FTIR MICROSPECTROSCOPY AS A TOOL FOR EVALUATING THE DIGESTIBILITY CHARACTERISTICS OF CEREAL GRAINS FED TO RUMINANTS

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ABSTRACT

Dry matter, crude protein and starch degradation characteristics of one corn (Pioneer 39P78) and four barley grain varieties (CDC Bold, CDC Dolly, Harrington and Valier) were evaluated in two *in situ* nylon bag trials. Trial 1 compared ground and rolled treatments of Harrington barley and Pioneer 39P78 corn, whereas Trial 2 evaluated ground and rolled treatments of the four barley varieties. Rumen degradability characteristics were compared with analytical results from thermal- and synchrotron-source FTIRM. Infrared absorbance spectra were collected from corn and four barley varieties using thermal-source FTIRM on the mid-IR beamline at the Canadian Light Source, Ltd. (Saskatoon, SK). Synchrotron-source FTIRM spectral data was collected for corn, Harrington barley and Valier barley on the U2B mid-IR beamline at NSLS-BNL (Upton, NY). CHO:Amide I peak area ratios were compared to the *in situ* rumen degradation results to determine if FTIRM spectral data could be related to the rate and extent of rumen degradation, and if thermal- and synchrotron-source FTIRM yielded different results. A grain x processing method interaction ($P<0.01$) was observed in both *in situ* trials where grinding produced a greater increase in the rate and extent of rumen degradation for Harrington barley than it did for corn (Trial 1) along with a greater increase in the rate and extent of rumen degradation for CDC Bold and CDC Dolly than for Harrington and Valier (Trial 2). Among barley varieties, increasing rate and extent of rumen degradation (CDC Bold>CDC Dolly>Harrington>Valier) corresponded to increasing starch:protein ratio as estimated by chemical analysis. This relationship was reversed for corn and Harrington barley where corn had a higher starch:protein ratio yet slower rumen degradation kinetics. For both thermal- and synchrotron-source FTIRM, CHO:amide I peak area ratios were greater ($P<0.05$) for corn than for Harrington barley. Comparison of CHO:amide I peak area ratios of barley varieties measured with thermal-source FTIR showed that varieties with higher ($P<0.05$) CHO:Amide I peak area ratios generally had higher rate and extent of rumen degradation. This indicates that starch:protein ratio estimated with FTIRM may be an indicator of rumen degradability characteristics when comparing varieties of the same grain, but not for different species of grains.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance
BNL	Brookhaven National Laborotory
CLS	Canadian Light Source, Inc.
CP	Crude protein
D	Insoluble, but potentially degradable fraction of material for *in situ* incubation (%)
DM	Dry matter
EDCP	Effective degradable crude protein (%)
EDDM	Effective degradable dry matter (%)
EDSt	Effective degradable starch (%)
FTIR	Fourier Transform infrared
FTIRM	Fourier Transform infrared microspectrpscopy
GB	Ground Bold
GC	Ground corn
GD	Ground Dolly
GH	Ground Harrington
GV	Ground Valier
IR	Infrared
ISDMD	In situ dry matter digestibility (%)
K_d	Rate of degradation of D fraction(% h^{-1})
K_p	Rumen passage rate (% h^{-1})
ME	Metabolizable energy
NE_m	Net energy of maintenance
NSLS	National Synchrotron Light Source
OCT	Optimum cutting temperature
OMD	Organic matter digestibility
PI	Processing index
R(t)	Rate at time t
RB	Rolled Bold
RC	Rolled corn
RD	Rolled Dolly
RH	Rolled Harrington
RV	Rolled Valier
S	Soluble fraction in the *in situ* incubation (%)
S/N	Signal to noise ratio
SR-FTIRM	Synchrotron-radiation Fourier Transform Infrared Microspectroscopy
T	Transmission
TDN	Total digestible nutrients
U	Undegradable fraction of material for *in situ* incubation (%)
VFA	Volatile Fatty Acid

1.0 Review of Literature

1.1 Introduction

Cereals produced for the grain-based products industry represent a significant portion of the gross farm cash receipts in Canada. Cereals for human consumption have numerous uses in the flour milling, pasta, baking, biscuit and cereal manufacturing sectors and are used in a wide range of products. In addition to supplying the domestic food market, cereal grains represent a large portion of Canada's agricultural export market share, and generate the bulk of the income for many farms across Canada and the western Provinces. For example, nearly one half of the 21 million tonnes of spring wheat produced in 2004 was sold on the export market as grain or grain products (Canada Grains Council, 2004).

In western Canada alone, over 18 million tonnes of wheat and 12 million tonnes of barley were produced in 2004/2005 growing season (Canada Grains Council, 2004). Each year, a portion of the grain produced will not meet the requirements and standards for human consumption or the export market. Poor quality can result from a shortened growing season, poor harvest weather conditions, storage, transportation, and other factors. As a result, grain quality is insufficient for the human food market, but still possesses excellent value for animal feed, into which it is then channeled. From the 2004/05 crop production, 4.5 million tonnes of wheat and an additional 9.4 million tonnes of barley was used for livestock feed (Canada Grains Council, 2004).

Based on availability, price, and nutritional characteristics, cereal grains represent the principal source of energy in the diets of feedlot and dairy cattle in North America. Particularly in Western Canada, barley is the primary staple of the cattle feeding industry. Corn and wheat are also utilized to some extent, usually dependent on market conditions, price and availability.

Despite providing an excellent source of available energy in ruminant rations, one of the major challenges of feeding cereal grains is avoiding the occurrence of digestive upset. Acidosis and bloat are two of the most common nutritional disorders affecting cattle fed high levels of cereal grain. Problems occur largely as a result of the build-up of excess products from the rapid digestion and fermentation of starch by the rumen microbial population. When the rate of starch degradation and fermentation exceeds the ability of the bacterial population to use the products, the build-up of acids and other products of fermentation leads to illness and reduction in cattle performance.

Because of their common use in ruminant diets, knowledge of the nutritional value and digestibility characteristics of cereal grains is essential. These factors have direct influence on the feeding characteristics of cereal grains. Therefore, correct estimates of the rate and extent of rumen degradation are needed in order to minimize the occurrence of nutritional disorders associated with high grain rations, such as acidosis and bloat.

Variation in the inherent physical structure and chemical composition of cereal grains has an effect on the rate and extent to which they will be degraded in the rumen environment, thus influencing their nutritional value and feeding characteristics. Therefore, there is a need to have effective techniques for estimating the rumen degradability characteristics of various grains. While effective techniques such as *in situ* rumen incubation have been established and used for a number of years, they are still labor intensive, time-consuming and prone to inconsistent results due to procedural variation (De Boer et al., 1987; Goslink et al., 2004). Recent studies by Yu et al. (2003a, 2004a, 2004d) have indicated that the use of Fourier Transform infrared microspectroscopy (FTIRM) may be a potential alternative method of estimating the rumen degradation characteristics of cereal grains. This technique may be capable of estimating the digestibility of cereal grains based on quantification of chemical constituents (Yu et al., 2004a) present in the sample as opposed to the traditional methods of *in situ* digestibility measurements.

The objective of this literature review is to provide background information on the role of cereal grains in ruminant nutrition and how the rate and extent of degradability affects animal production, as well as the factors affecting rumen

degradation characteristics of cereal grains. Traditional methods of estimating the rate and extent of rumen degradation will also be discussed, and how physical structure can be related to degradability characteristics. The underlying principles of the technique of synchrotron IR spectroscopy as well as its current and potential application in the analysis of structural and chemical composition of biological materials will also be discussed.

1.2 Role of Cereal Grains in Ruminant Nutrition

1.2.1 Physiology and structure

Cereals are cultivated grasses that are primarily grown for their seed production. The fruit of a grass is known as a 'caryopsis' and contains a single seed accounting for the majority of the entire fruit when mature (Evers et al., 1999). Basic structural forms of cereal caryopses are relatively similar among species and can be described with a generalized diagram (Figure 1.1).

1.2.1.1 Embryo

The embryo, also known as the germ, is comprised of the embryonic axis (shoot, mesocotyl and radicle) and scutellum. This structure contains the highest concentration of lipid within the seed, and also has the highest moisture content. With the exception of corn in which the extraction of oil is economically important, the embryo is generally not identified as a target for genetic improvement in cereal grain varieties (Evers and Millar, 2002).

1.2.1.2 Endosperm

The endosperm is the largest tissue of the cereal grain kernel, accounting for nearly 80% of the total kernel weight (McAllister and Cheng, 1996; Evers et al, 1999). It consists of two main areas, the starchy endosperm and aleurone layer (Kent and Evers, 1994). Starchy endosperm accounts for most of the mass of tissue occupying the center of the grain, and most references to 'endosperm' actually refer only to this component. Within the starchy endosperm, nutrients are stored in insoluble form, the

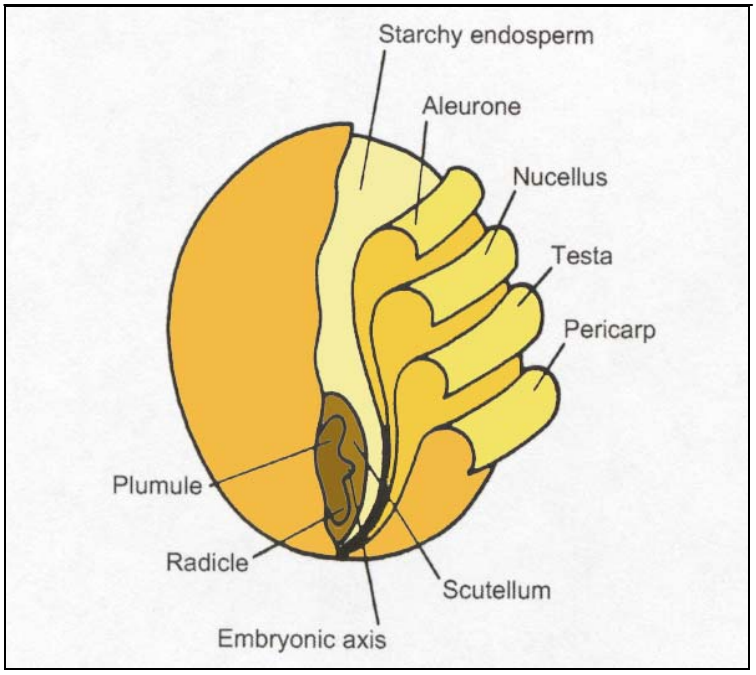

Figure 1.1. Generalized cereal grain kernel showing the main common characteristics. Adapted from Kent and Evers (1994).

primary component being the carbohydrate, starch. Typically, barley contains approximately 53 to 58% starch as a percentage of the entire kernel (Cone et al., 1989; Zinn, 1993) while corn consists of approximately 72% starch as a percentage of the entire kernel (Kent and Evers, 1994). Within the endosperm tissue, starch may account for 70% of the DM, with protein being the next most abundant constituent (Evers et al., 1999). Starch within the endosperm exists in granular form. In barley, these granules have a distinct bimodal size distribution consisting of large A-type granules 15 to 25 µm in diameter and smaller, irregularly-shaped B-type granules less than 10 µm in diameter (MacGregor and Fincher, 1993). A-type granules comprise only 10-15% of the total number, yet they account for nearly 90% of the starch by weight (Båga and Chibbar, 2003). Endosperm cell size is variable and tends to diminish towards the periphery of the endosperm region. Reduction in cell size is accompanied by an increase in cell wall thickness.

Besides starch, other carbohydrates present in endosperm tissue are predominantly located in the cell walls. Cell walls in the starchy endosperm of barley contain very little cellulose; rather, the structural carbohydrates found in the cell walls

are predominantly (1→3) and (1→4) β–D glucans (70%) and arabinoxylans (20%). There is a notable difference in the cell wall composition of aleurone cells which contain approximately 26% β–D glucan and 67% arabinoxylan (Duffus and Cochrane, 1993).

Endosperm starch, as with other natural starches, contains a mixture of amylose (glucose molecules linked by α 1-4 glucosidic linkages) and amylopectin (α 1-4 glucosidic linked chains with α 1-6 branch points). Corn grain contains starch granules that are composed of roughly 27% amylose and 73% amylopectin (Zinn et al., 2002). Several different types of barley exist which contain varying amounts of amylose and amylopectin. In a review of several published studies, MacGregor and Fincher (1993) reported that normal barley starch typically has an amylose content ranging from 23 to 28%. Waxy-type barley, or 'zero amylose' varieties have minimal amylose (up to approximately 5%), and high amylose varieties were found to contain 38 to 53% amylose.

In the mature kernel, starch granules are encased in a protein-rich matrix. An inverse gradient involving starch and protein exists in all cereals with the protein percentage per unit mass of endosperm tissue increasing towards the periphery (Evers et al., 1999). Cells located toward the outside of the kernel are also characterized by decreasing cell size and increasing cell wall thickness (Kent and Evers, 1994).

Endosperm tissue in wheat and barley is consistent throughout, and contains starch granules that are loosely associated with the protein matrix (McAllister and Cheng, 1996). In corn and sorghum, the starch endosperm is divided into two distinct regions: the floury (also called soft or opaque) and the horny endosperm (also called vitreous or hard). The floury endosperm is very similar in composition to that of wheat and barley; however the horny endosperm contains starch granules that are tightly embedded within the protein matrix and has a higher protein content in general (Eckhoff and Paulsen, 1996). This protein matrix is resistant to invasion by microbes and presents a barrier to digestion. Conversely, the protein matrix of barley and wheat is readily penetrated by proteolytic bacteria, rendering the starch available for microbial attack, and resulting in a very rapid rate of digestion. It is estimated that between 80

and 90% of barley and wheat starch is digested in the rumen compared to only 55 to 70% in corn and sorghum (Nocek and Tamminga, 1991).

Cereal proteins are divided into four major groups based on their solubilities: albumins, globulins, prolamins and glutelins. Variation in the proportion of these major protein groups exists between different types of cereal grains. For example, corn typically contains a higher percentage of prolamins and a relatively lower proportion of albumins and globulins than barley (Lookhart, 1991). In addition, the major storage protein fraction in maize, the prolamin fraction, is called zein, whereas the prolamins in barley are hordeins (Lookhart, 1991). Such differences in the relative proportion of these four protein fractions, as well as variation in amino acid composition, affect the physical and chemical properties of cereal grains and may contribute to the differences observed in the rate and extent of ruminal degradation.

Surrounding the starchy endosperm is the aleurone layer. This portion of the endosperm tissue consists of one to three layers of cells, depending on the cereal species. Wheat, corn, rye, oats and sorghum commonly have a single layer of aleurone cells, while barley and rice usually contain three (Kent and Evers, 1994; Evers et al., 1999). In the mature kernel, starch is present exclusively in the endosperm cells, but is not distributed uniformly. Unlike the starchy endosperm, aleurone cells contain little starch, but are rich in protein, lipid, vitamins and minerals (Evers et al., 2002). Starch synthesis occurs last in the cells around the periphery of the endosperm during kernel development; therefore, these so called 'sub-aleurone' cells are preferentially filled with protein rather than starch (MacGregor and Fincher, 1993).

1.2.1.3 Pericarp and seed coat and hull

The outermost tissues of the seed are the nucellus and testa, collectively referred to as the seed coat. The seed coat generally consists of one or two cellular layers that once formed part of the ovule of the mother plant, and are thus from an earlier generation than the endosperm tissue (Kent and Evers, 1994). A multilayered structure on the periphery of the seed is known as the pericarp, which consists of several complete and incomplete layers of cells. During seed development, the pericarp serves to protect and support the growing endosperm and embryo. Chloroplasts are present in

the innermost layers, and photosynthesis produces some starch accumulation. By maturity, all starch has disappeared and the pericarp consists of largely empty, squashed and broken down cells (Kent and Evers, 1994).

Certain cereal grains, including barley, oats and rice, possess modified leaves (paleas) commonly referred to as the hull. Barley grains posses an adherent hull which is removed only with difficulty in hulled varieties. On average, the hull comprises approximately 13% of the grain by weight, but may range from 7 to 25% depending on factors such as grain type, variety, grain size and latitude of production site (Evers et al., 1999). The high fiber content of the hull serves to reduce the digestible energy value of the hulled barley varieties (Yang et al., 1996)

1.2.2 Nutrient profile

Nutrients are not evenly distributed throughout the grain kernel. When whole grains are fed, this is of little consequence as the animal will consume all portions of the kernel. However, when particular fractions of grains remaining after extraction of components valued for human food are combined and used in animal feeding, the nutrient composition and distribution in that particular fraction becomes more important (Evers et al., 1999).

1.2.2.1 Energy content

Carbohydrates in the form of starch provide the most readily available source of energy from cereal grains. Corn generally has significantly higher starch content than barley on a DM basis. Various researchers have reported the starch content of corn as 66, 67, and 72% compared to that of 53, 57 and 58% for barley (Cone et al., 1989; Beauchemin et al., 1994; Zinn, 1993, respectively). Although corn generally contains a larger proportion of starch than barley, the availability of that starch to rumen microbes for fermentation and growth is much lower when comparing grains that are processed by dry-rolling (Ørskov, 1986; Theurer, 1986). A review of literature by Theurer (1986) noted that the ruminal digestion of barley starch was 93% compared to only 73% for corn starch. Because of this, corn is usually subject to processing by steam-flaking which utilizes heat, pressure and shear force to gelatinize some of the starch granules

and to increase the exposure of the remaining starch granules (Zinn et al., 2002). This serves to enhance the availability and degradability of corn starch and allows rumen microbes to extract more of the available energy from the feed. Conversely, barley starch is more readily available to rumen microorganisms, and there is no apparent advantage in steam-rolling barley for growing-finishing cattle in western Canada (Mathison, 1996). On the basis of total digestible nutrients (TDN), corn and barley are very comparable in the amount of energy that they provide per kg of feed (90 and 84%, respectively, which corresponds to a ME of 3.25 and 2.84 Mcal/kg) (NRC, 1996, NRC, 1982). In a review of a large number of studies reporting feeding trial data, Owens et al. (1997) found that the average metabolizable energy of dry-rolled barley exceeded that of corn (3.4 vs 3.26 Mcal/kg), but processing corn by steam rolling significantly increased the ME to 3.73 Mcal/kg.

1.2.2.2 Differences in digestibility

Cereal grains differ in their rate and extent of degradation in the rumen. In general, corn and sorghum are less rapidly fermented by rumen microorganisms than wheat and barley (Cone et al., 1989; McAllister et al., 1990b; McAllister and Cheng, 1996). McAllister et al. (1993) suggested that the variation in ruminal digestion between corn and barley was due to the structure and composition of the protein matrix within the endosperm tissue. In corn, the protein matrix is particularly resistant to degradation by rumen bacteria and rumen fungi appear to be the only ruminal microorganisms capable of penetrating this structure. Conversely, the protein matrix of barley is more readily degraded by proteolytic bacteria, and is therefore digested more rapidly (McAllister et al., 1990b). Variation in the association of starch and protein within the endosperm of cereal grains contributes to the differential effectiveness with which amylolytic bacteria can utilize cereal starch (McAllister et al., 1990a).

In an extensive review of the literature involving several feeding trials, Owens et al. (1997) determined that the average feed efficiency (feed:gain) of cattle fed barley was 6.25 compared to 6.57 for corn-fed cattle when processed by dry-rolling. Steam-roll processing of corn significantly improved its feed efficiency to 5.87 ($P<0.05$). This type of processing causes changes to the physical structure of the starch by gelatinizing

some of the starch granules and increasing its exposure, making it more readily available to digestion and fermentation by rumen microbes.

1.2.2.3 Problems with feeding cereal grains – nutritional disorders

There are several nutritional disorders that are commonly diagnosed in cattle fed high grain diets. Rumen acidosis and feedlot bloat along with their long-term effects are some of the most widespread and economically challenging nutritional disorders faced by cattle feeders.

1.2.2.3.1 Acidosis

Rumen acidosis is a widespread problem in feedlot cattle occurring as a result of grain overload or engorgement, and most frequently arises as animals are moved through the transition from forage to a cereal grain diet (McAllister and Cheng, 1996). The development of nutritional disorders such as acidosis in feedlot cattle involves a complex interaction among intake, diet composition, ruminal microorganisms, and the animal.

During any transition in the diet of an animal, there is a corresponding transition occurring within the rumen environment. First, the major substrates for fermentation are shifting from largely plant cell wall and fibrous material in forages to sugars and starch in cereal grains. Secondly, there is a changeover and adaptation occurring in the rumen microbial population from cellulolytic to starch-fermenting microorganisms. If the dietary shift is gradual, the microbial population will adapt to the new feed without any adverse effects on animal health. However, if the diet changes too rapidly, imbalances will occur between the substrate available for fermentation and the microbial population present to carry out digestion. This situation often leads to acidosis and other digestive problems.

Acidosis is characterized by a reduction in normal rumen pH which often occurs after an increase in the amount of rapidly fermentable carbohydrate in the diet. A large amount of fermentable substrate accommodates the proliferation of starch-fermenting, lactate-producing bacteria (predominantly *Streptococcus bovis* and *Lactobacillus* ssp.),

and the subsequent accumulation of fermentation acids in the rumen (Owens et al., 1998). Acidosis can be divided into two forms: acute and sub-acute, or chronic acidosis.

Acute acidosis usually occurs following consumption of large quantities of highly fermentable carbohydrate, often when the rumen environment of an animal is unadapted to a high-carbohydrate diet. Normally low concentrations of lactic acid in the rumen can increase substantially, along with increased concentrations of volatile fatty acids (VFA), both of which contribute to an increased acid load and reduction of rumen pH. Owens et al. (1998) suggest that a ruminal pH of 5.2 is indicative of acute acidosis. In severe cases, acute acidosis can be fatal. Animals that survive and recover from an episode of acute acidosis may exhibit chronic symptoms and subsequently poor performance. Further complications resulting from acute acidosis are liver abscesses and laminitis. Laminitis may cause sole hemorrhages and lameness leading to poor performance. Liver abscesses, on the other hand, can have a variable influence on performance.

Sub-acute acidosis may occur as a one-time event in which an animal experiences low rumen pH causing diarrhea, dehydration, rumen stasis and reduced feed intake. Alternatively, it may be a chronic issue in cattle fed high grain diets with adapted rumen microbial environments. These animals may experience periodic bouts of low rumen pH for several hours at a time. The exact definition of sub-acute acidosis is unclear. Owens et al. (1998) suggest that rumen pH measure below 5.6 is indicative of chronic acidosis, whereas other sources advise that a pH below 5.8, but above 5.0, indicates subclinical ruminal acidosis and that the incidence of subclinical acidosis increases when ruminal pH falls below 5.8 (Beauchemin and Yang, 2005). Animals may recover from a one-time occurrence with minimal effect on production; however when the acidotic state becomes cyclic in nature, animals are considered to be chronically affected. In contrast to acute acidosis, outward signs of illness are not usually present in animals affected with sub-acute or chronic acidosis, but rather, they exhibit decreased feed intake and lower performance (Galyean and Rivera, 2003).

1.2.2.3.2 Feedlot bloat

Bloat occurs when the gases produced by fermentation are trapped in the rumen causing over-distention. The normal process of eructation is inhibited, and the gases cannot be released as they are produced. As with acidosis and liver abscesses, feedlot bloat is largely a disease that results from the feeding of highly fermentable, grain-rich diets (Galyean and Rivera, 2003). Frothy pasture bloat, often affecting cattle grazing lush forages such as alfalfa or winter wheat, is caused by the high soluble protein content in the forage combined with rapid fermentation which produces the formation of a stable foam. In the case of feedlot bloat, microbial factors rather than plant components seem to be the primary foam-producing agents (Cheng et al., 1998). Consumption of highly fermentable grain-based diets by animals that do not have an adapted rumen microbial population promotes the proliferation of certain species of slime-producing bacteria (especially *S. bovis*) that create a stable foam consisting of gases mixed with rumen contents which cover the cardia and inhibit eructation (Cheng et al., 1998). As gas accumulates, the expanding rumen exerts pressure on the diaphragm and lungs, and impairs respiration (Cheng et al., 1998). This type of bloat is common in feedlot cattle and can lead to death if left untreated.

A second, less common type of bloat is free gas bloat which is also the result of interference with the normal eructation process. Physical obstruction or damage to the esophagus or cardia (Galyean and Rivera, 2003) are the common causes of free gas bloat, however it may also occur as a result of decreased rumen motility in response to excessive acid concentration.

1.2.2.3.3 Other complications

While acidosis and bloat are the two major nutritional disorders that affect feedlot cattle, other long-term complications may arise as a result of these initial maladies. Liver abscesses and laminitis are two such problems that are manifested in fed cattle that have been on high grain rations for a length of time. The occurrence of liver abscesses is generally associated with ruminal acidosis because it is thought that a continual high acid load in the rumen can lead to damage of the rumen epithelium and necrosis of rumen papillae (Ørskov, 1986). These damaged areas of the rumen tend to

provide routes of entry for microbes and bacteria into the portal blood system where they can be transported to the liver and cause liver abscesses (Galyean and Rivera, 2003). *Fusobacterium necrophorum*, which is normally found in both rumen contents and associated with the rumen wall, is the primary organism involved in the formation of liver abscesses in cattle (Nagaraja and Chengappa, 1998).

Laminitis is usually indicated by the presence of sole ulcers or hemorrhages and swelling of the coronary band above the hoof. One theory of its cause is the production of endotoxins by gram negative bacteria, such as E. coli, as a result of acidotic conditions in the rumen. These endotoxins are absorbed into the circulatory system where they cause vasoconstriction in the vascular system of the hooves leading to hypoxia and malnutrition of the laminar and cornary tissues of the hoof wall (Aiello, 1998).

1.2.3 Understanding digestibility

1.2.3.1 Economic implications

Although mortality is an economic concern for cattle producers, the incidence of death from nutritionally related disorders is typically low (Galyean and Rivera, 2003). Smith (1998) reviewed mortality data from several large feedlots in the United States indicating that the average mortality rate ranged from 0.17 to 0.42% per month; of this, digestive disorders accounted for 30 to 42%. Perhaps of more importance than the mortality itself is the morbidity associated with nutritional disorders in feedlot cattle. Morbidity in feedlot cattle may have greater consequences than animal death when taking into consideration the expenses of medication, labor involved with treatment, premature culling because of chronic conditions, and the expense of reduced performance both during and after illness (Smith, 1998). Due to the high economic cost of nutritional disorders, it is of great interest to cattle producers to minimize the incidence of these cases and to improve overall cattle growth, performance and efficiency.

1.2.3.2 Factors influencing digestibility

Rate and extent of starch digestion in the rumen are determined by the interaction of several factors, including source of dietary starch, diet composition, rate of feed intake, degree of processing, and degree of adaptation of the rumen microbial population (Huntington, 1997).

Processing is likely the most important physical factor influencing the digestibility of cereal grains. Whole cereal grain kernels are poorly digested by the animal due to the tough outer seed coat that prevents access of rumen microbes into the interior of the kernel. These whole grains will often pass directly through the digestive tract with little or no digestion occurring. A study by Beauchemin et al. (1994) showed that more than 75% of barley and wheat and 50% of corn kernels were excreted in the feces of cattle fed whole barley, wheat and corn grain. In the same study, whole grains incubated in the rumen had less than 30% DM disappearance even after 96 h of ruminal incubation. Physical disruption of the pericarp and seed coat through mastication or other mechanical processing methods (rolling, grinding, steam flaking, etc.) is essential to provide access for rumen microbes into the interior of the kernel for digestion of carbohydrates. Ruminants can more effectively digest unprocessed corn than barley or wheat because the pericarp is more extensively damaged by mastication during chewing and rumination (Beachemin et al., 1994). The magnitude of the processing effect on starch utilization is not as great with barley as with corn (Theurer, 1986). This is likely due to inherent differences in starch and protein digestibility. However, unprocessed barley is poorly digested and processing does dramatically improve the digestibility of barley grain. Once the pericarp is breached, the composition of the protein matrix and endosperm cell walls will dictate the rate at which ruminal bacteria access the starch granules (Wang and McAllister, 2000). Additionally, the number and combination of different microbial species present in the rumen will affect the rate of starch digestion. Anatomical differences exist among cereal grains with regard to starch granule and protein matrix structure, making the digestion of different structures a complex process which requires the use of several specialized enzymatic steps. Microflora in the rumen consist of a wide array of microbial species including bacteria, protozoa and fungi (McAllister and Cheng, 1996). A number of different species of ruminal bacteria are

believed to be responsible for most of the feed digestion in the rumen (Cheng et al., 1991 as reported by McAllister and Cheng, 1996). Each of these species is able to digest starch, but is not capable of individually producing the array of enzymes required to efficiently digest the entire grain kernel. Rather, complimentary bacterial species combine to form a complex microbial digestive consortium on the surface of cereal grains (McAllister et al., 1993). This sequential process involves the establishment of a primary microcolony of amylolytic bacteria which adhere to the surface of starch granules. These microbes produce metabolites that attract secondary colonizers, and a climatic microbial population capable of complete digestion is eventually established (McAllister and Cheng, 1996). Alteration of the sequential development of this microbial consortium, such as processing of the grain or abrupt change in diet, can affect both the rate and extent of cereal grain digestion in the rumen.

Rate of feed intake and the rate of feed passage through the digestive tract have an effect on the digestibility of feeds and availability of nutrients to the animal. In general, the rate and extent of feed particle digestion in the rumen are directly proportional and an increase in the rate of feed particle passage is directly proportional to increased feed consumption (Huntington, 1996). Especially with regard to digestion of forages, increased rate of passage through the rumen will reduce the digestibility of the feed because it escapes the rumen before microbial digestion is complete (Van Soest, 1994). Therefore, techniques which are able to alter the pattern and rate of feed consumption have the ability to influence the digestibility of a feed to a certain extent. Some examples of this are the use of feed additives, such as ionophores (Zinn, 1987), changing the physical consistency of the diet to encourage or discourage consumption by cattle, or altering consumption by employing various feeding management techniques (Pritchard and Knutsen, 1995).

1.2.3.3 Methods of manipulating digestibility

Some of the factors influencing the efficiency of cereal grain digestion by ruminants include kernel structure, cereal species, site of digestion, and degree of processing (McAllister and Cheng, 1996). Rumen fermentation rates have been manipulated by grain processing (both physical and chemical) and by feeding various

combinations of cereal grains in order to alter the rate and extent of ruminal digestion. From the literature, we know that there is a natural variation in the degradation rate between cereal species and cultivars. The other variable that is now starting to be considered in more detail is how to make use of kernel structure as a tool for manipulating rumen fermentation dynamics.

Method of processing has a large influence on the digestibility of cereal grains. The primary goal of grain processing, particularly with corn, is to shift the site of starch digestion from the small intestine to the rumen where energy can be used to enhance microbial growth (Theurer, 1986). Steam flaking will increase the NE_m of corn grain by 15% (Zinn et al., 2002). The increase in digestibility results from a combination of gelatinization of starch granules, and disruption of the protective protein matrix surrounding the starch granules. Thus, moist heat in combination with shear force serves to enhance the rate of starch digestion in corn (Zinn et al., 2002). Processing also has an effect on the digestibility of barley. It is important to keep in mind that the value of processing barley is somewhat dependant on the desired use of the end product. For ruminant feeding, the goal of processing is to disrupt the outer seed coat of the kernel in order to allow microbial access to the interior, but to avoid excessive production of small particles, or fines, which will cause digestive disturbances. The degree of processing can be quantified using a processing index (PI), which refers to the bulk density (i.e., volume weight expressed either as g/L or lb/bu) of the barley after processing expressed as a percentage of its volume weight before processing (Yang et al., 1999). A higher degree of processing produces finer particles which will have a lower volume weight and consequently, lower PI (Wang and McAllister, 2000).

1.3 Estimating Cereal Grain Digestibility

It has long been acknowledged that feeding ruminant animals based simply on quantity of feed is not sufficient to meet nutritional requirements for growth and production. Knowledge about the specific nutrient content of feedstuffs and the degree to which nutrients are made available to rumen microorganisms as well as the amount that escapes ruminal fermentation is essential for maximizing feed utilization and animal performance. In order to establish the amounts and ratios of nutrients necessary

for optimal microbial and animal response, one must first adequately predict the degree to which nutrients are made available in the rumen (Nocek, 1988). Different methods for estimating ruminal availability of nutrients in feed have been developed and utilized, with varying degrees of success and accuracy.

1.3.1 *In vitro* digestibility estimation

There are several methodologies used for *in vitro* measurement of feed digestibility. Enzymatic digestion techniques utilizing proteolytic enzymes offer several advantages over live microbial cultures. Relatively low cost, reduced time for analysis, less contamination of feed residue, and the convenience of carrying out experiments in test tubes rather than with live animals all enhance the practicality of this method. However, despite the many conveniences, the use of enzyme procedures may lack the desired accuracy for digestibility measurements. Use of single enzyme systems or commercial enzyme preparations may vary in relation to actual ruminal proteolytic activity (Nocek, 1988). In a comprehensive review of the literature, Nocek (1988) concluded that enzymatic digestion techniques may be most suitable for measuring relative differences between feedstuffs rather than providing absolute digestibility values.

To overcome some of the limitations of enzymatic digestion, *in vitro* methods utilizing rumen fluid as the digestion media were developed. 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 stimulate postruminal digestion. It is the most commonly used *in vitro* digestion method. This technique appears to more closely approximate true rumen digestion. Monson et al. (1969) reported a correlation of 0.81 for several forages between *in vivo* nylon bag digestibility measurements and *in vitro* measurements obtained by Tilly and Terry (1963). The disadvantages of this technique lie primarily in the number of steps and the length of time required for analysis (Van Soest, 1994), and that it lacks and the physical interactions of the true rumen environment.

1.3.2 *In situ* digestibility estimation by rumen incubation

Suspension of feed material within the rumen allows direct contact of the test feed with the authentic rumen environment. This is possibly the most accurate simulation of the rumen environment to which feed is exposed when consumed by the animal, and has been used for several years as the basis for predicting digestion in several feeding systems (Nocek, 1988).

Estimation of the rate and extent of rumen digestibility using the *in situ* technique is accomplished by analysis of the feed sample residue remaining after incubation in the rumen. This analysis partitions feed material into three fractions based on ruminal availability: soluble, potentially degradable and non-degradable. These are commonly referred to as the A, B, and C fractions, respectively (Nocek, 1988). Note that in the following chapters, the A, B, and C fractions are represented by the variables S, D and U, respectively. The in *situ* technique can be used to quantify the solubility fractions as well as the rate of digestion (K_d) of the B fraction. Estimation of the soluble (S or A) fraction is a measure of the amount of the test feed that disappears from the bag prior to rumen microbial digestion. Rumen bags containing the test feed sample are incubated in the rumen at a number of time points beginning at 2 hours and extending until the endpoint of digestion can be detected. For concentrate samples, 36 to 48 hours is adequate time to detect the endpoint of digestion. After being removed from the rumen, washed and dried, the residue remaining in the bags for each incubation time point is weighed, and from this measurement the degradable (B or D) and non-degradable (C or U) fractions as well as the rate of degradation (K_d) are estimated using a nonlinear statistical procedure. The rate of degradation of the B fraction is described by a first-order kinetic rate constant (Mertens, 1973 cited by Nocek, 1988), and assumes that the substrate will be degraded as a linear function of time in the rumen.

A study by Gosslink et al. (2004) compared the predictive value of four techniques (pepsin-cellulase technique, *in situ* nylon bag technique, the gas production technique, and the technique of Tilley and Terry (1963)) to determine the digestibility of organic matter (OMD) in ruminants. These researchers concluded that the *in situ*

technique exhibited the highest degree of accuracy in predicting OMD, but was also the most expensive and time consuming.

Another of the major drawbacks of the *in situ* technique is the potential for variability in experimental results. Ability to produce repeatable measurement of digestibility both among and within laboratories is heavily dependent on several procedural factors, including porosity and size of bags, substrate particle size, variability due to animal, washing technique, and even the human variability due to the technician carrying out the procedure. De Boer et al. (1987) conducted an extensive study to compare several points of variation in the *in situ* technique in order to develop a standardized procedure for *in situ* incubations which would provide the best repeatability, reduce the labor requirement, and increase the throughput capacity of the *in situ* method. However, despite the attempts at making a standardized, practical procedure, there is still large room for variability among digestibility measurements of feeds produced in different experiments and in different laboratories.

1.4 Synchrotron Infrared Microspectroscopy

Although traditional methods of measuring digestibility (*in vitro* and *in situ*) in animal feeds are functional and relatively accurate, they are also time-consuming and laborious. Another disadvantage of these techniques is the requirement for samples to be homogenized (ground) for 'wet' chemical analysis, thus eliminating the opportunity to gain chemical and structural information regarding isolated regions of the kernel, such as the endosperm.

Application of FTIRM technology (both thermal and synchrotron light source) may present a new alternative method for investigating the molecular structure of biological tissues at high ultra-spatial resolution, without destroying the inherent structure of the grain. Data obtained from this type of chemical analysis may be pertinent to discovering the unique chemical micro-structures of each grain type and how this relates to rumen digestibility characteristics.

1.4.1 Using IR spectroscopy for biological samples

Infrared (IR) spectroscopy has become a useful technique and a valuable analytical tool for examining the chemical composition of biological samples. The basic principle behind the technique is based on the vibrational motions of atoms and chemical bonds within organic molecules. When a beam of light containing the mid-IR radiation band is passed through a sample, light energy from the photons is absorbed by bonds and transformed into vibrational motions (Gough, 2003). Different bonds absorb light at different frequencies giving very specific absorption patterns to various biological components.

A key advantage of infrared spectroscopy is that it is sensitive to the structure and concentration of all tissue components (proteins, lipid, carbohydrate, phosphate, carbonates, nucleic acids, etc.) present in the sample. Each of these tissue components can be detected by their characteristic absorption bands at specific wavelengths within a single spectrum (Gough, 2003). The vibrational spectrum collected from a specific tissue location has unique properties that provide detailed information for all

components within the illuminated pixel. This information is valuable for the identification of functional groups and molecular conformation (Gough, 2003).

1.4.2 Advantage of synchrotron light

One of the advances in the IR spectroscopic technique is the use of a synchrotron light source in place of the conventional thermal light, allowing for the microscopic examination of chemical structures within biological tissue samples at much higher spatial resolution. Synchrotron radiation greatly enhances both spatial and spectral resolution over that obtained using a conventional thermal IR light source (Wetzel and LeVine, 2000). There are several advantages to synchrotron light over the thermal source. First, synchrotron IR light is 100 to 1000 times brighter than a conventional thermal source because the effective source size is small and the light is emitted into a narrow range of angles (Dumas et al., 2004; Miller, 2004), resulting in a concentrated, non-divergent beam. This feature gives it the ability to explore the chemistry within microstructures of plant tissue at high spatial resolutions of 3 to 10 μm since even a small spot can be highly illuminated because the beam is not spread out over a larger area (Wetzel et al., 1998). Secondly, the brightness of the synchrotron source allows smaller regions to be probed, while at the same time, maintaining an acceptable signal to noise (S:N) ratio (Dumas and Miller, 2003). Figure 1.2 compares the infrared spectra of a single human cell collected with an internal IR light source using a 6 x 6 μm^2 aperture with a synchrotron spectrum collected using a 3 x 3 μm^2 aperture. Clearly, there is a vast improvement in the spectral quality made possible with the brightness advantage of synchrotron light. A minimal amount of thermal noise (fluctuation) is generated with synchrotron radiation, making the source more constant and able to produce acceptable spectral data (Wetzel et al., 1998).

Finally, the brightness of the synchrotron infrared source permits rapid acquisition time for spectral data combined with superior quality and spatial resolution at the diffraction limit (Dumas et al., 2004). Using synchrotron microspectroscopy,

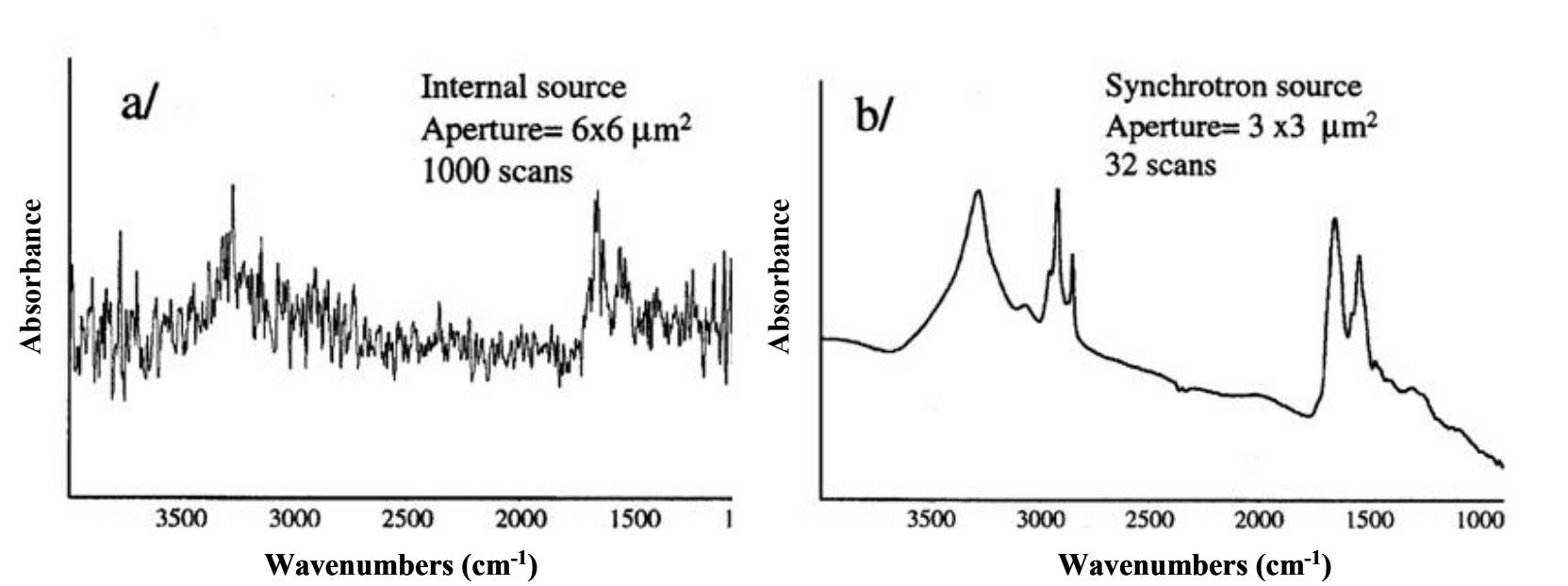

Figure 1.2. The IR spectra of an individual human cell (a) collected using a 6 x 6 μm^2 aperture with a blackbody IR light source, and (b) using a 3 x 3 μm^2 aperture with the synchrotron light source. Adapted from Dumas and Miller (2003).

molecular structures and specific functional groups within an organic compound can be identified based on the unique vibrational characteristics displayed when exposed to mid-IR radiation.

1.4.3 Sample preparation techniques

Sample preparation is perhaps the most critical part of a successful IR microspectroscopy experiment (Miller and Dumas, 2006). Infrared spectra collection in transmission mode requires the use of thin sections of tissue, typically 5 to 30 μm thick. Polymers, unmineralized biological tissues, and other organic materials are sectioned to a thickness of 10 to 15μm (Miller, 2004). These thin sections are generally prepared by fixing the sample in an embedding agent and then cutting with a cryomicrotome. In the case of seed sectioning, the process becomes somewhat more complicated due to the incidence of shattering when the dry, desiccated seed is cut. For analysis with light microscopy, samples are typically embedded in liquid hydrocarbon (such as paraffin wax or optimum cutting temperature solution), cooled to -10 or -20ºC and sectioned. This sample preparation method can also be used for IR microspectroscopy. As the hydrocarbon matrix serves to maintain the physical integrity of the sample during sectioning, the quality of the tissue sections for IR spectroscopy is superior to those sectioned without the use of an embedding agent. With IR spectroscopy, however, the use of such embedding agents is not ideal as they produce strong IR absorption bands which may interfere with the sample spectra (Jackson and Mantsch, 2000). In addition, the paraffinization process involves extraction with xylene and other solvents which remove lipid from the sample along with the paraffin, and may cause denaturation of protein (Jackson and Mantsch, 2000), thus distorting measurements of actual chemical composition of the seed.

Because of this undesirable interaction, a method of sample preparation and sectioning without the use of any chemical embedding agents is advantageous for IR spectroscopy. However, in practice, it can be difficult to obtain thin tissue sections that are smooth, intact and properly fixed to the slide. As a result, it may be necessary to weigh the benefits of obtaining a good sample section by using an embedding technique

with the disadvantage of observing additional spectral absorptions from compounds used in the embedding process.

Once sectioned, samples are fixed onto specialized IR microscope slides. No additional preparation techniques are required, and the fact that IR microspcetroscopy can utilize untreated, unstained tissue samples is a distinct advantage of this technique (Gough, 2003). Samples can be mounted directly onto IR microscope slides which have been glazed on one side with IR reflective material for analysis in reflectance mode. Alternatively, samples can be mounted on disks or 'windows' made of infrared-transparent material such as CaF_2 or BaF_2 for transmission mode microspectroscopy. Although regular glass slides used for light microscopy are transparent to visible light, they are not suitable for IR spectroscopy because all wavelengths of IR light below 2500 cm^{-1} are absorbed by glass (Jackson and Mantsch, 2000).

Sample thickness is one of the most important factors involved in obtaining quality IR spectra. When the sample tissue is too thick, the intensity of resultant absorbance bands in some spectral regions (particularly the Amide I and carbohydrate regions) will be such that problems due to detector nonlinearity will result (Jackson and Mantsch, 2000). Absorbance of IR light passing through a sample is equal to: $A = \log(1/T)$. Therefore, when A=1.0, only 10% of the incident IR light is passing through the sample to the detector. The sensitivity and accuracy of the detector at such low levels of light is reduced, making the reliability of the data questionable. Some sources suggest that ideally, absorbance values should be in the range of 0.4 to 0.6 absorbance units in order to ensure the most reliable and accurate detector readings (Jackson and Mantsch, 2000).

Transmission mode microspectroscopy is generally preferred over reflection because up to one half of the incident IR light is blocked by a mirror that collects the reflected light, thus reducing the amount of incident light illuminating the sample. Figure 1.3 illustrates the difference between a microscope configured for analysis in transmission and reflectance modes.

Another disadvantage of using reflectance mode is that the incident light must travel through the sample twice: the IR beam penetrates through the sample, reflects off the IR-reflective slide, and then passes back through the sample a second time before

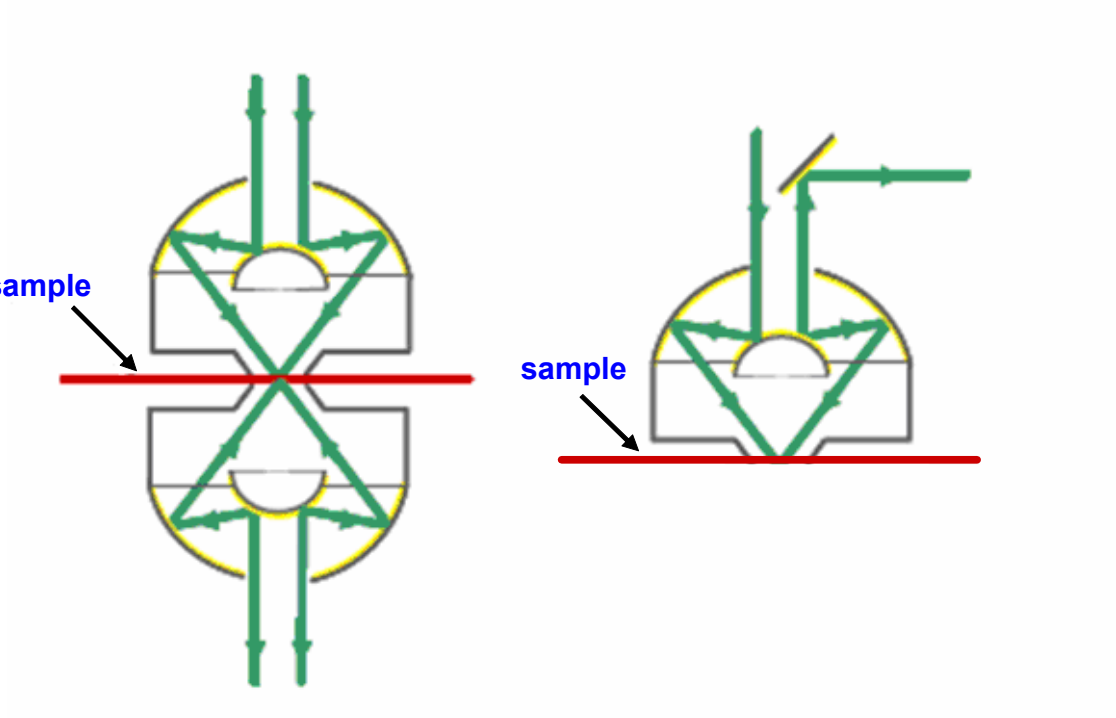

Figure 1.3. Path of the IR beam in transmission (left) and reflectance (right) modes. Adapted from Miller (2004).

traveling to the detector. Since the beam passes through the sample twice, the result is a "double-absorption" spectrum (Miller, 2004). Essentially, the beam is passing through twice the thickness of sample as the transmission beam which only passes through the sample once before being directed to the detector. Thus, in order to maintain an acceptable level of IR absorption (ie. below 1.0 absorbance units), reflectance mode analysis often requires sample sections to be cut thinner than that used for transmission measurement. The ideal sample thickness varies depending on the material being studied. For certain types of samples, 6 μm sections will produce acceptable data in reflectance mode. However, as was discovered during experiments in this project, barley and corn sections cut to 6 μm thickness were still too thick for satisfactory analysis in reflectance mode, resulting in poor high absorbance values and poor signal to noise ratio (S/N).

1.4.4 Interpretation of spectra: major absorption groups

Interpretation of spectral data relies heavily upon an understanding of the spectroscopic properties of the individual constituents of the sample tissue (Jackson and Mantsch, 2000). Assignment of spectral features in biological samples to specific frequencies should be offered as guidelines only. It is important to remember that assignment of IR absorption features in biological samples requires knowledge of both sample histology and pathology, in addition to spectroscopy (Miller et al., 2000). Bonds within molecules will absorb infrared light at the specific wavelength which has the same vibration frequency as the molecular dipole moment, thus making it possible to assign absorption peaks to specific functional groups. Major chemical structures such as proteins, lipids, starch, and phosphates have predictable absorption peaks at known wavenumbers; however, these peaks may shift slightly from tissue to tissue due to various interactions and physical properties of the sample being studied. The positions of vibrations in the spectrum are reported as inverse wavelength, or wavenumber. Figure 1.4 illustrates the nature of the stretching and bending vibrations of atoms and bonds within molecules when they absorb IR light.

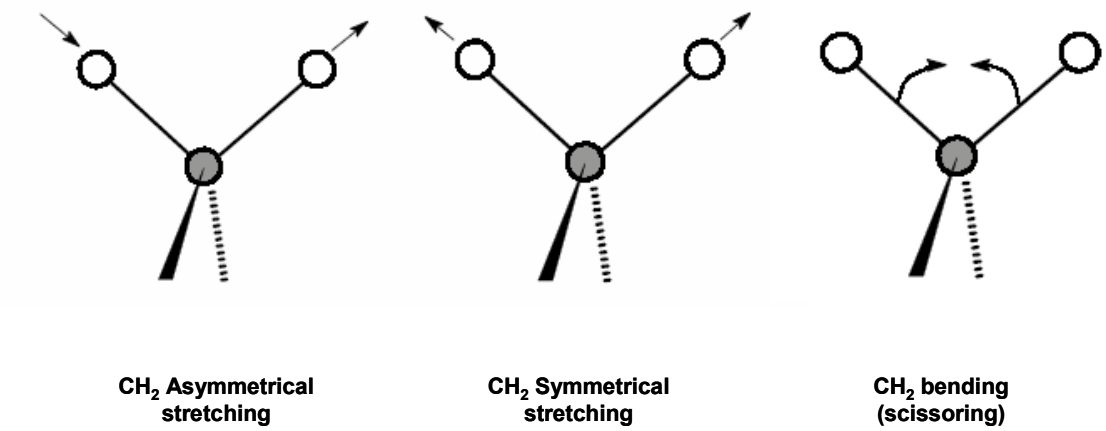

Figure 1.4. Stretching and bending vibrations of bonds in a CH_2 molecule. Source: http://cat.middlebury.edu

The wavelength of light absorbed by a vibrating bond depends upon the atoms in the bond, the type of bond, the type of vibration and any inter- and intramolecular interactions (Jackson and Mantsch, 2000). For complex samples such as plant tissues, knowledge of the biochemical constituents present will aid in identifying the origin of a particular bond absorption in the IR spectrum. For example, the stretching vibrations of C=O bonds absorb IR light at a wavelength of ca. 1650 cm^{-1}. Molecules that contain a significant number of C=O bonds, such as the amide group in the backbone of the protein structure, will exhibit an absorption band at this location. Therefore, in biological samples known to contain significant amounts of protein, the absorption due to bending vibrations of C=O is assigned to the amide group of protein molecules and is used as an indicator of Amide I (and thus protein) present in the sample. Obviously, if the sample being studied is non-biological and does not contain protein, possible absorptions due to C=O bending would be assigned to another compound known to be present in the sample.

A typical absorption spectrum from a section of barley tissue is shown in Figure 1.5. The region of the IR spectrum between wavenumbers 1750 and 2800 cm^{-1} is free from absorption of functional groups present in biological material with the exception of water present in wet tissue or fluids and carbon dioxide (Jackson and Mantsch, 2000). The dominant absorptions of interest in this spectrum are found in the 1650 –

1025 cm^{-1} region. Two distinct peaks at ca. 1650 cm^{-1} and ca. 1550 cm^{-1} correspond to the protein absorption bands. Amide I is the major protein absorption band located at 1650 cm^{-1}, and occurs predominantly from the C=O stretching vibration of the amide C=O group. A smaller protein absorption band, Amide II, is found between 1500 and 1560 cm^{-1} and is largely the result of N-H bending vibrations coupled with C-N stretching within the protein molecule (Jackson and Mantsch, 2000).

Carbohydrates comprise a large portion of the organic compounds in plant tissues and, therefore, will generally exhibit large absorption peaks. Precise assignment of polysaccharide absorptions is difficult due to the complexity of overlapping carbohydrate bands in the 1200-800 cm^{-1} region. The majority of these absorptions arise from stretching vibrations of the COH and COC groups (Colthup et al., 1990; Robert et al., 2005). The largest and broadest carbohydrate absorption peak is centered at approximately 1025 cm^{-1}, and is attributed to non-structural carbohydrate, or starch (Wetzel et al., 1998). This absorption band arises from the stretching vibration of C-O groups (Jackson and Mantsch, 2000). Yu et al. (2004) observed that a set of three peaks (at approximately 1160, 1080 and 1025 cm^{-1}) were present in the carbohydrate region of the IR spectrum of all analyzed pixels of corn endosperm tissue. Cell wall carbohydrate (cellulosic material) has been identified by other researchers as having absorption peaks at approximately 1400 cm^{-1} and 1246 cm^{-1} (Wetzel et al., 1998; Peitrazk and Miller, 2005) and 1033 cm^{-1} (Robert et al., 2005), while the aromatic ring structures within lignin molecules will absorb IR radiation at approximately 1510 cm^{-1} (Yu et al., 2004).

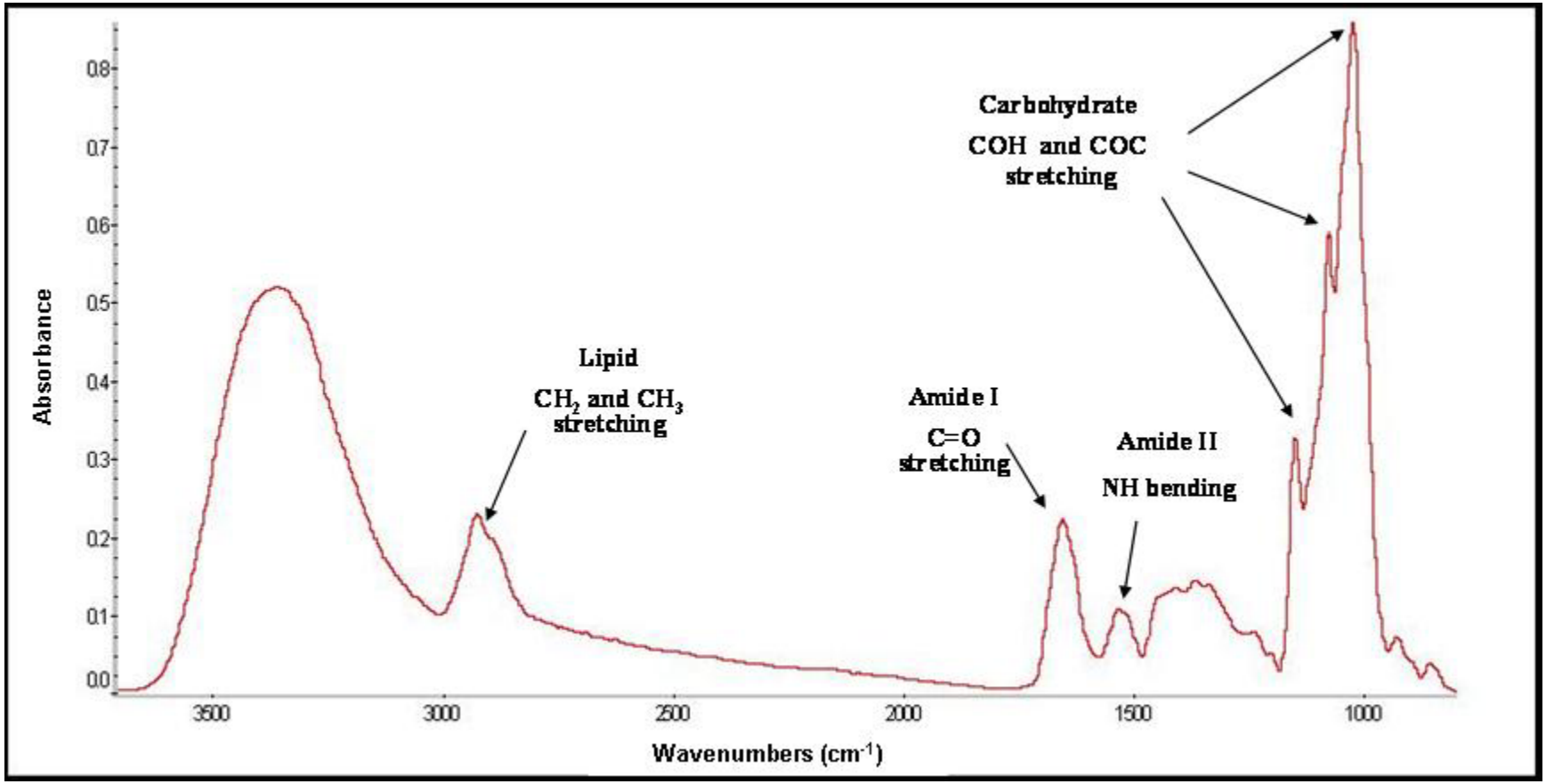

Figure 1.5. Typical IR absorbance spectrum of cereal grain endosperm tissue taken from a cross section of Harrington barley using synchrotron IR light (NSLS). Adapted from Jackson and Mantsch (2000) and Gough (2003).

The presence of lipid is indicated by absorptions from the symmetric and asymmetric stretching vibrations of CH_2 (2922 and 2852 cm^{-1}) and CH_3 (2956 and 2874 cm^{-1}) groups of the acyl chains (Jackson and Mantsch, 2000). Due to the greater number of CH_2 groups present in the acyl chains, the corresponding peak intensities are 10 to 20 times greater than that of CH_3 (Yu et al., 2004). Lipid content in the sample is also denoted by a strong absorption band at approximately 1735 cm^{-1} arising from the stretching vibration of the C=O ester groups present in lipid molecules (Jackson and Mantsch, 2000; Pietrzak and Miller, 2005).

A broad absorption band between 3400-3200 cm^{-1} is present in nearly all spectra and is attributed to OH and NH stretching. However, this spectral feature is not used for characterization of functional groups.

1.4.5 Biological application of SR-FTIRM

The high spatial resolution of a synchrotron IR source permits the sub-cellular chemical mapping and characterization of plant composition (Miller and Dumas, 2006). Determining the distribution of plant components such as lignin, cellulose and other carbohydrates, proteins and lipids can reveal specific information about the biochemistry of the plant tissue. This biochemical information can potentially be linked to physical, structural and nutritional information, helping to elucidate the factors which influence the nutritional and digestibility characteristics of cereal grains. Several recent studies have explored the application of synchrotron FTIRM to the study of biological samples. Molecular imaging of corn (Yu et al., 2004b) and barley (Yu et al., 2004d) has been carried out to reveal spatial intensity and distribution of chemical functional groups within the sample tissue. Such information is useful for relating nutrient utilization and digestion characteristics with the specific chemical makeup of the grain. Protein content and the composition of secondary protein structure (α-helix and β-sheet) has been studied in feather protein (Yu et al., 2004c). This is accomplished by analysis of the line shape of the Amide I protein absorption to estimate the relative content of α-helix and β-sheet secondary structures present in the sample. Characterizing the relationship between protein secondary structure and digestibility may provide a useful method of comparing the relative protein quality of different feed sources with respect

to both human and animal nutrition. Yu et al. (2004a) also used SR-FTIRM to estimate and compare the starch and protein content of Harrington and Valier barley with reference to differences in their physical digestibility characteristics. Synchrotron FTIRM is a technique which is useful for examining plant tissues to ultimately gain a better understanding of the physiochemical basis of quality traits that are currently measured by more empirical methods (Pietrzak and Miller, 2005).

1.5 Conclusions, Hypothesis and Objectives for Research

Rumen digestibility characteristics of cereal grains and their effect on the growth, health and economic performance of cattle are of importance to producers and the feed industry. Traditional methods of evaluating nutrient availability of feeds for cattle, specifically the *in situ* nylon bag technique, have proven to be relatively accurate and reliable, but require a large time and labor commitment.

The hypothesis of the current research was that Synchrotron Fourier Transform infrared microspectroscopy (SR-FTIRM) could be used as an analytical tool to help predict the physical digestibility characteristics of cereal grains for ruminant animals, with a specific focus on barley. It is suggested that the starch to protein ratio, measured using FTIRM, can be used as an indicator of nutrient digestibility in the rumen. This technique may be a rapid, labor-efficient method that can potentially represent a reasonable alternative to *in situ* incubation as a method of estimating the nutrient digestion kinetics of cereal grains in the rumen environment.

To obtain sufficient experimental data to support the analysis of this hypothesis, the research was conducted in a two-step approach. First, *in situ* rumen digestibility characteristics of corn and barley were determined at two levels of processing to analyze the nature of the response of different species of grain and different varieties of a single species to the effects of processing and grain type on digestibility characteristics. After analysis of the physical digestibility, the second step was to evaluate the chemical composition of the grain samples using IR microspectroscopy to obtain spatial information on the relative concentration and location of starch and protein within the grain kernel, and to determine if it could be related to the physical nutrient digestibility parameters determined by the *in situ* technique. Data from these

two types of analysis (chemical and infrared) were evaluated and compared to determine if they were able to provide similar predictive information about the digestibility characteristics of cereal grains.

2.0 *In situ* Degradation Characteristics of Selected Varieties of Corn and Barley

2.1 Introduction

Cereal grains are the principal source of energy in feedlot and dairy rations fed in western Canada. The total energy content of the ration is primarily determined by the starch content of the grain. Starch is rapidly degraded by rumen microbes to produce volatile fatty acids which are used for energy production and other metabolic processes. Providing such concentrated sources of energy in the diet of high producing ruminants is essential. However, supplying large quantities of rapidly degradable starch to rumen microbes can have detrimental effects on normal rumen function and the health of the animal. Digestive disorders resulting from poor management of high starch feeds can have a negative effect on animal health and overall performance. The relative risk of causing digestive disturbances is directly related to the rate at which starch is fermented by the microbial population in the rumen. Starch sources that are less rapidly degraded are preferable for ruminant feeding because they are less likely to cause an accumulation of acidic fermentation products which are detrimental to rumen function.

Starch is contained within the endosperm of cereal grains. The endosperm tissue is by far the largest portion of the cereal kernel and accounts for over 80% of the total kernel weight (McMasters et al., 1971). Barley endosperm tissue is relatively consistent throughout with starch granules that are loosely associated with the protein matrix (McAllister and Cheng, 1996). Conversely, corn has two distinct regions: the floury and the horny endosperm. The floury endosperm is very similar in composition to that of barley, but the horny endosperm contains starch granules that are tightly embedded within the protein matrix. This protein matrix is resistant to invasion by microbes and presents a barrier to digestion, whereas the protein matrix of barley is readily penetrated by proteolytic bacteria, rendering the starch available for microbial attack, and resulting in a very rapid rate of digestion. It is estimated that between 80 and 90% of barley and

wheat starch is digested in the rumen, compared to only 55 to 70% in corn and sorghum, respectively (Nocek and Tamminga, 1991).

This variation in fermentation rate occurs not only between cereal species, but also among cultivars of the same species. Studies with different cultivars of barley have shown large variation in starch content, quantity of soluble and degradable carbohydrate fractions, and the rate of degradation (Khorasani et al., 2000), as well as differences between malting and feed-type barley varieties (Yu et al., 2003).

With respect to ruminant feeding, the rate and extent of starch digestion is of interest because of its direct effect on animal health and performance. Varying rate and extent of dry matter and starch digestion can have implications for ruminant feeding, including required processing and inclusion rates of cereal grains in commercial diets. Different types of cereal grains such as corn and barley exhibit varying rates of ruminal degradation due to differences in the intrinsic chemical structure of the endosperm tissue. The ability to measure the rate and extent of digestion in different cereal grains provides useful insight into the nutritional and feeding qualities of a particular grain sample. With this knowledge, grains that have more desirable feeding qualities may be selected and used in the formulation of rations specifically tailored for use in cattle feeding programs.

Digestibility measurements have traditionally been obtained through a variety of enzymatic and biological analyses. One of the most accurate and widely used methods of digestibility measurement for feeds is the *in situ* nylon bag technique described by De Boer et al. (1987). Although acceptable results can be obtained with this technique, it is time consuming and requires hours of labor and laboratory analysis. Furthermore, the analysis of nutrient composition is conducted through a variety of chemical procedures, all of which require homogenization, or grinding, of the entire sample. This method does not reveal any spatial information regarding nutrient distribution within the sample, and eliminates the possibility of analyzing specific areas of the tissue in isolation from the whole kernel.

Application of infrared technology (thermal or synchrotron source) presents a possible alternative to traditional methods of nutritional evaluation for cereal grains. Fourier Transform infrared microspectroscopy (FTIRM) has the ability to identify and

spatially resolve the molecular structures and chemical components of biological tissues based on the unique vibrational characteristics apparent when samples are exposed to mid-IR radiation. Unique properties of vibrational spectra yield detailed information for all components within the illumined pixel, permitting quantitative assessment of tissue components at the cellular and sub-cellular level (Gough, 2003). Another important feature of IR microspectroscopy is the capability to examine untreated, unstained tissue (Gough, 2003), permitting analysis of the sample without compromising the integrity of chemical structures contained within.

Use of a synchrotron light source for IR microspectroscopy provides the ability to explore the chemistry within microstructures of plant tissue at much higher spatial resolution than with a conventional thermal light source (10-12 μm vs. 40-50 μm, respectively), while maintaining good spectral quality (Yu et al., 2004). The advantage of synchrotron light is the brightness of the infrared source which permits rapid acquisition time and superior quality of spectra with spatial resolution at the diffraction limit (Dumas et al., 2004). Recent work by Yu et al. (2004) has shown that synchrotron FTIRM can be used as a non-destructive technique to investigate the molecular structure of barley tissue at high ultra-spatial resolution.

The specific objective of this study was to evaluate the utility of FTIRM as a technique for analyzing the nutrient content of cereal grains relative to their nutritional qualities for ruminant feeding. In order to accomplish this, it was necessary to determine the physical digestibility characteristics of cereal grains using the traditional method of analysis, the *in situ* nylon bag technique. This included a direct comparison of corn and barley, as well as a second comparison of four different varieties of barley.

2.2 Materials and Methods

2.2.1 Barley and corn samples

Four varieties of barley (*Hordeum vulgare*) were chosen for this experiment. Harrington (2-row malting barley), Valier (2-row mid-season spring barley with improved feed characteristics), CDC Bold (2-row hulled semi-dwarf feed barley) and CDC Dolly (2-row feed barley variety, often used as a check variety in production trials) were grown in university research plots near Saskatoon, SK, Canada. Barley samples were provided by B. Rossnagel, Crop Development Center (CDC), University of Saskatchewan (Saskatoon, SK, Canada). Corn (Pioneer 39P78) (a hybrid variety for grain production) was obtained from V. Racz, Prairie Feed Resource Centre (Saskatoon, SK, Canada).

2.2.2 Sample selection and preparation

Prior to processing, Harrington, CDC Bold, CDC Dolly and Valier barley samples were screened on 3.18, 2.97, 2.78, 2.59 and 2.38 mm screens using a Dockage Tester (Carter-Day Co., Minneapolis, MN) at the Crop Development Centre (Saskatoon, SK). This was done to remove small and large grain that would be differentially affected by processing. The screen size which retained the largest proportion of the grain population is shown in Table 2.1. Grain remaining on these screen sizes was retained for further processing and use in the *in situ* trials. Screen size groups varied due to the kernel size variation among barley varieties.

Table 2.1. Screen sizes from which barley grain samples were used for *in situ* nylon bag incubation

Barley variety	Screen size (mm)
CDC Bold	< 3.18 > 2.97
CDC Dolly	< 3.18 > 2.97
Harrington	< 3.18 > 2.97
Valier	< 2.77 > 2.59

The sample of Valier used in this experiment had smaller, less plump seeds compared to the other variety samples, likely a factor of the hot and dry growing conditions in which this particular sample was grown (Brian Rossnagel, personal communication). Small kernels in the corn sample were visually identified and removed.

Barley and corn samples were tempered prior to processing using the tempering method for test milling of wheat (method 26-10A) according to the AACC (1995). Grain was tempered for 24 hours to a moisture content of 11-12% by the addition of distilled water from the equation:

$$\text{Water added (mL)} = \left(\frac{(100 - \text{original moisture of sample})}{(100 - \text{desired moisture})} \right) - 1 \times \text{sample wt. (g)}$$

Corn and CDC Bold, Harrington, CDC Dolly and Valier barley samples were finely processed using an ultra centrifugal mill (Retsch ZM 100, Haan, Germany) through a 2 mm screen. Coarse processing was carried out using a Sven Roller Mill (Apollo Machine and Products Ltd., Saskatoon, SK) with 20 cm rollers adjusted to a gap size of 1.7 mm for Harrington, CDC Bold and CDC Dolly, and a 1.6 mm gap for Valier. Roller gap size was adjusted when necessary between each barley sample to ensure that all kernels were at least cracked. Roller gap size was approximately proportional to the average plumpness of 100 kernels of each variety as measured with a caliper. After rolling, samples were visually examined and uncracked kernels were removed or manually cracked to ensure a more consistent degree of processing. Particle size of processed barley was determined by dry sieving with an oscillating sieve shaker (RX-86 Sieve Shaker, W.S. Tyler, Mentor, OH) with sieves arranged in descending mesh size (3.35, 2.36, 2.0, 1.4, 1.0 mm and pan). The acceptable degree of processing was determined to contain less than 3% fines characterized by the portion of sample collected in the pan (i.e. passing through 1 mm screen) (Mathison, 1996).

2.2.3 *In situ* rumen incubation

2.2.3.1 Animals and diets

Two yearling Black Angus heifers weighing approximately 400 kg were surgically fitted with a 13 cm (inside diameter) flexible rumen cannula (Bar Diamond Inc., Parma, ID) at the Western College of Veterinary Medicine, Saskatoon, SK. Heifers were housed in 3 m x 3 m pens in the Livestock Research Building at the University of Saskatchewan during the *in situ* incubation experimental trials. During the experimental period, heifers were fed a diet containing 42.5% rolled corn, 42.5% rolled barley and 15% barley silage on a DM basis at 2.2% of body weight. It has been suggested that animals used for *in situ* experimentation should be fed the same ingredient sources as being tested in order to establish a diverse rumen microbial population (Nocek, 1988). Pure cultures of amylolytic bacteria exhibit differential abilities to digest cereal starches (McAllister et al., 1990); therefore, both grains were included in the ration to insure a bacterial population that would be well adapted to digesting both corn and barley samples for the *in situ* trials.

Feed was provided in two equal portions at 0800 and 1600 h. The ration included 55 g of mineral (CO-OP® 3:1 Beef Cattle Mineral, Reg. # 640314, Co-op Feeds, Ltd. Saskatoon, SK) daily. Animals used in this experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

2.2.3.2 Rumen incubation

Rumen degradation characteristics of corn and four barley varieties (CDC Bold, Harrington, CDC Dolly, and Valier) were determined in two separate *in situ* incubation trials using the nylon bag technique according to the procedure of McKinnon et al. (1995). Trial 1 was a comparison of the degradation characteristics of corn and Harrington barley and included four treatments: rolled corn (RC), ground corn (GC), rolled Harrington (RH), and ground Harrington (GH). Trial 2 involved four varieties of barley (Harrington, CDC Bold, CDC Dolly and Valier) processed by grinding and rolling for a total of eight treatments (RH, GH, RB, GB, RD, GD, RV and GV). Each trial included four repetitions, or runs, and all bags from a single run were incubated in the same animal.

Seven-gram samples of test grains were weighed into 10 cm x 20 cm coded bags (Nitex 03-41/31 monofilament open mesh fabric, Screentec Corp., Mississauga, ON) with a pore size of 41 μm and open area of 31%. Sample bags were held in the ventral sac of the rumen by placing them in a 45 cm x 45 cm polyester mesh bag attached to a 90 cm rope and secured to the outside of the fistula opening. The bag was anchored by adding plastic bottles filled with sand as weights to keep the incubated samples in the liquid strata of the rumen and to prevent them from rising to the top of the rumen mat. Bags were added to the rumen according to the 'gradual addition/all out' schedule, and samples were incubated for 0, 2, 4, 8, 12 and 24 hours in Trial 1, with an additional time period of 36 hours in Trial 2. To ensure that sufficient sample residue remained after incubation to perform the necessary chemical analysis, the number of bags filled for each time period increased with the length of incubation. Estimates of the original amount of sample required were made by using degradation predictions from previously published data for Harrington and Valier barley (Yu et al., 2003). An explanation of the number of bags required for a single incubation run in Trial 1 is included in Appendix A. A maximum of 26 bags were incubated in the rumen of each animal at one time; therefore, because a total of 56 bags were to be incubated in each run, a staggered incubation procedure was devised in order to incubate all bags for the specified time period without exceeding the limit of 26 per incubation time. To do this, all treatment bags for each incubation time in a single run were pooled together (ie. all 2-hour GC, RC, GH and RH bags for run 1 were pooled). A set number of bags from each time period were randomly chosen from the pooled groups for each incubation. The number of incubation bags required for each time period as well as the total number of bags required in Trial 2 is detailed in Appendix A. A total of 26 bags were incubated at once, therefore it took six 36-hour incubation periods to incubate 144 bags in a single run of Trial 2 (there were a total of 160 bags for each run, but the 0-hour bags were not incubated). This procedure was repeated for all four runs in both Trial 1 and Trial 2.

Table 2.2 shows a summary of the experimental parameters for the *in situ* incubation experiments.

Table 2.2. Summary of experimental parameters for the two *in situ* incubation trials

	Trial 1	Trial 2	
Treatments	RC	RH	RB
	GC	GH	GB
	RH	RV	RD
	GH	GV	GD
Runs	4	4	
Incubation times (h)	0,2,4,8,12,24	0,2,4,8,12,24,36	
Bags required	240 (60 per run)	640 (160 per run)	

RC: rolled corn; GC: ground corn; RH: rolled Harrington; GH: ground Harrington; RV: rolled Valier; GV: ground Valier; RB: rolled CDC Bold; GB: ground CDC Bold; RD: rolled CDC Dolly; GD: ground CDC Dolly

Following incubation, all bags were removed from the rumen and rinsed under a cold stream of tap water to remove excess ruminal contents and to stop microbial activity. Bags were then hand-rinsed ten at a time with 1000 mL of cool tap water (McKinnon et al., 1991). This rinse was repeated six times for each group of bags until the rinse water was clear. Bags representing 0 h were also washed in this way to determine the soluble fraction (S). Excess water was gently squeezed out and bags were laid flat on metal trays and dried in a forced air oven at 55°C for 48h. Dry sample residues were weighed and removed from the bags. Residues were pooled according to run, treatment and incubation time to obtain a sufficient amount of sample for chemical analysis.

2.2.3.3 Chemical analysis

Samples of the original, undigested grain treatments and the rumen residues were ground through a 0.5 mm screen using an ultra centrifugal mill (Retsch ZM 100, Haan, Germany). Pooled residues for each treatment and incubation time as well as original barley and corn samples were analyzed for dry matter (AOAC official method 954.02) and crude protein (AOAC official method 984.13) according to the procedures of the AOAC (1990). Because of the limited amount of sample for some incubation times, certain modifications were made to the original procedures. In the case of dry matter, 0.5 g of sample was dried in ceremic ashing crucibles (4 cm diameter) rather than 1.0 g in metal containers (6 cm diameter). Before proceeding with this modification, preliminary comparisons were conducted to determine if it would affect the DM analysis result. It was observed that samples ground at 0.5 mm did not show any significant change in the moisture content when analyzed using 0.5g samples rather than 1.0 g.

Total starch content of grains and rumen residues was analyzed using the Megazyme® Total Starch Assay Kit (Megazyme International Ireland Ltd., Wicklow, Ireland). Duplicate samples were enzymatically digested to hydrolyze starch molecules to glucose monomers. Glucose concentration of each sample solution was measured with a spectrophotometer (Ultrospec III, Pharmacia LKB Biochrom, Ltd., Cambridge, UK). Total starch was determined by applying a conversion factor of 0.9 to the glucose

concentration to account for the mass of the water molecule released in the dehydration reaction which forms glycosidic linkages between glucose molecules in starch.

Crude protein (CP) was determined by the standard Kjeldahl method of nitrogen (N) determination in feeds and forages according to methods of the Association of Official Analytical Chemists (AOAC, 1990). This two-step process involves the digestion of the sample in sulfuric acid with a catalyst to hydrolyze the protein molecules and results in the conversion of nitrogen to ammonia. This is followed by steam distillation and titration to determine the nitrogen content in the solution. Samples were analyzed in duplicate, and repeated if the error was in excess of 3%.

2.2.3.4 Rumen degradation parameters

In situ digestion kinetics were estimated using the proc NLIN procedure of the statistical package SAS 9.1 (2003) using iterative least squares regression (Gauss-Newton method). The percentage of DM, CP and starch residue from *in situ* incubation was fitted to the first-order kinetics equation of Ørskov and McDonald (1979):

$$R(t) = U + D \times \exp(-K_d \times (t-T_0))$$

where R(t) = residue of the incubated material after *t* h of rumen incubation (g/kg); U = undegradable fraction (g/kg); D = potentially degradable fraction (g/kg); T_0 = lag time (h); and K_d = degradation rate (% h^{-1}). This equation estimates the disappearance of DM, CP or starch for a given time period, but it is unable to predict the amount that will be truly degraded in the rumen over time (i.e. effective degradability). Thus, by using the nonlinear parameters estimated by the above equation (U, D and K_d), effective degradability (ED) of DM, CP and starch were calculated as:

$$\text{EDDM (g/kg)} = S + D \times K_d/(K_p + K_d),$$

where S = soluble, or 'wash-out' fraction (g/kg), and K_p = estimated rate of outflow from the rumen (% h^{-1}). A K_p value of 6% h^{-1} was adopted to represent the rumen turnover rate (Yu et al., 2003). A similar approach was taken to estimate EDCP and EDSt.

2.2.3.5 Statistical Analysis

Analysis of variance data for non-linear variables (S, soluble fraction; D, potentially degradable fraction; U, undegradable fraction, and K_d, rate of disappearance) and ruminal DM, CP and starch disappearance was carried out using the mixed procedure of SAS (2003). Sums of squares were separated into effects of grain variety and processing method. The model used for the analysis was:

$$Y = \mu + \alpha + \beta + (\alpha\ \beta) + \varepsilon$$

where Y is an observation of the dependent variable; μ the population mean for the variable; α the effect of grain type/variety; β the effect of processing method; and ε the random error associated with the observation. When F-tests were significant ($P<0.05$), means were separated by LSD (SAS, 2003). Least square means and the associated standard error are reported.

2.3 Results and Discussion

Grinding causes a reduction in particle size that tends to increase the rate and extent of digestion beyond that which occurs when feed is masticated and ruminated by the animal. Such overprocessing of grain in commercial situations can lead to a reduction in feed value by decreasing feed intake and causing digestive upsets (Wang and McAllister, 2000); therefore, grinding is not used as a common processing method for ruminant feeding. However, the inclusion of ground samples in digestibility experiments can be of value when assessing the rates of *in situ* dry matter, protein and starch degradability of cereal grains. Processing effects on the structure and particle size of cereal grains relative to the rate of *in situ* degradation have been established by previous work. The objective of this experiment was not to compare the differences in rate of degradation among processing methods as this has been reported by previous researchers (Theurer, 1986; Wang and McAllister, 2000; Ramsey et al., 2001); rather, the intent was to examine the effect of endosperm structure on the rate and extent of digestion of cereal grains and if the processing affected different grain varieties in a similar manner. Since the premise of this study was to determine if the inherent chemical structure of the endosperm tissue had an effect on the rate of degradation, it was hypothesized that grinding should destroy most of the macro-protein-starch

structures that would differentiate the rate of digestion of one barley variety from another. Therefore, with ground samples it was anticipated that all varieties would show more similar rates of degradation. Differences were expected to be observed among the rolled treatments because this processing method does not destroy the inherent physical structures of the endosperm tissue.

Ground barley and corn samples were processed through a 2 mm screen as reported by Mustafa et al. (2000) and Boss and Bowman (1996). Other researchers have used a 3 mm grind for *in situ* samples (Lehman et al., 1995; Ramsey et al., 2001), but did not report any significant differences in digestibility due solely to the particle size. Ramsey et al. (2001) found that the sample washout loss with a 3 mm grind did not differ from results obtained in previous studies carried out by the same group with 2 mm ground samples.

Particle size is not the only source of variation encountered with this experimental method. Other factors to consider when comparing degradability results from different studies include bag and pore size, sample: bag area ratio, researcher's technique and individual animal differences. In this experiment, the sample:bag surface area ratio was 0.0175 g/cm^2 with a pore size of 41 μm which is similar to that reported by other researchers. For example, the following sample:bag surface area ratios and bag pore sizes, have been reported: 0.0125 g/cm^2 and 51μm, 0.050 g/cm^2 and 57 μm, 0.0286 g/cm^2 and 57.5 μm, and 0.026 g/cm^2 and 50 μm (Lehman et al., 1995; Yang et al., Khorasani et al., 2000; 1996; and Ramsey et al., 2001, respectively).

2.3.1 Trial 1: Comparison of Corn and Harrington Barley

Starch and crude protein content (DM basis) as well as the ratio of starch:protein content in the entire kernel for corn and the four barley cultivars used for the *in situ* experiment are shown in Table 2.3. Harrington and Valier barley had similar chemical composition with regard to starch and crude protein content, analogous to the findings of Yu et al. (2003). Starch content of Harrington and Valier was also similar to the average of 60 barley varieties reported by Khorasani et al. (2000). Numerically, CDC Bold had the highest starch content of the four barley varieties (63%), but the lowest CP (11%), which resulted in the highest starch:protein ratio. CDC Dolly was intermediate

Table 2.3. Nutrient composition of corn and barley samples used for experimental work

	Grain				
Item	Corn	CDC Bold	CDC Dolly	Harrington	Valier
DM (%) [yz]	90.59	88.27	88.39	88.92	89.20
Starch (% DM) [z]	66.52	63.32	58.74	57.78	56.61
CP (% CM) [z]	11.49	11.10	12.78	14.06	14.01
Starch:Protein [x]	5.79	5.70	4.59	4.11	4.04

[z] Samples run in duplicate, accepted at an error rate of <3%.

[y] Moisture content (%) of grain after processing and prior to incubation

[x] Ratio refers to starch and protein content of entire kernel

between CDC Bold and Harrington and Valier for both starch and protein content (58.7% and 12.8%, respectively). Corn had higher starch content than the four barley varieties, and lower protein than all barley varieties except CDC Bold. Protein within the endosperm tissue of cereal grains is arranged in a matrix that surrounds and protects the starch granules, making them less available for digestion by rumen bacteria (McAllister et al., 1993). This may be a major factor responsible for differences in rumen starch degradation among different grain varieties. If more protein within the grain kernel relates to a stronger protein matrix, higher protein content should indicate a larger degree of 'protection' which would be related to a reduction in the rate and extent to which starch is able to be degraded. Therefore, a lower starch to protein ratio may be indicative of a greater degree of protection of starch granules within the protein matrix, resulting in a lower rate and extent of rumen degradation (Yu et al., 2004a). 'Wet' chemical analysis requires homogenization of the sample tissue before analysis, so the starch:protein ratio reported in Table 2.3 reflects the relative amounts within the entire kernel, not a specific region of tissue (i.e. the endosperm).

In situ rumen kinetics of DM, CP and starch for rolled and ground samples of corn and Harrington barley are shown in Table 2.4. The variables K_d, S and D represent an attempt to describe rumen disappearance of DM, CP and starch over a 24 h period as a result of bacterial fermentation activity. The S variable represents the soluble, or 'wash-out' fraction (g/kg) of the feed (measured for each of DM, CP and starch), and is defined as 100 minus the residue remaining in the 0 h bags after the washing procedure (i.e. the soluble portion of sample washed out of the bags without being subjected to rumen incubation). Feed material which is potentially rumen degradable corresponds to the D (g/kg) variable, while K_d represents the rate, expressed as % h^{-1}, at which material (DM, CP or starch) is degraded in the rumen environment. These variables are then used to predict the effective degradability of DM, CP and starch in the rumen assuming a fixed rumen passage rate of 6% h^{-1} (Yu et al., 2003). From the point of view of this work, the rate and extent of rumen degradation (ie. effective degradability) are the two most critical factors.

As expected, the rate of DM degradation (K_d) was significantly lower ($P<0.05$) for corn than for barley (Herrera-Saldana et al., 1990; Boss and Bowman, 1996), and lower

Table 2.4. Trial 1 *in situ* rumen degradation kinetics for corn and Harrington barley

	Corn		Harrington			P-value		
Item	Rolled	Ground	Rolled	Ground	**SEM**	grain	processing	g x p
Rumen degradation characteristics of DM								
K_d (%/h)	8.85 c	9.64 c	12.11 b	31.29 a	1.225	<.0001	<.0001	<.0001
S (%)	8.91 c	19.9 b	8.96 c	29.61 a	0.474	<.0001	<.0001	<.0001
D (%)	50.50 c	61.31 b	65.90 a	57.46 b	1.616	0.0012	0.3644	<.0001
EDDM (%)	38.18 d	57.44 b	52.65 c	77.79 a	0.715	<.0001	<.0001	0.0002
Rumen degradation characteristics of crude protein (CP)								
K_d (%/h)	4.94 b	5.39 b	7.32 b	14.19 a	0.692	<.0001	0.0003	0.0008
S (%)	8.77	18.14	3.95	20.42	3.945	0.0728	0.3663	0.1815
D (%)	63.06	75.89	78.52	77.26	7.232	0.6707	0.2478	0.4544
EDCP (%)	35.03	54.06	45.88	74.63	2.572	<.0001	<.0001	0.9394
Rumen degradation characteristics of starch (St)								
K_d (%/h)	6.78 b	8.87 b	12.87 b	39.58 a	1.028	<.0001	<.0001	<.0001
S (%)	5.57	16.41	7.35	33.23	1.272	<.0001	<.0001	0.4206
D (%)	68.91	75.92	79.19	66.2	4.776	0.0988	0.2877	0.4976
EDSt (%)	39.72	61.29	61.03	90.69	0.911	<.0001	<.0001	0.5700

Means with different letters in the same row are significantly different ($P<0.05$)

K_d = rate of rumen degradation of the D fraction; S = soluble or 'wash out' fraction; D = insoluble but potentially degradable fraction; EDDM = effective degradability of DM

for rolled than for ground samples. There was, however, a significant interaction of the effects of grain type and processing method ($P<0.05$) on the rate of rumen DM degradation. Both GC and GH showed faster rates of degradation than did RC and RH (9.6 and 31.3 vs 8.9 and 12.1 % h^{-1}, respectively), but the magnitude of the processing effect was different for each grain. Grinding significantly increased the K_d of DM for barley from 12.1 % h^{-1} for RH to 31.3 % h^{-1} for GH ($P<0.05$), while for corn, there was no statistical difference between RC (8.9 % h^{-1}) and GC (9.6 % h^{-1}). A similar interaction between grain type and processing method was seen in the ruminal degradation rate (K_d) of both crude protein and starch as well. The nature of these interactions is illustrated in Figure 2.6. In general, the effect of processing on the rate of DM, CP and starch degradation was greater for Harrington than for corn.

Intuitively, ground corn should be more quickly degraded and to a greater extent than rolled corn, as seen for barley, but the lack of difference in their rate of degradation is not necessarily surprising. Dry-rolled and ground corn are known to have a lower rate of digestibility than corn processed with heat and steam (Theurer, 1986). A nine-year summary of published trials with corn grain by Huntington (1995) reported that steam flaking increased starch digestibility an average of 7% over dry processing. As opposed to barley, reduction in particle size does not have as much effect on the starch degradability of corn as does the application of moisture, heat and pressure during processing (Yu et al., 1998).

There was a significant grain type by processing interaction observed for EDDM (Figure 2.6). The nature of this interaction was such that the EDDM of ground Harrington increased over rolled Harrington to a greater degree than seen with corn. A significant interaction was not detected for EDCP or EDSt. For both parameters, a greater ($P<0.05$) response was observed with fine processing and for Harrington relative to corn (Figure 2.6, Table 2.1). These results indicate that measuring DM degradation kinetics may be indicative of the rate and extent of starch digestion. However, this is opposite to the observations of Ramsey et al. (2001), who found no correlation between the rate of DM and starch disappearance in either ground or rolled grain. These researchers concluded that measurement of DM disappearance was not adequate for estimating ruminal starch disappearance in barley grain.

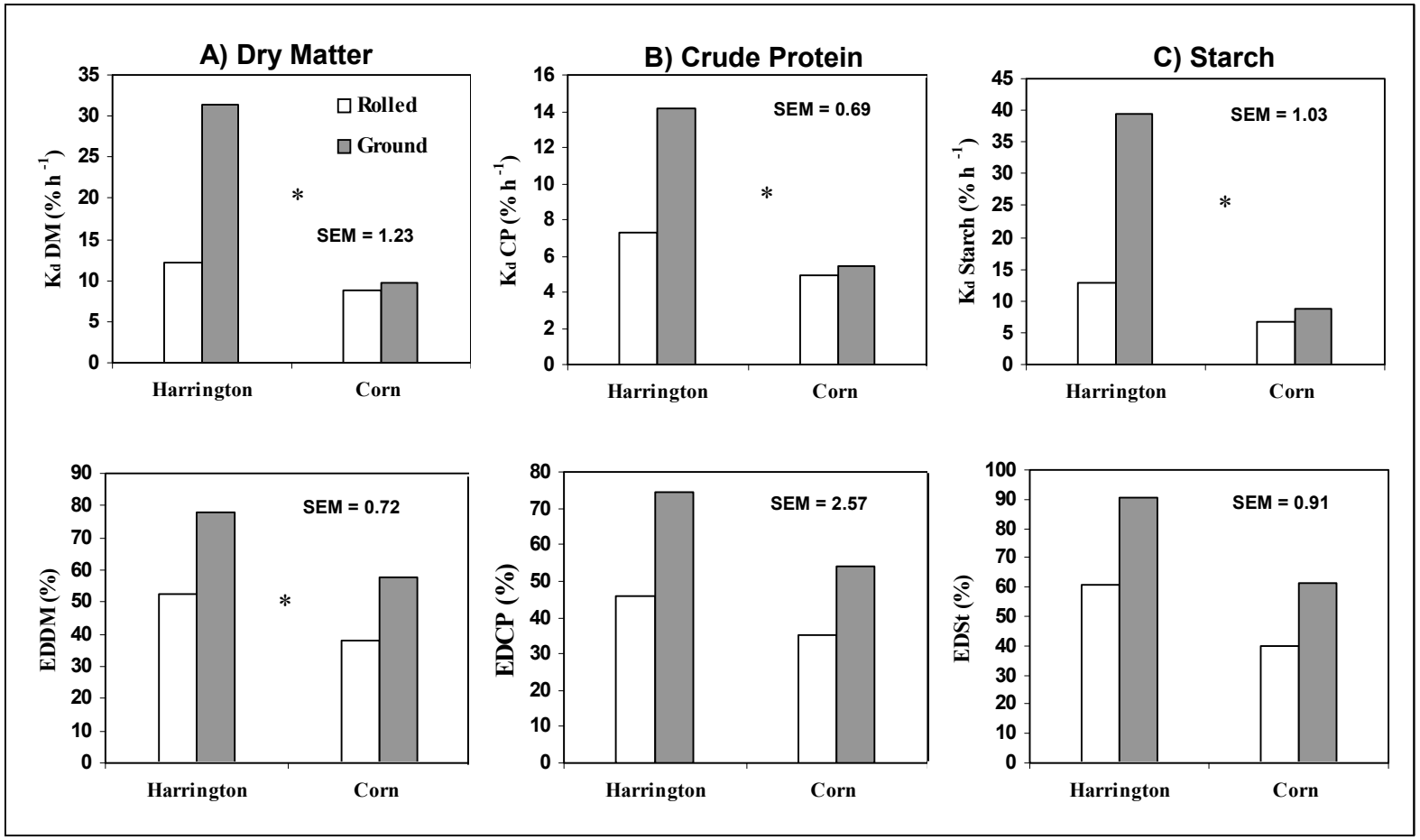

Figure 2.6. Comparison of the rate of degradation (Kd) and effective degradability (ED) of A) dry matter, B) crude protein (CP); and C) starch. * Significant interaction ($P<0.05$) between the effects of grain type and processing method.

Solubility of DM increased with reduced particle size in the ground samples compared to the rolled treatments. The soluble fraction (S) of DM was not different between RC and RH (8.91 vs 8.96%), but ground samples had a significantly higher S fraction than the rolled, and ground barley was significantly higher than ground corn ($P<0.05$). With this technique, the soluble fraction of the grain is estimated by measuring the amount of the sample that is 'washed out' of the non-incubated (0h) bags during the washing procedure. Grain particle size and bag pore size are two factors that will influence this value. In this experiment, the measured pore size of the bag material was 41μm, and any sample particles smaller than this would potentially be washed out of the bags. Since ground barley showed significantly higher 'wash-out' fraction than ground corn, it would indicate that barley has a greater tendency to shatter and that grinding produced more excessively small particles (i.e. less than 41 μm) than with corn. Difference in oil content between corn and barley may affect the observed response to grinding. Although crude fat analysis was not performed on these particular samples, corn grain generally contains approximately twice as much oil as does barley (4.0 vs. 2.2%, respectively) (NRC, 1996) which may have influenced the response to grinding.

As previously stated, the objective for including rolled and ground treatments in this experiment was to determine the processing effect on the rate and extent of rumen degradation, and whether minimal (rolling) or excessive (grinding) processing has a differential effect on the inherent degradability characteristics of cereal grains in the rumen. The results indicated a significant ($P<0.05$) grain type by processing interaction on the rate of DM, CP and starch degradation indicating that corn and barley respond differently to processing. However, it is important to note that the interaction was related to the magnitude of the response, but not the direction of the response. That is to say, grinding caused an increase in the rate and extent of rumen degradation in both corn and barley, but the magnitude of the response was different for each grain type. Therefore, since a similar response was observed for both processing methods, we elected to focus on the main effect of grain type by removing the ground treatment. In commercial situations, ground grain is not fed to ruminants, so from a practical point of

view we wanted to focus more specifically on rolled grain as it is utilized in ruminant rations.

Table 2.5 compares the rumen degradation kinetics of rolled corn and Harrington barley. Overall, the main observations were that 1) DM, CP and starch in barley was degraded more quickly ($P<0.05$) than in corn, and 2) the extent of degradation (effective degradability) was higher for barley than for corn, both of which were similar to previous *in situ* digestibility experiments using ground samples (Herrera-Saldana et al., 1990; Boss and Bowman, 1996). Solubility of DM and starch was not different between Harrington and corn, but the soluble fraction of CP was significantly lower for rolled Harrington than for corn (3.95 vs. 8.77%). Effective degradability of CP and starch was lower for corn (35.0 and 39.8%) than for Harrington barley (45.9 and 61.0%) as a result of a slower rate of degradation, leaving more rumen undegradable material and reducing the overall degradability. Effective rumen degradability of starch (EDSt) was over 20% greater ($P<0.05$) for RH than RC (Table 2.5). This agrees with Ørskov's (1986) observation that a greater proportion of barley starch is fermented in the rumen than that of corn. Despite corn having a higher overall starch content than Harrington barley (66.5 vs. 57.8%, respectively) (Table 2.3), a larger fraction of corn starch escapes ruminal fermentation. McAllister et al. (1993) reported that bacterial attachment to, and digestion of, starch granules in the rumen are hindered by the surrounding protein matrix, and that this interaction has a larger effect on the digestion of starch in corn than in barley. This could be a causative factor in the differences seen between the rumen degradation rate of corn and Harrington barley in this experiment.

Table 2.5. Trial 1 *in situ* rumen degradation kinetics for rolled samples of corn and Harrington barley

	Grain type			
Item	Corn	Harrington	SEM	P-value
Rumen degradation characteristics of dry matter				
K_d (%/h)	8.85 a	12.11 b	1.330	0.0046
S (%)	8.91	8.96	0.401	0.9276
D (%)	50.50 b	65.90 a	2.007	0.0033
EDDM (%)	38.18 b	52.65 a	0.807	0.0001
Rumen degradation characteristics of crude protein				
K_d (%/h)	4.94 b	7.32 a	0.8711	0.0411
S (%)	8.77 a	3.95 b	5.215	0.0435
D (%)	63.06	78.52	9.842	0.1908
EDCP (%)	35.03 b	45.88 a	3.504	<.0001
Rumen degradation characteristics of starch				
K_d (%/h)	6.80 b	12.87 a	1.173	0.0039
S (%)	5.57	7.35	1.077	0.1964
D (%)	68.91	79.19	5.774	0.2466
EDSt (%)	39.82 b	61.03 a	1.098	0.0006

Means with different letters in the same row are significantly different (P<0.05)

2.3.2 Trial 2: Comparison of four barley varieties

Effects of barley variety and processing method (rolled vs ground) on the *in situ* DM degradation characteristics of DM, CP and starch are presented in Table 2.6. Again, the rationale for including both processing methods was to determine if these different barley varieties would exhibit varying responses to digestion in the rumen at different levels of processing. Table 2.6 shows that there were variety and processing effects as well as variety by processing interactions for nearly all parameters measured. Fine processing (grinding) doubled the rate of digestion of DM from 15.3% h^{-1} for rolled samples to 33.3% h^{-1} for ground samples (average K_d of four varieties), and increased the extent of DM degradation by approximately 20% (average EDDM = 78.2 vs 59.4% for grinding and rolling, respectively). Variety differences were primarily seen between two groups: CDC Bold and CDC Dolly differing from Harrington and Valier. However, of most importance is the interaction between barley variety and processing methods observed for both rate and extent of DM, CP and starch degradation. Processing produced a similar directional response in the K_d and effective degradability of DM, CP and starch for all barley varieties, whereas the magnitude of the response varied between varieties. These interactions are illustrated in Figure 2.7. Grinding caused the rate of degradation of CP to double for CDC Bold and CDC Dolly, but did not produce any significant increase in the K_d of CP over the rolled treatment for Harrington or Valier (Table 2.6). A wide variation existed in the rate of CP digestion in ground samples, ranging from 30.5% h^{-1} for CDC Bold, to 12.4% h^{-1} for Harrington (Table 2.6). Ground Harrington and Valier were statistically the same for rate of CP degradation, and significantly lower than ground samples of both CDC Dolly and CDC Bold. The effect of grinding on the rate CP degradation may have implications with respect to the rate of starch digestion. As noted by McAllister et al. (1993), the protein matrix appears to serve as a protective barrier to starch digestion; therefore as the rate of disappearance of CP increases, the rate of starch disappearance should follow the same pattern. This trend appears to occur in this experiment as the rate of starch degradation for CDC Bold and CDC Dolly is higher ($P<0.05$) than that of Harrington. The K_d of

Table 2.6. *In situ* rumen degradation characteristics of DM, CP and starch of ground and rolled samples from four barley varieties

	Barley variety											
	Bold		Dolly		Harrington		Valier			P-value		
Item	Rolled	Ground	Rolled	Ground	Rolled	Ground	Rolled	Ground	SEM	v	p	v x p
Rumen degradation characteristics of DM												
K_d (%/h)	16.61 c	38.35 a	15.77 c	36.95 a	14.92 c	28.40 b	13.71 c	29.35 b	1.534	0.0006	<.0001	0.0295
S (%)	10.50 d	37.22 a	9.25 d	30.23 b	9.42 d	29.28 b	6.68 e	27.71 c	0.815	<.0001	<.0001	<.0001
D (%)	70.48 a	49.31 c	71.75 a	57.73 b	70.23 a	57.26 b	69.67 a	59.08 b	1.179	0.0006	<.0001	0.0004
EDDM (%)	62.2 c	79.82 a	61.01 cd	79.88 a	59.30 d	76.53 b	55.01 e	76.51 b	0.950	<.0001	<.0001	0.0313
Rumen degradation characteristics of crude protein (CP)												
K_d (%/h)	12.24 c	30.50 a	11.77 c	25.57 b	10.11 c	12.38 c	10.15 c	15.71 c	1.589	<.0001	<.0001	<.0001
S (%)	7.04 d	27.32 a	4.71 de	21.19 b	2.74 ef	23.35 b	0.56 f	16.41 c	1.353	<.0001	<.0001	0.0354
D (%)	77.79 ab	60.78 d	79.64 a	72.67 bc	78.73 a	71.82 c	78.09 ab	78.92 a	2.00	0.0009	<.0001	0.0019
EDCP (%)	59.16 d	77.75 b	57.19 d	79.92 a	52.01 e	71.55 c	48.49 f	72.97 c	1.207	<.0001	<.0001	0.0224
Rumen degradation characteristics of starch (St)												
K_d (%/h)	16.98 bc	46.32 a	16.56 bc	46.24 a	16.67 bc	29.69 b	14.75 c	37.80 a	3.601	0.0317	<.0001	0.0393
S (%)	11.39 e	44.04 a	8.40 f	35.89 b	9.56 ef	33.09 c	4.01 g	28.02 d	1.148	<.0001	<.0001	0.0002
D (%)	81.85 a	55.32 e	85.15 a	63.62 d	81.01 b	65.96 d	85.25 a	71.28 c	1.315	<.0001	<.0001	<.0001
EDSt (%)	71.86	92.95	70.75	92.18	68.94	76.80	64.44	89.41	4.459	0.1483	<.0001	0.2606

Means with different letters in the same row are significantly different (P<0.05)

v - the effect of variety; p - the effect of processing

K_d = rate of rumen degradation of the D fraction; S = soluble or 'wash out' fraction; D = insoluble but potentially degradable fraction; EDDM = effective degradability of DM

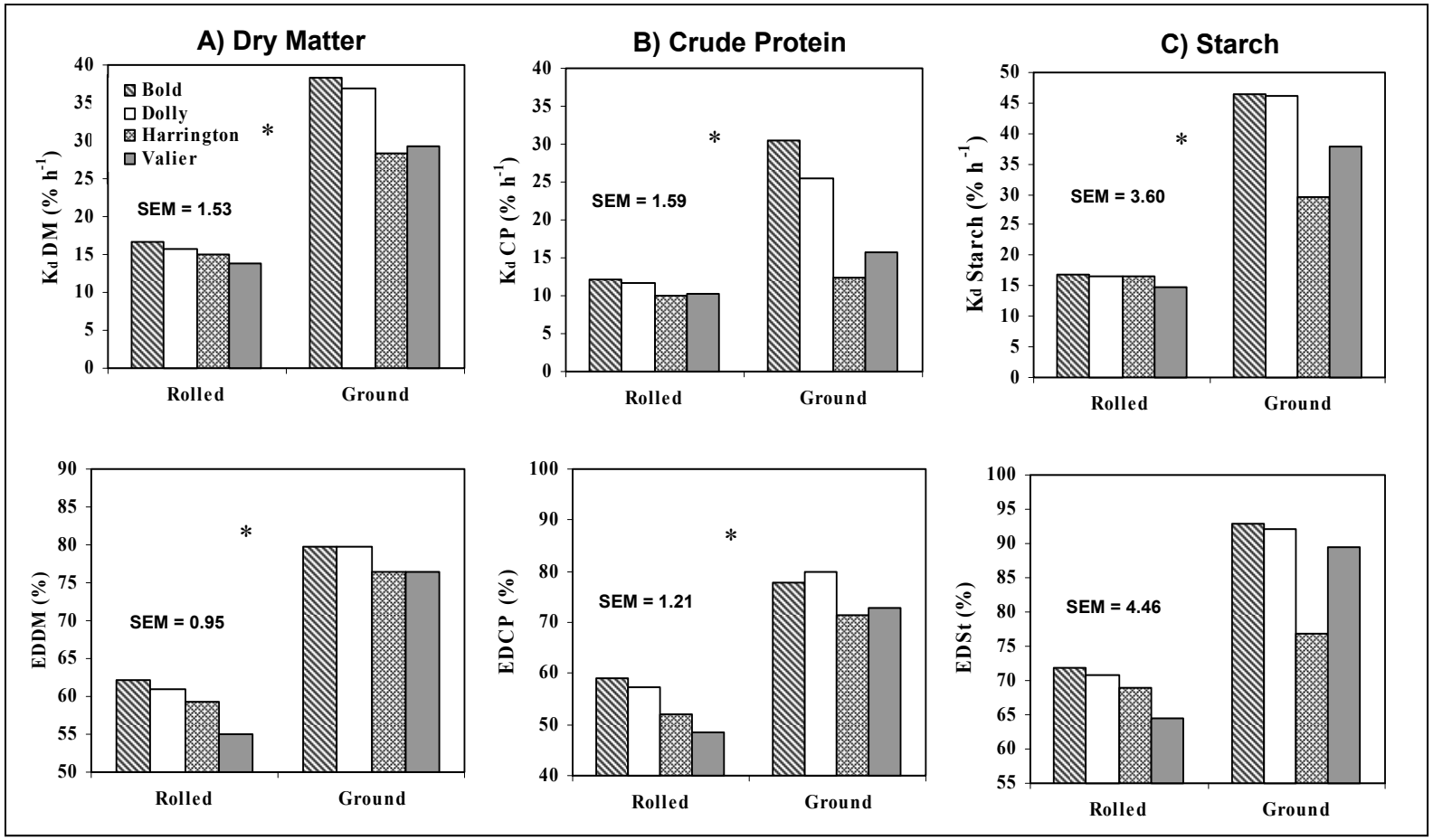

Figure 2.7. Comparison of the *in situ* rate of degradation (K_d) and effective degradability (ED) of A) dry matter, B) crude protein (CP); and C) starch of four barley varieties: CDC Bold, CDC Dolly, Harrington and Valier. * Indicates a significant interaction ($P<0.05$) between the effects of grain type and processing method

starch for ground Valier is numerically lower than that of CDC Bold and CDC Dolly, but is not statistically different.

Grinding also increased the EDCP of Valier to a greater extent (>11%) than seen with the other barley varieties. In total, the EDCP of ground Valier increased by nearly 50% over the rolled sample, while CDC Bold, CDC Dolly and Harrington were increased by 30, 37 and 39%, respectively. Processing also increased the rate of starch degradation, but once again, the magnitude of the effect was not equal for all varieties. CDC Bold and CDC Dolly showed an increase in the K_d of starch by a factor of 1.7 and Valier increased by a factor of 1.5, while the starch K_d for ground Harrington was only increased by a factor of 0.8 over the rolled treatment.

Rates of DM disappearance from ground barley samples ranged from 28.4 to 38.5% h^{-1}, similar to rates reported by Lehman et al. (1995; 23 to 35% h^{-1}), Yu et al. (2003; 29 to 32% h^{-1}) and Khorasani et al. (2000; 38% h^{-1}). Ramsey et al. (2001) observed much higher rates of DM degradation ranging from 33 to 58% h^{-1}. It seems that all these studies show a high degree of variability in the measurements, increasing the range in digestibility rate and making it more difficult to find statistical differences.

In contrast to the results of this experiment, none of Ramsey et al. (2001), Boss and Bowman (1996) or Yu et al. (2003) was able to detect an effect of barley variety on the rate of DM disappearance of ground barley grains. Lehman et al. (1995) did observe an effect of barley variety on the rate of DM disappearance, however, their study compared 22 different varieties of barley, thus increasing the probability of detecting statistical differences.

The expectation in this experiment was that grinding would tend to reduce the variability in rate and extent of rumen degradation among barley samples by destroying the physical structure of the endosperm tissue, thus making the starch more uniformly available to rumen bacteria for the process of fermentation. However, statistical differences were observed among ground samples that did not occur among the rolled samples. Ground samples of CDC Bold and CDC Dolly showed higher ($P<0.05$) rates of rumen disappearance than did Harrington and Valier for both DM (38.3 and 36.9 % h^{-1} vs. 28.4 and 29.3 % h^{-1}, respectively) and CP degradation (30.5 and 25.6 % h^{-1} vs. 12.4 and 15.7 % h^{-1}, respectively) (Table 2.6). This result was consistent with the

degradation rates of 32 % h^{-1} for Harrington and 29 % h^{-1} for Valier observed by Yu et al. (2003).

Solubility (S) of DM from ground barley ranged from 28.4 to 36.9%, with a mean value of 31% (Table 2.6.); which was similar to the 33.7% soluble fraction of DM measured by Lehman et al. (1995), and slightly lower than the 35.5% soluble fraction obtained by Yu et al. (2003). Others have reported a noticeably higher proportion of the 'wash-out' or soluble DM fraction. Ramsey et al. (1995) measured an average of 51% in the wash-out fraction of ten barley varieties, while in a comparison of sixty barley cultivars by Khorasani et al. (2000), the DM solubility was found to range from 35.2 to 59.4%, with a mean of 44%. The difference in solubility measurements may be the result of varying experimental procedures and techniques, including sample particle size, bag pore size and washing technique. The hand-washing technique used in this experiment may have produced different solubility results when compared to other methods which use mechanical washing procedures.

Pore size of the rumen incubation bags may have more influence on DM solubility. Considerably higher DM solubility values were obtained by Khorasani et al. (2000) and Ramsey et al. (2001) who used bags with pore sizes of 57.5 μm and 50 μm, respectively, in comparison to the 41 μm pore size of the bags used in this trial.

In this study, both rolled and ground treatments of Valier had lower ($P<0.05$) solubility of DM, CP and starch than the other varieties. This resulted in lower ($P<0.05$) EDDM and EDCP for Harrington and Valier than for CDC Bold and CDC Dolly (Table 2.6.). Even with the smaller particle size of ground samples, the observed variation in rate and extent of digestion indicates that each barley variety possesses distinct physical and chemical characteristics involved in determining the rate and extent of physical degradation and digestion in the rumen, and gives each variety its own unique degradation kinetics.

In general, there was a significant variety by processing interaction ($P<0.05$) on the rate of DM, CP and starch degradability observed among the four barley varieties included in this study. The nature of this interaction tended to be that grinding caused an increase in the rate and extent of digestibility for all varieties, but to a greater extent for CDC Bold and CDC Dolly compared to Harrington and Valier.

Table 2.7 shows the rumen degradation kinetics of four barley varieties for rolled samples only. As with the corn vs. Harrington barley trial, the objective was to isolate the effect of barley variety on *in situ* rumen degradation characteristics by analyzing variables at a single level of processing. Rolling was chosen as it is the most common form of processing for barley in ruminant rations.

The rates of DM degradation for rolled treatments ranged from 13.7% h^{-1} for Valier to 16.6% h^{-1} for CDC Bold, and showed no statistical difference between varieties, similar to the observation of Ramsey et al. (2001; 6.0 to 16.0% h^{-1}). This is in contrast to results observed with ground treatments where CDC Bold and CDC Dolly exhibited greater rates of DM degradation than Harrington or Valier. Neither the present study nor that of Ramsey et al. (2001) was able to detect any statistical difference in the rates of DM and starch degradation between different varieties of rolled barley. Similarly, Yu et al. (2003) did not find any differences in the rate of DM, CP or starch degradation between samples of rolled Harrington and Valier barley. However, in the study by Yu et al. (2003) as well as the present experiment, it should be noted that numerically, Valier had the lowest rate of digestion (K_d) for DM and starch. Valier was also statistically lower ($P<0.05$) than the other three barley varieties for EDDM, EDCP and EDSt (Table 2.7). Significant differences ($P<0.05$) were also detected in the soluble fraction of DM, CP and starch between rolled barley samples. Valier had lower ($P<0.05$) solubility of DM, CP and starch than both CDC Bold and CDC Dolly, and was similar to Harrington for CP and starch solubility (Table 2.7). Previous reports have indicated that the protein matrix must first be disrupted before starch granules can be released and made available for microbial digestion (McAllister and Cheng, 1996). Therefore, a reduction in protein solubility may have influenced a subsequent reduction in the degradation of starch. The relationship between Harrington and Valier barley for rumen degradation kinetics observed in the present study confirmed previous work conducted by Yu et al. (2003). Both studies found Harrington to be numerically, but not statistically ($P>0.05$), higher than Valier for rate of DM and starch rumen degradation (K_d), and significantly higher ($P<0.05$) in S (DM and CP), EDDM and EDCP.

Table 2.7. Trial 2 *in situ* rumen degradation kinetics for rolled samples of CDC Bold, CDC Dolly, Harrington and Valier barley

	Barley variety					
Item	Bold	Dolly	Harrington	Valier	SEM	P-value
Rumen degradation characteristics of dry matter						
Kd (%/h)	16.61	15.77	14.92	13.71	0.985	0.2555
S (%)	10.50 a	9.25 a	9.42 a	6.68 b	0.642	0.0030
D (%)	70.48	71.75	70.23	69.67	1.368	0.6765
EDDM (%)	62.20 a	61.01 a	59.30 a	55.01 b	1.154	0.0022
Rumen degradation characteristics of crude protein						
Kd (%/h)	12.24	11.77	10.11	10.15	0.799	0.1953
S (%)	7.04 a	4.71 ab	2.75 bc	0.53 c	0.872	0.0013
D (%)	77.79	79.64	78.73	78.09	2.068	0.8661
EDCP (%)	59.16 a	57.19 a	52.01 b	48.49 c	1.327	0.0001
Rumen degradation characteristics of starch						
Kd (%/h)	16.98	16.56	16.67	14.76	0.860	0.2994
S (%)	11.39 a	8.40 ab	6.84 bc	4.01 c	1.252	0.0142
D (%)	81.85	85.15	83.46	85.25	1.635	0.3919
EDSt (%)	71.86 a	70.76 ab	68.00 b	64.44 c	1.323	0.0048

Means with different letters in the same row are significantly different ($P<0.05$).

K_d = rate of rumen degradation of the D fraction; S = soluble or 'wash out' fraction; D = insoluble but potentially degradable fraction; EDDM = effective degradability of DM

Although the rate of DM, CP and starch degradation measured in this experiment showed no statistical differences among the four barley varieties (P = 0.255, 0.195 and 0.299, respectively), the numerical order was the same as that of the ground samples with CDC Bold showing the fastest K_d values followed by CDC Dolly, Harrington and Valier (Table 2.7). Valier was statistically lower ($P<0.05$) in EDDM, EDCP, EDSt and S of DM than the other three varieties (Table 2.7), indicating that it has less extensive rumen fermentation than the other barley varieties even though the actual rate of degradation was statistically similar.

Despite the lack of significant differences in K_d, the ranking of barley varieties in the order of decreasing K_d corresponded to the order of decreasing starch:protein ratio in the kernel as estimated by chemical analysis (Table 2.3) (CDC Bold > CDC Dolly > Harrington > Valier). This observation helps to support the theory that a lower starch:protein ratio is indicative of lower DM degradation in the rumen. The assumption is that higher protein content, which is reflected in a lower starch:protein ratio, gives greater 'protection' to starch granules in the endosperm and reduces the rate of rumen degradation by hindering the access of rumen microbes to the starch granules.

Besides rate of rumen DM degradation, most of the rumen degradation kinetics (with few exceptions, i.e. K_d of starch), were consistently ranked in the same order: CDC Bold, CDC Dolly, Harrington and Valier, with CDC Bold having the highest, and Valier the lowest values for K_d, of DM and CP, S and EDDM; for starch, S and EDSt.

2.4 Conclusions

The results of the *in situ* digestibility study shows that the rate and extent of rumen disappearance varies both between cereal grain types (i.e. corn and barley) as well as among varieties of barley. Corn exhibited slower rates of *in situ* DM, CP and starch degradation than barley at two different levels of processing (rolling and grinding), confirming previous research indicating that the availability of corn starch for rumen fermentation processes is less than that of barley. Although fine processing (grinding) served to increase the rate and extent of digestion for both corn and barley, the magnitude of the response was differentially affected. Ground Harrington (GH) showed a significant increase in rate and extent of DM degradation over the rolled form, while there was no significant difference ($P<0.05$) detected in the rate of degradation between rolled and ground corn (Table 2.4.). This indicates that the physical structure of the grain kernel is not the only factor which influences digestibility. Chemical constituents and molecular structure must also be involved in the total determination of DM, CP and starch digestion.

In situ digestibility comparison of four different varieties of barley also revealed differences in the rate and extent of DM, CP and starch digestion. Valier consistently proved to be less extensively degraded and at a slower rate than the other three varieties, verifying previous reports of slower DM degradability than other barley varieties (Yu et al., 2003).

The starch:protein ratio calculated from total starch and CP analysis on whole kernel samples of each grain was also of interest in this study. From our experimental observations, it seems that a lower starch:protein ratio is related to reduced rate and extent of rumen degradation measured by *in situ* methods. However, it is important to note that this was only observed when comparing varieties of barley. It was not true for the comparison of corn to barley.

With respect to ruminant feeding programs, favorable digestive characteristics, such as a slower rate of ruminal starch degradation, could become a criterion for selection in barley breeding programs. Such selection and breeding programs with the specific goal of developing varieties of feed barley that exemplify the desirable digestibility rates and characteristics for ruminants would benefit from a technique that

would provide rapid analysis and evaluation of barley grain varieties for these desirable characteristics.

These findings indicate that there is potential for further investigation into the molecular and biochemical nature of cereal grains and their relationship to digestion within the rumen. Physical factors such as kernel structure and degree of processing which affect digestibility can be evaluated using *in situ* techniques, but physical factors represent only one element of the total mechanism involved in cereal grain digestion. Based on the known physical digestibility, further research would be valuable to explore the use of other technologies that may help to clarify the biochemical and molecular factors which are also important contributors to the overall digestibility of cereal grains.

3.0 Infrared Microspectroscopy of Cereal Grains

3.1 Introduction

Studies on determination of nutrient availability are traditionally carried out using the *in situ* method. While this technique is valid, it is expensive and time-consuming, making it impractical and unrealistic for large numbers of samples (Khorasani et al., 2000). This method provides a quantitative measure of the fermentation rate of different samples of plant tissue, but it gives no indication of the specific chemical and structural factors that influence these varying rates.

One of the new technologies available to scientists is the use of synchrotron microspectroscopy for the examination of chemical microstructures within biological tissue samples. The ability to use infrared synchrotron radiation as the light source for microspectroscopy has made this technique a valuable analytical tool for examining the chemical composition of biological samples. Synchrotron light is up to 1000 times brighter than a conventional thermal infrared light source (such as the Globar light source found internally in most commercial FTIR spectrometers), giving it the ability to explore the chemistry within microstructures of plant tissue at high spatial resolutions of 3 to 15 μm, while at the same time maintaining high signal to noise ratio (Yu et al., 2003a, 2004a, 2004b). The advantage of synchrotron light is the brightness of the infrared source which permits rapid acquisition time and superior quality of spectra with spatial resolution at the diffraction limit (Dumas et al, 2004). Using synchrotron microspectroscopy, molecular structure and specific functional groups within an organic compound can be identified based on the unique vibrational characteristics displayed when exposed to mid-IR radiation.

Another important feature of IR microspectroscopy is the capability to examine untreated, unstained tissue (Gough, 2003), allowing analysis of the sample without compromising the integrity of the inherent chemical structures. This provides a distinct

advantage over the traditional methods of chemical analysis which require homogenization (grinding) of the sample which destroys any spatial information regarding the location of specific functional groups within the sample.

Unique properties of vibrational spectra yield detailed information, including identification of functional groups and molecular conformation for all components within the illumined pixel. This permits quantitative assessment of tissue components at the cellular and sub-cellular level (Gough, 2003).

Synchrotron FTIRM has been used to characterize plant composition in a number of different forage and grain species. Difficulties in tissue preparation and sectioning have limited the number of studies to some extent, but this technique is still useful for revealing specific information on the biochemistry and distribution of plant components such as lignin, cellulose and other carbohydrates, protein and lipids (Miller and Dumas, 2006). Several studies have been carried out with various cereal grain species. For example, the starch content and relative fraction of α-helical protein structure has been characterized in hard versus soft wheat (Wetzel et al., 2003); the composition of cell wall polysaccharides has been observed in both wheat and barley (Robert et al., 2005; Philippe et al., 2006); endosperm cell composition and structure have been related to grain hardness in wheat (Barron et al., 2005); and distribution of proteins, lipids and carbohydrates across the cotyledon and in the hilum region of soybean seeds have been demonstrated (Pietrzak and Miller, 2006).

Previous work by Yu et al. (2004d, 2004b) has shown synchrotron FTIRM to be a useful technique for providing spectral, chemical and functional group characteristics in both barley and corn, as well as being able to distinguish differences in the biochemical components of endosperm tissue between two different varieties of barley (Yu et al., 2004a). Recent work (Yu et al., 2003b) has shown that synchrotron FTIR microspectroscopy can be used as a non-destructive technique to investigate the molecular structure of biological tissues at high ultra-spatial resolution. Data obtained from this type of chemical analysis may prove useful for helping to identify the unique chemical micro-structures of each grain type and how this relates to specific digestibility characteristics.

Based on the knowledge of physical digestibility characteristics of corn and barley grain determined by previously described *in situ* studies, the objective of this experiment was to assess the capabilities of internal thermal source IR light and external synchrotron-source FTIRM as a tool for predicting plant composition in relation to physical digestibility characteristics. Further objectives were to measure the relative concentration of starch and protein in isolated regions of endosperm tissue in corn and barley, and to determine whether the ratio of starch:protein in endosperm tissue of cereal grains measured with IR microspectroscopy can be related to the physical nutrient degradation kinetics determined by the *in situ* nylon bag technique.

3.2 Materials and Methods

3.2.1 IR Microspectroscopy

3.2.1.1 Grain selection

Five kernels of barley from each of four barley varieties (CDC Bold, CDC Dolly, Harrington and Valier) were selected for IR analysis based on the diameter and weight parameters described in Figure 3.8. Grain corn (cv. Pioneer 39P78) was obtained from the Prairie Feed Resource Centre (University of Saskatchewan, Saskatoon, SK, Canada). This variety is a hybrid grain corn with a relatively low heat unit requirements (2050 heat units), making it suitable for production in western Canada. Similar to the barley, five corn kernels were chosen for IR analysis. They were assessed visually to remove any extreme or unusual kernels, and then weighed individually. Kernels chosen for analysis were selected from the weight range of 190 to 210 mg.

3.2.1.2 Slide preparation

Samples of corn and barley were prepared for FTIRM analysis by cutting thin sections from the kernel and mounting them onto IR microscope slides. Dry, desiccated seeds tend to shatter when cut. In order to minimize this problem, kernels were imbibed in distilled water for 24 hours at 4ºC in order to soften the tissue for sectioning. The tips of the barley kernels were removed with a scalpel blade to enhance water penetration into the interior (S. Miller, personal communication). Corn kernels were

Table 3.8 Seed populations from which kernels were selected for IR analysis

Variety	Screen size (mm)	Weight (mg)
CDC Bold	<3.18 > 2.97	54.0 - 54.9
CDC Dolly	<3.18 > 2.97	56.0 - 56.9
Harrington	<3.18 > 2.97	53.0 - 53.9
Valier	< 2.77 > 2.59	47.6 - 48.5

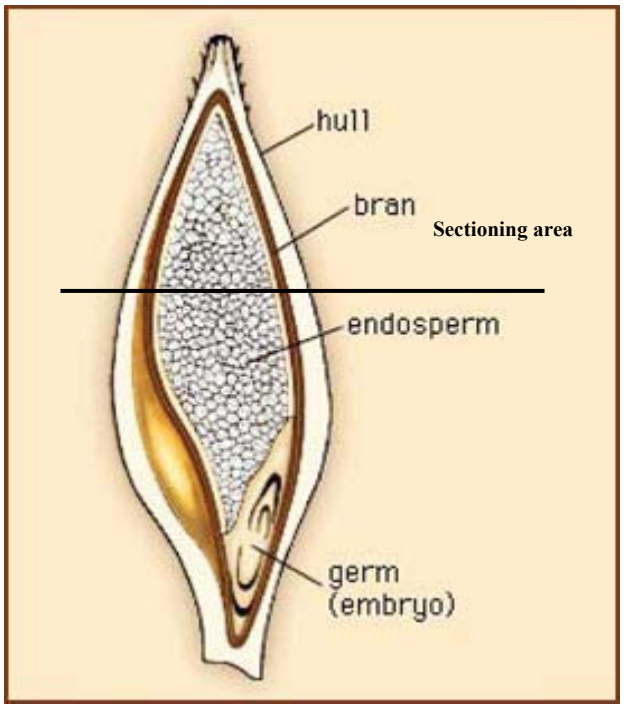

Figure 3.8 Longitudinal section of a barley kernel indicating location of cross sections to be taken for IR analysis. Source: www.barleyfoods.org/gfx/barley-kernel.jpg

rubbed with sandpaper to remove the top portion (dent area) of the structure, as cutting tended to crack the kernel. After soaking in distilled water, samples were frozen at -20ºC and cut into 6 µm sections using a cryostat (Leica CM3050S, Leica Microsystems, Wetzlar, Germany). Kernels were mounted in OCT compound (Miles Inc., Elkhart, IN) for sectioning. Sections for analysis were cut approximately one-third of the distance from the top of the barley kernel (Figure 3.8). Corn kernels were cut in a similar manner. This area was chosen for two reasons: first, to avoid cross sections that bisect the embryonic region of the kernel, and second, to collect an area of endosperm tissue that would best represent the maturity of the whole kernel. Starch granules at the tip of the kernel tend to fill up last during the growth and maturation stages of the plant, and are therefore less dense than those located lower in the kernel. Frozen sections were dry mounted onto Low-e IR microscope slides (Kevley Technologies, Chesterland, OH) for microspectroscopy in reflectance mode. Reflectance mode microspectroscopy was used for experiments conducted at the Canadian Light Source Inc. (Saskatoon, SK, Canada) and the Karlsruhe Research Center (ANKA, Karlsruhe, Germany). For synchrotron FTIRM at the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY), tissue samples were prepared in a slightly different manner. Five seeds from corn (Pioneer 39P78) and Harrington and Valier barley were embedded in paraffin (procedure detailed in Appendix B) and cut into thin cross-sections of approximately 6µm using a Microm 330 microtome (Microm Laborgerate GmbH, Sandhausen, Germany). Sections were immediately mounted onto 13 mm x 1mm BaF_2 disks (Spectral Systems, Hopewell Junction, NY) for synchrotron FTIRM in transmission mode, and deparaffinized by extraction with a series of xylene and ethanol solvents (Appendix B).

3.2.1.3 FTIRM studies of cereal grain tissue

3.2.1.3.1 Thermal-source IR microspectroscopy

Thermal-source FTIR microspectroscopy was performed on the 01B1-1 mid-infrared beamline at the Canadian Light Source Inc. using a Hyperion microscope with programmable mapping stage, a Bruker IFS 66v/S Series IR spectrophotometer (Bruker Optics Inc., Billerica, MA, USA) and the internal Globar IR light source. Fifty discrete

spot samples were collected from the endosperm region from each of five sections (representing five different seeds) of corn (39P78) and four barley varieties (CDC Bold, CDC Dolly, Harrington and Valier), for a total of 250 spectra from each grain variety (Figure 3.9). Spectral data was collected in the mid-infrared range of 4000-800 cm^{-1} at a resolution of 4 cm^{-1} and an aperture setting of 50×50 μm. The microscope stage was purged with dry air. Each spectrum consisted of thirty-two co-added scans. Visible images were obtained using an Exwave HAD CCD camera (Sony, Japan). Automated stage control, spectrum data collection and processing were performed using OPUS version 4.2 (Bruker Optics Inc. Billerica, MA, USA).

3.2.1.3.2 Synchrotron-source IR microspectroscopy: reflectance mode

Synchrotron source FTIR microspectroscopy data in reflectance mode was collected on the IR-1 beamline at ANKA, Karlsruhe Research Center, Karlsruhe, Germany. Ten discrete spot sample spectra were collected from the same tissue sections used in the CLS experiment. This experiment examined three sections representing three different kernels from each of corn, CDC Bold, CDC Dolly, Harrington and Valier, for a total of 30 spectra per variety (Figure 3.9). Spectral data was collected using a nitrogen purged IR microscope coupled with a Bruker IFS 66v/S spectrophotometer (Bruker Optics Inc., Billerica, MA, USA). Infrared absorption spectra were recorded between 4000 and 800 cm^{-1} using a 15x IR objective and a 12 μm circular aperture. Individual spectra were produced from 256 co-added scans. A new background spectrum was collected after every 10 sample spectra. The IR beam was manually focused at each spot sample location to optimize the signal amplitude at each individual point. Visual images were taken using a Sony DDC-IRIS high resolution video camera. Stage control, data collection and processing were performed using OPUS version 4.2 software package (Bruker Optics Inc.).

3.2.1.3.3. Synchrotron-source IR microspectorscopy: transmission mode

Infrared analysis of corn, Harrington and Valier barley using synchrotron-source FTIRM was carried out on the U2B beamline at the Albert Einstein Synchrotron Bioscience Center (NSLS-BNL, Upton, NY). Spectral data was collected in

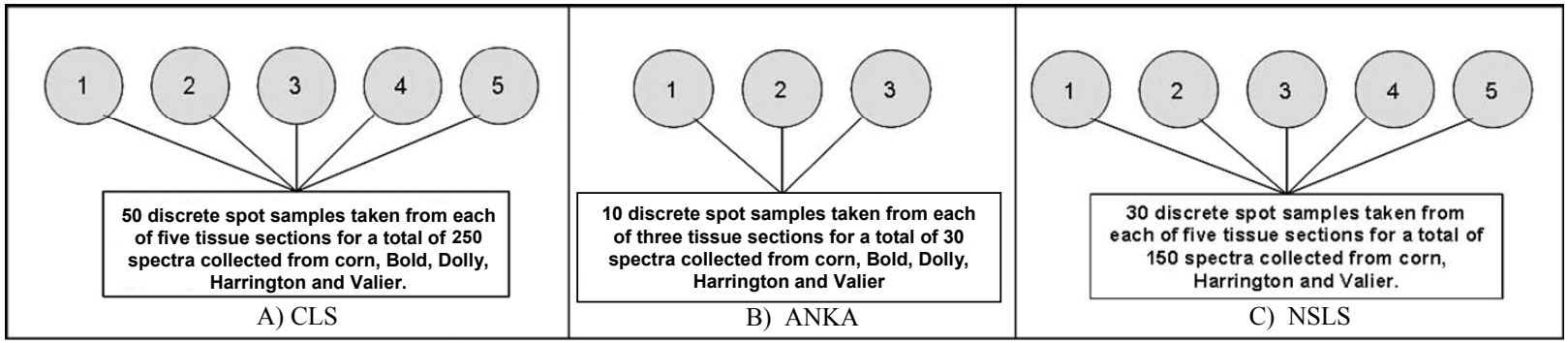

Figure 3.9. Schematic diagram of the spot sample spectra collected from A) CLS experiment B) ANKA experiment: and C) NSLS experiment

transmission mode using a Nic-Plan IR microscope with a 32x IR objective and a 14 μm round upper aperture. Microscope stage and samples were continuously purged using dry N_2 to minimize IR absorption from water vapor and CO_2 in ambient air. The microscope was connected to a Magna-IR 860 spectrometer (Nicolet Instruments, Madison, WI) and a mercury cadmium telluride (MCT-A) detector. The bench was configured to collect IR spectra from 4000 to 800 cm^{-1} at a resolution of 4 cm^{-1}. Background and sample spectra were produced from 128 and 64 co-added scans, respectively. A new background spectrum was collected after approximately ten sample spectra had been taken, and the IR focus was manually adjusted at each point to maximize beam signal strength.

Thirty spectra from distinct locations within the endosperm region were collected from each of five tissue sections of corn, Harrington and Valier (Figure 3.9). Spot sample location was confined to the region extending 100-600 μm inside the aleurone layer (Figure 3.10). Automated stage control, spectrum data collection and data processing were done using OMNIC Atlμs™ 7.2a software (Thermo Fisher Scientific Inc, Waltham, MA, USA). Additional spectral data was collected from pixels in a structured grid pattern for use in chemical imaging. Area maps (approx. 100 μm x 500μm, 15 pixels x 40 pixels) extending from the outer seed coat, through the aleurone layer and into the endosperm tissue were collected. Mapping steps were equal to aperture size (~14 μm).

3.2.1.4. Data treatment and analysis

Each sample spectrum was corrected by subtracting the corresponding background spectrum and displayed in absorbance mode. Automatic baseline correction was applied using the respective software (OPUS 4.2 for CLS and ANKA data, and OMNIC Atlμs™ 7.2a for NSLS-BNL data) to generate the final spectra. Individual spectra were visually examined for spectral quality based on absorption values and thermal noise present in the spectrum. Spectra with absorbance values greater than 1.4 and 1.6 for barley and corn, respectively, were removed from the data set. Large absorbance values indicate that the tissue is too thick for the IR light to penetrate through to the detector. In such cases, the majority of the IR light is absorbed by the sample, and the amount of

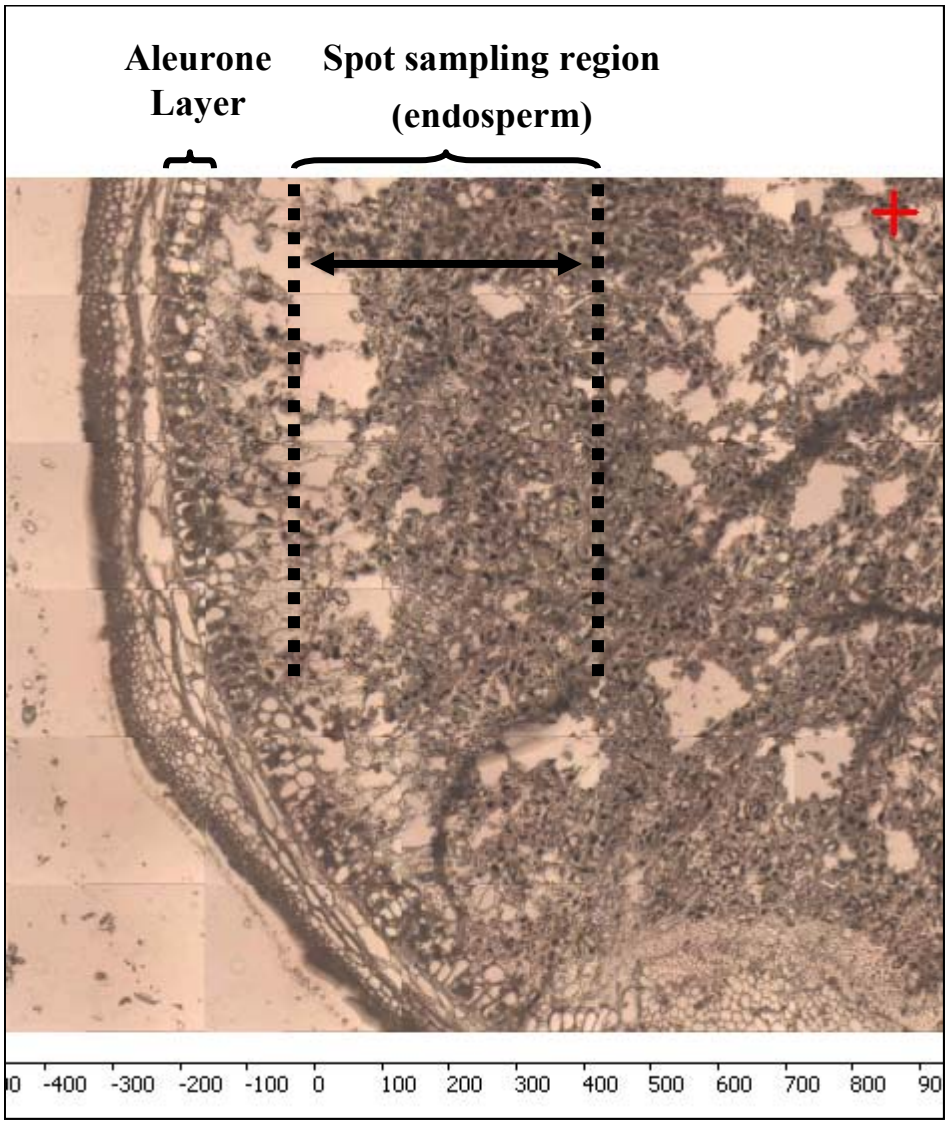

Figure 3.10. Photomicrograph of a cross section of Harrington barley showing the endosperm region where spot sample spectra were collected: 100-600 μm inside the aleurone layer.

IR light that passes through the sample and reaches the detector is so small that the detector is unable to accurately estimate the actual amount of component in the sample (detector nonlinearity). Such data is not necessarily reliable, and should be used with caution. Absorbance is equal to log (1/ % transmission). Therefore, when absorbance is 1.0, 10% of the IR light is passing through the sample to the detector; when absorbance is 2.0, 99% of the light is being absorbed by the sample and only 1% reaches the detector. If the light absorbance in the sample tissue is too high and only a small portion of the incident light passes through, the detector cannot accurately estimate the relative absorbance values attributable to various functional groups. All that can be determined is that there is a significant proportion of the particular structure that is causing the saturation present in the pixel (i.e. amide, carbohydrate, etc.). Some sources suggest that the optimal absorbance values for data analysis are in the range of 0.4-0.6 (Jackson and Mantsch, 2000). However, this was not easily attainable due to the nature of the corn and barley tissues used in these experiments. Based on the quality of spectral data collected, the absorbance limit in the CHO region (1200-900 cm^{-1}) was arbitrarily set as 1.4 for barley and 1.6 for corn. Using these limits, a sufficient number of spectra were retained for statistical analysis that would otherwise have been discarded due to the CHO absorbance though the remainder of the spectrum was of acceptable quality.

The second derivatives of several representative spectra from each grain variety were analyzed to determine the wavenumber limits for the Amide I and CHO peak area calculation (Figure 3.11). Spot sample spectra were individually analyzed using the same wavenumber limits to measure the area under the functional group absorption bands (Figure 3.12): Amide I (1580-1710 cm^{-1}) and carbohydrate (1065-950 cm^{-1}), generally attributed to starch absorption (Wetzel et al., 1998; Yu et al., 2003; Yu et al., 2004b). Starch:protein peak area ratios were obtained by dividing the area under the CHO functional group absorption peak (1065-950 cm^{-1}) by the area under the Amide I functional group peak (1580-1710 cm^{-1}). Chemical imaging of functional groups to show relative spatial distribution and absorption intensity of starch and protein was done using OMNIC 7.2a software (Thermo Electron Corporation). False color maps were generated to illustrate the spatial distribution of starch (CHO: 1066-950 cm^{-1}) and

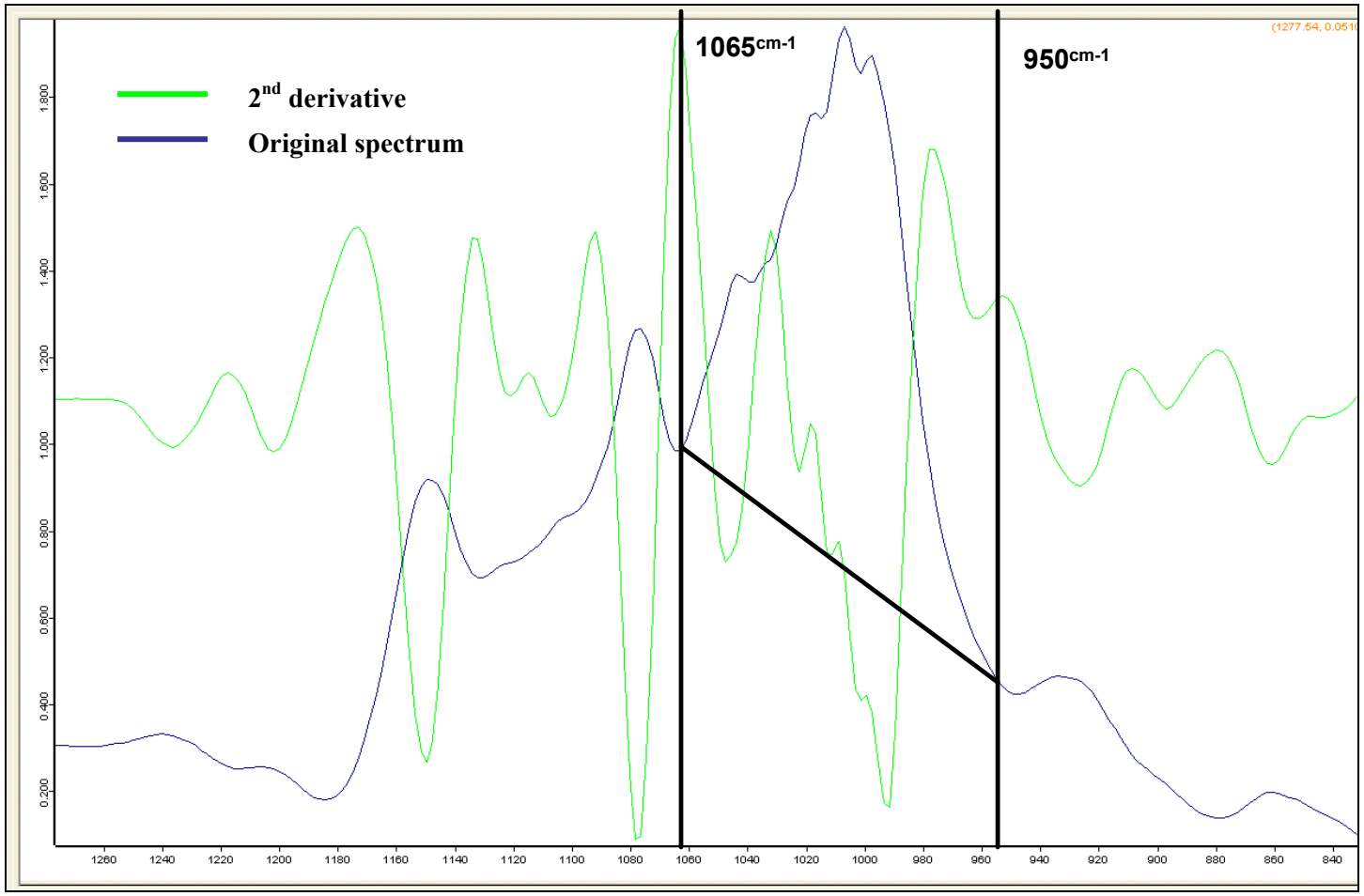

Figure 3.11. Determination of wavenumber range for CHO peak area calculation using second derivative spectrum to locate the edges of the peak. Peaks in the second derivative spectrum correspond to nadirs in the original spectrum. The CHO peak area was measured between 1065 cm^{-1} and 950 cm^{-1}.

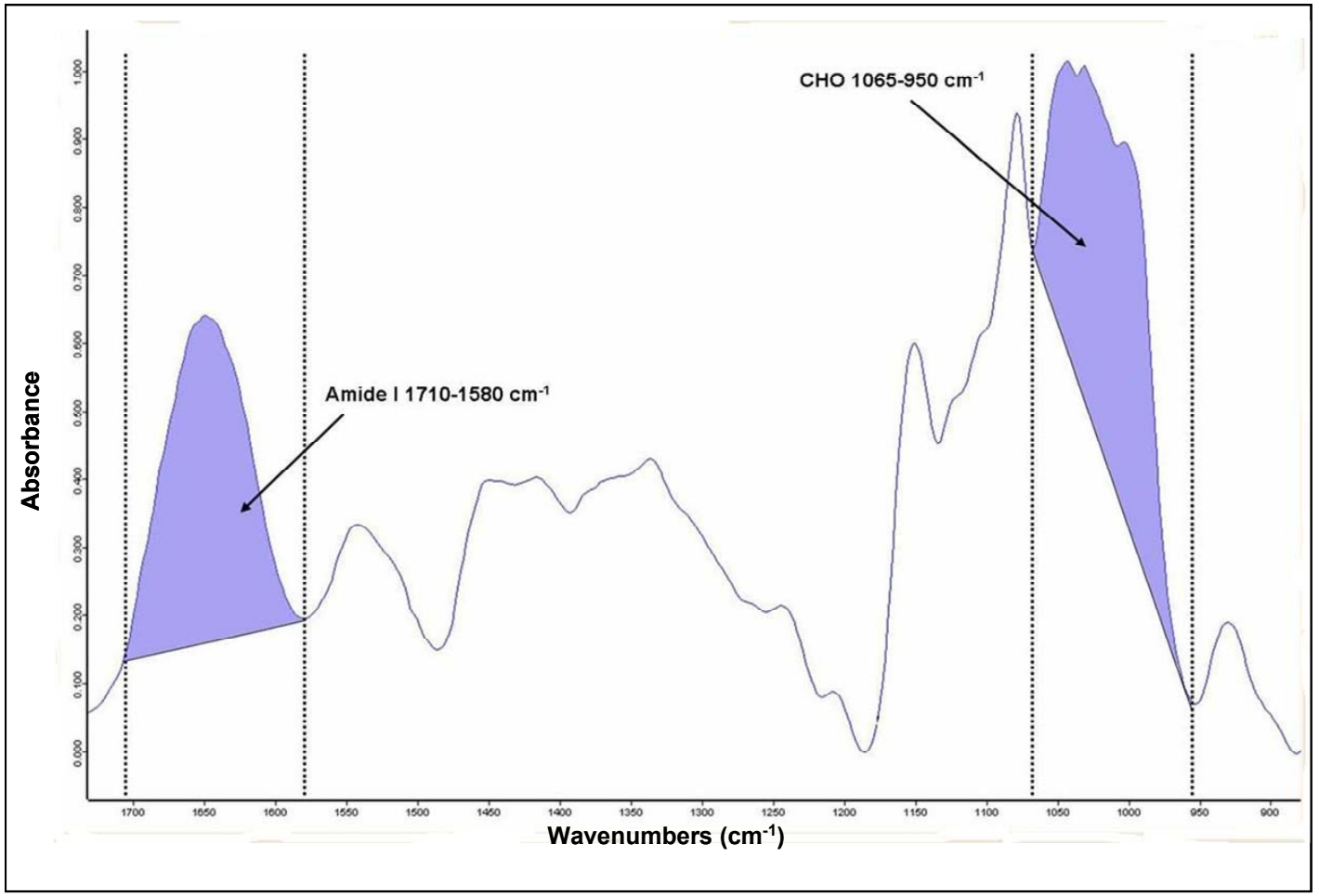

Figure 3.12. Internal IR-source FTIRM spectrum of CDC Bold barley in the 1700-900cm^{-1} region illustrating the calculation of peak area under the absorption peak of Amide I (1710-1580cm^{-1}) and CHO (starch) (1065-978cm^{-1}).

protein (Amide I: 1580-1710 cm^{-1}) within the tissue. Imaging data is qualitative in nature and can be used for making visual comparison of the structural components in the tissue layers of different grains. The spot sample spectral data was used to quantitatively measure and analyze differences in the relative content of chemical components between grain varieties.

3.2.1.5. Statistical analysis

Spot sample spectral data collected in the CLS and NSLS experiments were statistically analyzed to determine significant differences in the absorption intensity (indicating relative amount in sample) of starch and protein in the endosperm tissue of corn, CDC Bold, CDC Dolly, Harrington and Valier (CLS experiment) and corn, Harrington and Valier (NSLS-BNL data). Spot sample spectral data was analyzed as a completely randomized nested design with the effect of seed according to the model:

$$Y = \mu + \alpha + \beta(\alpha) + \varepsilon$$

where Y is an observation of the dependent variable; μ is the population mean; α is the effect of grain variety; β is the effect of the seed section; and ε is the random error associated with the observation. Analysis of variance data for peak area of Amide nested within grain variety I, CHO and the ratio of CHO:Amide I was produced using the Mixed procedure of SAS (2005). Sum of squares were separated into the effects of seed section (1 to 5) and grain variety (C, B, D, H and V for CLS data, and C, H and V for NSLS data). When F-tests were significant ($P<0.05$), means were separated by the LSD method (SAS, 2003). Least square means and the associated standard error are reported. When data was unbalanced, pooled standard error was calculated and reported.

3.3 Results and Discussion

Considerable potential exists for variation in size and weight among grain kernels within a population. Variation exists between kernels originating from different plants, between kernels from different tillers on the same plant, and between kernels from different locations on the same spikelet. Therefore, since the sample size for this experiment was limited (5 kernels from each variety), selecting a random sample was

not possible because the small sample size would not necessarily provide an accurate representation of the entire population. As a result, seed selection was intentionally biased in order to obtain a uniform group of kernels for analysis. The intent was to avoid selecting extreme observations that would significantly skew the data due to the small sample size. A sub-sample of the screened portion from each barley variety used for *in situ* experimental work was further selected by weighing out individual kernels. This was done to obtain a small pool of kernels with the same weight and density. Valier was lighter and less plump than the other three varieties, likely as a result of different weather and soil conditions during the growing season.

A photomicrograph of a cross-section of Harrington barley illustrating the physical structures of the kernel from the outside pericarp, through the seed coat and aleurone layer and into the endosperm region is shown in Figure 3.10. Holes and gaps noticeable in the tissue are likely due to slight tearing and structural disturbance that occurred during sectioning as the microtome blade cut through the tissue. Because the sections are so thin and fragile, it is necessary to ensure that no folding occurs as they are affixed to the slide or window. Vertical lines in Figure 3.10 indicate the region of endosperm tissue from which spot sample data was collected, extending from 100-600 μm beneath the aleurone layer.

The complex lower region of the mid-infrared spectrum between 1800-950 cm^{-1} is known as the 'fingerprint region' as nearly every organic compound produces a unique pattern of absorbance in this area. Comparison of the spectral features in this region can be helpful in revealing compositional differences between samples.

A single representative FTIRM spectrum, collected using an internal IR light source, from each of the four barley varieties is shown in Figure 3.13. Each spectrum represents a single pixel, randomly chosen from the 250 taken from each variety, and is meant to show the typical absorptions present in the 1750-900 cm^{-1} region of the barley spectrum. Two predominant features exist in the IR spectrum relating to protein and are seen in spectra from all four barley varieties. An absorption band assigned to protein Amide I is centered at 1650 cm^{-1} and is attributed to the stretching vibration of C=O bonds in the protein backbone (Wetzel et al., 1998; Miller, 2004). Amide II is represented by the weaker absorption band at approximately 1550 cm^{-1}, and is

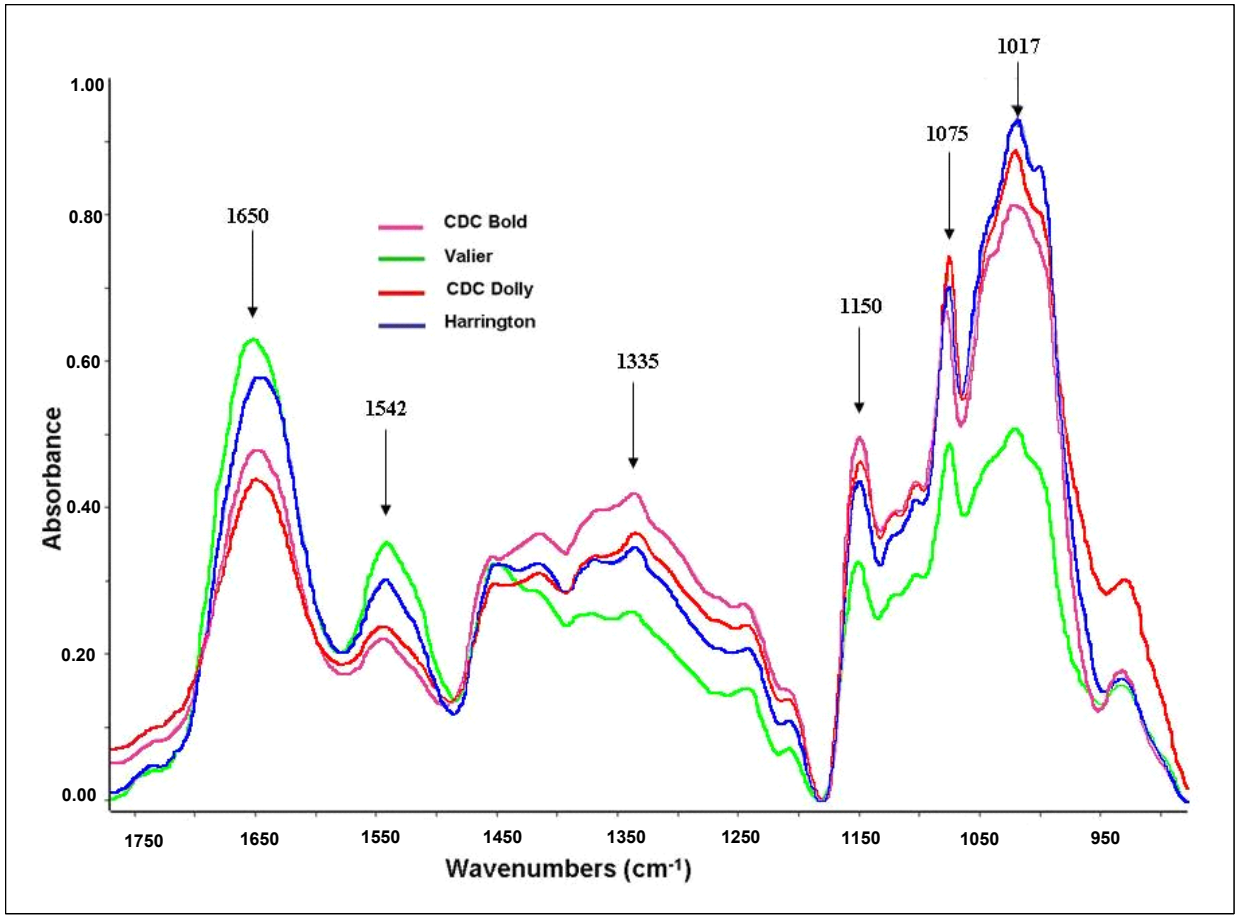

Figure 3.13. Enlarged 1850-800 cm^{-1} region of FTIRM spectra of one random spot sample from each of Harrington, CDC Bold, CDC Dolly and Valier barley endosperm tissue obtained with internal-source IR light. Harrington absorption peaks are evident at 1650, 1542, 1335, 1150, 1075 and 1017 cm^{-1}.

characteristic of bending vibrations of the N-H bonds coupled with C-N stretching (Jackson and Mantsch, 2000). The Amide II absorption is the product of complex vibrations involving several different functional groups; therefore it is less useful for estimating protein content than Amide I (Colthup et al., 1990). Figure 3.14 shows an enlarged image of the 1200-900 cm^{-1} region typically occupied by various OH and CO bond absorptions from both structural (cellulosic) and non-structural (starch-type) polysaccharides (Yu et al., 2004b). The spectral region containing carbohydrate absorption assignments is complex and not fully understood. There are three prominent absorptions consistently seen in the spectra of barley and corn endosperm tissue. The first, centered at 1150 cm^{-1}, is attributed to the C-O-C stretching vibrations of glycosidic linkages (Kačuráková and Wilson, 2001; Robert et al., 2005; Philippe et al., 2006). This is a fairly distinct and sharp absorption common to all four barley spectra, as is the absorption peak centered at 1075 cm^{-1}. This absorption likely arises from C-O-H bending vibrations of polysaccharide-type compounds (Cael et al., 1973), but the exact nature of the functional group responsible for this particular peak is undetermined. The largest of the three carbohydrate peaks (1150, 1075 and 1017 cm^{-1}) seen in the absorption spectrum is centered at approximately 1017 cm^{-1}. Non-structural carbohydrate such as starch is most often attributed to absorption at 1025 cm^{-1} (Wetzel et al., 1998, Yu et al., 2004b), however, there appears to be no distinct peak at this location evident in these spectra.

The third major CHO absorption peak centered at 1017 cm^{-1} (measured between 1065-950 cm^{-1}) in these spectra is very broad and has noticeable shoulders on either side of the main peak. This could be a result of the larger aperture size used with the internal IR light source in this experiment. Since the 50 μm aperture encompasses an area that is larger than the dimension of starch granules (10-25 μm) (MacGregor and Fincher, 1993), the spectra may reflect the presence of other carbohydrates such as cell wall components (β–D glucans and arabinoxylans). It may also be an indication of the heterogeneous nature of the carbohydrates present in the endosperm tissue.

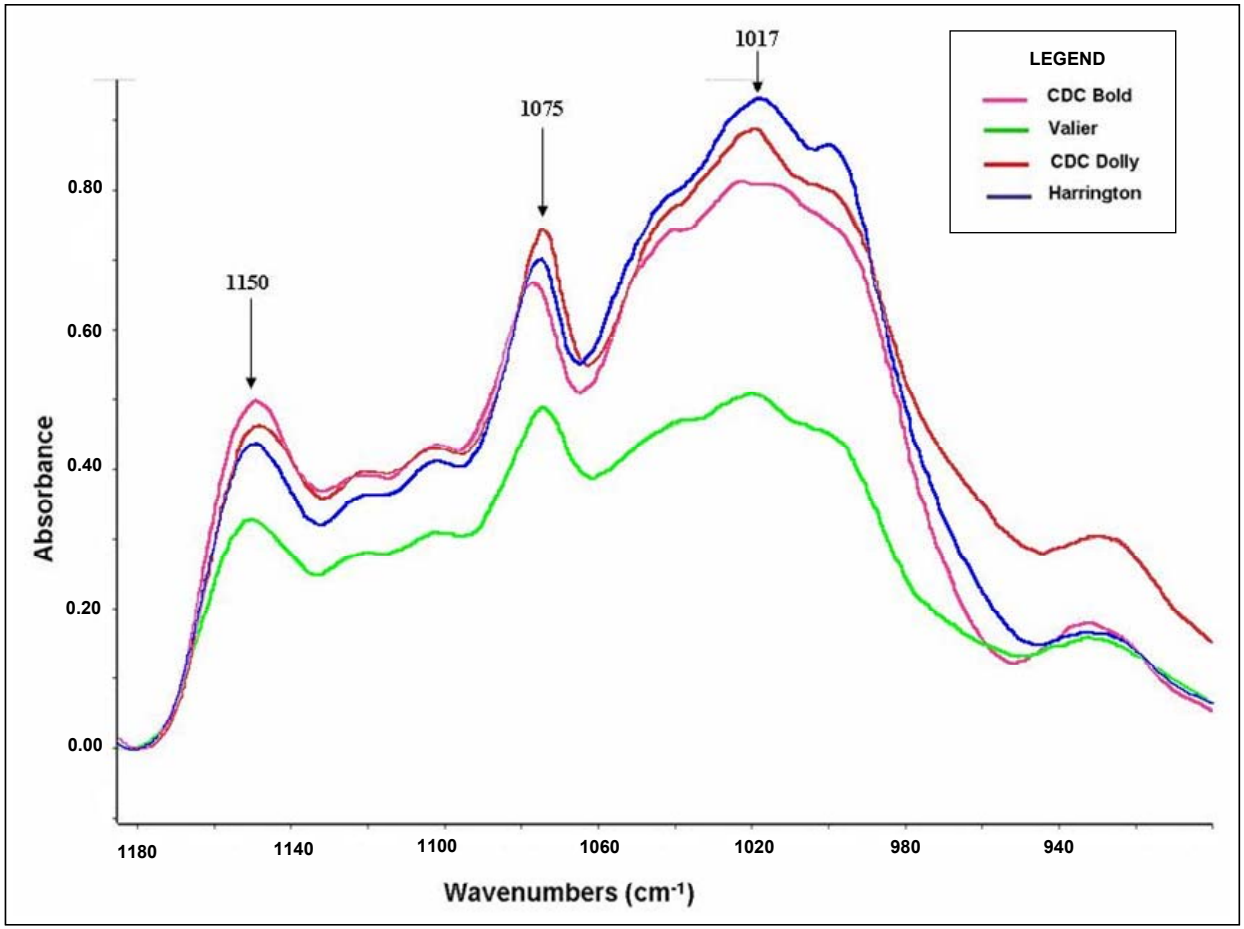

Figure 3.14. Enlarged image of the CHO region (1200-900 cm^{-1}) region of the FTIRM spectra of one random spot sample from Harrington, CDC Bold, CDC Dolly and Valier barley endosperm tissue obtained with internal-source IR light. Harrington CHO peaks are evident at 1150, 1075 and 1017 cm^{-1}.

3.3.1 Thermal-source IR microspectroscopy

Least square means of peak area for Amide I and CHO (starch), and ratios of CHO to Amide I are shown in Table 3.9. Data from this table was obtained from individual spot sample spectra taken from 50 randomly selected points within the endosperm region from five different sections of each grain (corn, CDC Bold, CDC Dolly, Harrington and Valier) for a total of 250 spectra per grain variety. Discretion must be used when evaluating the absorbance area measurements for the Amide I and CHO peaks. Direct comparison is difficult because they are not absolute values, but rather represent the relative content of functional groups present at each sampling point. Absorbance units show the amount of IR energy absorbed by the sample, which is directly proportional to sample thickness. Direct comparison of the area under the absorbance peak between two or more discrete spectra does not account for changes in sample thickness between pixel locations. Differences in the IR absorbance that occur as a result of variation in tissue thickness can be accounted for by using peak height or area ratios (Miller, 2004). Therefore, the ratio of CHO:Amide I peak area is the most accurate and practical way of comparing spectra collected from various spatial locations on different tissue samples. There was no difference between Amide I (1710-1580cm^{-1}) peak area absorptions for corn and Harrington (Table 3.9), possibly indicating similar protein content in the endosperm tissue of each grain. In contrast, the amount of protein measured by chemical analysis (Table 2.3) showed that Harrington had slightly higher protein content than corn (14.1 vs 11.5%, respectively), keeping in mind that this was an analysis of the entire kernel, rather than solely the endosperm region.

Carbohydrate absorption peak area centered at 1017 cm^{-1} was significantly higher ($P<0.05$) for corn than Harrington barley (Table 3.9). Again, this was consistent with the chemical starch analysis in which corn showed higher starch content than Harrington based on whole kernel analysis; however, there was a noticeable degree of difference in the magnitude of starch content measured using the two different techniques (FTIRM and chemical analysis).

Starch content determined by the total starch assay (Table 2.3) showed that corn was higher in starch content than Harrington barley (66.5 vs 57.8%, respectively), while

Table 3.9. CLS Globar light source spot sample data: LS means of peak area for Amide I and CHO, and ratio of CHO to Amide I for corn and Harrington.

	LS means (Absorbance units)[z]		
Grain Type	Amide I	CHO	CHO:Amide I
Harrington	24.35 (± 7.56)	22.03 (± 6.63) [b]	0.993 (± 0.431) [b]
Corn	23.96 (± 7.53)	43.67 (± 10.56) [a]	1.964 (± 0.732) [a]
Pooled SEM	0.526	0.598	0.036
P-value[y]	0.5998	<0.0001	<0.0001

Means with different letters in the same column are significantly different (P<0.05)

[z] Represents the mean of spot sample spectra (n=26 to 46) from five different tissue sections of each grain

[y] P-value indicates effect of grain type

the IR measurements of CHO peak area for corn are nearly double that of barley (Table 3.9). Because the chemical analysis was a reflection of the starch content in the entire kernel, whereas the IR technique was measuring starch in endosperm tissue only, this may indicate differences in the distribution of starch throughout the kernel. The amount of starch observed in the endosperm tissue using IR analysis was much greater for corn, possibly indicating that starch is proportionally higher in the endosperm tissue of corn compared to barley. Since these peak area measurements do not take tissue thickness into consideration, the numerical difference between them is not necessarily an absolute value, and may reflect some inconsistency in the texture and thickness of the tissue sections. The assumption is also being made that the CHO absorption peak centered at 1017 cm^{-1} is attributed solely to starch. This assumption is based on the knowledge that the majority of the carbohydrate in the endosperm region is starch (Kent and Evers, 1994), and that only minor portions of the total carbohydrate are other forms (such as cell wall or structural components).

Because of the lack of certainty for estimating the absolute amount of chemical constituents in a sample and the difficulties in making a comparison of peak absorption measurements between two different samples, FTIRM may not be the most appropriate technique to use if quantitative measurement of a particular functional group is required. However, it does appear to be useful for preliminary analysis of samples for the purpose of ranking them in order of increasing/decreasing content of a particular constituent of interest.

Due to the limitations of comparing peak absorbance of single components, the ratio of absorption values is of more interest and diagnostic value. The advantage of this method is that by using ratios, any variation in spectral intensity resulting from differences in tissue thickness is eliminated (Yu et al, 2004b). Therefore, the CHO:Amide I ratio was calculated and analyzed in these experiments. The ratio of CHO:Amide I peak area absorption was higher ($P<0.05$) for corn than Harrington barley. This observation is consistent with the higher starch content of corn estimated by the chemical analysis (Table 2.3). Although there appears to be consistency between the two methods, the results do not agree with the observations from the *in situ* rumen degradation trial for the same grains where corn showed both a lower rate and extent of

DM degradation than Harrington. McAllister et al. (1993) suggested that the protein matrix surrounding starch granules in the endosperm is a major factor responsible for differences in the rate of rumen starch digestion of cereal grains. The structure of the protein matrix provides a certain amount of 'protection' to starch granules, shielding them from enzymatic attack of rumen microorganisms. Assuming that more protein would increase this level of protection, it follows that a lower starch:protein ratio (i.e. more protein per unit of starch) would tend to reduce the rate of rumen degradation. However, when comparing the *in situ* rumen degradation of corn and Harrington barley with the starch:protein ratio determined by both chemical and IR techniques, it appears that these two grains behave in the exact opposite manner to what would be predicted based on the starch:protein ratio. Corn starch is more slowly digested even though there is relatively less protein present in the entire corn kernel as well as in the endosperm tissue. This would indicate that it may be the nature or composition of the protein rather than the quantity which serves to 'protect' the starch granules from digestion and slows the rate and extent of starch degradation by shielding the starch from microbial attack. For the purpose of classification, cereal proteins are divided into four major groups based on their solubilities: albumins, globulins, prolamins and glutelins. Differences in the amount of each protein fraction in corn and barley exist. Albumins and globulins, soluble in water and dilute salt solutions, respectively, comprise approximately 25% of the total protein content of barley, but only account for 6% of corn protein (Lookhart, 1991). In addition, glutelins, which are soluble in acid or alkali, account for nearly 20% more of the total protein content in corn than in barley. Finally, the major storage protein fraction in corn, the prolamin fraction, is called zein; prolamins in barley are termed hordeins (Lookhart, 1991). Differences in the amino acid content and structure that exist between the proteins of different grain types (i.e. corn and barley) may influence how polypeptides react to the rumen degradation processes, contributing to the observed differences in starch degradability in corn and barley.

Table 3.10 presents the least square means comparison of peak areas from spot sample spectra taken from the endosperm region of CDC Bold, CDC Dolly, Harrington and Valier barley. The values in Table 3.10 represent the mean peak areas from spectra

Table 3.10. CLS Globar light source spot sample data: peak area comparison of CHO and Amide I of CDC Bold, CDC Dolly, Harrington and Valier barley.

	LS means (Absorbance units)[z]		
Variety	Amide I	CHO	CHO:Amide I
Bold	20.62 (± 8.10) [c]	20.57 (± 6.87) [b]	1.109 (± 0.481) [a]
Dolly	25.82 (± 8.27) [b]	22.43 (± 7.72) [a]	0.947 (± 0.394) [b]
Harrington	24.35 (± 7.56) [b]	22.03 (± 6.63) [a]	0.993 (± 0.431) [b]
Valier[y]	28.11 (± 9.83) [a]	19.21 (± 7.08) [b]	0.767 (± 0.378) [c]
Pooled SEM	0.595	0.521	0.031
P-value[x]	<0.0001	<0.0001	<0.0001

Means with different letters in the same column are significantly different (P<0.05)

[z] Represents the mean of spot sample spectra (n=26 to 46) from five different tissue sections of each grain

[y] LS mean obtained from spectra collected from four tissue sections

[x] P-value indicates effect of variety

(n=26 to 46) collected from each of five different sections of tissue from each barley variety. There is a wide variation in the absorption intensity of Amide I and CHO even among spectra from the same tissue section. For example, the peak area absorbance of CHO and Amide I from 46 spectra taken from a single section of Harrington barley ranged from 8.7 to 38.2 and 7.7 to 51.7, respectively (data not shown), indicating the heterogeneous nature of the protein and starch distribution within the endosperm tissue. As previously discussed, differences in the absorbance of Amide I and CHO observed between varieties are not necessarily indicative of the difference in relative amount of chemical components.

Harrington had higher ($P<0.05$) absorbance intensity for CHO and lower absorbance intensity for Amide I than Valier (22.0 vs. 19.2, and 24.4 vs. 28.1, respectively). Similarly, Yu et al. (2004a) observed that Valier tended to have lower starch and higher protein absorbance intensity than did Harrington.

CDC Bold had the largest CHO:Amide I ratio (1.109), followed by CDC Dolly and Harrington (.947 and .993, respectively) and Valier (.767). Yu et al. (2004a) also found that Harrington had greater ($P<0.05$) ratio of starch to protein than Valier. This order follows the same ordinal ranking as the starch:protein ratio determined by chemical analysis (Table 2.3). More importantly, it is also consistent with the results of the *in situ* rumen degradation kinetics reported in Chapter 2 where CDC Bold had the fastest rate of rumen degradation, followed by CDC Dolly, Harrington and Valier (Table 2.6). Yu et al. (2004a) suggested that a greater association of the protein matrix with starch granules in the endosperm of barley may serve to limit the access of ruminal microorganisms to starch granules, thus reducing the rate and extent of rumen degradation. From this suggestion as well as observations of the current study, a lower starch to protein ratio in barley seems to be associated with a slower rate of rumen degradation. This hypothesis holds true when comparing cereal grains within the same family, but does not, however, appear to apply when comparing grains from different families (i.e. barley varieties can be compared, but not barley to corn).

From this experiment, it seems that analysis with internal IR-source FTIRM is able to rank barley varieties according to the rate of rumen degradability based on measurement of the CHO to Amide I ratio. However, this observation is based on

limited data from only four barley varieties compared with a single corn sample. To more accurately test this hypothesis, IR analysis for other cereal grains such as wheat and oat would be useful for comparison with the corn and barley results from this study. It would also be beneficial to increase the number of corn samples in order to observe variation among different varieties of corn as seen with the barley varieties in this experiment.

3.3.2 Synchrotron-source IR microspectroscopy: reflectance mode

Synchrotron FTIRM analysis of corn, CDC Bold, CDC Dolly, Harrington and Valier was conducted at ANKA. Initially, the experimental protocol was to analyze the exact same 50 spot samples that were collected with the internal IR light source at the CLS by uploading saved X,Y coordinates from each point into the OPUS software program at ANKA. The objective was to directly compare the spectra obtained with synchrotron and internal IR-source light to determine the differences between their analytical and functional capabilities by removing the variable of tissue variation. However, the microscope configuration used at ANKA did not have the same stage reference points and dimensions as the instrument used at the CLS. Consequently, the saved coordinates did not correspond with the same point on the microscope slides, so new spot sample points were randomly chosen.

Figure 3.15 shows a visual example of the quality of the spectral data collected in the ANKA experiment. Many of the spectra collected showed high absorbance values in the OH stretch region (3100-3600 cm^{-1}) and the carbohydrate region (1200-900 cm^{-1}) with some detector saturation. Spectra also showed poor signal to noise ratio (S/N) indicating that the tissue was too thick for optimal analysis. Waves and oscillations were seen in the baseline of many of the ANKA spectra (between 2800-2000 cm^{-1}), likely due to the fact that portions of the tissue sections were cracking and lifting off of the IR slides. Any inconsistencies on the surface of the tissue section, or space between the tissue and the IR slide can cause oscillations in the background of the IR spectrum (Miller, 2004) and reduce the quality of the data.

Due to the poor quality of the spectral data collected at ANKA, these spectra were not used for direct analysis, but rather for qualitative evaluation. In a general

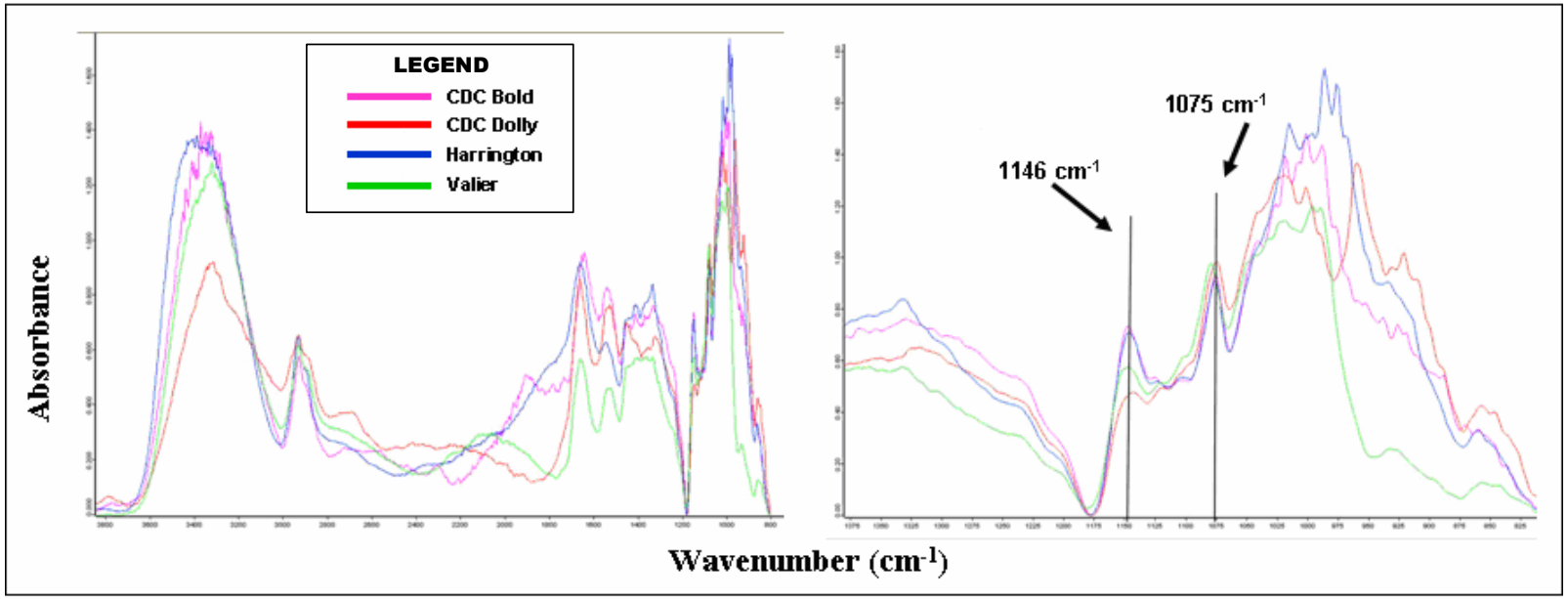

Figure 3.15. Spot sample spectra of four barley varieties obtained with synchrotron-source light at ANKA. A) Entire mid-IR spectral range showing oscillations in the baseline (2000-2600 cm^{-1}) and high absorbance in the OH stretch (3000-3600 cm^{-1}) and CHO (1200-900 cm^{-1})regions and B) Enlarged image of the 1300-900 cm^{-1} region showing two common CHO absorptions at 1146 and 1075 cm^{-1}.

assessment of the collected data, there does not appear to be a prominent absorption peak at 1025 cm^{-1} which is commonly attributed to starch (Wetzel, 1998, Yu et al., 2004a). However, the two smaller CHO absorption peaks, located at approximately 1146 and 1075 cm^{-1}, are consistently present in most of the spectra. These absorptions are consistent with the first and second carbohydrate peaks present in the typical IR spectrum of cereal grain tissue obtained with thermal-source IR light. Although there are similarities in this data to the CLS thermal-source data, the poor spectral quality reduces its reliability and accuracy. Problems with structural integrity of the tissue sections as well as the use of reflectance mode for IR analysis were identified as the key factors contributing to the poor spectral quality.

Mathematical manipulations, such as second derivative calculation, smoothing, and Fourier Transform self-deconvolution, can be applied to enhance the original spectral data. However, these techniques cannot improve the quality of the data, and extreme caution must be exercised when using any type of data manipulations. When starting with poor quality data, rather than assisting in elucidating information from the original data, these manipulations would essentially cause further distortion of any of the information contained in the original spectra.

3.3.3 Synchrotron-source IR microspectroscopy: transmission mode

3.3.3.1 Chemical imaging

Figures 3.16 to 3.18 (A) show visual images of the tissue regions from which functional group maps were taken from corn, Harrington and Valier. Maps were positioned to include the various layers of tissue from the outer pericarp and seed coat, through the aleurone layer and into the endosperm region of the tissue. Single-pixel spectra were collected every 14 μm according to the aperture size of the microscope. The overlaid grid represents each individual pixel location where a spectrum was collected in order to generate the overall mapping area. Color images of the mapped region of tissue show the spatial distribution and intensity of the ratio of CHO to Amide I peak area absorptions, where higher intensity represents a larger content of starch relative to protein. Red represents regions of highest intensity while green/blue represent the lowest absorption intensity. Areas of black on the false color maps

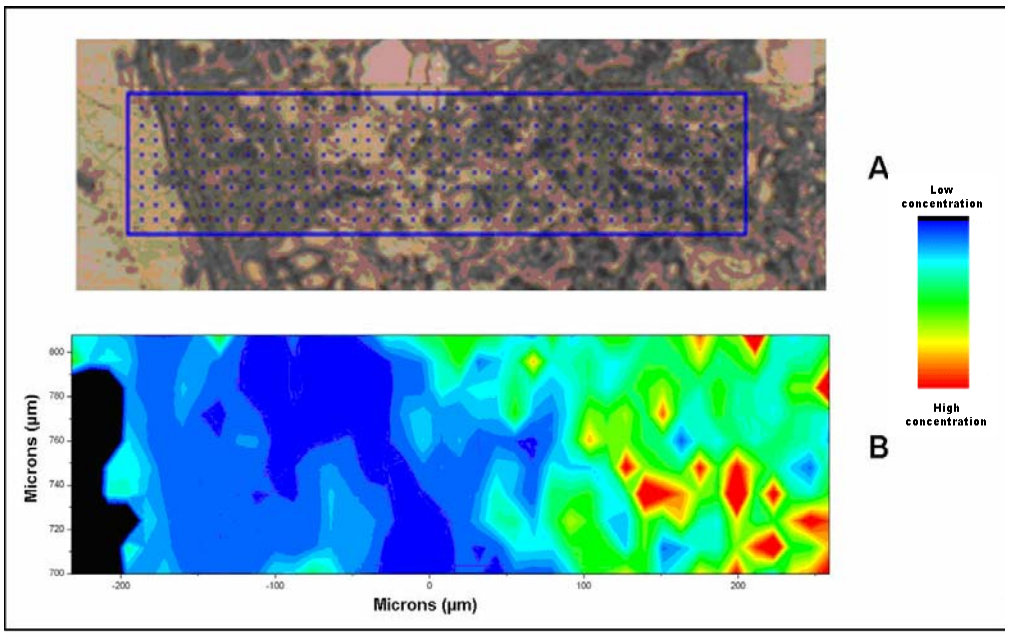

Figure 3.16. Visible image of Harrington barley with overlay of the mapping grid shown in blue. B) False color map showing the ratio of the area under the CHO (1065-950 cm^{-1}) and Amide I (1710-1580 cm^{-1}) peaks.

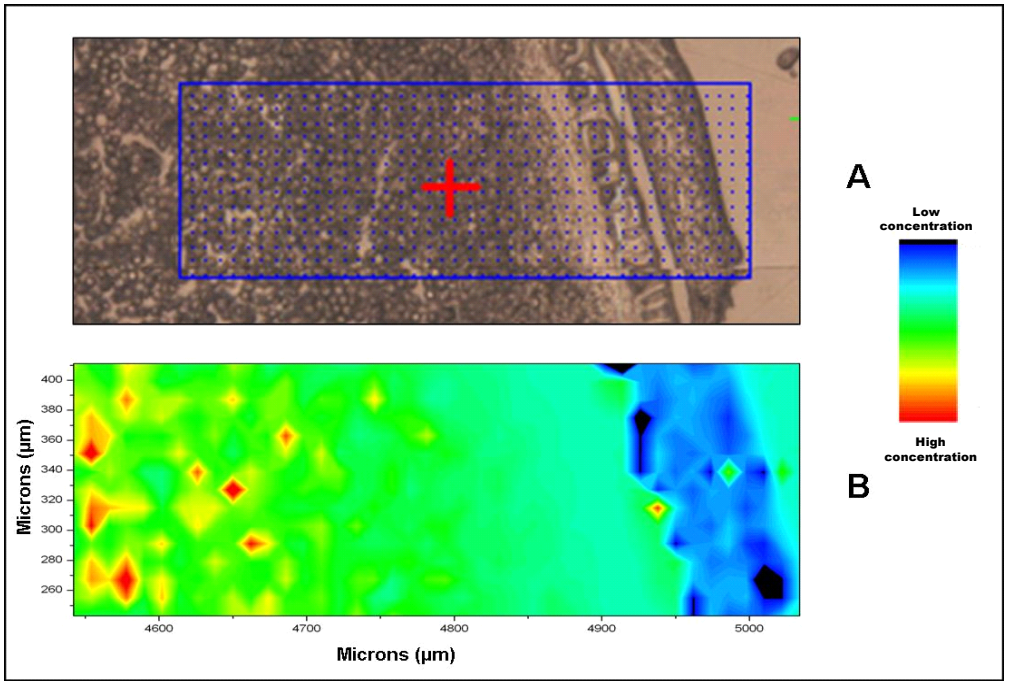

Figure 3.17. Visible image (A) of 39P78 corn with overlay of grid showing mapping points. B) False color chemical image depicting the ratio of CHO (1065-950 cm^{-1}) to Amide I (1710-1580 cm^{-1}) peak area absorption.

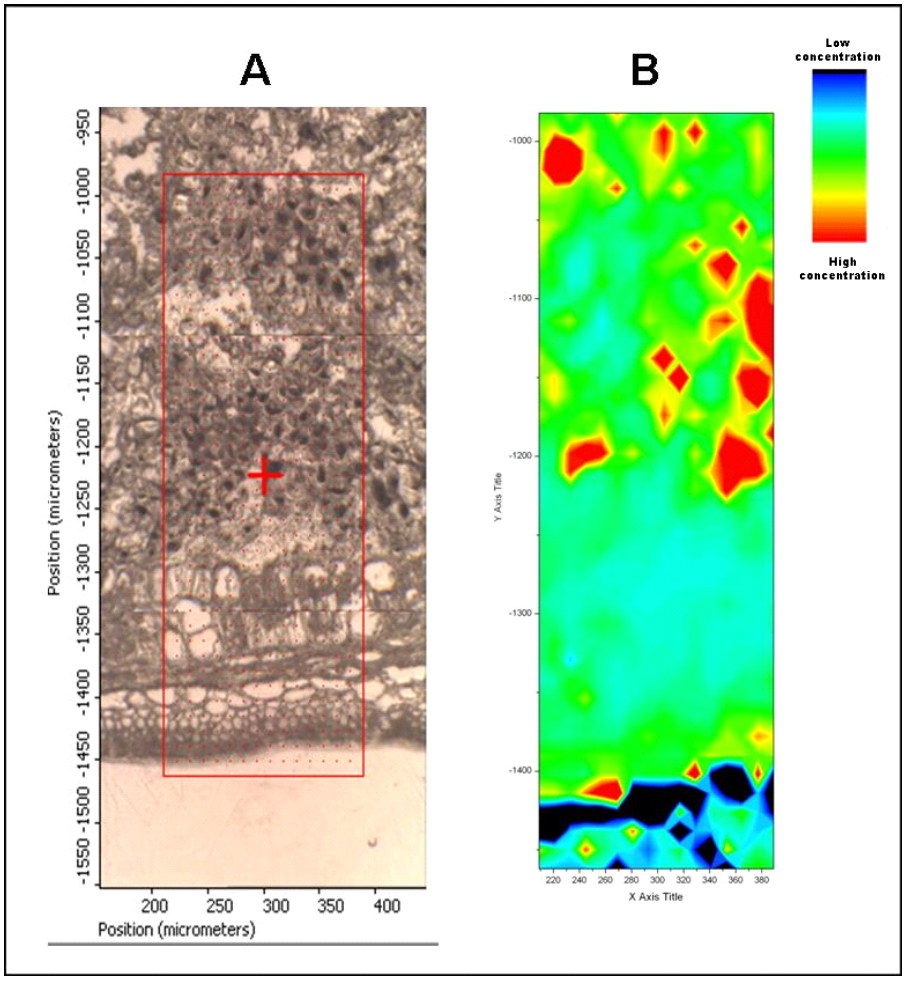

Figure 3.18. Visible image (A) of Valier barley with overlay of grid showing mapping points. B) False color chemical image of the ratio of CHO (1065-950 cm^{-1}) to Amide I (1710-1580 cm^{-1}) peak area absorption.

represent flawed data points that were removed from the data matrix.

In all three grains, the chemical maps show variation in the distribution of functional groups within the tissue sampling area. Starch content is relatively low in the regions of pericarp, seed coat, and aleurone, but gradually increases into the starchy endosperm tissue. Groups of starch granules can be identified by the high absorption intensity spots in the endosperm region. Based on this data, care was taken in determining the location of the sampling region for spot sample data collection.

The value of chemical imaging is that it provides insight into the relative distribution of chemical constituents in various regions of the grain kernel. Such spatial information is lost in chemical analysis of the whole kernel, and the ability to isolate specific regions of the kernel (endosperm, cell wall, etc.) for individual analysis is impossible. Use of IR images offers the ability to relate the concentration of chemical constituents to anatomical structure. IR mapping may provide the knowledge to predict digestibility of CHO and other tissue components based on where they are located in the kernel relative to other components (i.e. starch and protein, where there is more protein, starch will presumably be less digestible)

3.3.3.2 Spot sample analysis

Due to poor results from the reflectance mode synchrotron analysis conducted at ANKA, a second synchrotron-source FTIRM experiment was performed to compare the results with internal IR-source data previously collected at the CLS. Because the synchrotron-source analysis of samples in reflectance mode (carried out at ANKA) resulted in poor data with unsatisfactory spectral quality, further data using synchrotron-source IR light was collected in transmission mode. This required modifications to the sample preparation methodology, and use of BaF_2 spectral windows rather than IR reflectance slides.

Samples for this experimental procedure were again cut to 6 μm thickness, but instead of sectioning frozen, untreated seeds, the samples were embedded in paraffin before sectioning. The purpose of using a hydrocarbon matrix is to maintain the physical integrity of the sample during sectioning (Jackson and Mantsch, 2000). Methodology for this procedure is included in Appendix B. The method is a modified

version of the general paraffin embedding process used for animal tissue at Prairie Diagnostic Services, Saskatoon, SK (Phil Dillman, personal communication). In the final step of the procedure, grain kernels are soaked in Paraplast for an extended period of time (38.5 hours) to allow the paraffin to penetrate the outer seed coat. Paraffinization helped to reduce the amount of shattering and tearing as the sections were cut, producing a more even and homogenous section and maintaining the integrity of the thin tissue. This was likely one of the problems with the dry-mounted tissue used in the reflectance mode experiments at ANKA, and was a contributing factor in the poor spectral quality. Paraffinzation of the grain kernels for the NSLS experiment appeared to enhance adhesion of the tissue sections to the BaF_2 windows, helping them to remain flat on the slide, and preventing parts of the tissue from lifting off of the slide. This was important as an uneven sample surface can scatter the incident IR light, further reducing collection efficiency and causing artifacts in the IR spectrum (Miller, 2004).

Concerns with using embedding agents, such as paraffin, have been raised in that it can actively absorb IR light and produce noticeable absorption bands in the sample spectrum. However, the most intense absorbance features of paraffin are limited to the C-H stretch region (2800-3000 cm^{-1}) (Miller and Dumas, 2006), and do not overlap the IR absorption features of the sample that are of interest in this experiment, namely carbohydrate and protein absorption (1700-900 cm^{-1}). Although there is some concern that the solvents used in the paraffinization process may extract lipid components from the sample (Mike Jackson, personal communication), such extraction would cause minimal change to the compositional profile of the barley tissue as lipid comprises only 2-4% of the whole grain, and only 1-3% of the endosperm tissue (Morrison, 1993). Furthermore, the lipid content of the grain kernels was not of primary interest in this experiment. Rather, the main focus was on starch and protein within the endosperm tissue. Future experimental procedures where the objective is to examine lipids in barley grain may require different methods of sample preparation.

Due to experimental time limitations at the NSLS synchrotron facility, only three grain types were analyzed in this experiment. Corn and Harrington were chosen as they were used for direct comparison in the previous *in situ* digestibility and internal IR-source experiments. Valier barley was chosen as the third variety based on its

performance in the *in situ* digestibility trial in which it exhibited significantly different digestibility characteristics compared to the other three barley varieties.

Infrared spectra of corn, Harrington and Valier, obtained with a synchrotron light source and recorded in the 1750-900 cm^{-1} region of the IR spectrum, are shown in Figure 3.19. These three spectra are from a single pixel located in the endosperm region of their respective tissue sections. Although the general shape of all three spectra is similar, indicating the presence of similar functional groups, there are differences in the strength of absorption, as reflected in the variation in peak area and height. Corn shows stronger absorption peaks for Amide I (1656 cm^{-1}) and the three CHO peaks (1150, 1077, and 1025 cm^{-1}), while Harrington and Valier are more similar in both line shape and degree of absorption, indicating that the endosperm tissue is more similar in nature between the barley varieties rather than the barley compared to corn. The corn spectrum had sharper, more defined Amide I absorption than the two barley varieties. The peak at 1536 cm^{-1} is likely not a reflection of the Amide II absorption, which is typically centered at approximately 1550 cm^{-1}, but rather a complex peak resulting in the overlap of several vibrational modes which obscure the relatively small Amide II absorption vibrations. The large absorptions in the CHO region may also contribute to the overlapping effect which overwhelms both the Amide I and II absorptions.

A smaller but consistent absorption peak at 1333 cm^{-1} is also observed in spectra of all three grains. This absorption is rarely mentioned in previous reports of IR analysis of cereal grains, however, Cael et al. (1973) noted the presence of an absorption peak at 1334 cm^{-1} in the Raman spectrum of amylose and α-D-glucose which they assigned to a combination of the bending vibrations of C-O-H groups as well as a CH_2-related deformation mode.

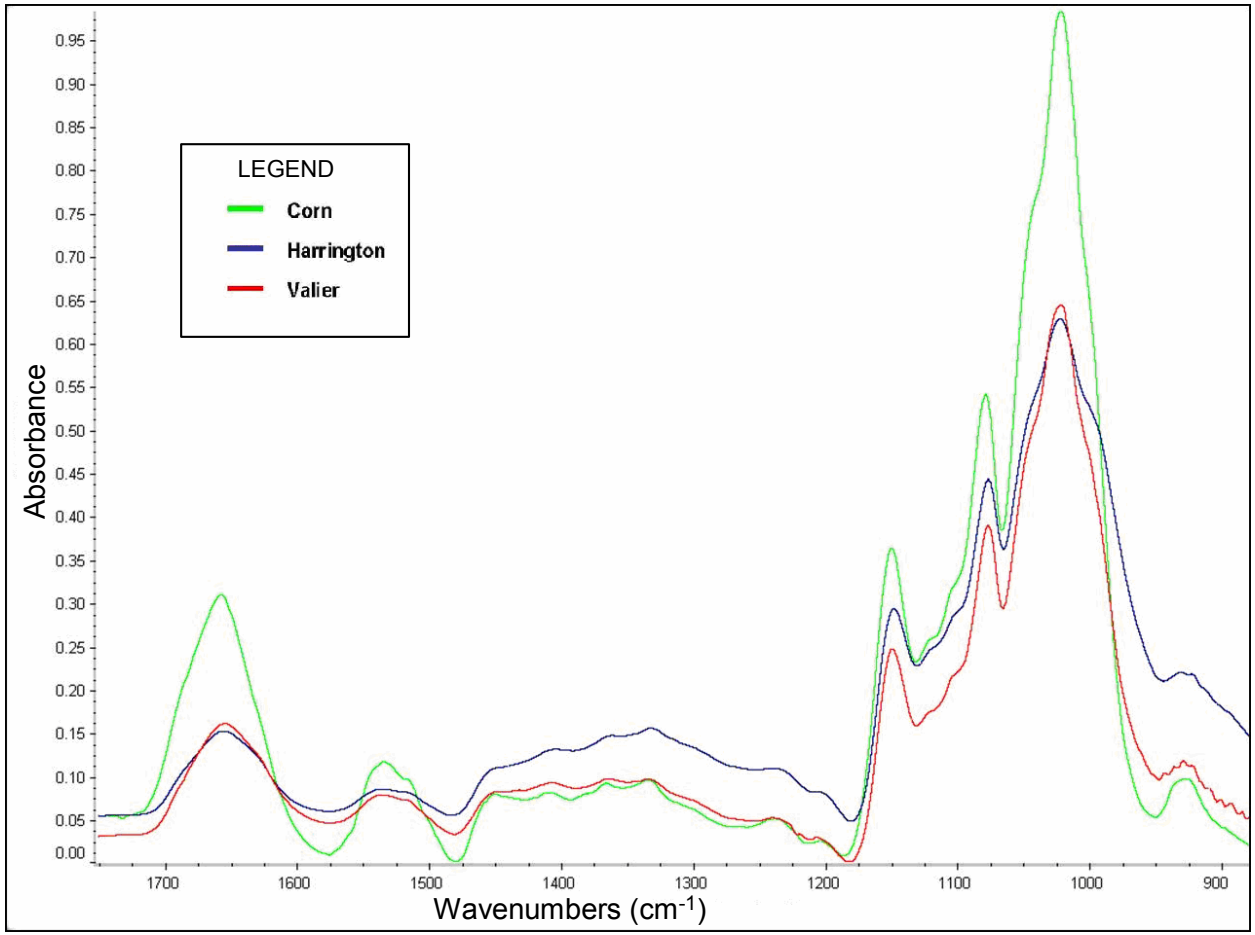

Figure 3.19. Enlarged 1750-900 cm^{-1} region of FTIRM spectra of one random spot sample from Corn, Harrington and Valier endosperm tissue obtained with synchrotron-source IR light at NSLS. Harrington absorption peaks are evident at 1656, 1536, 1333, 1149, 1077 and 1025 cm^{-1}.

Whereas barley spectra collected with internal-source IR light exhibited a broad CHO absorption band centered at 1017 cm^{-1} (Figure 3.12), the same CHO peak is shifted slightly to the right and centered at approximately 1025 cm^{-1} in the synchrotron spectra (Figure 3.19). Similarly, prior synchrotron studies conducted on barley tissue, including our own experimental data collected at ANKA, detected a carbohydrate absorption peak attributed to starch centered at 1025 cm^{-1} (Wetzel et al., 1998; Wetzel et al., 2003; Yu et al., 2004b), but did not report a peak existing at 1017 cm^{-1}. There appears to be consistency in the location of the third carbohydrate (starch) absorption peak at 1025 cm^{-1} when comparing data collected using the same type of IR light source. The type of IR light source used for analysis may have an effect on where the third CHO peak is centered. However, this cannot be determined strictly from one experiment, and there is no available literature which includes barley analysis using internal-source IR light with which to compare our experimental results. Further internal-source IR analysis of several barley tissue samples would be required to confirm this observation.

Pixel size determined by the aperture setting may be responsible for the differences seen in the CHO absorption profile between the internal thermal-source IR and external synchrotron-source data. As previously discussed, the 50 μm aperture used for data collection with the internal-source IR light encompasses a larger area of tissue, allowing for greater variation of functional groups that are present and able to absorb the incident light. On the other hand, the smaller 12 μm aperture setting of the synchrotron experiment serves to limit the area of tissue being analyzed, and may serve to make the selected pixel more homogeneous.

Peak area measurements and ratios from spot sample spectra collected using synchrotron source IR light at the NSLS for corn and Harrington barley are shown in Table 3.11. Similar to the CLS internal IR-source data (Table 3.9), CHO absorbance for corn was more than 65% greater than for Harrington ($P<0.05$). However, as previously discussed, this difference may be partially due to differences in sample tissue thickness and can not be taken as an absolute difference in concentration. There does, however, seem to be a certain degree of continuity in the CHO and Amide I peak absorption measurements obtained with the thermal and synchrotron-source analysis.

Table 3.11. NSLS synchrotron data: Comparison of least square means of peak area for Amide I and CHO, and ratio of CHO to Amide I for corn and Harrington barley.

	LS Means (Absorbance units) [z]		
Grain Type	Amide I [y]	CHO[x]	CHO:Amide I
Harrington	6.34 (± 3.91) [b]	30.81 (± 13.09) [b]	6.68 (± 5.51) [b]
Corn	8.56 (± 4.46) [a]	50.98 (± 13.42) [a]	8.09 (± 4.98) [a]
SEM	0.293	1.005	0.384
P-value [w]	<0.0001	<0.0001	<0.0001

Values in the same column with different letters are significantly different (P <0.05)

[z] Mean of spot sample spectra (n=30) from five different tissue sections for each grain type. Total n for each grain = 150.

[y] Amide I peak area measured between 1710 to 1581 cm^{-1}.

[x] CHO peak area measured between 1066 and 950 cm^{-1}

[w] P-value represents effect of grain type

Peak area absorbance for Amide I was greater for corn than for Harrington (8.56 vs. 6.34, respectively), in contrast to the CLS internal IR-source data in which Harrington had similar Amide I absorbance to corn. In addition, the magnitude of Amide I absorption is noticeably different between spectra collected with thermal and synchrotron source IR light. Internal IR-source spectra have Amide I absorptions in the range of 23 to 24 absorbance units (Table 3.9) while synchrotron source spectra are in the range of 6.3 to 6.5 absorbance units (Table 3.11). This reduction in Amide I absorption intensity, paired with relatively consistent CHO absorption between the two methods, results in the synchrotron data having much higher CHO to Amide I ratios than the corresponding internal IR light source data. These differences in the relative absorbance values are likely due to the fact that the microscope aperture size used during spectral data collection changed from 50 μm for thermal IR-source technique to 14 μm for synchrotron work. Individual pixel size was decreased in the synchrotron data, making it easier to focus on a single tissue component, such as an individual starch granule, while excluding unwanted areas around the region of interest. The larger 50 μm aperture used for data collection with internal-source IR light encompasses a larger area which includes more protein contained in the matrix between starch granules. Alternatively, the smaller aperture setting utilized with the synchrotron is closer to cellular dimensions and can be focused more specifically on regions of tissue, such as single starch granules, excluding other material surrounding it. As a result, the synchrotron spectra show a large proportion of CHO which nearly overwhelms the protein (amide) absorption all together. Figure 3.20 shows the comparison of two spectra taken from Harrington barley using both internal IR and synchrotron light sources. The synchrotron spectrum in red shows much higher absorptions in the CHO region, and less in the Amide I region when compared with the spectrum collected using the internal IR light source.

Corn consistently showed a higher starch to protein ratio than Harrington barley when analyzed by chemical analysis (Table 2.3), internal-source IR (Table 3.9) and synchrotron IR (Table 3.11). Despite this, it exhibited the slowest rate of rumen degradation when evaluated using the *in situ* nylon bag technique. This disparity could be the result of differences in the structure of corn protein, which influences its ability

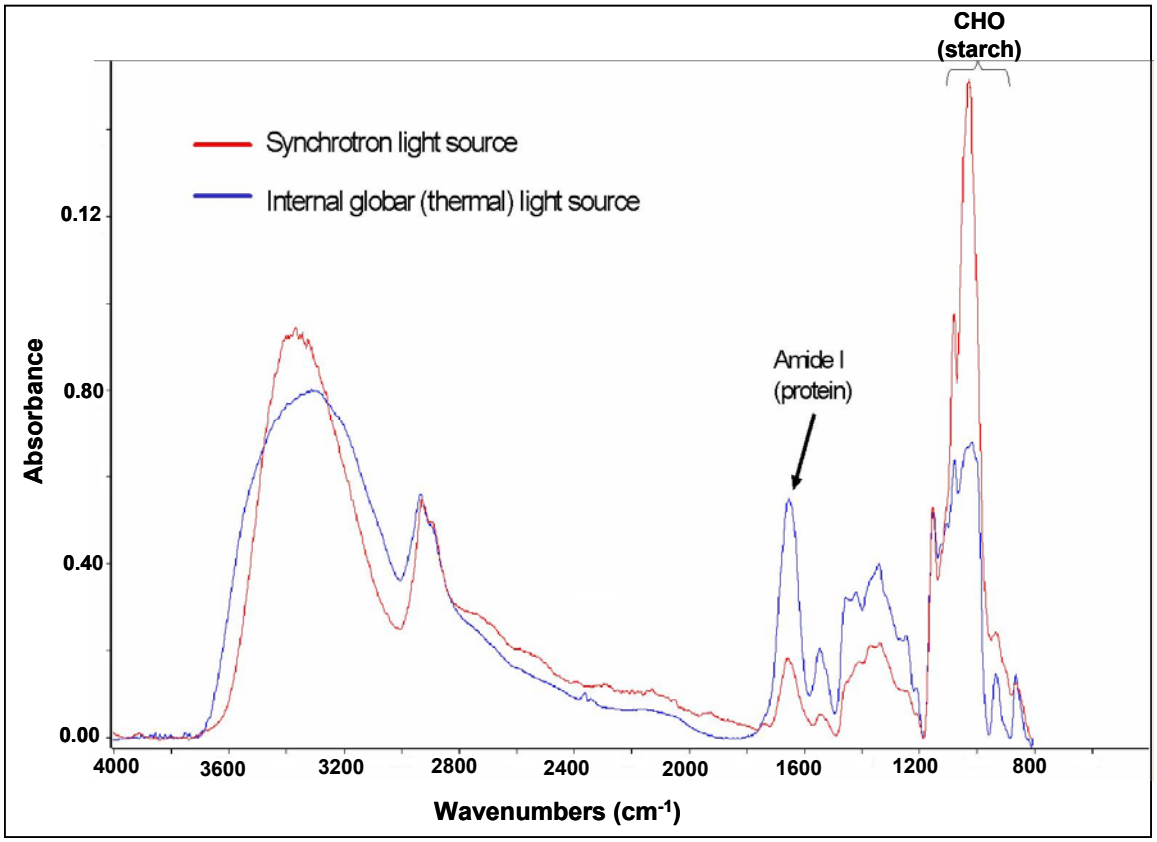

Figure 3.20. Comparison of spot sample spectrum of Harrington barley collected using two IR light sources: internal thermal source (blue) and synchrotron source (red).

to effectively shield starch granules from the digestive process. Rather than the quantity of protein relative to starch, it may be that protein structure is more important to its ability to protect starch granules from digestion. Differences in the composition of the total protein content of corn and barley exist. For example, barley contains a larger proportion of albumins and globulins, and less glutelins than corn. The proportion of prolamins present in the two grains is relatively similar, but the type of prolamin differs between corn and barley. Zein proteins present in corn may exhibit different degradation characteristics than the hordeins found in barley due to variation in amino acid content and structure.

A direct comparison of peak area measurements obtained from synchrotron-source FTIRM for Harrington and Valier barley is presented in Table 3.12. The two barley varieties were analyzed separately from the corn in order to make the comparison consistent with previous analysis of *in situ* digestibility data and internal-source IR data in which the barley varieties were grouped separately. Mean peak area measurements for Amide I and CHO did not differ between Harrington and Valier ($P>0.05$), nor was there any statistical difference in the ratio of carbohydrate to Amide I (6.68 vs.6.26, respectively). Numerically, Harrington had a higher carbohydrate to Amide I ratio than Valier, consistent with the observations of synchrotron-source FTIRM analysis by Yu et al. (2004b) as well as internal-source data from this project (Table 3.10). Based on results from the *in situ* trials, Harrington and Valier tended to be the most similar in their digestibility characteristics (Table 2.6), so it is not completely unexpected that no significant differences are seen in the IR peak area absorbencies of Amide I and CHO. Increasing the scope of the analysis by including additional barley varieties, particularly varieties such as CDC Bold and CDC Dolly which showed faster and greater *in situ* rumen degradation characteristics, would be of interest and could lead to observable differences in CHO to Amide I ratio as determined by synchrotron-source FTIRM.

A consistent relationship between Harrington and Valier barley was observed in the chemical analysis (Table 2.3), internal-source FTIRM (Table 3.10) and synchrotron-

Table 3.12. NSLS synchrotron data: Comparison of least square means of peak area for Amide I and CHO, and ratio of CHO to Amide I for Harrington and Valier barley.

	LS Means (Absorbance units) [z]		
Variety	Amide I [y]	CHO [x]	CHO:Amide I
Harrington	6.34 (± 3.91)	30.81 (± 13.09)	6.68 (± 5.51)
Valier	6.62 (± 4.25)	31.96 (± 13.54)	6.26 (± 3.93)
SEM	0.313	1.039	0.361
P-value [w]	0.5399	0.4339	0.4069

Values in the same column with different letters are significantly different ($P<0.05$)

[z] Mean of spot sample spectra (n=30) from five different tissue sections for each grain variety. Total n for each grain = 150.

[y] Amide I peak area measured between 1710 to 1581 cm^{-1}.

[x] CHO peak area measured between 1066 and 950 cm^{-1}

[w] P-value represents effect of variety

source FTIRM (Table 3.12). This nature of this relationship was such that Harrington showed similar or higher amounts of protein (chemical analysis) or Amide I (FTIRM), and higher CHO content than Valier, resulting in a higher starch to protein ratio. Differences in the internal-source FTIRM experiment were significant, whereas the synchrotron data showed similar trends, but was not statistically different between the two varieties. Despite this, the evaluation of the synchrotron and internal IR-source data of Harrington and Valier barley shows them to be consistent and repeatable in the evaluation of chemical components present in barley endosperm tissue. With further experimentation to validate the observations of this study, the quality of information gained from the internal light source data may be useful for preliminary analysis of the chemical composition of plant material. With specific reference to cereal grains, FTIRM may prove useful for measuring the relative content of starch and protein within a grain sample. This type of chemical information may help to estimate the rate and extent of rumen degradation in different varieties of barley, and provide insight into the nutritional value of the grain with respect to the digestibility characteristics.

3.4 Conclusions

Based on the observations of the FTIRM analysis using both thermal and synchrotron-source IR light, a lower starch to protein ratio measured using FTIRM techniques seems to correspond with a slower rate of rumen degradation measured by the *in situ* method. This hypothesis, however, appears to hold true only when making comparisons between barley varieties. When corn is included in the comparison, neither data obtained from chemical analysis nor FTIRM (both thermal and synchrotron-source light) support the hypothesis that a lower starch:protein ratio corresponds to slower physical *in situ* degradation characteristics. When estimated using both the chemical analysis and FTIRM analysis, corn had a higher starch:protein ratio (indicating less protein per unit starch), both in the whole kernel and endosperm tissue, than all the barley varieties, yet still exhibited the slowest rate of rumen degradation in the *in situ* trials. This may indicate that it is not necessarily the amount of protein present in the endosperm, but rather the form of the protein which provides the protective qualities alluded to by McAllister et al. (1993). It becomes an issue of protein structure and function as opposed to protein quantity.

Statistical differences were observed between four barley varieties in the peak area absorption for both CHO and Amide I when using internal IR-source FTIRM. Data from this analysis ranked CDC Bold, CDC Dolly, Harrington and Valier from highest to lowest in CHO:Amide I ratio, similar to the ranking of starch:protein ratio estimated by chemical analysis for the four barley varieties, as well as corresponding with the rate and extent of ruminal DM degradation estimated by the *in situ* technique.

No differences in the peak area absorption of CHO or Amide I were apparent between Harrington and Valier barley using synchrotron FTIRM, nor were there any significant differences observed in the CHO:Amide I ratio. Analysis of CDC Bold or CDC Dolly in this study along with Harrington and Valier would have provided a useful comparison and continuity with the previous internal IR-source FTIR experiment. Based on the observed differences in rate and extent of DM, CP and starch digestibility from the *in situ* trials, coupled with the variation in IR absorption peak areas measured using internal-source IR light, a comparison of IR synchrotron data for CDC Bold and

CDC Dolly, as well as additional barley varieties, would certainly be of interest in validating the observations of this experiment and to gain a better understanding of the value of synchrotron IR analysis of cereal grains.

4.0 General Conclusions

4.1 The Value of FTIRM for Analysis of Cereal Grain Tissue with Respect to Nutritional Value for Ruminant Feeding

The basic objective of this project was to assess the potential for using Fourier Transform Infrared Microspectroscopy (FTIRM), both with thermal and synchrotron light sources, as an analytical tool to predict *in situ* digestibility characteristics in cereal grains. To achieve this, the experimental procedure was designed in a two-step approach: first, to evaluate the physical digestibility characteristics of cereal grains, and second, to assess the same samples using IR technology.

An *in situ* digestibility trial with the purpose of evaluating the effects of grain type (corn vs. barley), grain variety (CDC Bold, CDC Dolly, Harrington and Valier), and processing method (rolled vs. ground) on rumen degradation kinetics was initially conducted. Results from this experiment demonstrated that differences in the rate and extent of DM, CP and starch degradation existed both between different grain types, as well as among varieties of the same species. Corn consistently showed slower rate and extent of DM, CP and starch degradation than Harrington barley at both levels of processing. Although this was not unexpected based on the literature, the differential effect of grinding on the rate and extent of ruminal degradation for corn and barley was of particular interest. Fine processing tended to increase the rate of DM degradation over the rolled treatment to a much larger degree for barley than corn. This indicates that chemical composition and molecular structure, in addition to physical structure, are all involved in the determination of the rumen digestibility characteristics of cereal grains. When comparing the *in situ* rumen degradation kinetics of different varieties of barley, significant differences in the rate and extent of degradation were also observed. Valier consistently exhibited a lower rate and extent of DM, CP and starch degradation when compared to the other three barley varieties. Similar results were observed at both levels of processing, which again indicates that chemical composition

and molecular structure are important components involved in the mechanism of cereal degradation, and that the intrinsic molecular structure of each variety is unique. The importance of the chemical composition of cereal grain kernels is of interest not only with respect to the whole kernel, but also for specific regions and structures within the kernel. Chemical analysis procedures typically used for feed grain analysis can estimate the content of starch and protein in the whole kernel, but do not provide any information regarding the location or concentration of these constituents within the kernel structure. From the observations of the *in situ* experiments, it is clear that the chemical composition of the grain kernel is relevant to learning about specific digestibility traits. For this reason, the relationship of the starch to protein ratio of different varieties of barley to their physical digestibility was of interest, both in the whole kernel and in specific regions.

Study of cereal grain tissue using FTIRM was identified as a potential technique for evaluating the chemical constituents in a sample with respect to spatial distribution and concentration. The goal was to measure starch and protein in isolated endosperm tissue using FTIRM, and to determine whether the ratio of starch to protein measured with this technique could be related to the *in situ* digestibility of cereal grains. Comparison of the starch to protein ratio estimated by laboratory chemical analysis (whole kernel) to estimates obtained from internal-source IR microspectroscopy showed that FTIRM was able to rank the grains in the same order as chemical analysis based on the value of the starch:protein ratio.

The relationship between chemical and structural information obtained from both conventional and synchrotron IR analysis with physical digestibility data from *in situ* incubations was also investigated. Corn consistently showed the highest starch:protein ratio when analyzed by total starch assay (Table 2.3), internal-source IR (Table 3.9) and synchrotron IR (Table 3.11). Despite this, it still exhibited the slowest rate of rumen degradation when evaluated using the *in situ* nylon bag technique, contrary to our hypothesis that a relatively higher content of protein would decrease the rate and extent of ruminal starch digestion. This disparity is likely due to differences in the structure of corn protein, which influences its ability to effectively shield starch granules from the digestive process. Amino acid content and specific protein structure unique to different

cereal grain types may also influence the digestibility characteristics of the protein present, and hence the starch. As previously mentioned, there are significant differences in the composition of the structural proteins, the prolamins, which exist between corn and barley. When comparing only barley grains, it was observed that the rate and extent of degradation increased with increasing starch:protein ratio. The four barley varieties were ranked in the same order of decreasing starch:protein ratio, with CDC Bold showing the highest ratio, followed by CDC Dolly, Harrington and Valier, by both the chemical analysis and the internal-source IR measurement. Synchrotron IR measurements were only available for two of the original four barley varieties, Harrington and Valier, but again showed that Harrington had a higher starch:protein ratio than Valier, although the results were not statistically significant. These values were in agreement with the *in situ* digestibility observations where CDC Bold tended to be the fastest and most extensively degraded variety, followed by CDC Dolly, Harrington and Valier.

From this it appears that evaluation of the starch to protein ratio as an indicator of rumen degradation characteristics may be applicable when comparing different varieties of the same grain (i.e. barley), but not when evaluating the relationship between different species of cereal grains. If it is indeed structure rather than quantity that is the main factor, then the starch:protein ratio is not an adequate predictor of the digestibility characteristics of cereal grains when making comparisons between grain species that possess different types of protein. However, based on the comparison of four barley varieties, the relationship between starch:protein ratio and digestibility characteristics may be valid among grains within the same species if we assume that they possess the same basic protein structure.

IR spectroscopy is a technique which has potential for a range of analytical applications in the evaluation of biological materials, including cereal grains used in cattle feeds. Further research which includes analysis of a larger number of grain varieties and species would be of great benefit to discover the value of this technique for practical application for feed analysis.

If through further studies, the relationship between starch:protein ratio estimated by FTIRM analysis and the *in situ* digestibility characteristics of cereal grains can be

verified, this technique may be suitable as a screening method for determining which barley varieties are acceptable candidates for selective breeding programs. Use of such a technique would have the ability to reduce the time required for growing and multiplying grains in order to have enough sample to carry out an *in situ* digestibility trial – a process which would otherwise require several years to multiply enough seed for the experiment.

4.2 Objectives for Future Research

The objective of the current project was to assess the nature of the starch to protein ratio of corn and different barley varieties and its relationship to physical *in situ* digestibility characteristics. Much additional information is available from the IR spectra collected, but analysis of all the possibilities was beyond the scope of this project. Indeed, there are several aspects that would require further study. Some possibilities for further analysis of the synchrotron spot sample and mapping data that has been collected in this experiment include:

1. Specific analysis of the protein content, including measurement and comparison of Amide I and Amide II absorptions.
2. A detailed examination of protein composition. This would involve possible peak fitting and analysis of second derivative spectra to determine the compositional fractions represented by α-helix and β-sheet in the Amide I absorption band. With respect to differences in the nature of protein structure between grain varieties, this type of analysis may be useful for the discovery of specific protein structure that affects physical digestibility.
3. Principal component analysis (PCA) of the carbohydrate absorption region (1200-900 cm^{-1}) to identify regions of different carbohydrate composition.
4. Comparison of endosperm spectra with those of purified starch samples (waxy, high amylose, etc.) in order to elucidate the position of various absorption bands associated with the different chemical forms of starch.

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Appendix A

Nylon bags required for a single run of Trial 1 (corn vs Harrington).

Incubation time (h)	Treatments			
	Corn		Barley	
	Ground	Rolled	Ground	Rolled
0*	2	2	2	2
2	2	2	2	2
4	2	2	2	2
8	2	2	2	2
12	3	3	3	3
24	4	4	4	4
	13	13	13	13
Total bags per run				52

Total number of bags incubated in a single run of Trial 2, and the number of bags from each time period that were incubated at one time. 0-hour bags were not incubated in the rumen and are not included in the summation.

Incubation (h)	# bags	% of total	# bags incubated at once	treatments	bags/ incubation Run	Runs	Total # bags
0	2*	–	–	–	–	–	–
2	2	0.11	3	8	16	4	64
4	2	0.11	3	8	16	4	64
8	2	0.11	4	8	16	4	64
12	3	0.17	4	8	24	4	96
24	4	0.22	5	8	32	4	128
36	5	0.28	7	8	40	4	160
Total	18		26		144		576

* not incubated in the rumen

Appendix B

Paraffin Processing Procedure for Seed Samples (corn and barley)

1. Gross Trimming

End caps of the corn and barley kernels were cut off and the seeds were placed into standard processing cassettes.
A 2-3 mm piece through the center of the seed was cut out and these were placed into standard processing cassettes.

2. Paraffin Processing (Shandon Pathcentre Tissue Processor)

1. 80% ethanol – 1 hour
2. 95% ethanol – 1 hour
3. Absolute ethanol – 1 hour
4. Absolute ethanol – 1 hour
5. Absolute ethanol – 1 hour
6. Absolute ethanol – 1 hour
7. Absolute ethanol – 1.5 hour
8. Xylene – 1 hour
9. Xylene – 1 hour
10. Xylene – 1 hour
11. Paraplast – 1 hour
12. Paraplast – 1 hour
13. Paraplast – 1 hour
14. Paraplast – 38.5 hours (extended time required for paraffin to penetrate seed coat)

3. Paraffin Embedding

Paraffin blocks were made using the standard embedding technique in the Shandon Histocenter Embedder (Thermo Shandon, Pittsburg, PA). Cut surfaces of the seeds were embedded down in the paraffin blocks.

4. Paraffin Sectioning

Paraffin blocks were rough trimmed to expose the surface of the seed, then placed on ice for approximately 30 minutes prior to sectioning. Tissue sections were cut at 6 µm thickness using a Microm 330 microtome (Microm Laborgerate GmbH, Sandhausen, Germany). Cut sections were floated in a 45ºC waterbath and then mounted on BaF_2 transmission windows.

5. Section Drying

Sections mounted on to the slides or windows were then dried in a 60 C oven overnight.

6. Deparaffinization of Sections

Paraffin wax was removed from the slides using the standard technique commonly employed in histology laboratories. This consists of:

1. Xylene – 5minutes
2. Xylene – 5minutes
3. Absolute ethanol – 5 minutes
4. Absolute ethanol - 5 minutes
5. 95% ethanol – 5 minutes
6. 70% ethanol – 5 minutes
7. distilled water – (5 changes of 2 minute each)