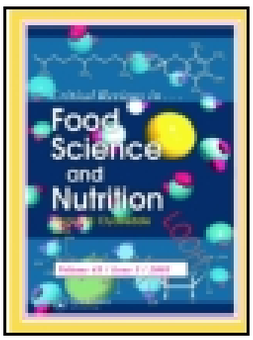

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Recent Research in Flaxseed (Oil Seed) on Molecular Structure and Metabolic Characteristics of Protein, Heat Processing-Induced Effect and Nutrition with Advanced Synchrotron-Based Molecular Techniques

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Running head: Molecular Structure and Metabolic Features of Flaxseed (Oil Seed)

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CONTENTS

ABSTRACT:

Advanced synchrotron radiation based infrared microspectroscopy is able to reveal feed and food structure feature at cellular and molecular levels and simultaneously provides composition, structure, environment and chemistry within intact tissue. However, to date, this advanced synchrotron-based technique is still seldom known to food and feed scientists. This article aims to provide detailed background for flaxseed (oil seed) protein research and then review recent progress and development in flaxseed research in ruminant nutrition in the areas of (1) dietary inclusion of flaxseed in rations; (2) heat processing effect; (3) assessing dietary protein; (4) synchrotron-based Fourier transform infrared microspectroscopy as a tool of nutritive evaluation within cellular and subcellular dimensions; (5) recent synchrotron applications in flaxseed research on a molecular basis. The information described in this paper gives better insight in flaxseed research progress and update.

Keywords: Synchrotron Radiation, Protein Molecular Structure, Oil seed, Nutrient Utilization and Availability, Processing

I. DIETARY INCLUSION OF FLAXSEED IN RATIONS

1.1 Oilseeds in Ruminant Rations

Oilseeds and oilseed meals are commonly included in ruminant rations (Stake et al. 1973; Ward et al. 2002). Oilseed meals are striped of most of their fat content and are a good source of protein and fibre while whole oilseeds are typically a good source of energy as well as unsaturated fats. Recent interest in providing ruminants with a greater supply of dietary unsaturated fatty acids comes in part from a human health standpoint. There is an effort to ameliorate the fat profile of milk and meat, by decreasing the ratio of saturated fatty acids to unsaturated fatty acids, to foster better cardiovascular health. This is based on the fact that some of the unsaturated fats, as a consequence of ending up in the bloodstream after feeding, are

deposited in fat deposits and milk of an animal. Unsaturated fats will eventually become saturated if in the rumen long enough through a process called biohydrogenation. Unfortunately, not all of the unsaturated fat will be absorbed as such, extensive biohydrogenation occurs in the reducing environment found in the rumen which partially negates the effort of trying to get unsaturated fats into the diet to begin with. When fed whole, the oil from an oilseed is expected to be protected against biohydrogenation, due to its physical structure, but results are contradictory (Keele et al. 1989; Scollan et al. 2001a,b).

There are some considerations for dietary fat in ruminants, in particular the effects it has on the protozoa and bacteria in the rumen. One such effect is the suppression of protozoal activity, which decreases the number of protozoa in the rumen (Czerkawski et al. 1975). Protozoal suppression can lead to an improvement in bacterial protein synthesis (BPS) because there are fewer protozoa engaged in breaking down the bacteria or other dietary constituents supporting a futile nitrogen (N) cycle of nutrient breakdown and resynthesis (Jouany 1996). Fats and oils also have effects on the bacteria in the rumen, which results in impairment of organic matter (OM) digestion, energy digestion, and bacterial protein synthesis (BPS), and is dependent on the concentration of fats present (Ikwuegbu and Sutton 1982). The interactions between fats and oils on the major rumen inhabitants and interactions among the inhabitants themselves make the effects of fat/oil dietary inclusion diverse and not always predictable. The reduction in protozoal activity, for example, would increase the efficiency of BPS yet at the same time the fats are believed to inhibit BPS in bacteria. The inhibition of BPS is due to the toxicity that the free fatty acids, released when triglycerides are degraded, exhibit towards some bacterial species within the rumen. The result is a shift in the bacterial population in the rumen (Broudiscou et al. 1988). Depending on the extent of either of these occurrences, different results would be expected. These changes to rumen function restrict the use of fats and oils as energy supplements in ruminant rations despite some of the benefits they might provide.

1.2 Flaxseed Shape and Morphology

Commercial flaxseed varieties are varied in size and colour. On average they are 5 mm long, 2.5 mm wide and 1.5 mm thick (Peterson 1958). Figure 1 shows a micrograph cross section of flaxseed. The seed cross section, taken from the center of the seed, shows the epiderm, known

as mucilage, seed coat, and a thin layer of endosperm surrounding two cotyledons. The endosperm and the cotyledons have different protein solubilities (Painter and Nesbitt 1969) and oil compositions (Dorrell 1970). Not visible in Figure 1 is any discrete aleurone tissue. In the case of flaxseed, there are aleurone cells distributed throughout the endosperm and cotyledons. The shape of the aleurone grains within the aleurone cells differs between endosperm and cotyledon (Winton and Winton 1932). Oil is distributed throughout the endosperm and cotyledons. The oil is contained within the cells as microscopic droplets (Peterson 1958).

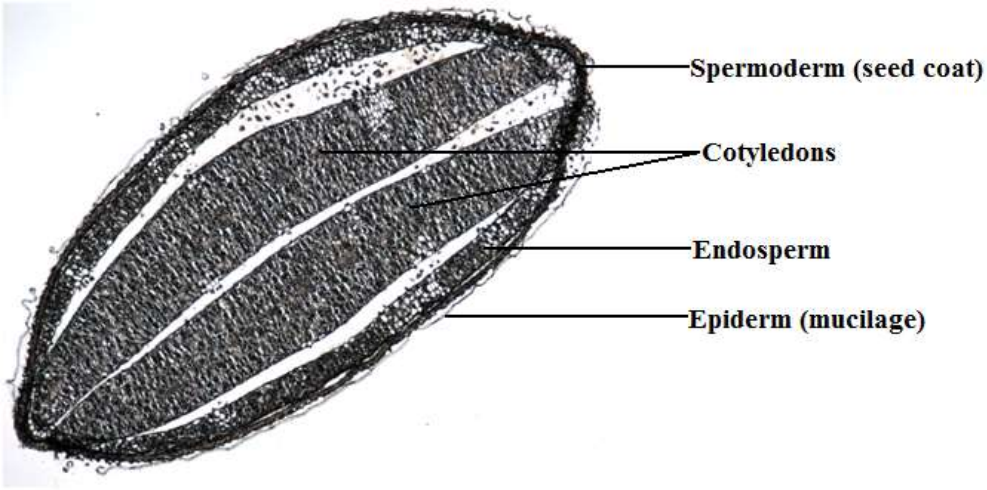

Figure 1. Cross section of a flaxseed.

1.3 Flaxseed Oil and Protein Characteristics

Canadian flaxseed contains, on average, 41% oil and 26% protein. Cultivar and growing conditions, however, play a significant role in the composition of flaxseed. Oil is a major component of flaxseed and it consists of several fatty acids, including palmitic acid (16:0) (5%), stearic acid (18:0) (3%), oleic acid (18:1n-9) (17%), linoleic acid (18:2n-6) (15%) and ALA (18:3n-3) (59%) (Bhatty 1995). ALA exhibits sensitivity to temperature post flowering, whereby the ALA content is favored by cooler post flowering temperatures at the expense of oleic acid content. It is of interest to note that the concentration of individual fatty acids in flaxseed oil is highly variable between seeds when environmental factors are equal (Bhatty 1995).

Flaxseed averages 26% protein on a moisture-free basis, while flaxseed meal consists of 44% CP on a moisture-free basis (Bhatty 1995). Similar to other oilseeds, the major storage proteins of flaxseed are albumins and globulins, which are classified based on their solubility in aqueous solvents. Globulins are the major storage protein and comprise roughly 58-66% of the total protein in flaxseed. Albumin is a much smaller protein in size and comprises around 20% of the protein content of flaxseed (Oomah and Kenaschuck, 1995). The secondary structure profile of the globulin storage protein consists of 3% α-helix, 17% β-sheet, and 80% aperiodic (Madhasudhan and Singh, 1985a). The protein secondary structure profile of albumin is very different from that of globulin, and is comprised of 26% α-helix, 32% β-sheet, and 42% is aperiodic (Madhasudhan and Singh, 1985b). Considering the secondary structure profiles of the two major protein types, it is evident that that the predominant secondary structure, other than aperiodic, in flaxseed protein is the β-sheet. Oleosins are another important protein group found in flaxseed. Oleosins can make up 2-8% of total protein in oilseeds. The major function of oleosins is oil storage (Huang 1992). A large part of the function of these oil storage proteins is enabled by an elongated, anti-parallel β-sheet containing many hydrophobic residues. β-sheets make up about 30% of this protein in peanuts (Huang 1992). Oleosins are required for the formation of the oil bodies and, therefore, the storage of oil. These proteins, as a result of their amphipathic nature, are responsible for the oil/protein matrix of flaxseed which plays a role in protein and fat digestibility (Huang 1992).

1.4 Benefits of Flaxseed Feeding

Flaxseed shows promise as a feed ingredient for several reasons. One is the potential for improvements in herd health when flaxseed is added as a supplement to the diet. These improvements include a reduction in pregnancy loses as well as an increased conception rate that occurs when cows are supplemented with flaxseed (Ambrose et al. 2006). Other examples of improvement when supplementing cattle finishing diets, for example, are increases in performance and efficiency, as well as an increased marbling in meat (Maddock et al. 2006). These are possibilities that could both lead to increased profitability for the producer. However, there is still much work to be done with respect to the effects of processing and how the other ingredients in the ration may interact with the flaxseed.

1.5 Dietary Flaxseed and Its Effects

The fatty acids found in the diet are generally different than those absorbed by the small intestine. This is a result of the extensive biohydrogenation that occurs in the rumen and, generally speaking, the fatty acids on their way out of the rumen will be more saturated than those from the diet. The process of biohydrogenation can be avoided to some extent, however, by various chemical treatments, such as with formaldehyde or by heating or even by feeding whole oilseeds (Kennelly 1995). The ability to incorporate some flax-based fatty acids into milk and meat creates another opportunity for producers to create a value-added product with benefits to the consumer's health. In human nutrition, it is widely believed that modern western diets are deficient in n-3 fatty acids and too rich in n-6 fatty acids, and also contain much greater amounts of saturated fats than historic diets. The ratio of n-6 to n-3 fatty acids in the modern diet is typically around 20-30:1, whereas it is estimated that the ratio on which man lived throughout history is much lower, around 1-2:1 (Simopoulos 1999). There are various reasons for the shift in this ratio, but the consequences are clear with respect to human health, increased mortality from cardiovascular disease (Simopoulos 1999). The increased mortality is a result of the physiological effects that the n-6 and n-3 fatty acids produce. These physiological effects appear antagonistic to one another as a diet rich in n-6 fatty acids and low in n-3 fatty acids skews the physiological state to one that favors clotting, vasospasm and vasoconstriction, whereas the n-3 fatty acids are shown to have hypolipidemic, antithrombotic and anti-inflammatory effects (Simopoulos 1999). The ALA content of flaxseed makes it an excellent source of n-3 fatty acids for the purpose of using dietary fats to modify milk and meat fat profiles. Scollan et al. (2001a) demonstrated that by feeding whole bruised flaxseed, they were able to modestly improve the n-6:n-3 ratio in both meat and adipose lipid profiles, while maintaining total muscle fatty acid content. Petit (2002) also demonstrated a similar shift in this ratio for milk fat from cows fed whole flaxseed. The implications for flaxseed as a feed ingredient are evident as it can rectify, to some extent, the fatty acid profile of the consumer's diet towards what the historical values would be. Combining this with growing consumer knowledge of functional foods and their ties to human health, flaxseed dietary supplementation should only increase in popularity as time passes.

II. ASSESSING DIETARY PROTEIN

In ruminant nutrition, it is sometimes beneficial to increase the amount of dietary protein that bypasses the rumen. Thermal processing provides the means to shift the site of protein digestion to favor the small intestine, which permits the ruminant to absorb an amino acid profile that more closely resembles that of the diet. The protein remains available for absorption in the small intestine because it must pass through the abomasum which has a much lower pH than the rumen and contains different proteolytic enzymes. In addition to this, the small intestine also has its own complement of proteolytic enzymes. The result of the shift in the site of protein digestion can be of benefit to the animals under certain production and dietary conditions (Yu et al. 2002). At present, several means are used to evaluate how heat treatment will affect the degradation behaviour of protein.

2.1 Bypass Crude Protein

The major benefit to thermal processing feeds is an increase in the availability of protein to the small intestine. One measure of an increase in nutrient availability is the ruminally undegraded protein (RUP) or BCP which represents that fraction of the crude protein in a feed which will not be fermented in the rumen. BCP is based on the first order kinetics model outlined by Orskov and McDonald (1979), where $R(t) = U + D \text{ Exp}^{-Kd(t-T0)}$ and is calculated as $\%BCP = U + D * K_p / (K_p + K_d)$ and BCP (%CP) = 1.11 * CP * %BCP using the assumption that the passage rate (K_p) is 6%/h (Yu et al. 2000). Several papers report that for different feeds, thermal processing increases BCP (Yu et al. 2000; Broderick and Craig 1980; Stern et al. 1985). It should be noted that in increasing the amount of bypass crude protein, a corresponding drop in microbial crude protein production would be expected and the amount of absorbable BCP must increase enough to offset this difference (Yu et al. 2002). When feeds are heated to excess, increases in BCP are the result of increases in indigestible intake protein (IIP) and are related to increases in acid detergent insoluble fibre nitrogen (ADIN) content. This protein is not available to the animal and, consequently, has been demonstrated to result in poorer performance in dairy calves (Reddy and Morill, 1993).

2.2 Metabolizable Protein

Another assessment of changes in protein availability is metabolizable protein (MP). From the NRC (2001) model, MP is true protein that is digested post-ruminally with the resulting amino acids absorbed by the small intestine and is comprised of digestible RUP (ARUP), digestible ruminally synthesized microbial CP (AMCP), and digestible endogenous CP (AECP) and is defined by the equation MP=ARUP+AMCP+AECP (Yu 2005). As shown in the equation, if thermal processing were to decrease AMCP by a greater amount than ARUP is increased, a decrease in the total MP would be expected. Despite this concern, several experiments have demonstrated that MP will increase as the degree of processing increases in various feedstuffs (Yu 2005a; 2005b). When feedstuffs are heated to excess, the consequences for MP are obvious as the ARUP fraction and AMCP fractions will decrease because less RUP would be digestible, and as a consequence, there would be less MCP produced in the rumen.

2.3 The DVE/OEB System

A more recent model, known as the DVE/OEB system, has been developed to evaluate dietary protein based on the strengths of previous protein evaluation models while incorporating additional elements, including the role of energy balance in protein supply. The DVE/OEB system consists of two main parts. Each feed has a DVE value representing the protein that is absorbable in the small intestine. DVE is calculated as the sum of absorbable bypass crude protein (ABCP) and absorbable microbial crude protein (AMP), less a correction for endogenous protein losses (ENDP). The second part of the system is the OEB feed value which illustrates the balance between the potential microbial protein synthesis from ruminally degradable intake protein (N_MP) and the potential microbial protein synthesis from anaerobic fermentation (E_MP). When OEB is positive, there is a potential for N loss from the rumen due to a lack of energy available to rumen microbes, whereas if the OEB value is negative, there is a lack of N available in the rumen and microbial production may be restricted (Tamminga et al. 1994; Yu et al. 2002). The DVE/OEB system provides a measure of the protein value of feeds, including the microbial protein provided by the individual feed (Tamminga et al. 1994). The reader is directed to Tamminga et al. (1994) for a detailed explanation of the DVE/OEB system.

The values for a feedstuff using the DVE/OEB system are going to change as a consequence of thermal processing. Looking at the formulas for both DVE and OEB, ABCP and BCP are present. These values are changed upon thermal processing. The question then becomes, what is the overall effect of thermal processing on the DVE/OEB value of a feed? Yu et al. (2002) reviewed the effects of thermal processing on the DVE/OEB system for several different feedstuffs. Generally speaking, the DVE value increases with increases in temperature as well as with increasing cooking time. While endogenous protein losses are relatively constant upon heating, AMP shows a slight decrease with a larger concomitant increase in ABCP, the net result being an increase in the DVE value for that feedstuff. The OEB value, on the other hand, generally decreases as temperature and cooking time are increased. BCP is again responsible for the majority of the change in the OEB value because as BCP increases, the value for N_MP decreases. E_MP on the other hand decreases with temperature and time, due to the reduction of FOM, again due to changes in BCP, and therefore contributes less and less to the lowering of the OEB value.

2.4 The Cornell Net Carbohydrate and Protein System

Using the Cornell net carbohydrate and protein system (CNCPS) provides us with yet another means of evaluating ruminant dietary protein and the effects that thermal processing will have on it. The CNCPS uses *in vitro* data to characterize protein. Sniffen et al. (1992) describes in detail the classification scheme that follows. In the CNCPS, dietary protein is divided into three categories or sub-fractions, each as a percentage of CP (Figure 2). Fraction PA represents non-protein nitrogen (NPN) and consists of ammonia, peptides and amino acids. Fraction PC represents protein unavailable to the animal and is determined by the amount of protein insoluble in acid detergent. Fraction PC consists of protein associated with lignin, tannins, and the protein tied up in Maillard products. Fraction PB represents true protein and is further subdivided into sub fractions to permit the estimation of ruminal degradation rates. Fraction PB1 is made up of buffer soluble CP which is rapidly degraded in the rumen. Fraction PB2 represents that protein which is insoluble in buffer but is not bound to NDF. Fraction PB2 undergoes some degree of ruminal fermentation while the remainder, depending on the rates of degradation and passage, escapes to the small intestine. The final fraction, PB3, represents that protein associated with cell walls and is insoluble in neutral detergent, yet soluble in acid detergent solution. Due to the

association with the cell wall, fraction PB3 ends up being slowly degraded in the rumen and as such, much of it escapes ruminal degradation.

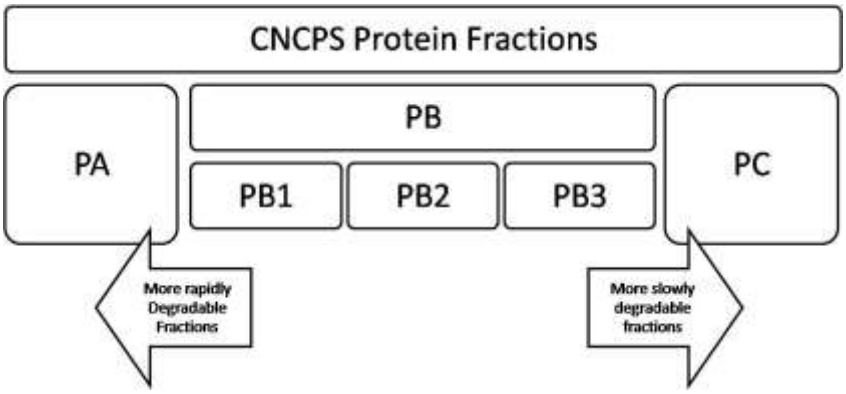

Figure 2. Schematic representation of protein sub-fractions in CNCPS system.

The effects of thermal processing on the protein sub-fractions are typified by shifts in the fractions, where the rapidly degradable fractions are decreased while the more slowly degradable fractions increase. This would be expected if more protein bypassing the rumen was anticipated. In a study conducted by Yu (2005c) which compared raw and roasted flaxseed, this is exactly what was observed, fractions PA and PB1 decreased in proportion while fractions PB2, PB3 and PC increased in proportion. In a study by Shannak et al. (2000) using soybeans, dry heating induced a similar decrease in protein fractions PA and PB1, while showing an increase in the PB2 and PB3 fractions and no change in the PC fraction. In contrast, in the same study using moist heating, the PA protein fraction increased while the PB1 decreased markedly. PB2 and PB3 meanwhile increased and PC showed a slight decline. It should be concluded from this that although a general shift of fractions to the more slowly degradable ones upon heating can be expected, the changes in the fraction profile depend on the feedstuff under investigation as well as the method of applying heat. If excessive heating were to be applied and caused protein damage, major changes in the PC fraction would be expected. The PC fraction represents ADIN, and is considered a marker for heat damage and the Maillard reaction (Van Soest and Mason, 1991).

III. HEAT PROCESSING SEEDS/FEEDS

3.1 Means and Purpose of Heating Feeds

Heat processing feeds is done in an effort to manipulate the digestive behaviour of the macronutrients contained within the feed. For protein, in terms of ruminant nutrition, the objective of heat treating a feed is to increase the amount of dietary protein that is passed out of the rumen, (bypass crude protein; BCP) without negatively affecting the digestibility of the protein in terms of the whole gastrointestinal tract (Yu et al. 2002). There are many ways to apply heat to a feed, but in general they are distinguishable by traits including the presence of moisture, as in autoclaving but not in toasting. They are also distinguishable in that some impart physical changes to the feed, such as extrusion or steam flaking. Changes in the rate and extent of rumen degradation depend on multiple factors, including the type of treatment used, the severity of the treatment in terms of time and temperature, and the pH of the feed during heating. These changes are further complicated by the type of diet and the relative proportions of its constituents (Yu et al. 2002). There are several reactions that take place in a feed when heat is applied due to the diverse nature of the organic molecules that make up plant material comprising feeds. These reactions are chemical in nature and bonds are broken or formed whether they are, for example, covalent molecular bonds or non-covalent hydrogen bonds.

3.2 Heating and Protein

As described by Yu et al. (2002), the primary means of altering the protein degradation characteristics upon heating is through denaturation. Denaturation of protein can occur at every level of protein structure. Quaternary structure is affected as heating breaks the non-covalent intermolecular bonds that hold the protein subunits together. Tertiary structure is affected because heating causes intramolecular non-covalent bonds and disulfide bonds to break, causing the protein to unfold. Secondary structure is affected by heat in a manner similar to tertiary structure. However, the non-covalent bonds involved are hydrogen bonds necessary to stabilize the α-helical and β-sheet conformations. Finally, primary structure may be altered by the breaking of the covalent bonds that hold the polypeptide chain together. The extent to which

these changes occur is dependent on the severity of the heating conditions during treatment, not solely temperature but also moisture and pH. Typically, once the primary structure begins to be affected, the process is more appropriately termed protein degradation.

3.3 Heating and Maillard Reaction

One major chemical reaction that takes place when feedstuffs are treated with heat is the Maillard reaction. The Maillard reaction is responsible for a diverse range of chemical products that can influence the nutritional value of a food or feed (Martins et al. 2001). Among the most important processes of non-enzymatic Maillard reactions relates to the reaction of amino acids, peptides and proteins with reducing sugars and vitamin C (Arnoldie 2001). The Maillard reaction is dependent on the presence of reducing sugars such as glucose and fructose, both monosaccharides, but the reaction may also proceed in the presence of disaccharides such as lactose. Sugars that are not reducing, like sucrose, bound glycoproteins, and glycolipids, require hydrolysis before they can take part in product formation (Arnoldie 2001). The Maillard reaction is complex in nature and the reaction pathways that lead to end products are numerous. The non-enzymatic browning involves a condensation reaction between primary amines and reducing sugars in the presence of water. The main reactive amines found in proteins are the epsilon amino groups of lysine. Another, reactive amine, is the α-amino group at the N' terminus of the polypeptide chain (Martins et al. 2001). The condensation product of the initial reaction is an N-substituted glycosylamine, which undergoes rearrangement to form an Amadori rearrangement product whose subsequent degradation is pH dependant (Martins et al 2001). The end products that are formed from the Maillard reaction are not considered digestible and it is widely held that the products are detrimental to the nutritional value of the protein, despite the fact that some intermediates are fully available in laboratory rats (Yu et al. 2002).

IV. SYNCHROTRON RADIATION -BASED FOURIER TRANSFORM INFRARED MICROSPECTROSCOPY AS A NOVEL TOOL OF FEED EVALUATION

4.1 Infrared Spectroscopy

Infrared spectroscopy measures the frequencies of IR light absorbed by a sample. It is through this absorption of energy that the constituent molecules in a sample of interest are identified. The commonly used device to make such measurements is a FTIR spectrometer. The FTIR spectrometer uses an interferometer which splits the incoming light into two, with one path remaining a constant distance and the other path bouncing off a moving mirror. As a result, the light travels a varying distance before it is finally recombined, run through the sample and then onto the detector (Wade 2003). The light recombines to form an interferogram containing all IR wavelengths simultaneously (Wade 2003). Initially, the interferogram is said to be in the time domain with the signal corresponding to the energy seen by the detector as the mirrors move. Fourier transform, a computer algorithm, is then applied to show the strength of absorption as a function of frequency. In other words, it is expressed in the frequency domain (Wade 2003).

Infrared or vibrational spectroscopy allows an investigator to identify which molecular bonds are present in a sample. It does so through the IR induced excitation of the bonds at wavelengths of IR light characteristic of the bond of interest. The IR frequency required to cause excitation of a molecular bond is dependent on the masses of the atoms involved in the bond and the strength of that bond or, more accurately, the stronger the bond and the lighter the atoms involved, the greater the energy required to induce bond excitation (Moore and Dalrymple, 1976). To illustrate consider the following. Triple bonds will vibrate at a higher frequency than double bonds, and double bonds at a higher frequency than single bonds (Wade 2003). The two types of vibrations/excitations of importance in identifying organic compounds are stretching and bending, and since bending motions are easier to induce than stretching motions, they absorb light of lower frequencies or energy (Moore and Dalrymple, 1976). In addition, the bond may be stabilized or destabilized somewhat by interactions with the environment. This is demonstrated by watching the shift of the carbonyl band in polar and non-polar solvents. When surrounded by a polar environment, the hydrogen bonding that takes place serves to stabilize the bond, thus requiring IR light at a higher frequency (more energetic) to induce vibration. Other interactions include electrostatic and dispersion interactions, which may also influence vibration (Novikov et al. 1998). Not all molecules are able to be excited by IR light of any frequency. IR active bonds have a dipole moment, meaning they have some degree of charge separation between the atoms

that make up the bond. This distribution of charge in the molecule is required to interact with the electric field of the IR electromagnetic radiation (Wade 2003). When the electric field is in the same direction as the dipole moment, the bond is compressed. When the electric field is changed to the opposite direction, the bond is stretched. When this stretching and compressing occurs at a frequency that matches the natural vibration state of a molecular bond, energy may be absorbed (Wade 2003). The sensitivity of IR spectroscopy to the small interactions involving the bond of interest makes it a measurement not only of that bond, but its interactions with the surrounding environment

4.2 Development of Synchrotron-Based Fourier Transform Infrared Microspectroscopy

Fourier Transform Infrared (FTIR) microspectroscopy is the result of the combination of microscopy with infrared (IR) spectroscopy, and enables researchers to do comparisons between the histological structures in a tissue sample and the corresponding spectroscopic chemical information (Wetzel and LeVine, 2001). These comparisons can be conducted because the microscope permits us to focus the incidental light in the experiment onto small enough regions of a sample and to isolate the histological structures in the sample. Initially, FTIR microspectroscopy was conducted on a bench top apparatus using a thermal (globar) device to generate IR light, which would then be directed through a microscope, then through the sample and finally onto the detector. This thermal source of IR light had limitations in its application, mainly due to its low brightness and the diffraction effects that occur when the aperture is reduced to sizes that correspond with the wavelength of the incoming light. The diffraction of the IR light further reduces the amount of light that can reach the detector, and consequently reduces the signal to noise ratio (S:N), in addition to scattering the light beyond the area defined by the masks (Wetzel and LeVine, 2001). This, of course, would limit the spatial resolution of the experiment as well as increase the time required to collect data. Eventual improvements in instrumentation, both IR optically efficient microscopes and spectrometers, would eventually make the light source the limiting factor in these experiments (Wetzel and Levine, 2001).

Eventually, a superior light source, the synchrotron, was applied to IR microspectroscopic experiments that would drastically improve both their spectral and spatial resolution. This new technique is called synchrotron-based FTIR (S-FTIR). The reasons for

improved spectral and spatial resolution are the intensity of the light, which is 100-1000 fold greater, the lack of noise found in thermal sources, which makes it more stable, and it is relatively non-divergent, which allows it to be highly focused with less energy lost (Wetzel and LeVine, 2001). The first two characteristics are related to increasing the S:N which generates better spectra with fewer scans. The third characteristic is related to spatial resolution (Wetzel and LeVine, 2001). The consequence of this was the ability to generate maps of the structural-chemical makeup of tissues *in situ* with each pixel representing an area that was smaller than the cells that comprised the tissue (6 µm X 6 µm) and revealed the chemical heterogeneity of the samples at those dimensions (Wetzel et al. 1998).

4.3 Important IR Bands in Feed Research

Many different molecular bonds make up the biological components of a feed. Some bonds, in this context, are considered characteristic of specific biological components. The peptide bond is characteristic of protein and depicted in Figure 3. It is considered to be a very stable, rigid and planar bond. In the IR spectrum, two bands arise from the peptide bond in protein. These are the amide I and amide II bands (Figure 4). The amide I band arises from C=O stretching vibrations (80%) in addition to some contribution from C-N vibrations, with its peak in the region of ca. 1650 cm^{-1} in the IR spectrum (Jackson and Mantsch, 1995; 2000; Miller 2002; Marinkovic et al. 2002; Marinkovic and Chance, 2005; Yu 2004). The amide II band peak is found around 1550 cm^{-1} and arises from N-H bending vibrations (60%) as well as C–N stretching vibrations (40%) (Jackson and Mantsch, 1995; 2000; Yu 2004; Marinkovic and Chance, 2005). To look at changes or differences in protein structure due to processing of a feed sample, these bands would be expected to serve as an appropriate probe indicating changes to molecular structure. The amide II band, however, is considered less useful in evaluating protein secondary structure due to the complex vibrations involving many functional groups that comprise it (Haris and Chapman, 1992, Jackson and Mantsch, 1995; 2000, Marinkovic and Chance, 2006).

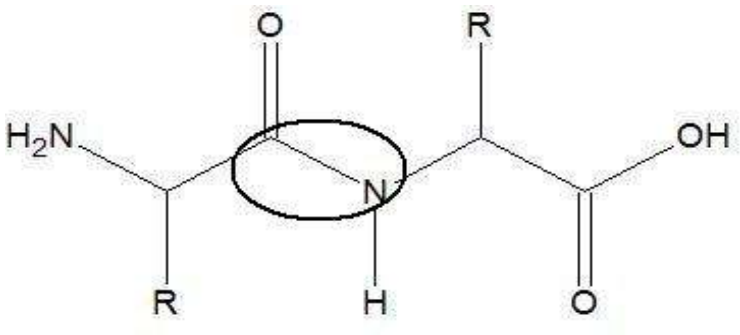

Figure 3. Representation of the peptide bond (*circled*) between two amino acids. R represents one of the amino acid side chains.

Lipids, as both free fatty acids and triglycerides, are also composed of characteristic functional groups (C=O ester, CH_2, CH_3) which lead to bands in the IR spectrum that are characteristic of lipids. The C=O ester band is found near ca. 1738 cm^{-1}, while the two other functional groups generate peaks near 1470 cm^{-1} (CH bending), 2961 cm^{-1} (CH_3 asymmetric stretching), 2925 cm^{-1} (CH_2 asymmetric stretching), ca. 2871 cm^{-1} (CH_3 symmetric stretching), and ca. 2853 cm^{-1} (CH_2 symmetric stretching) (Wetzel and LeVine 1999; Miller 2002; Jackson and Mantsch 2002; Yu 2004). To investigate changes in lipid structure or composition these bands could serve as appropriate probes in a manner similar to how the amide I band would be used for protein. There are molecular differences in lipid structures. Free fatty acids are depicted with the 3 C glycerol, Triglyceride from the same fatty acids. Fatty acids chains also vary themselves in composition in terms of chain length, saturation, and double bond locations (Voet and Voet, 1995a)

Carbohydrates (CHO) are composed of a variety of bonds between carbon (C), hydrogen (H) and oxygen (O). The resulting IR absorption bands are quite complex making their interpretation complex as well. CHO generates strong bands in the ca. 1550-800 cm^{-1} spectral region, in particular around 1185-800 cm^{-1} (Wetzel et al. 1998; Yu 2004). There are some bands (ca. 1420 cm^{-1}, 1370 cm^{-1}, 1335 cm^{-1}), however, that can be used to indicate the presence of structural (cellulose) CHO in a sample (Wetzel et al. 1998). The band near 1420 cm^{-1} has been more specifically associated with β-glucan (Wetzel et al. 1998). Other bands considered to be indicative of different CHOs include ca. 1025 cm^{-1} (non-structural CHO starch), ca. 1510 cm^{-1} (aromatic lignin), ca. 1246 cm^{-1} (cellulosic compounds), ca. 1100 cm^{-1} (cellulose) (Wetzel et al.

1998; Yu 2004). There are different bonds that lead to a polymer of the same subunits with a very different structure. For example, there are difference in the molecular structure of common bonds found in starch by α-1-4 linkage and those found in cellulose by β-1-4 linkage. The different bonds have effects on the polymer structure (Voet and Voet, 1995b)

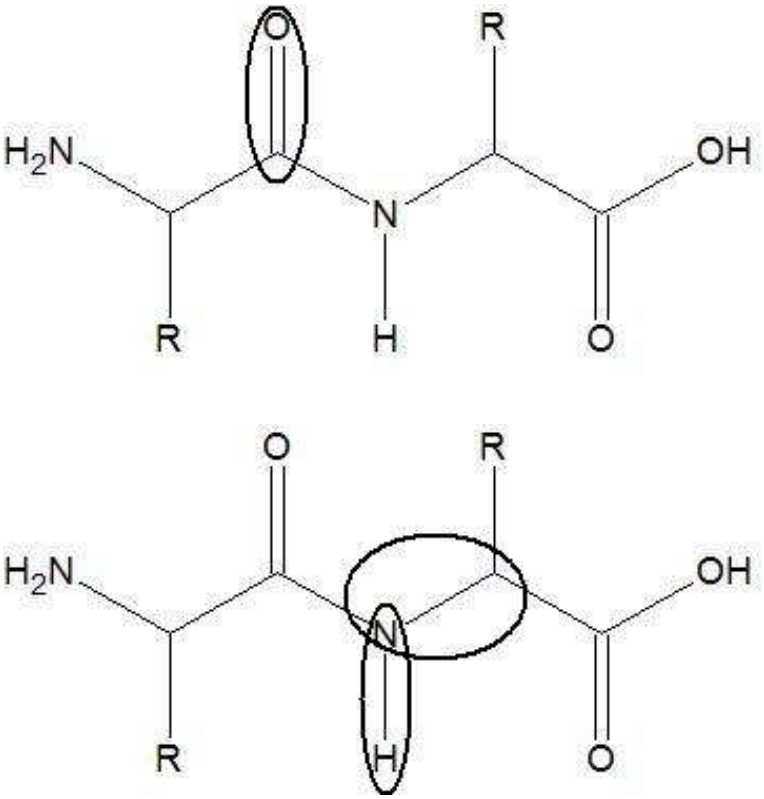

Figure 4. Major bonds responsible (*circled*) for the amide I band (*top*) and amide II band (*bottom*) in the mid-IR spectrum.

4.4 Assessing Feeds with IR Spectroscopy

It has been suggested that the protein matrix that surrounds each starch granule is important with respect to how quickly the starch in various cereal grains will degrade (McAllister et al. 1993). In a study conducted by Yu et al. (2004a), two different barley varieties

were selected based on similarities in traditional chemical values (starch, CP, etc.), but also on differences in their digestion characteristics. Harrington, a quickly and extensively degraded malting barley, and Valier, a slowly and limited degrading feed type barley, were selected to probe whether differences in the starch-protein matrix were responsible for their differences in digestive behaviour. The starch-protein matrix was assessed by the starch to protein band intensity ratio between the two varieties. The study showed that the starch to protein band intensity ratio was the same for both Harrington and Valier. Harrington, however, showed a greater range in that ratio, and it was concluded that this was a result of greater heterogeneity of the starch-protein matrix in the endosperm. It was concluded that the association of starch with protein, as defined by the band intensity ratios, was not the sole cause of differences in degradation behaviour. It would appear. however, that heterogeneity of the starch-protein matrix itself may have some potential as an assessment of sub-cellular structure as it influences degradation behaviour.

S-FTIR can be used to characterize solely the protein in the endosperm of a feed by focusing specifically on those bands that result from the peptide bonds of protein. These are known as the amide I and amide II bands, as previously mentioned. Prior to the Yu et al. (2004a) study, it had been shown that the amide I to amide II ratio decreased in scrapie-infected tissue (Miller and Dumas, 2006; Wang et al. 2005). Scrapie is known to result in secondary structure changes in prion proteins from α-helices to β-sheets. Therefore, a difference in this ratio is indicative of some difference in protein structure. It is possible that protein structure plays a role in degradability, so this ratio may permit the evaluation of protein structure in a manner which could be linked to degradability. Yu et al. (2004a) results showed in fact, that there was a difference in both band intensities between two varieties of barley (Harrington and Valier) with different rumen degradation rates. The ratio was slightly smaller for Harrington, and it was concluded that there was some structural difference between the two varieties that led to the results. There is little published data, however, on the relationship between these values and nutrition.

Because the amide I and amide II bands are predominantly from protein, they exhibit shifts in their location in the spectrum depending on the secondary structure in which the peptide bonds are located. The frequency shift permits them to be used to determine the ratios of

secondary structure that make up the protein in a feed (Wetzel et al. 2003). The amino acids in a protein are found in α-helix, β-sheet conformations, but also in β-turn and random coil conformations. The former conformations are periodic while the latter are aperiodic. Each of these secondary structures provides a different environment for the amide I bonds, resulting in shifts in the location of the peak for the amide I band in the spectrum (Marinkovic et al. 2002; Marinkovic and Chance, 2006). This means that the bands are actually made up of multiple elements blurred together, where the contribution of each is concentration dependant and requires mathematical treatment to isolate the contribution of each element (Marinkovic and Chance, 2006; Marinkovic et al. 2002). To separate the individual peaks, the spectrum first undergoes Fourier self-deconvolution (FSD). Then, to quantify the area of each of the peaks in the FSD spectrum, multi-peak fitting software is used, thus providing us with relative values for the amount of each secondary structure (Yu 2006b).

It is important to note that concentration values for protein secondary structures, obtained from mid-IR spectroscopic data, are only relative and not exact determinations (Yu 2006a). Important factors in IR spectroscopy are responsible for this fact, and include different molar absorptivities, the number of component bands, component band shapes and noise (Yu 2006a). In addition to this, researchers do similar analyses but select different-sized spectral regions, different baselines and different mathematical manipulations of the spectra (Yu 2006a). The relative values that are derived are still useful, however, for comparing treatments, but there are several considerations one needs to be aware of regarding these determinations.

Due to the actual physical differences in these secondary structures, there may be some difference in rumen degradability of the protein in a feed. Examining the protein secondary structures of feather, a poorly degraded protein source, showed a much lower α-helix to β-sheet ratio than that found in more easily degraded protein sources (Yu et al. 2004b). Subsequently, the study between the two barley varieties showed no difference in the α-helix:β-sheet ratio. There were differences when the α-helix and β-sheet were compared to other elements of secondary structure, leading to the conclusion that there were, in fact, detectable structural differences between the two cultivars (Yu et al. 2008). In a separate study, Yu et al. (2005) used two types of flaxseed (golden and brown), both raw and roasted, to determine if cooking would change the α-helix:β-sheet ratio. The results showed that roasting did reduce the α-helix:β-sheet ratio of the

golden flaxseed, but not the brown flaxseed, indicating that different feeds will exhibit different sensitivities to changes in protein secondary structure upon heating. It was further shown (Yu et al. 2005) that the changes in protein secondary structure were accompanied by changes in protein degradability in flaxseed. The picture that remains is that protein secondary structure differences are indicative of differences or changes in protein degradability, but caution should be used when comparing different feeds as they may respond differently to treatment.

4.5 Multivariate Statistical Methods for Spectral Comparison

The previously mentioned measurements focus on direct measurements of specific characteristics of the IR spectrum of a feed that are compared using univariate methods of analysis. Multivariate analysis permits the researcher to use all or large portions of the spectral data at once to do comparisons, regardless of what the bands in the spectra specifically represent (Yu 2006b). Multivariate methods used in spectroscopic research include principal component analysis (PCA) and hierarchical cluster analysis (CLA). The purpose of these procedures is to group the spectra according to their similarity to one another, but they use different methods to do comparisons.

CLA calculates a distance matrix which contains information about the similarity of the spectra. Then it groups the most similar spectra based on the distance matrix to create a cluster which is followed by the recalculating the distances of the remaining spectra to the new cluster (Yu 2006b). CLA, when used in feed research, is able to distinguish between different varieties of the same feedstuffs in addition to different treatments of the same variety (Yu 2005d).

PCA, meanwhile, constitutes a statistical data reduction method that transforms the original variables in the data set into a new and uncorrelated set of values called principal components that still account for the original variance in the data (Yu 2005d). Each spectrum is assigned a score (eigenvector) defining its relationship to each principal component. The eigenvector is then used to create 2-D or 3-D scatter plots, on which proximity of spectra to one another indicates similarity between the spectra. For a more detailed explanation of PCA, the reader is directed to Dunteman (1989). As with CLA, PCA has also been shown to be able to distinguish between different varieties of the same feedstuffs in addition to different treatments

of the same variety (Yu 2005d). While PCA and CLA used in this manner do not provide structural information per se, they imply molecular differences or similarities in the mid-IR spectroscopy data that is used.

4.6 The Need for S-FTIR Use in Feed Research

S-FTIR ultimately provides us with information relevant to the nutritional characteristics of a particular feed because these characteristics are influenced not only by total composition of a feed, but also by morphological characteristics and biological component matrices (Yu 2006b). The information about the latter two is typically lost during most chemical analyses and this loss of information, or the inability to account for it, is believed to be accountable for the failure of models such as the NRC 2001 model to fully predict nutritive or energy values for a given feed (Yu et al. 2004a). In addition, S-FTIR is a rapid and non-destructive technique so the sample remains intact and unaltered by the experimental process, allowing further assaying of the sample if desired. The problems for the investigator that remain are connecting the elements of structure that are revealed by S-FTIR (or changes to those elements) to their influence on digestive behaviour and determining if the sample, from a sub-cellular area, is representative of the feed as a whole. Another concern is that the sample preparation process, prior to spectroscopic analysis, will itself affect the spectrum from a given sample so caution must be exercised to ensure the samples are treated identically.

V. RECENT SYNCHROTRON APPLICATIONS IN FLAXSEED (OIL SEED) RESEARCH

5.1 Heat-Induced Protein Structure of Oil Seeds (Flaxseed) and Subfractions in Relation to Protein Degradation Kinetics and Intestinal Availability in Dairy Cattle

In recent research by Doiron et al. (2009a), the aims were to reveal protein structures of feed tissues affected by heat processing at a cellular level, using the advanced synchrotron technology - synchrotron-based Fourier Transfer Infrared Microspectroscopy (S-FTIR) as a novel approach, and quantify protein structure in relation to protein digestive kinetics and

nutritive value in the rumen and intestine in dairy cattle. The parameters assessed included: 1) Protein structure α- helix to β-sheet ratio; 2) Protein subfractions profiles; 3) Protein degradation kinetics and effective degradability; 4) Predicted nutrient supply using the DVE/OEB system in terms of the intestinally absorbed protein supply (DVE) and degraded protein balance (OEB) to dairy cattle. In this study, Vimy flaxseed protein was used as a model feed protein and autoclave-heated at 120°C for 20, 40 and 60 min as treatments T1, T2 and T3, respectively. The hypothesis was that heat-induced protein structure changes affected the protein quality, fermentation and digestion behaviour in the rumen and intestines. The synchrotron-based protein chemistry research was performed at the National Synchrotron Light Source in Brookhaven National Laboratory. The results showed that using the synchrotron-based analytical technique (S-FTIRM), the heat-induced protein structure changes was revealed and identified. The heating at 120°c for 40 and 60 min increased protein secondary structure α- helix to β-sheet ratio. There were linear effects of heating time on ratio. The heating also changed chemical profiles, which showed soluble CP decreased upon heating with concomitant increases in non-protein nitrogen, neural and acid detergent insoluble nitrogen. The protein sub-fractions with the greatest changes were PB1 (fraction PB1 is a rapidly degradable protein fraction in rumen) which showed a dramatic reduction, and PB2 (fraction PB2 is fermented in the rumen at a lower rate than buffer-soluble fractions and some of the PB2 fraction escapes to the lower gut) showing a dramatic increase demonstrating a decrease in the overall protein degradability. In situ results showed a reduction in rumen-degradable protein (RDP) and dry matter (RDDM) without differences between the treatments. Intestinal digestibility by three-step in vitro showed no changes to the RUP. Modelling results showed that the heating increased total intestinally absorbable protein (feed DVE value) and decreased degraded protein balance (feed OEB value), but there were no differences between the treatments. There were linear effect of heating time on the DVE and cubic effect on the OEB value. Our results showed that the heating changed chemical profiles, protein secondary structure α- helix to β-sheet ratio and protein sub-fractions, and decreased RDP and RDDM and increased potentially nutrient supply (DVE value) to dairy cattle. The protein secondary structure α- helix to β-sheet ratio had significantly positive correlation with total intestinally absorbed protein supply - DVE value and negative correlation with degraded protein balance.

5.2 Detecting Molecular Changes in Vimy Flaxseed Protein Structure Using Synchrotron FTIRM and DRIFT Spectroscopic Techniques: Structural/Biochemical Characterization

Mid-IR techniques were used to characterize any changes that occurred on a molecular level in flaxseed that had been heated using an autoclave. This study (Doiron et al. 2009b) aims to investigate the effects of autoclave heating on differences in diffuse reflectance infrared Fourier transform (DRIFT) and synchrotron-based Fourier transform infrared microspectroscopy (SFTIRM) based measurements of the protein α-helix to β-sheet ratio for flaxseed (*Linum usitatissimum*), cv. Vimy. Hierarchical cluster analysis (CLA) and principal components analysis (PCA) were also conducted to identify molecular differences in the DRIFT spectra. Flaxseed samples were kept raw for the control or autoclaved in batches at 120°C for 20, 40 or 60 min for treatments 1, 2 and 3, respectively. DRIFT analysis of protein secondary structure ratios showed a decrease ($P < 0.05$) in the α-helix to β-sheet ratio for the whole seed while the results from synchrotron SFTIRM spot data from the endosperm tissue in flaxseed showed autoclaving had the opposite effect ($P>0.05$). CLA and PCA were successfully used to make distinctions between the different treatment spectra and showed enhanced sensitivity upon selection of a smaller spectral window to include only the amide I and II portion of the IR spectrum. Our results indicated that autoclaving had a great enough effect on the mid-IR spectrum of flaxseed to identify the altered α-helix to β-sheet ratio and subsequently differentiated between the treatments using PCA and CLA suggesting greater sensitivity of mid-IR spectral methods in identifying the effect of heat treatment on protein secondary strucutre. Future study is needed to quantify the relationship between protein secondary structure and protein functionality (Doiron et al. 2009b).

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