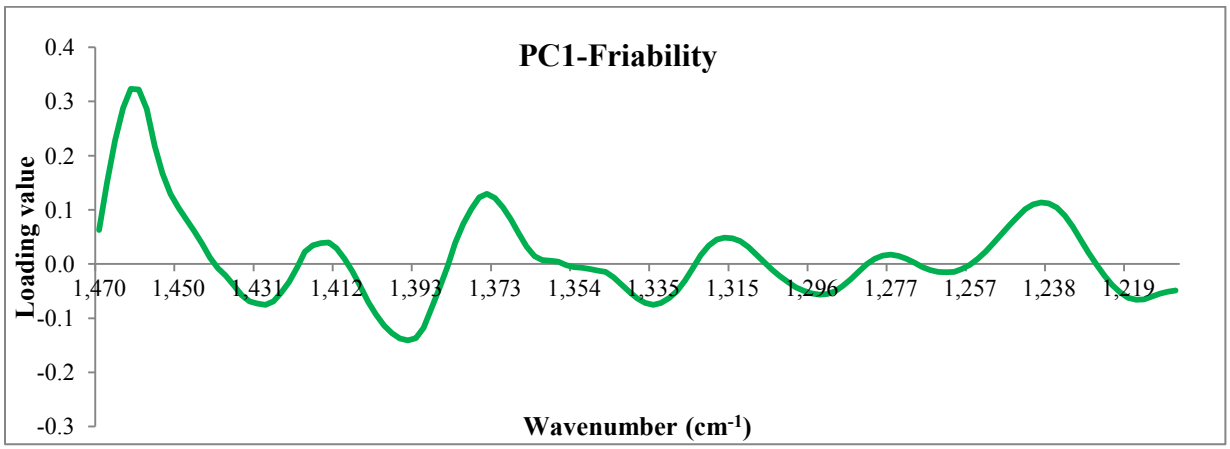
(f)


Scores
PC-2 (14%)
PC-1 (71%)
0h
4h
12h

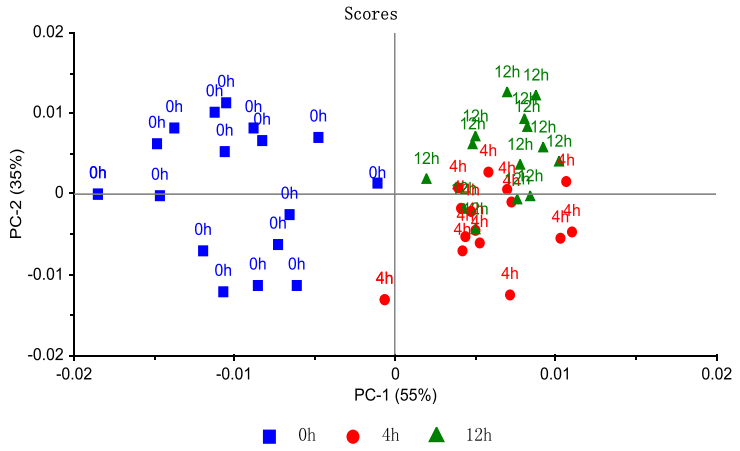
(g)


Scores
PC-2 (14%)
PC-1 (71%)
High
Low

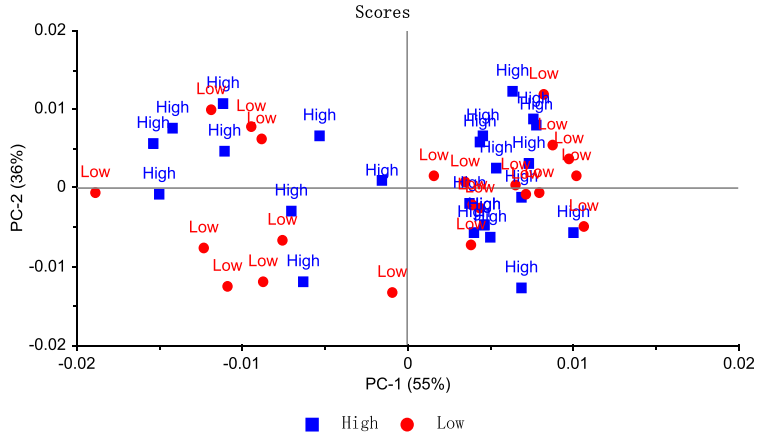
(h)


PC1-Friability
Loading value
Wavenumber (cm-1)

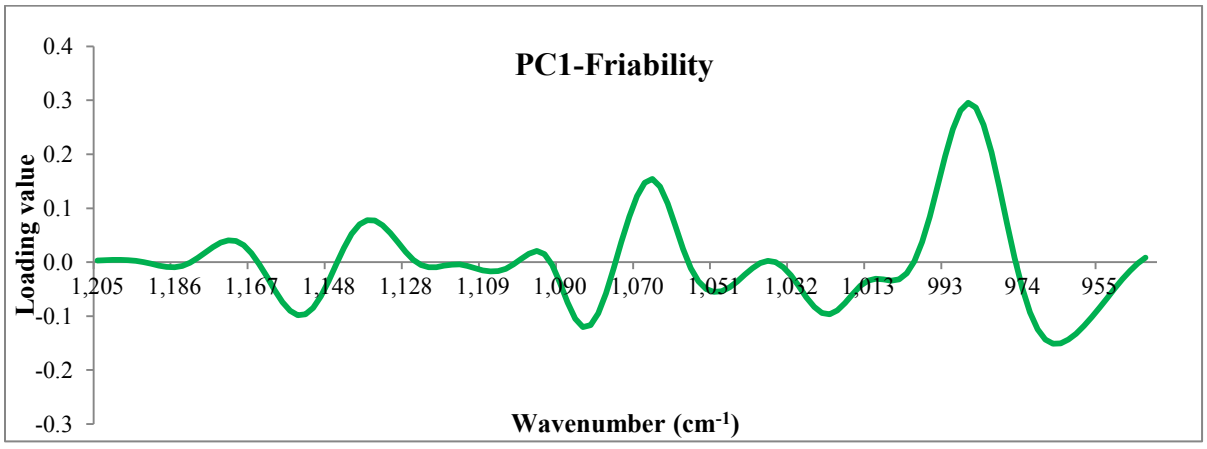
(i)


Scores
PC-2 (35%)
PC-1 (55%)
0h
4h
12h

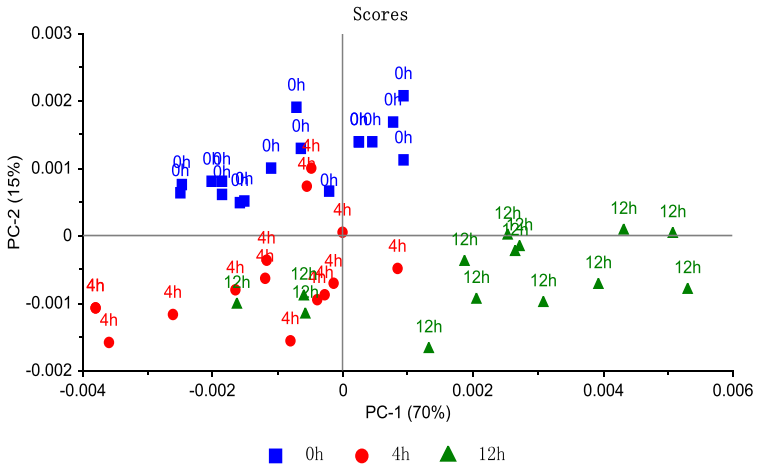
(j)


Scores
PC-2 (36%)
PC-1 (55%)
High
Low

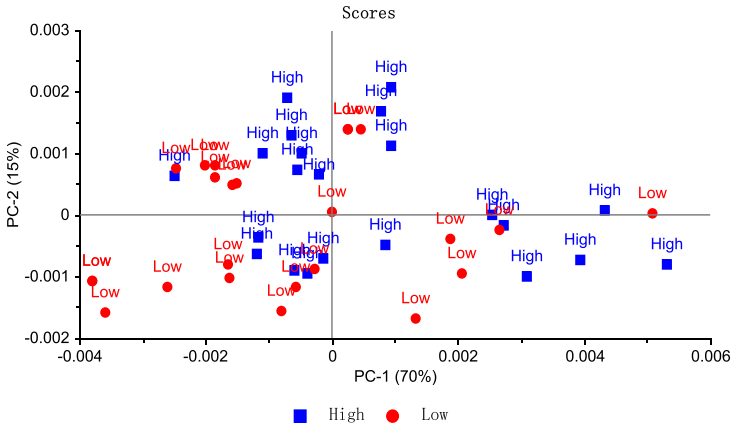
(k)


PC1-Friability
Loading value
Wavenumber (cm-1)

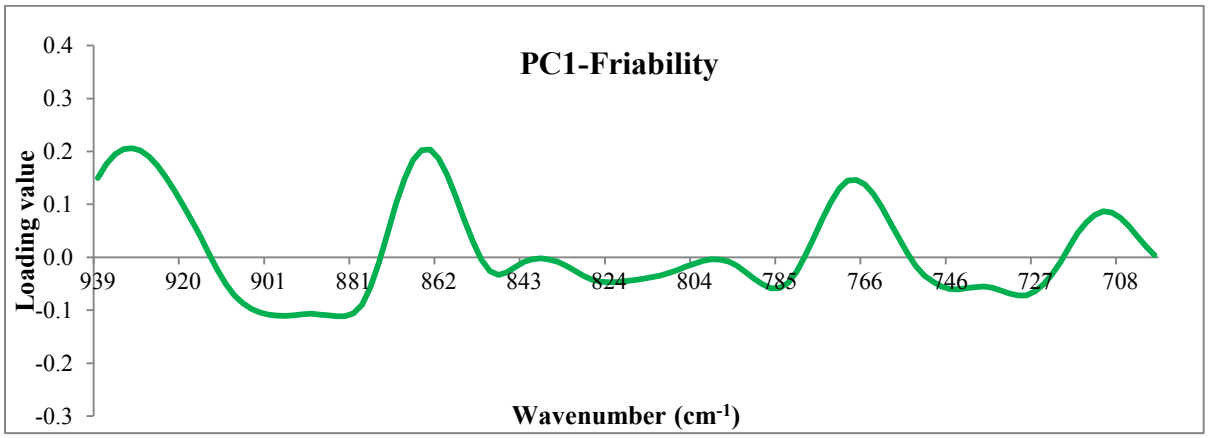
(l)


Scores
PC-2 (15%)
PC-1 (70%)
0h
4h
12h

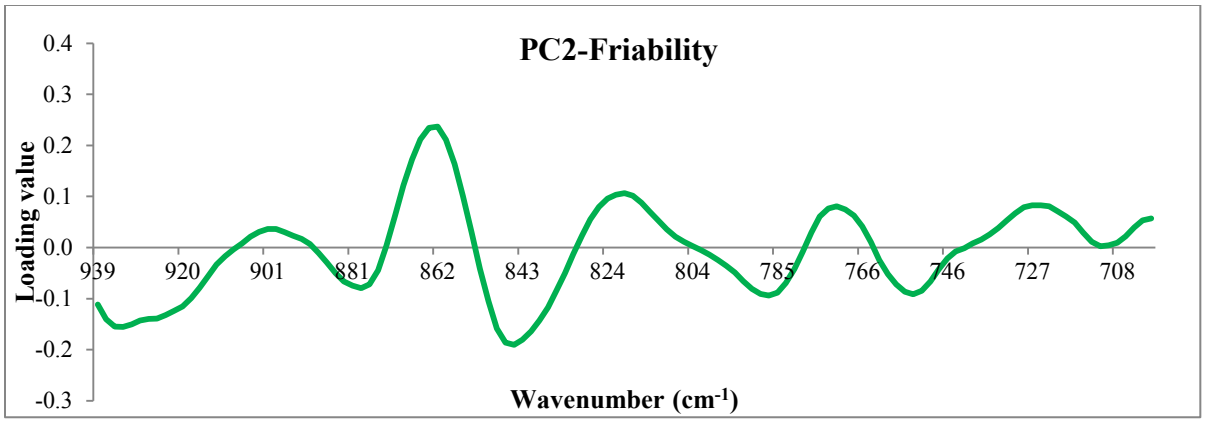
(m)


Scores
PC-2 (15%)
PC-1 (70%)
High
Low

(n)


PC1-Friability
Loading value
Wavenumber (cm-1)

(o)



(p)

Appendix-4 Scores and loadings plots of principle component analysis (PCA) based on five sub-divided regions of ATR-FTIR spectra at 0 (original barley grain), 4 and 12 h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative, using samples selected for high and low friability (FRI)

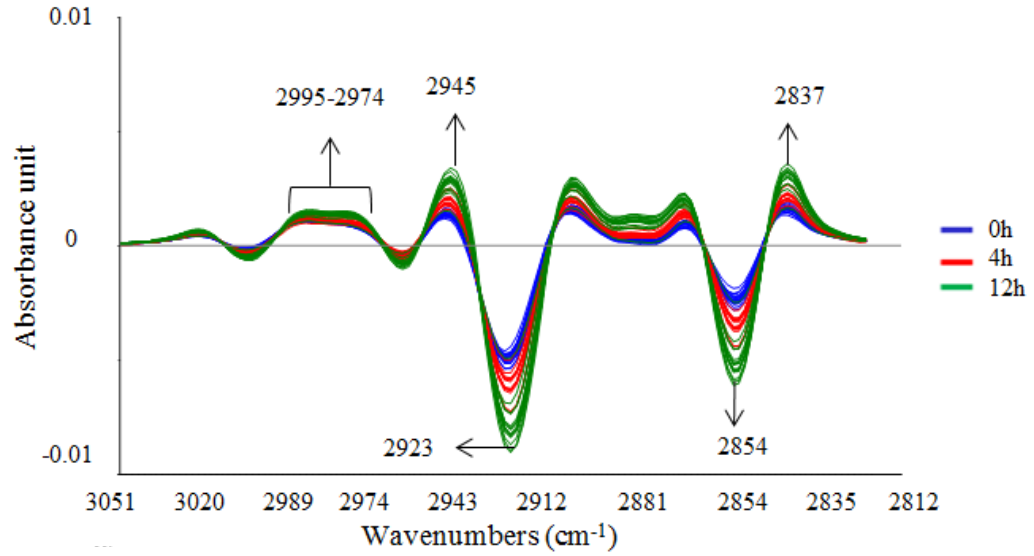
Region 1 3050-2812 cm^{-1}: a) Scores plot of PCA-spectra grouped by incubation time point; b) Scores plot of PCA-lack of spectra grouping by level of FRI; C) PC-1 loadings plot of PCA-origin of variation in the spectra in region 1.

Region 2 2626-1471 cm^{-1}: d) Scores plot of PCA-spectra grouped by incubation time point; e) Scores plot of PCA-lack of spectra grouping by level of FRI; f) PC-1 loadings plot of PCA-origin of variation in the spectra in region 2.

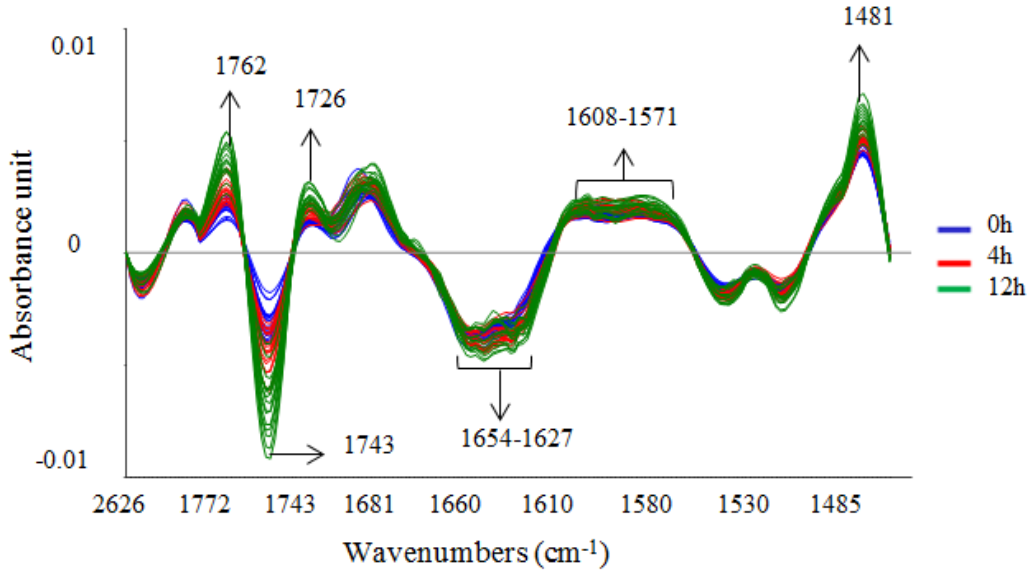
Region 3 1469-1207 cm^{-1}: g) Scores plot of PCA-spectra grouped by incubation time point-; h) Scores plot of PCA-lack of grouping by level of FRI; i) PC-1 loadings plot of PCA-origin of variation in the spectra in region 3.

Region 4 1205-941 cm^{-1}: j) Scores plot of PCA-spectra grouped by incubation time point-DP; k) Scores plot of PCA-lack of spectra grouping by level of FRI; l) PC-1 loadings plot of PCA-origin of variation in the spectra in region 4.

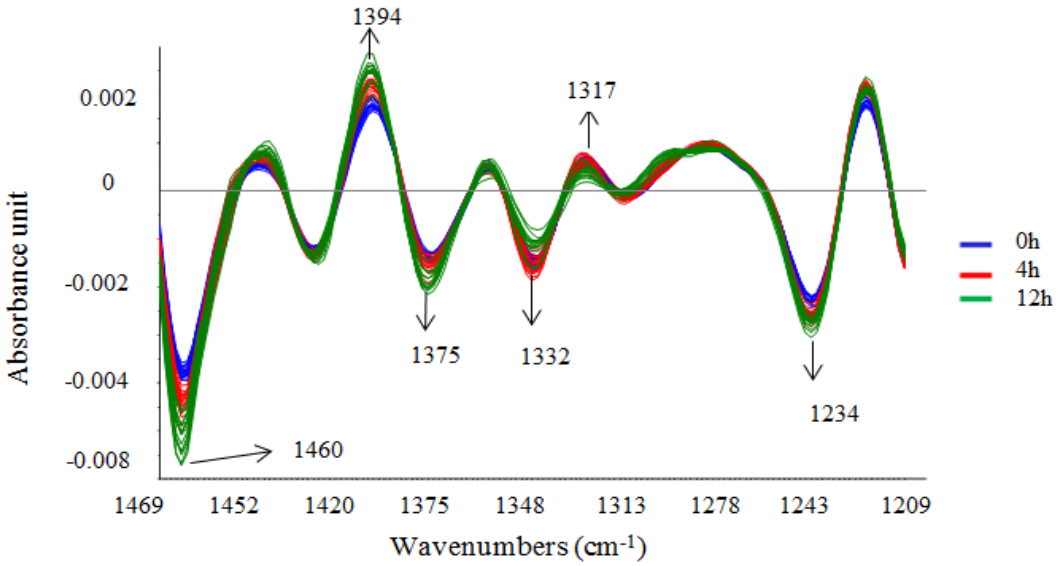
Region 5 937-650 cm^{-1}: m) Scores plot of PCA- spectra grouped by incubation time point-DP; n) Scores plot of PCA-lack of spectra grouping by level of FRI; o) PC-1 loadings plot of PCA-origin of variation in the spectra in region 5. p) PC-2 loadings plot of PCA-origin of variation in the spectra in region 5.


0.01
0
-0.01
Absorbance unit
2995-2974
2945
2837
2923
2854
3051
3020
2989
2974
2943
2912
2881
2854
2835
2812
Wavenumbers (cm-1)
0h
4h
12h

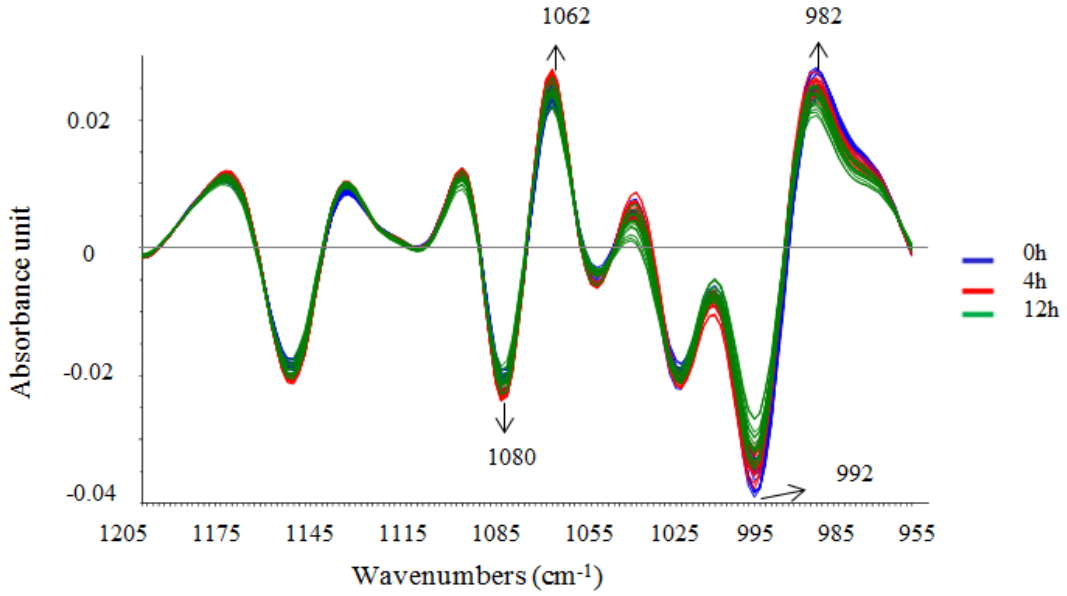
(a)


0.01
0
-0.01
Absorbance unit
1762
1726
1608-1571
1481
1743
1654-1627
2626
1772
1743
1681
1660
1610
1580
1530
1485
Wavenumbers (cm-1)
0h
4h
12h

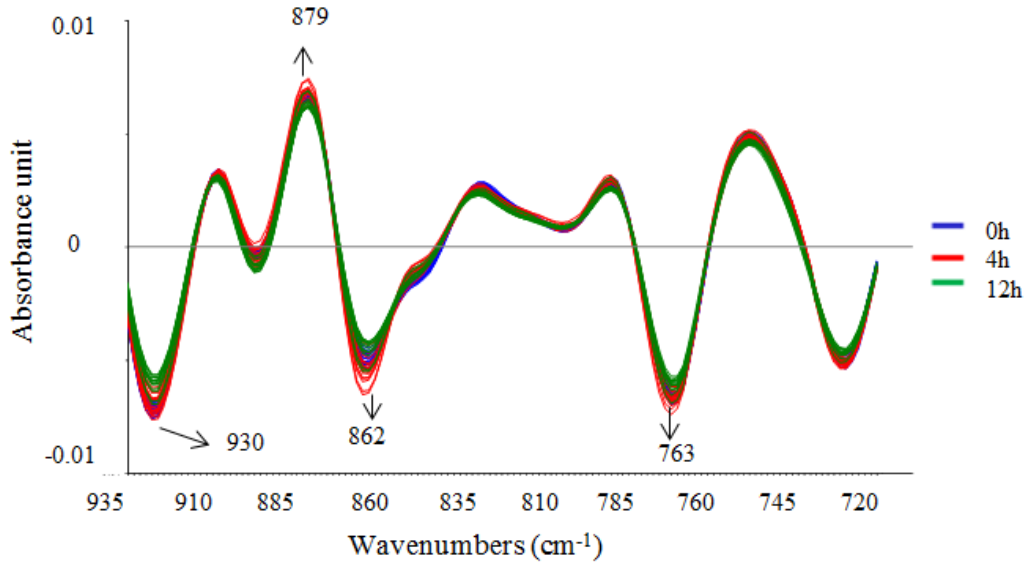
(b)


1394
1317
0.002
0
-0.002
-0.004
-0.008
Absorbance unit
1375
1332
1234
1460
1469
1452
1420
1375
1348
1313
1278
1243
1209
Wavenumbers (cm-1)
0h
4h
12h

(c)

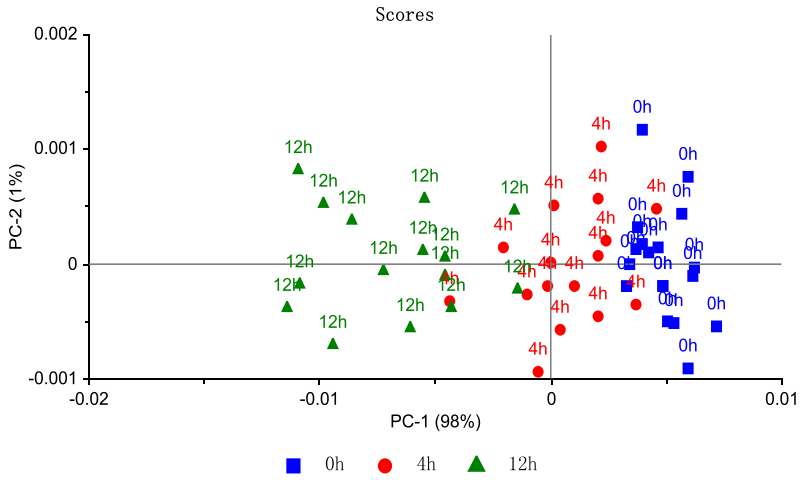

1062
982
0.02
0
-0.02
-0.04
Absorbance unit
1080
992
1205
1175
1145
1115
1085
1055
1025
995
985
955
Wavenumbers (cm-1)
0h
4h
12h

(d)

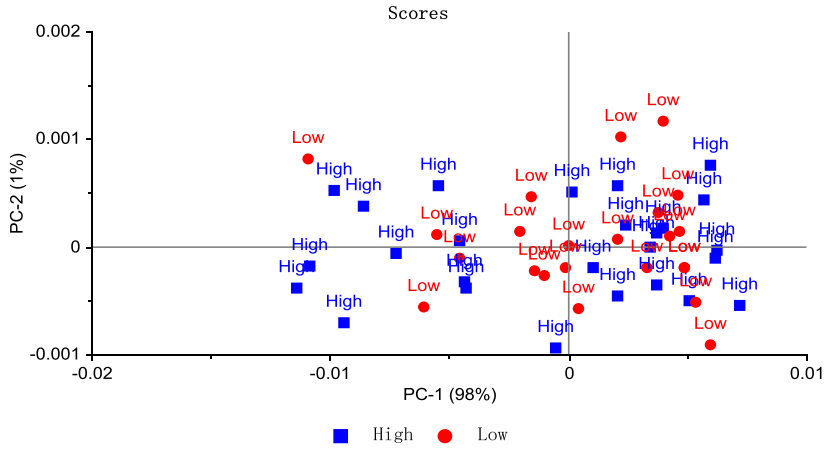


(e)

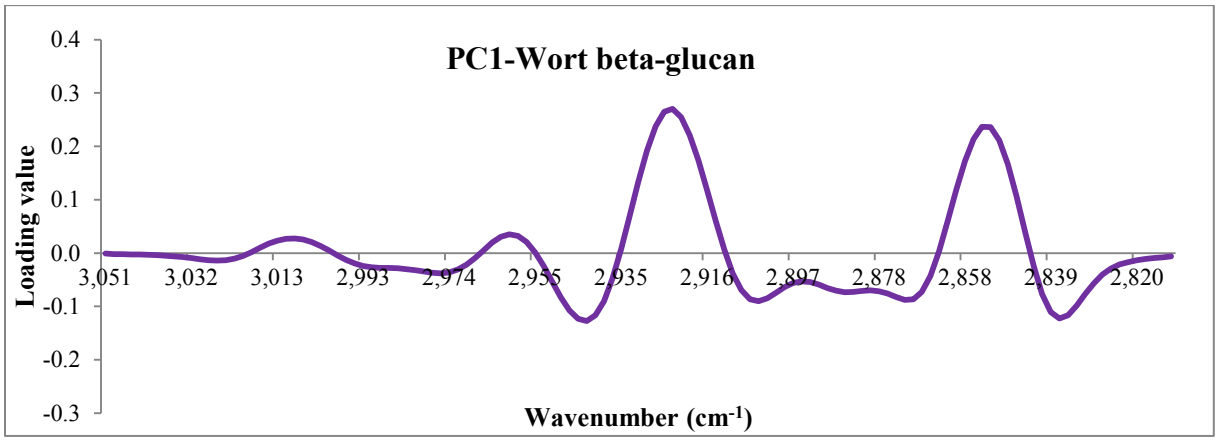
Appendix-5 Five sub-divided regions of ATR-FTIR spectra of barley samples selected for high and low values of seed hardness transformed by Standard Normal Variate and Savitzky-Golay second-order derivative. (a) Region 1: 3050-2812 cm^{-1}; (b) Region 2: 2626-1471 cm^{-1}; (c) Region 3: 1469-1207 cm^{-1}; (d) Region 4:1205-941 cm^{-1}; (e) Region 5: 937-650 cm^{-1}. Spectra of each region were separated according to incubation time point (0, 4 and 12h) of *in situ* rumen digestion at certain wavenumbers. Each spectrum (line) represents a sample.


Scores
PC-2 (1%)
PC-1 (98%)
0h
4h
12h

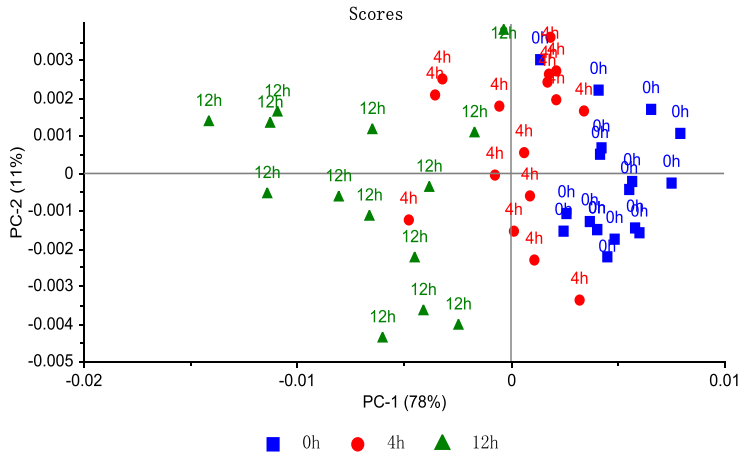
(a)


Scores
PC-2 (1%)
PC-1 (98%)
High
Low

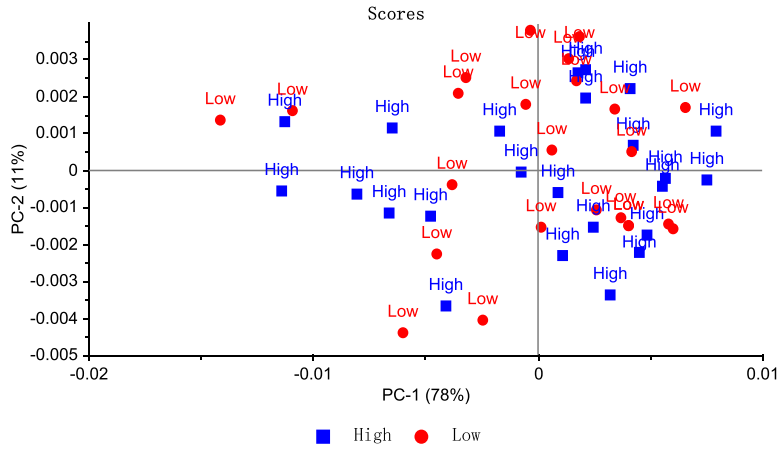
(b)


PC1-Wort beta-glucan
Loading value
Wavenumber (cm-1)

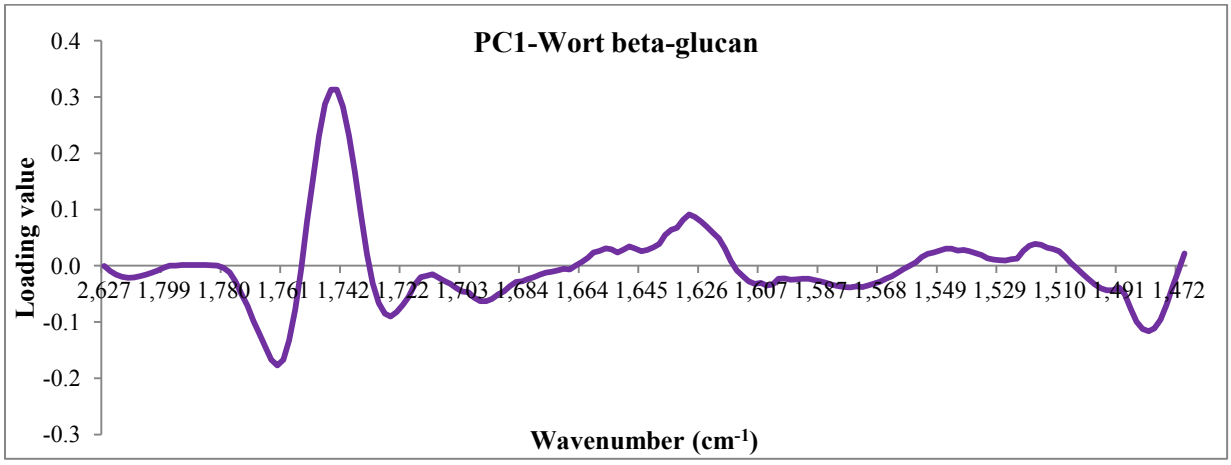
(c)


Scores
PC-2 (11%)
PC-1 (78%)
0h
4h
12h

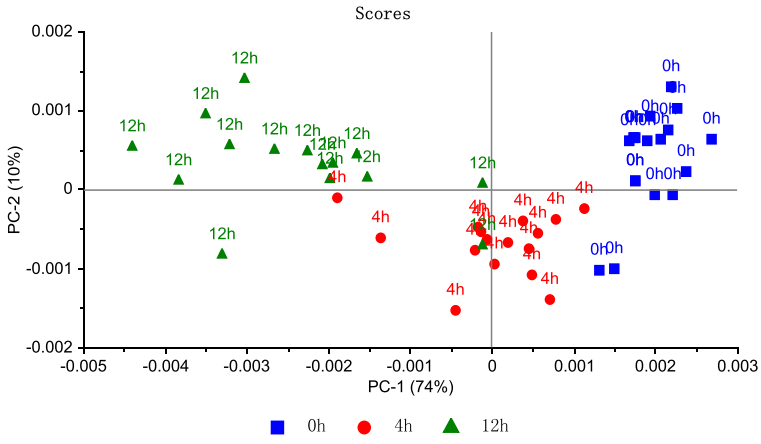
(d)


Scores
PC-2 (11%)
PC-1 (78%)
High
Low

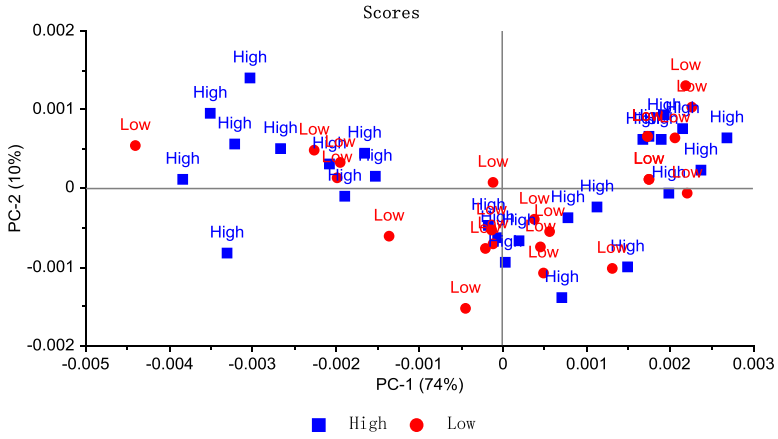
(e)


PC1-Wort beta-glucan
Loading value
Wavenumber (cm-1)

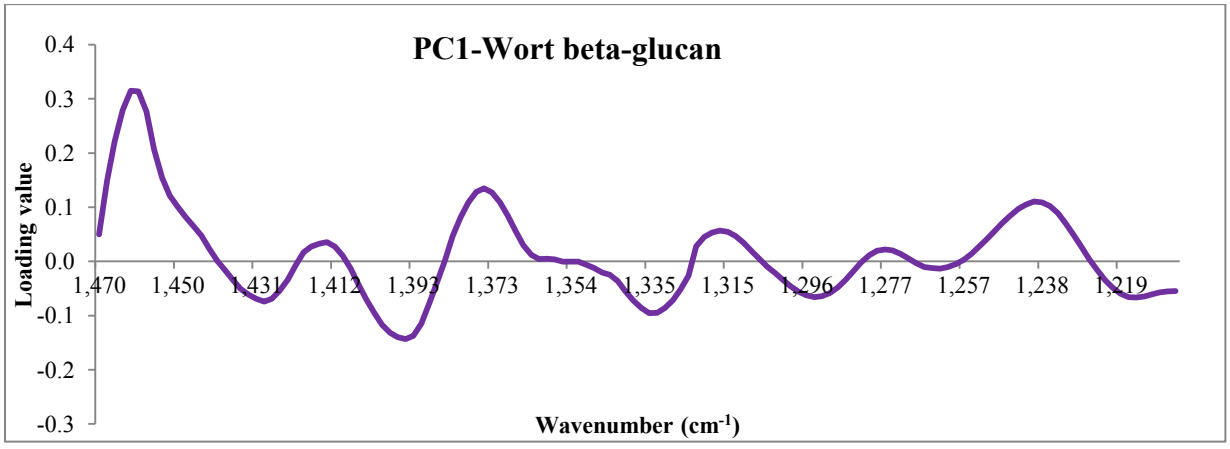
(f)


Scores
PC-2 (10%)
PC-1 (74%)
0h
4h
12h

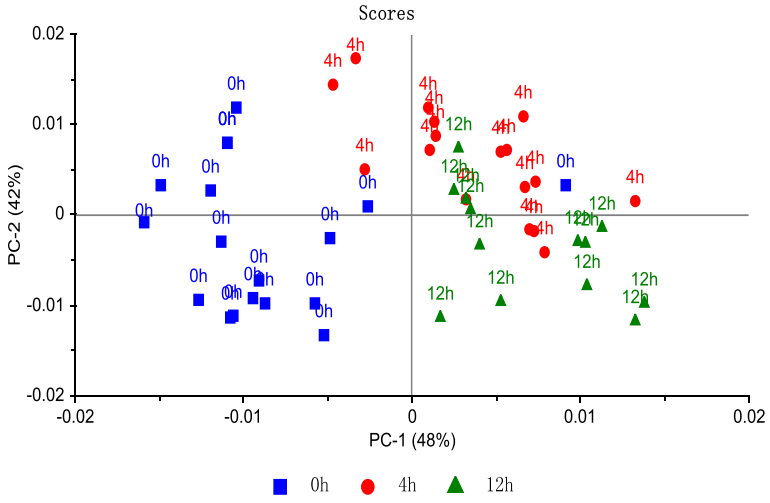
(g)


Scores
PC-2 (10%)
PC-1 (74%)
High
Low

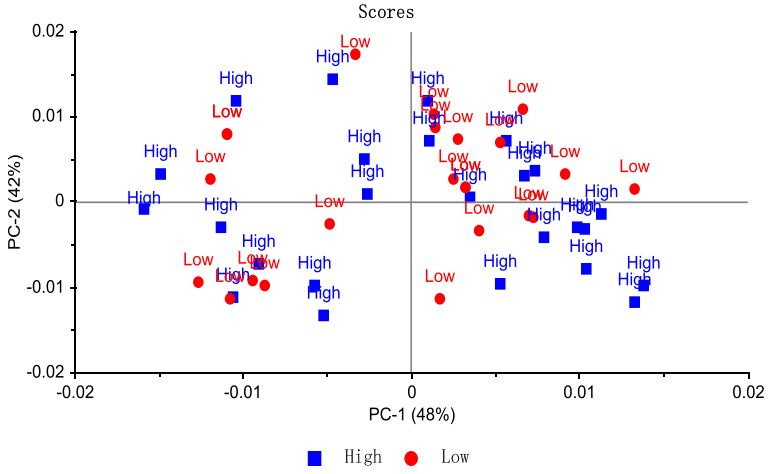
(h)


PC1-Wort beta-glucan
Loading value
Wavenumber (cm-1)

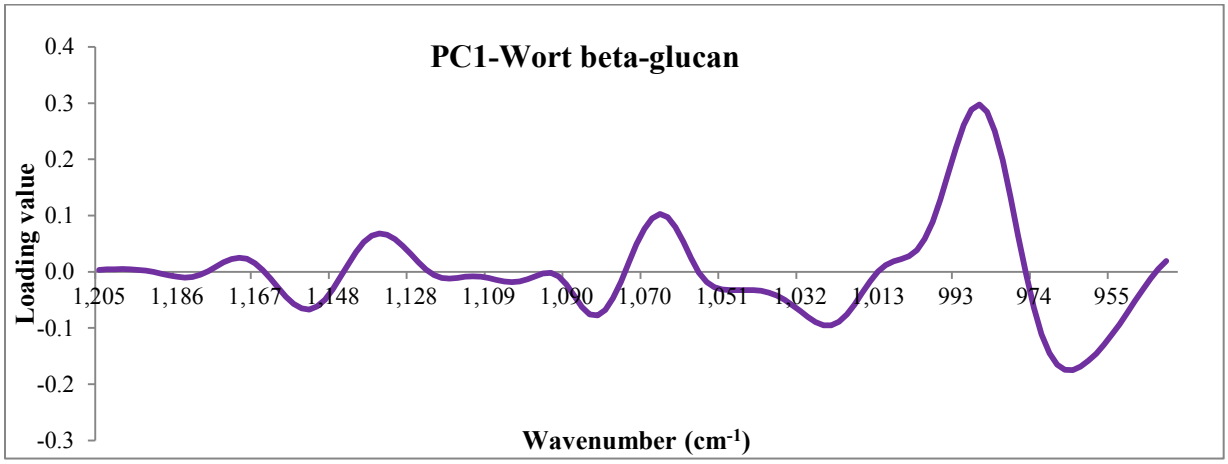
(i)


Scores
PC-2 (42%)
PC-1 (48%)
0h
4h
12h

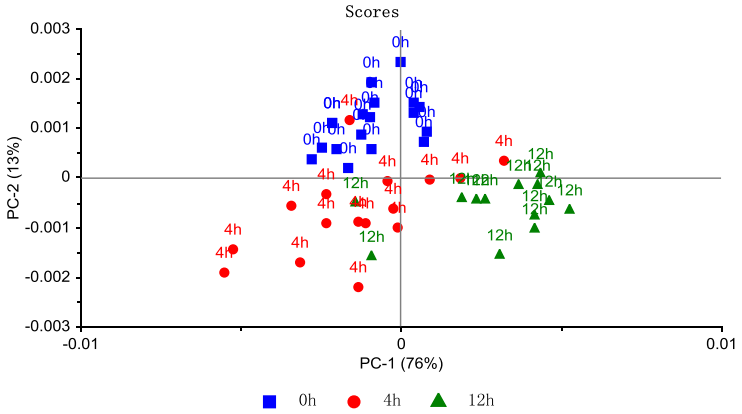
(j)


Scores
PC-2 (42%)
PC-1 (48%)
High
Low

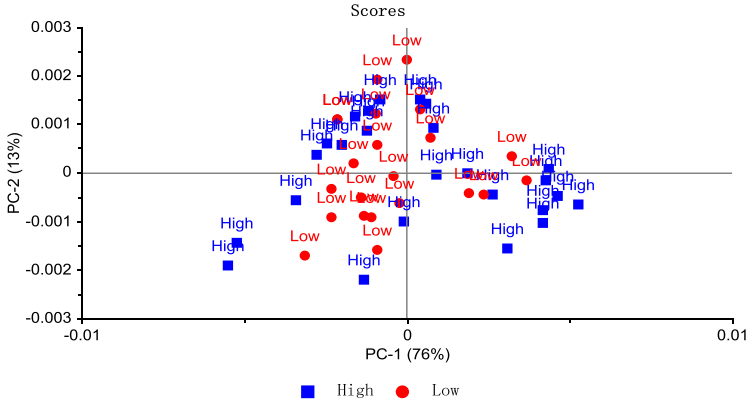
(k)


PC1-Wort beta-glucan
Loading value
Wavenumber (cm-1)
1,205
1,186
1,167
1,148
1,128
1,109
1,090
1,070
1,051
1,032
1,013
993
974
955

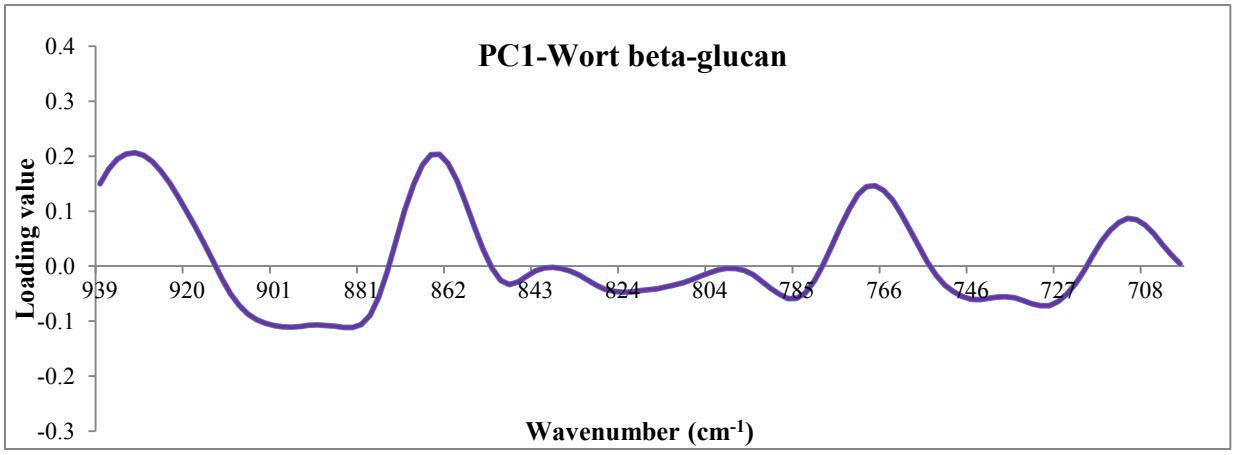
(l)


Scores
PC-2 (13%)
PC-1 (76%)
0h
4h
12h

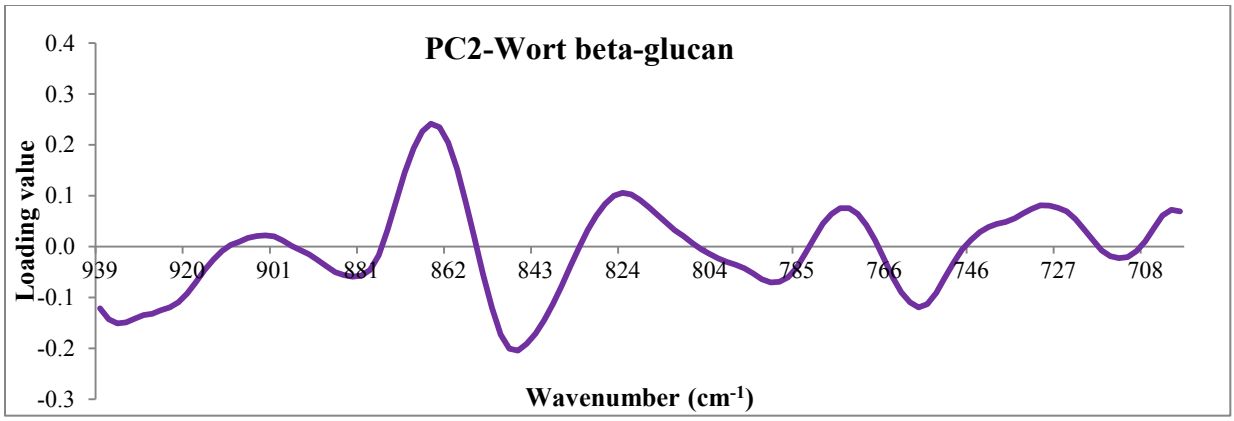
(m)


Scores
PC-2 (13%)
PC-1 (76%)
High
Low

(n)


PC1-Wort beta-glucan
Loading value
Wavenumber (cm-1)

(o)



(p)

Appendix-6 Scores and loadings plots of principle component analysis (PCA) based on five sub-divided regions of ATR-FTIR spectra at 0 (original barley grain), 4 and 12 h of *in situ* rumen digestion, transformed by Standard Normal Variate and Savitzky-Golay second-order derivative, using samples selected for high and low wort beta-glucan (WBG)

Region 1 3050-2812 cm^{-1}: a) Scores plot of PCA-spectra grouped by incubation time point; b) Scores plot of PCA-lack of spectra grouping by level of WBG; C) PC-1 loadings plot of PCA-origin of variation in the spectra in region 1.

Region 2 2626-1471 cm^{-1}: d) Scores plot of PCA-spectra grouped by incubation time point; e) Scores plot of PCA-lack of spectra grouping by level of WBG; f) PC-1 loadings plot of PCA-origin of variation in the spectra in region 2.

Region 3 1469-1207 cm^{-1}: g) Scores plot of PCA-spectra grouped by incubation time point-; h) Scores plot of PCA-lack of grouping by level of WBG; i) PC-1 loadings plot of PCA-origin of variation in the spectra in region 3.

Region 4 1205-941 cm^{-1}: j) Scores plot of PCA-spectra grouped by incubation time point-DP; k) Scores plot of PCA-lack of spectra grouping by level of WBG; l) PC-1 loadings plot of PCA-origin of variation in the spectra in region 4.

Region 5 937-650 cm^{-1}: m) Scores plot of PCA- spectra grouped by incubation time point-DP; n) Scores plot of PCA-lack of spectra grouping by level of WBG; o) PC-1 loadings plot of PCA-origin of variation in the spectra in region 5; p) PC-2 loadings plot of PCA-origin of variation in the spectra in region 5.

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