PREDICTION OF DRY MATTER, CRUDE PROTEIN DEGRADABILITY, AND AMINO ACID COMPOSITION OF CORN SILAGE AND GRASS SILAGE BY NEAR INFRARED REFLECTANCE SPECTROSCOPY (NIRS)

By

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE STUDIES ANIMAL SCIENCE

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March, 2003

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ABSTRACT

This research program was designed to meet three objectives. The first was to ascertain the feasibility of using near infrared reflectance spectroscopy (NIRS) to predict ruminal degradability of dry matter (DM) and crude protein (CP) in corn silage (CS) and CP degradability in grass silage (GS) as determined by the in situ technique. The second objective was to develop calibration models to predict intestinal disappearance of DM and CP in CS and intestinal digestibility of CP in GS as determined by the mobile bag technique. The last objective was to investigate the feasibility of using NIRS to predict the essential amino acid (AA) composition of CS and GS.

In situ data showed substantial variation in soluble and degradable DM and CP fractions as well as AA composition of CS. Based on the RPD statistic used to evaluate calibration equations, NIRS provides a viable option for the prediction of soluble DM and CP for CS, effectively degraded CS CP and CS CP disappearance from the intestinal and total digestive tract. It was not possible to produce robust calibration equations to predict rates of CS DM or CP degradability. Further study is required to ascertain the usefulness of NIRS in predicting AA composition of CS.

Interpretation of spectral data showed that DM solubility and degradability of CS is linked to N-H bonding. There was a strong relationship between soluble DM, potentially degradable DM and effective degradability of CS CP. A review of the major wavelengths used in each calibration model indicated that fiber did not play a major role in CS DM digestibility.

For the GS study, samples were classified according to increasing content of neutral detergent fiber (NDF) as this constituent is related to plant maturity. The content of soluble CP in GS significantly (P<0.01) decreased with increasing maturity but there was no significant
difference (P>0.05) in potentially degradable CP. Likewise the rate of degradation of the potentially degradable CP fraction did not change according to NDF content. The amount of ruminally undegradable CP from GS significantly (P<0.01) increased with advancing maturity but there was no difference in intestinal digestibility of ruminally undegradable CP according to NDF content. Likewise, there was no difference in essential AA content, expressed on a CP basis, due to stage of maturity.

Ruminally undegraded CP was inversely related to CP ruminal disappearance after 12 h and/or 24 h incubation. Pearson correlation coefficients were -0.83 and 0.86, respectively.

NIRS was not successful in predicting CP solubility or degradability fractions for GS as determined by the in situ technique. Prediction of essential AA content of GS was promising as RPD statistics for each equation, except Met and Lys, approached 2.3.

This thesis presents data for the development of several NIRS calibration models, which have not been previously explored in the scientific literature. These include models to predict intestinal digestibility as well as AA composition of forage. The concluding chapter presents recommendations for experimental methodology as well as for future research in the area of NIRS model development.
**TABLE OF CONTENTS**

ABSTRACT ................................................................................................................................. III

LIST OF TABLES ..................................................................................................................... VIII

LIST OF FIGURES ..................................................................................................................... IX

LIST OF ABBREVIATIONS ....................................................................................................... X

ACKNOWLEDGMENTS .............................................................................................................. XII

DEDICATION ............................................................................................................................. XIII

CHAPTER 1 .............................................................................................................................. 2

INTRODUCTION AND LITERATURE REVIEW ................................................................... 2

1.0 INTRODUCTION ............................................................................................................... 2

1.1 DETERMINATION OF RUMINAL DEGRADATION AND INTESTINAL DIGESTIBILITY OF DM AND CP ............................................................................................................. 3

  1.1.1 In Vivo Measurements ............................................................................................... 3

  1.1.2 In Vitro Measurements ............................................................................................ 4

  1.1.3 In Situ Measurements ............................................................................................. 4

  1.1.4 Mobile Bag Technique ............................................................................................ 6

  1.1.5 Chemical Analysis Techniques ............................................................................... 7

  1.1.6 NIRS Technique ................................................................................................... 7

1.2 NIRS .................................................................................................................................. 8

  1.2.1 Theory of NIRS ...................................................................................................... 8

  1.2.2 Development of NIRS .......................................................................................... 9

  1.2.3 NIRS – Transformation of Spectra ....................................................................... 10

  1.2.4 NIRS – Population Structuring ............................................................................ 12

  1.2.5 NIRS – Calibration .............................................................................................. 15

  1.2.6 NIRS - Statistics .................................................................................................. 18

1.3 NIRS – PREDICTING FORAGE DM AND CP DEGRADABILITY .................................. 20

  1.3.1 Grass Forage ......................................................................................................... 20

  1.3.2 Grass and alfalfa forage ....................................................................................... 23

  1.3.3 Barley Forage ....................................................................................................... 26

  1.3.4 Mixed Forages ..................................................................................................... 29

  1.3.5 Corn ....................................................................................................................... 31

1.4 NIRS – PREDICTING FORAGE DM AND CP INTESTINAL DIGESTIBILITY ............... 33

1.5 NIRS – PREDICTING FORAGE AMINO ACID COMPOSITION ................................ 33

1.6 OBJECTIVES .................................................................................................................... 34

1.7 REFERENCES ................................................................................................................... 48
CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS ........................................................................ 113

4.0 INTRODUCTION .......................................................................................................................... 113

4.1 RUMINAL DEGRADABILITY AND INTESTINAL DIGESTIBILITY OF DM AND CP .............. 113
  4.1.1 Corn silage ............................................................................................................................ 113
  4.1.2 Grass Silage ........................................................................................................................ 116
  4.1.3 The in-situ procedure ......................................................................................................... 118
  4.1.4 The mobile nylon bag procedure ...................................................................................... 121

4.2 AMINO ACID EVALUATION .................................................................................................... 122

4.3 FUTURE RESEARCH IN NIRS AND FORAGE QUALITY .................................................... 122

4.4 REFERENCES .......................................................................................................................... 125
List of Tables

TABLE 1.1. SUMMARY OF 14 STUDIES INVESTIGATING FEASIBILITY OF BUILDING NIRS CALIBRATION MODELS TO PREDICT DM AND CP DEGRADABILITY USING IN SITU DERIVED REFERENCE VALUES. .......................................................... 35

TABLE 2.1 CHEMICAL COMPOSITION OF PRE-ENSILED AND ENSILED CORN SILAGES SAMPLE .......................................................................................................................... 74

TABLE 2.2 DM AND CP DEGRADATION CHARACTERISTICS OF CORN SILAGE IN TOTAL AND CALIBRATION SAMPLE SETS........................................................................... 75

TABLE 2.3 RUMINAL DISAPPEARANCE, INTESTINAL AND TOTAL TRACT DISAPPEARANCE OF DM AND CP IN TOTAL AND CALIBRATION SETS OF NON-ENSILED AND ENSILED CS................................................................. 76

TABLE 2.4. AMINO ACID COMPOSITION (%DM) OF CORN SILAGE IN TOTAL AND CALIBRATION SAMPLE SETS.......................................................................................................................... 77

TABLE 2.5. STATISTICAL EVALUATION OF CALIBRATION EQUATIONS FOR RUMINAL DM AND CP DEGRADATION AND INTESTINAL DISAPPEARANCE OF DM AND CP. .......................................................... 78

TABLE 2.6. STATISTICAL EVALUATION OF CALIBRATION EQUATIONS FOR AA COMPOSITION (% DM). .......................................................................................................................... 80

TABLE 3.1. DEMOGRAPHICS OF 114 GRASS SILAGE SAMPLES COLLECTED FOR NIRS CALIBRATION .......................................................................................................................... 103

TABLE 3.2. PROXIMATE COMPOSITION OF GRASS SILAGE SAMPLES USED FOR NIRS CALIBRATION .......................................................................................................................... 104

TABLE 3.3. PROTEIN DEGRADATION CHARACTERISTICS OF GRASS SILAGE SAMPLES USED FOR NIRS CALIBRATION .......................................................................................... 105

TABLE 3.4. CORRELATION COEFFICIENTS (R) BETWEEN PROXIMATE COMPOSITION OR SINGLE POINT RUMINAL INCUBATION AND CP DEGRADABILITY. .......................................................... 106

TABLE 3.5. AMINO ACID COMPOSITION OF GRASS SILAGE SAMPLES USED FOR NIRS CALIBRATION .......................................................................................................................... 107

TABLE 3.6. NIRS CALIBRATION EQUATION PERFORMANCE FOR GRASS SILAGE CP DEGRADABILITY AND AA COMPOSITION .................................................................................. 108
List Of Figures

FIGURE 1.1. DISAPPEARANCE OF CP FROM ONE SAMPLE OF GRASS SILAGE INCUBATED IN THE RUMEN FOR 0 TO 96 H. .................................................................41

FIGURE 1.2 INFRARED PORTION OF THE ELECTROMAGNETIC SPECTRUM.................................42

FIGURE 1.3. AVERAGE SPECTRA OF CORN SILAGE AND GRASS SILAGE COLLECTED BY SCANNING DRIED, GROUND SAMPLES ON A SCANNING MONOCHROMATOR. AVERAGE OF TWO SCANS PER SAMPLE. LOG 1/R IS EQUIVALENT TO ABSORPTION.................................43

FIGURE 1.4. SPECTRA OF POULTRY MANURE GROUND THROUGH A FINE, MEDIUM OR COARSE MESH SCREEN.................................................................44

FIGURE 1.5. SPECTRA OF POULTRY MANURE GROUND THROUGH A FINE, MEDIUM OR COARSE MESH SCREEN. SPECTRA WERE TRANSFORMED USING THE STANDARD NORMAL VARIATE (SNV) ALGORITHM. .................................................................44

FIGURE 1.6. SPECTRA OF POULTRY MANURE GROUND THROUGH A FINE, MEDIUM OR COARSE MESH SCREEN. SPECTRA WERE TRANSFORMED USING THE STANDARD NORMAL VARIATE (SNV) AND DE-TRENDING PROCEDURES.................................................................45

FIGURE 1.7. PLOT OF 10 SAMPLES OF CORN SILAGE SHOWING ORIENTATION IN TWO-DIMENSIONAL SPACE AS DEFINED BY LOG 1/R VALUES AT 1940 AND 2280 NM..................45

FIGURE 1.8. HISTOGRAM OF H (MAHALANOBIS) VALUES FOR 77 SAMPLES OF GROUND CORN SILAGE........................................................................46

FIGURE 1.9. NIRS CALIBRATION FLOWCHART............................................................................47

FIGURE 4.1. RUMINAL DM DISAPPEARANCE FROM TWO COWS (2 BAGS PER COW). ..........125
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>immediately soluble fraction</td>
</tr>
<tr>
<td>AA</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>ADF</td>
<td>acid detergent fiber</td>
</tr>
<tr>
<td>ADIN</td>
<td>acid detergent insoluble nitrogen</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>B</td>
<td>potentially degradable fraction</td>
</tr>
<tr>
<td>c</td>
<td>rate of degradation of the B fraction. Also referenced as kd.</td>
</tr>
<tr>
<td>C</td>
<td>defined as the difference between 100 and the sum of the A and B fractions</td>
</tr>
<tr>
<td>CP</td>
<td>crude protein</td>
</tr>
<tr>
<td>CPA</td>
<td>rapidly degraded fraction of the crude protein</td>
</tr>
<tr>
<td>CPB</td>
<td>potentially degradable fraction of crude protein</td>
</tr>
<tr>
<td>CPED6</td>
<td>effective degradability of crude protein assuming a theoretical ruminal turnover rate of 6 % h⁻¹</td>
</tr>
<tr>
<td>CPIT</td>
<td>crude protein disappearing in the intestinal tract</td>
</tr>
<tr>
<td>CPkd</td>
<td>rate of degradation of potentially degradable crude protein</td>
</tr>
<tr>
<td>CPR</td>
<td>crude protein remaining</td>
</tr>
<tr>
<td>CS</td>
<td>corn silage</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>D</td>
<td>de-trend</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>DMA</td>
<td>rapidly degraded fraction of dry matter</td>
</tr>
<tr>
<td>DMB</td>
<td>potentially degradable fraction of dry matter</td>
</tr>
<tr>
<td>DMED6</td>
<td>effective degradability of dry matter assuming a theoretical ruminal turnover rate of 6 % h⁻¹</td>
</tr>
<tr>
<td>DMkd</td>
<td>rate of degradation of potentially degradable dry matter</td>
</tr>
<tr>
<td>EAA</td>
<td>essential amino acid</td>
</tr>
<tr>
<td>ED (4)</td>
<td>effective degradability assuming a theoretical ruminal turnover rate of 4 % h⁻¹</td>
</tr>
<tr>
<td>EP</td>
<td>escape protein</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>GS</td>
<td>grass silage</td>
</tr>
<tr>
<td>Ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>MLR</td>
<td>multiple linear regression</td>
</tr>
<tr>
<td>MP</td>
<td>microbial protein</td>
</tr>
<tr>
<td>MPLSR</td>
<td>modified partial least squares regression</td>
</tr>
<tr>
<td>MSC</td>
<td>multiplicative scatter correction</td>
</tr>
<tr>
<td>MT</td>
<td>math treatment</td>
</tr>
</tbody>
</table>
N  nitrogen
NDF  neutral detergent fiber
NIRS  near infrared reflectance spectroscopy
NR  not reported
NRC  National Research Council
PC  principal component
PCA  principal component analysis
PCR  principal component regression
PLSR  partial least squares regression
Pro  proline
R  reflectance
RPD  ratio of standard deviation of the reference set to the standard error of cross validation (or standard error of performance)
RSQ  coefficient of determination (R²)
RUP  ruminally undegraded protein
RUPDig  intestinal digestibility of ruminally undegraded protein
SD  standard deviation
SEC  standard error of calibration
SECV  standard error of cross validation
SEP  standard error of performance (or prediction)
Ser  serine
SEV  standard error of validation
SNV  standard normal variate
SNVD  standard normal variate and de-trend
TAA  total amino acid
Thr  threonine
Trp  tryptophan
Tyr  tyrosine
Val  valine
VR  variance ratio
WL  wavelength region
ACKNOWLEDGMENTS

As with any major accomplishment, completion of this thesis is really due to the team of family, friends, advisors and colleagues who have supported me in order that I might realize my goal.

The unfailing support of my husband Bruce, our children, Kate, Eric, Curtis and my parents, Alberta and the late Bruce Fisher is lovingly recognized for without their words and acts of encouragement and understanding, completion would remain a goal instead of an accomplishment.

My graduate supervisor, Dr. Jim Shelford, unfortunately did not live to see completion of the thesis. Jim had a depth of knowledge regarding forage and dairy production on which I depended and as with anything valuable, you only know how much you miss something when it is gone. I can only hope that this work is an honor to his memory.

My appreciation goes to Dr. Jim Thompson who stepped in to fill the role of graduate supervisor. Together with Dr. Eunice Li Chan and Dr. Doug Veira, their editorial comments were instrumental in completion of this thesis. A special word of thanks to Dr. Lorne Fisher who questioned my assumptions and conclusions and who taught me not to start a sentence with the word “Table”. To Dr. Marina von Keyserlingk, a note of gratitude, not only for her encouragement and support, but for her organizational skills regarding experimental work at the UBC campus. I would also like to recognize Dr. John Hall, and Dr. Ray Peterson, who retired, never failed to respond to a desperate email containing statistical questions.

I would like to thank the management team of Pro Form Feed’s Ltd. for their faith in my decision to invest in NIRS technology and their financial support of the forage research program. It was my work family, however, who lent their moral support over the years and I gratefully acknowledge Dave Dyble, Walt Goerzen, Carol Kennedy, Shelagh Niblock, Glenn Smith, and Vern Thiessen for their friendship.

Last, but certainly by no means least, heartfelt thanks goes to Rich Vanderwal, Liz Noordam, Rob Walsh, Steve Chiasson, Colin Radom, Marty Darrow, Sandi Standing, Kristine Carlson, Bumin Shangguan and Niansheng Yang from the Abbotsford Veterinary Clinic as well as Dr. Tom Scott, Dr. Valerie Stevens and Dr. Shabtai Bittman of the Pacific Agri-food Research Center in Agassiz. Their encouragement and support to complete this thesis and move on in life is one of the primary reasons the goal has been realized.

Financial support for this research, provided by Pro Form Feeds Ltd. and the British Columbia Ministry of Agriculture, Fisheries and Food (Research Partnership Program) is gratefully acknowledged.
DEDICATION

This thesis is dedicated to the memory of two men who made a difference during their time here on earth.

To my father, Walter Bruce Fisher

and

To my supervisor, Dr. James A. Shelford.
CHAPTER 1
INTRODUCTION and LITERATURE REVIEW

1.0 INTRODUCTION

In North America, producers of meat and milk face ever increasing pressure to recognize and integrate practices concerning environmental stewardship while improving economic efficiency. Studies have shown that the importation of N in the form of feed and fertilizer to farms far exceeds N exports as meat and milk, and that imported feed represents over 60% of this excess N (CAST, 2002). As a result, there is increasing emphasis on decreasing the amount of imported N onto the farm. Increasing the amount of forage in the diet is an effective means of accomplishing this goal but relies on the forage being of high quality and the availability of adequate forage analyses (CAST, 2002).

Traditionally, nutritionists have relied on forage analysis for dry matter (DM), crude protein (CP), acid detergent (ADF) and neutral detergent (NDF) fiber as inputs into computer programs used to balance rations for ruminant animals. These programs make no attempt to account for the dynamic process of protein degradability in the rumen, which is important for predicting the amount of escape or bypass protein and for estimating the amount of microbial protein output from the rumen. Ultimately, protein production in the form of milk and meat will be fueled by the absorption of amino acids supplied in large amount, by microbial protein synthesized in the rumen and by intestinal digestion of dietary CP escaping ruminal degradation (O'Connor et al., 1993). Research has shown that intestinal digestibility of forage CP escaping ruminal degradation can vary between 50 and 99% (Van Straalen and Tamminga, 1990).

New developments in ration balancing software have focused on the development of mathematical models (NRC, 2001; Boston et al., 2000) which attempt to predict the output of metabolizable energy and protein, based on degradation of feedstuffs in the rumen. These models also require inputs regarding intestinal digestibility of dietary protein, which escapes ruminal
degradation. Successful implementation of this advanced ration balancing software requires the ability to measure or predict the degree of degradation of feedstuff DM and CP in the rumen and the digestibility of CP in the intestinal tract.

1.1 Determination of Ruminal Degradation and Intestinal Digestibility of DM and CP

Methods found in the literature for estimating ruminal degradable fractions of DM and CP are the in vivo, in situ and in vitro techniques, regression equations based on chemical constituents such as protein or fiber and, near infrared reflectance spectroscopy (NIRS). Methods for determining intestinal digestibility are the in vivo and mobile nylon bag techniques.

1.1.1 In Vivo Measurements

In vivo measurements of forage digestion are laborious, time consuming and expensive (Rymer, 2000; Cochran and Galyean, 1994; Robinson et al., 1992). Cochran and Galyean (1994) described many parameters that must be considered in the design of a trial measuring nutrient digestibility using the in vivo technique. These included housing, number of animals, length of feeding period, and feeding frequency. The technique requires rigid and consistent protocols for sample (feed and feces) collection to reduce analytical and technical errors. The use of the in vivo technique to determine protein degradability is further complicated by the need to use abomassally or duodenally cannulated cattle or sheep (Robinson et al., 1992). Determination of intestinal digestibility, as measured by nutrient disappearance in the small intestine, requires animals having cannulas in the duodenum and ileum.

Estimates of protein degradability in the rumen obtained with the in vivo technique can be flawed by errors in measuring the proportion of undegradable protein due to escape of feed protein and microbial protein (Van Straalen and Tamminga, 1990). This method does not account for the endogenous secretion of N (Robinson et al., 1992). The inability to distinguish between dietary escape CP and microbial escape CP also interferes with the determination of intestinal digestibility of
Differentiation is possible between the two sources of escape CP upon entrance to the duodenum but not upon collection at the terminal ileum (Tamminga and Chen, 2000).

The in vivo method is labor intensive, time consuming and requires large amounts of the forage to be tested. In view of these disadvantages, Rymer (2000) concluded that the in vivo technique is not appropriate for routine evaluation of feedstuffs.

1.1.2 In Vitro Measurements

Weiss (1994) and Broderick (1994) offer comprehensive reviews of in vitro methods for the determination of DM and CP degradability. The original Tilley and Terry (1963) method and variations thereof, are still commonly used for the determination of DM disappearance over time. The method is a two-phase process in which a small amount of feed is first incubated in buffered rumen fluid and then digested with pepsin in weak acid. The amount of DM left in the residue is subtracted from the amount of DM of the original sample to determine in vitro dry matter digestibility. A disadvantage of this method is the need for cannulated animals, as the choice of donor animal can contribute a large source of potential variation, within or between laboratories conducting this analysis (Weiss, 1994). There is a wide range of procedures to determine CP degradability. Some of these methods are based on incubation of feedstuffs with proteolytic enzymes while others measure the solubility of proteins in various solvents (Kohn and Allen, 1995). Broderick (1994) concluded that each in vitro method has inherent flaws, which limits its ability to quantify the rate and extent of ruminal protein degradation.

1.1.3 In Situ Measurements

The in situ method, also known as the in sacco or nylon bag technique, has been used in degradability determinations since 1938 (Orskov, 2000) and is still the method of choice as it is thought to give reliable estimates of in vivo nutrient degradation across feedstuffs (Nocek, 1988). It
allows for the determination of fractional rates of degradation in the rumen and for the assessment of many types of feedstuffs at one time (von Keyserlingk, 1994). The in situ technique has become the most widely used method for determining protein degradability, and often serves as the reference method to validate in vitro or NIRS techniques (Hvelplund and Weisbjerg, 2000).

The in situ method requires the use of cannulated animals as several grams of a feedstuff, placed within a Dacron bag, is incubated in the rumen over time. Nutrient disappearance at each time point is calculated as the difference between the initial content of a nutrient in the feedstuff and its content in the residue remaining after incubation. Nutrient disappearance is commonly expressed as a percentage of the initial concentration (Figure 1.1). Nutrient disappearance information is used to generate a degradation curve characterized by the equation:

\[ P = A + B \left( 1 - e^{-ct} \right) \]  \hspace{1cm} \text{Equation 1.1}

where \( P \) is degradation at time \( t \), \( A \) is the soluble fraction, \( B \) is the potentially degradable fraction and \( c \) (kd) is the rate constant for \( B \). These terms are not used consistently throughout the scientific literature. For example, the soluble fraction \( A \) can be referred to as washing loss (Ørskov, 2000), the washable fraction (De Boever et al., 2002) and disappearance at 0 h (Johnson, 2000). The \( B \) fraction, representing the amount of DM or CP which could be potentially degraded in the rumen if ruminal turnover was not a consideration, is referred to as slowly degraded (Mathison et al., 1999), the potentially fermentable (Xu et al., 1996) and the insoluble potentially digestible fraction (Elizalde et al., 1999).

Effective degradability (ED) is calculated by the equation:

\[ ED = A + B\left( c/c+k \right) \]  \hspace{1cm} \text{Equation 1.2}

where \( k \) is the fractional rumen outflow rate (Ørskov and McDonald, 1979).

There are several factors that can affect in situ results and repeatability of results between laboratories (Hvelplund and Weisbjerg, 2000; Michalet-Doreau and Ould-Bah, 1992; Nocek, 1988). The ratio of sample size to bag surface, bag porosity, and sample grind size can influence precision.
of the data obtained with the in situ method (Weiss, 1994). Other factors impacting repeatability of the data collected by the in situ procedure include diet of the cannulated animal, length of incubation, and the method used to wash Dacron bags post-incubation (Nocek, 1988). Standardization practices for bag design and pore size, grind size, and minimum incubation time have been adopted in recent years (Hvelplund and Weisbjerg, 2000).

A large source of variation associated with the in situ procedure is contamination of the nylon bag residue with ruminal microflora. The question of which method to employ to correct for this microbial contamination remains unresolved. Broderick and Merchen (1992) reviewed a number of chemical markers, both internal and external in nature, which have been used to quantify microbial protein. All of these procedures add error into the in situ procedure, and as noted by Weiss (1994), the choice of an unsuitable method of correction for microbial protein, may do more damage than good.

1.1.4 Mobile Bag Technique

The mobile nylon bag technique for determining nutrient digestibility in the intestine was initially developed for pigs (Sauer et al., 1983) and adapted for use in ruminants by many laboratories including that of de Boer et al. (1987). A nylon bag containing approximately 1 g of sample, is first incubated in the rumen for 12 h, then removed and inserted in the intestinal tract via a duodenal cannula. The bag is collected from the feces and washed. Intestinal digestibility is calculated as the difference between total tract disappearance of the nutrient under investigation and disappearance of that nutrient after the 12 h ruminal incubation.

Due to the disadvantages of the in vivo procedure discussed previously, the mobile bag technique has been accepted as the method of choice for determining intestinal digestibility of feedstuffs (Hvelplund and Weisbjerg, 2000).
1.1.5 Chemical Analysis Techniques

For many years, forages have been characterized by chemical analyses as reviewed by Cherney (2000). Empirical equations to predict digestibility and/or degradability are commonly used by feed testing laboratories. One example is the equation developed to predict total digestible nutrients (TDN) (NRC 2001) which is based on ADF content. Several authors have reported prediction of protein degradability from feed composition. Madsen and Hvelplund (1985) and Waters and Givens (1992) observed a linear relationship between the effective degradability of CP and CP content of grass silage. Waters and Givens (1992) also noted that the potentially degradable (sum of A and B) fraction was negatively correlated with NDF content of primary growths of ryegrass. Antoniewicz et al. (1995) developed regression equations based on in situ analysis from seven samples of grass to predict potentially degradable CP and found a strong relationship ($r^2 = 0.93$) with sample CP content. In contrast, von Keyserlingk (1994) found no relationship between any of the ruminally degraded CP fractions and CP content of grass or corn silage but did note a relationship between effective degradability of CP in grass silage (22 samples) and initial NDF content. These equations tend to be population dependent (Weiss, 1994) and work well for samples of a similar nature. This limits their widespread use for predicting degradability of unknown samples or samples collected from different years, geographic location or different forage types.

1.1.6 NIRS Technique

Norris et al. (1976) introduced the use of the NIRS technique for the analysis of forages. However, it was Bouger in 1760 who first reported the concept of diffuse reflectance, and Seelinger in 1888 who realized that diffuse reflectance could originate from the surface and from the interior of a substance (Osborne et al., 1993). The theory of NIRS is complex and explained in depth by Dahm and Dahm (2001), Miller (2001), and Osborne et al. (1993).
1.2 NIRS

1.2.1. Theory of NIRS

NIRS relies on the disciplines of physics (Dahm and Dahm, 2002) and chemistry (Miller, 2001). Diffuse reflectance (R) is measured and related to absorbance (A) through the relationship $A = \log I/R$. The Beer Lambert Law describes the linear relationship between absorption of electromagnetic radiation and analyte concentration. NIRS extends this concept by relating the absorbance measured as $R$ at many wavelengths to analyte concentration.

In addition to water, plants consist of protein, fat and carbohydrates, which in turn consist of amino acids, fatty acids, and sugar molecules, respectively. These molecules have a structure defined by atomic composition, conformation, isomeric structure, and stereochemistry (Miller, 2001). When electromagnetic radiation is directed at a substance, it can be absorbed or transmitted, depending on molecular structure of the substance. Since electromagnetic radiation is a form of energy, each molecule gains energy and will undergo a transition from one energy state to another. Radiation from the infrared portion of the electromagnetic spectrum causes the transition between vibrational energy levels. The energy of a light particle, or photon, depends on its wave properties, expressed as wavelength, wavenumber or frequency. Quantum theory dictates that a molecule will only absorb light energy that corresponds to the difference between two of its vibrational energy levels (Miller, 2001). Vibrational energy levels include a fundamental energy level, which usually occurs within the mid-infrared region between 2600 and 15000nm. The visible region of the electromagnetic spectrum is from 300 to 750 nm, while the near infrared region is from 750 to 2600 nm (Figure 1.2). As light energy is directly related to the wavelength of light, it can be concluded that a particular molecule can absorb only certain wavelengths. This makes analytical spectroscopy possible.
1.2.2 Development of NIRS

During the 1950's, the United States Department of Agriculture initiated a research program to develop rapid analytical technologies for agriculture (Osborne et al., 1993). As a result of this program, the first instrument, which combined NIRS spectra with correlation statistics, was introduced to the market in 1971. The Canadian Grain Commission began evaluating this technique for assessing wheat quality and in 1975, NIRS was adopted by the Canadian Grain Commission as the official method for protein testing of wheat (Williams, 1975). Early NIRS instruments such as GAC and the InfrarAlyser 2.5 A, both manufactured by Dickey-john (Osborne et al., 1993) were filter instruments; that is, data were collected for light absorption at discrete wavelengths determined by the wavelength filters installed on the instrument. Norris et al. (1976) demonstrated the potential use of filter instruments in forage analysis by developing calibration equations to predict forage CP, ADF and NDF, DM digestibility and DM intake using the wavelengths 1680, 1940, 2100, 2180, 2230 and 2310 nm. These wavelengths were originally selected for prediction of oil, protein and moisture contents of grains and oilseeds (Norris et al., 1976). Norris et al. (1976) reported that these six wavelengths were suitable for the prediction of protein and fiber. However, these authors recommended that three of the original filters (1680, 2230 and 2310 nm) be replaced by filters to collect data for 1672, 1700 and 2336 nm, respectively, if NIRS was to be used for forage analysis (Norris et al. 1976).

As computer technology advanced, NIRS was adopted for use in many industries including grain, dairy products and alcoholic beverages (Osborne et al., 1993). During the 1980's, the scanning monochromator was introduced which allowed collection of spectral data over a continuous range from 400 to 2500 nm. These instruments are based on diffraction gratings that disperse light, usually from a quartz halogen lamp, into wavelengths (Osborne et al., 1993). It was also during this period that statistical methods were developed for data reduction and calibration (Osborne et al.,...
These developments in instrumentation and data management supported the acceptance of NIRS in the food, agricultural, chemical and pharmaceutical industries. Today, the use of fiber optic probes with NIRS technology has enabled advancements in the area of non-invasive diagnosis in medicine (Rempel and Mantsch, 1999).

Absorption data collected as log 1/R at different wavelengths by a scanning monochromator are represented graphically in Figure 1.3. The continuous spectrum is composed of many overlapping absorption bands (Shenk and Westerhaus, 1994). Height, location and width define these bands. Height is measured at the peak and the location of the band is measured as the wavelength at its peak. The width of a band is measured at 50% of peak height. Some absorption bands will appear only as shoulders (Shenk and Westerhaus, 1994). A typical NIRS spectrum (Figure 1.3) contains 7 to 10 peaks with many shoulders.

Generally, a fundamental vibration caused by a given grouping of atoms will always occur in the same area of the spectrum, although its exact position can be affected by variables such as particle size and temperature. Much work has been conducted (Osborne et al., 1993) towards the assignment of bands to functional groups resulting in correlation charts (Miller, 2001) and tables (Williams, 2001; Osborne et al., 1993).

1.2.3 NIRS – Transformation of Spectra

According to Barnes et al. (1989), individual spectra, collected as log 1/R, vary from one another due to three main reasons; 1) scatter of radiation at particle surface, 2) variable spectral pathlength through the sample, and 3) chemical composition of the sample. Radiation scatter is due to the physical nature of the sample particles while spectral pathlength is dependent on particle size (Barnes et al., 1989).

Particle size variation is considered as interference, as it does not generally relate to chemical composition (Shenk and Westerhaus, 1994). Particle size differences shift the spectrum according to
grind size (Figure 1.4). Derivative transformations of spectra remove base line shifts but do not correct for the effects of multiplicative scatter. Barnes et al. (1989) proposed using the standard normal variate method (SNV) of transforming spectral data. This technique calculates the mean and the standard deviation of all Log1/R values recorded at each wavelength and then transforms the data using the formula:

$$\text{SNV}_{(1 \text{ to } n)} = \frac{(y_{1 \text{ to } n} - \bar{y}_{\text{mean}}) \sqrt{\sum (y_{(1 \text{ to } n)} - \bar{y}_{\text{mean}})^2}}{N-1}$$  \hspace{1cm} \text{Equation 1.3.}$$

where $y$ equals the log 1/R value for each sample at n wavelength, $\bar{y}_{\text{mean}}$ equals the mean absorbance value at n wavelength and $N$ = number of wavelengths for which log1/R data are collected.

Spectra transformed by SNV have a mean of zero, a variance equal to one and are, therefore independent of the original linear scale and sample set characteristics (Dhanoa et al., 1994). This transformation serves to minimize the noise in the spectrum due to particle size as shown in Figure 1.5 and enables the calibration model to be based on chemical information.

NIRS spectra, as shown in Figure 1.3, tend to be linear in nature between 1100 and 2500 nm. However, a spectrum can become curvilinear especially for samples of densely packed powder such as finely ground forages or manure. The amount of curvature will vary with particle size and packing density (Barnes et al., 1989). The statistical procedure of de-trending uses least squares to fit a straight line to the absorbance data. The predicted absorbance data for each wavelength are then subtracted from the original absorbance data thereby creating a new matrix of absorbance data (Figure 1.6).
1.2.4 NIRS – Population Structuring

NIRS relies on an empirically derived mathematical relationship between the amount of electromagnetic radiation absorbed at defined wavelengths in the NIR region and the composition of a set of reference samples. The relationship is determined by multivariate regression analysis where the dependent variable (Y) is regressed on multiple independent variables, which are the absorbance (log1/R) values.

The number of independent variables is contingent on the parameters set by the scientist when collecting the spectra. As many as 2,100 points could be collected if absorption data were collected for every wavelength between 400 nm and 2498 nm. Much of these data are redundant and neighboring absorbance values in spectra are highly correlated (Shenk and Westerhaus, 1994). Advancing computer technology has enabled rapid processing by computer programs designed to facilitate statistical processing of spectral data. One of the common first steps is to reduce the quantity of spectral data through principal component analysis (PCA) (Esbensen et al., 1994). For example, if absorption data are collected at 1940 and 2280 nm from each of 10 samples of ground corn silage, each sample can then be represented in two-dimensional space by its absorption at 1940 and 2280 nm (Figure 1.7). In practice however, spectral data are often collected for a minimum of 700 wavelengths (for example, every 2 nm from 1100 to 2500 nm) and therefore, are represented in high-dimensional space. The PCA procedure provides a way of reducing the dimension of space representing the data (Osborne et al., 1993) by projecting each point onto a new variable known as a principal component (PC). The new variable is a linear combination of the original data. In this way patterns in spectra due to factors such as particle size, moisture content and/or chemical differences that contribute to variation between spectrum are identified. The first new variable (PC1) is determined using the statistical procedure of least squares; that is, the line that minimizes the squared distance between actual and projected. Therefore, PC1 will follow the direction that maximizes the
variance in the spectral data set. In the case of NIRS spectra, this variation is usually ascribed to factors that affect light scatter such as particle size (Shenk and Westerhaus, 1994). The second variable (PC2) will be orthogonal to PC1 and lies in the direction of the second largest variance which, in NIRS spectra, is often attributed to differences in sample moisture content (Shenk and Westerhaus, 1994). Each additional PC will be orthogonal to the last, and represent successively smaller variances until all information has been represented. The maximum number of new variables (PCn) derived from a data set is equivalent to the number of spectra collected or the number of wavelengths for which absorption data were collected, whichever is smaller. Once PC analysis is complete, each spectrum will be represented by a new set of co-ordinates equivalent to the number of new variables (PCn). The new coordinates for each sample in this PC dimensional space are called scores (Esbensen et al., 1994).

Defining a set of samples having accurate reference values that will adequately represent future populations of samples to be analysed is important in developing a NIRS calibration. It is possible to obtain a large number of samples and perform wet chemical analyses on every sample. This approach of analyzing every sample, however, would be very expensive if calibrations are to be developed for predicting degradability as determined by the in situ procedure. The program SUBSET, which uses correlation between spectra to identify spectrally unique samples was developed by the National NIRS Forage Research Project Network in order to address this problem (Windham et al., 1989). In the first step of the SUBSET procedure, correlation coefficients were calculated between the spectra for each pair of samples. Samples having a correlation coefficient less than 0.95 with any other sample were eliminated as being too dissimilar to be included in the population. The procedure then identified any sample with a correlation greater than 0.997 with any other sample in the population as being too similar for inclusion in the sample set. The SUBSET procedure was not always easy to apply, especially if the sample population was homogenous (Windham et al., 1989).
Shenk and Westerhaus (1991) proposed using scores generated by PCA to identify samples for inclusion in the calibration sample set. In the CENTER procedure, each sample is given an H value, which represents its Mahalanobis distance from the average spectra (Figure 1.8). In this way spectral outliers are identified as samples with large H values which lie far away from the average spectra. Shenk and Westerhaus (1991) suggest a limit of 3.0 for H. Careful consideration must be given to inclusion of samples with H values greater than 3.0 as extreme samples will influence wavelength selection and coefficient size during subsequent calibration procedures (Shenk and Westerhaus, 1991). Spectral outliers may be due to a number of factors including grinding (uneven particle size), and/or inconsistent scanning procedures. Spectral outliers may belong to a different population of samples or be contaminated by foreign substances such as weeds or dirt. If the sample appears to be a valid member of the population, the high H value may be due to differences in chemical composition and the sample should be retained in order to broaden the base of the calibration equation.

The second procedure, SELECT, described by Shenk and Westerhaus (1991) eliminates samples with similar spectra. Similar to CENTER, the procedure uses Mahalanobis distance, but this time the distance is calculated between pairs of samples. For this algorithm, Shenk and Westerhaus (1991) suggest a cut off value of 0.6; that is, if the distance between two spectra (representing two samples) is less than 0.6, one of the spectra (samples) is eliminated. The procedure is based on the assumption that only one sample is needed to represent all neighboring samples (Shenk and Westerhaus, 1991). These authors estimate laboratory cost savings of some 60% when the SELECT procedure is used to eliminate redundant samples (Shenk and Westerhaus, 1991).

According to Shenk and Westerhaus (1994), selecting a representative sample set is of primary importance to successful calibration development. A good collection of spectra should be symmetrically distributed along the PC dimensions. The SELECT procedure is useful in the identification of gaps in spectral information. It is also important for the identification of new
samples whose spectra can fill these gaps and strengthen the calibration equations developed for this sample set.

1.2.5 NIRS – Calibration

Once the calibration sample set has been selected, and the appropriate laboratory analyses completed, the process of developing calibration equations can begin. Decisions will be required regarding mathematical treatments for transforming spectral data, method of regression analysis and lastly, wavelength segments to include in the model. Unfortunately, there is no standard combination of these factors that works well in all situations and it is only by trial and error that a combination may be determined for each sample set under investigation.

Mathematical treatments for the transformation of spectral data, such as SNV and de-trend, have been discussed previously. Another method of mathematical transformation commonly used is derivatization, which helps to resolve the problem of overlapping peaks and baseline shifts (Osborne et al., 1993). In NIRS calibration, derivative transformation refers to differences between spectral data taken at closely spaced wavelengths (Mark, 1991). Therefore a first derivative would be calculated as

\[ X = A_{(2)} - A_{(1)} \]  

Equation 1.4

where \( X \) is the independent variable and the \( A \)'s represent \( \log 1/R \) values at two wavelengths. The distance between these wavelengths is called the gap. In addition, a smoothing procedure may be applied in which \( \log 1/R \) values are summed over wavelength segments, and these sums are used for the derivatization procedure. For example, a mathematical treatment of 1,4,4,1 (derivative, gap, smooth, second smooth) would sum \( \log 1/R \) data over four wavelength points (smooth) and then calculate the difference at every fourth sum (gap) to calculate the first derivative. A second derivative would be calculated as the difference of two adjacent first derivative measurements (Osborne et al., 1993).
As noted previously, NIRS relies on the mathematical relationship between the composition of a set of reference samples and the amount of light absorption (Log 1/R) at defined wavelengths. This relationship is defined as

\[ Y = B_0 + B_1X_1 + B_2X_2 + \ldots + B_pX_p \]  

Equation 1.5

where Y is the laboratory reference value, \( B_0 \) is the equation intercept, \( B_1 \) through \( B_p \) are coefficients or scaling factors relating changes in X to Y, and p is the number of X variables, in this case, corresponding to the number of wavelengths. The values of X are the log1/R or absorbance values.

Investigators exploring the feasibility of using NIRS to predict a constituent can choose from several methods of multiple regression. Among these are step-up, stepwise, partial least squares (PLSR), modified partial least squares (MPLSR) and principal component regression (PCR). Step-up and stepwise regression techniques begin by choosing the wavelength (X) that gives the best single term equation, and then continue in steps to choose wavelengths to add as successive variables. The failure of an added term (addition of another wavelength) to show statistical significance is usually the criterion to stop the regression process (Osborne et al., 1993). Although these regression methods work well for filter instruments with a small number of wavelengths, they are not as easy to utilize with the voluminous spectral data provided by a scanning monochromator. As discussed previously, it is not unusual to have 700 log 1/R values available as independent variables for equation development. The challenge resides in choosing the wavelength which best serves as the starting point for regression development. Once that wavelength is chosen, there are 699 wavelengths and combinations thereof which can be added to the equation. It is a time consuming and laborious process and as a result, software programs such as WinISI Version 1.05 (Infrasoft International LLC, Port Matilda, PA, USA) no longer utilize step regression to develop multivariate (calibration) equations.

Principal component (PCR) and partial least squares (PLSR) regression, called global or full spectrum regression, are easy to use and efficient in their use of spectral information (Osborne et al.,
Both methods produce a small number of factors that account for a majority of the variability in spectral data. As discussed previously, PC analysis accounts only for variability in spectral information and does not relate this variability to laboratory reference data. Principal component regression uses the PC scores calculated through PCA as the independent variable for multiple regression. The PLSR algorithm (Martens and Naes, 1989) reduces spectral data to a few factors that relate to sample reference values as well as to spectral information (Shenk and Westerhaus, 1994). Since PLSR has already related spectral variability to reference data, calibration equations developed using PLS require fewer factors (Osborne et al., 1993) and is a preferred method for equation development (Shenk and Westerhaus, 1994). The PLSR computes and adds factors to the equation iteratively; that is one factor, called a term, is constructed and added to the equation (Shenk and Westerhaus, 1991). As with step regression algorithms, it is important to avoid the temptation to add numerous terms to the equation. Performance statistics will improve as terms are added, but the model may lack robustness (Osborne et al., 1993).

The cross validation procedure, which allows one set of samples to act as calibration and validation set, is used in conjunction with PCR and PLSR algorithms to avoid overfitting (Shenk and Westerhaus, 1991; Osborne et al., 1993). Originally, cross validation was performed by removing one sample from the calibration group of samples, and a calibration equation developed using the remaining samples. This equation is then used to predict the composition of the removed sample and a prediction error is calculated. The cross validation procedure continues until every sample has been predicted. As terms are added to the equation, the prediction error (standard error of cross validation, SECV) will decrease, reach a minimum and then start to increase (Osborne et al., 1993). The point at which SECV is at a minimum indicates the optimum number of terms to be included in the equation. With large data sets, performing the cross validation procedure using single samples is onerous. As a result, cross validation is most often performed using groups of samples. One group is separated, and a calibration equation developed using the remaining groups of samples. In the
same way, the optimum number of terms to be included in the equation will be determined by the minimum SECV.

The last variable to be manipulated in the calibration exercise is that of choosing the wavelength segment. This decision will be dependent on the wavelength segments for which spectral data were collected. The collection of data from 400 to 2500 nm, allows the operator to investigate a minimum of two or three wavelength segments. These segments are dependent on operator preference and may include 400 to 2498 nm, 800 to 2498 nm or 1100 to 2498 nm, for example. The robustness of the equation developed using each segment is evaluated using calibration statistics such as SECV, as discussed below.

### 1.2.6 NIRS - Statistics

The discipline of statistics plays a pivotal role in NIRS. As discussed above, transformation of spectra and population structuring use established statistical procedures such as Mahalanobis distance and principal component analysis. NIRS also relies on measurements of uncertainty referred to as the standard error of difference (Shenk and Westerhaus, 1994) as computed by:

\[
\text{Standard Error of Difference (SED)} = \sqrt{\frac{\sum (D)^2}{n}}
\]

where D is the difference between two measurements such as the difference between the predicted value and the laboratory reference value for each sample. Examples of this statistic are the standard error of calibration (SEC), the standard error of validation (SEV) which is also called the standard error of prediction or performance (SEP) and the standard error of cross validation (SECV).

Predicting the original set of samples used to develop the calibration model generates the SEC statistic. It is calculated as the difference between the predicted and the reference values for each sample. It is not a very useful statistic and has limited use.
In many research studies, samples are divided into calibration and validation sets. This can be done manually by choosing every “i”th sample, or manually choosing a set of samples which represents the full range in composition, or statistically using Malahanobis distance as discussed previously. In the latter instance, spectrally unique samples are collected into the calibration set and those not unique are designated as the validation set. A calibration is developed using the data from the calibration set of samples, and this calibration is then used to predict samples in the validation set. The difference between predicted and laboratory reference values for the validation samples is called the SEV or SEP. As discussed by Williams and Sobering, (1996), this approach can be misleading especially if validation samples have been chosen to represent the full range in composition. These authors (Williams and Sobering, 1996) recommend the cross validation procedure, which, as discussed previously, involves dividing the calibration set into groups. One group is removed and a calibration equation developed using the remaining groups. This equation is then used to predict the separated group. In this case, the difference between the predicted value and the laboratory reference for those samples in the separated group will be the SEV. At the end of the cross validation process, there will a number of SEV values equivalent to the number of groups formed for cross validation. These SEV values are averaged to produce a single value for SECV.

The coefficient of determination, $R^2$ is often used as a measure of success in predicting the reference value $Y$ using multivariate regression.

$$R^2 = 1 - \frac{\text{residual sum of squares}}{\text{total sum of squares}} = \frac{(SEC)^2}{(SD)^2} \quad \text{Equation 1.7}$$

The $R^2$ statistic equates to the ratio of variability of $Y$ about the fitted line to the variability of $Y$ around its mean (Fearn, 2000). In a similar manner, the variance ratio (VR) is computed as $(SECV)^2 / SD^2$ when using the cross validation procedure and the statistic $1-VR$ is often reported when SECV statistics are reported.
Williams (1987) introduced the RPD (ratio of standard deviation of the reference data to the 
SECV) statistic as a means of evaluating calibration equations. The RPD statistic, calculated as the 
quotient of the SD of the reference set divided by the SECV, should be as high as possible. Williams 
(2001) recommends that RPD values between 0 and 2.3 are not acceptable, between 2.4 and 4.9 are 
acceptable for screening purposes, between 5.0 and 6.4 enable the equation to be used for quality 
control and above 6.5 the equation can be used in process control.

In summary, the development of a calibration equation using NIRS begins with collecting 
samples which are representative of the population for which analysis will be required in the future 
(Figure 1.9). These samples will be scanned and the mathematical transformations performed to 
reduce the quantity of spectral data. Using the Mahalanobis distance statistic, samples to be sent to 
the laboratory for chemical analyses are selected. Once the reference data are entered for each 
sample in the calibration set, multivariate regression will be used to find the line of best fit. Statistics 
such as SECV, 1-VR and RPD will be used to evaluate the usefulness of the equation.

1.3 NIRS – Predicting Forage DM and CP Degradability

Since the original work of Norris et al. (1976), the use of NIRS for routine analysis of forage 
has become standard practice for many commercial and research laboratories. The current scientific 
literature reports research regarding NIRS and forage quality from laboratories in many countries 
including Australia, Canada, France, Germany, Holland, Norway, Poland, the United Kingdom and 
the United States. Over the past decade, 14 reports have been published outlining results from 
research investigating the feasibility of using NIRS to predict forage DM and/or CP degradability as 
determined by the in situ technique (Table 1.1).

1.3.1 Grass Forage

Waters and Givens (1992) initially reported the feasibility of using NIRS to predict ruminal 
grass CP degradability. Nineteen samples of fresh ryegrass were dried, ground and spectral
information for each sample collected between 1100 and 2500 nm. The spectral data were transformed using a first derivative procedure (1,10,5,1). The method of multiple regression employed in this study was not reported. Due to the small sample size, no validation procedure was implemented and equation robustness was evaluated based on the $R^2$ and SEC statistics. The $R^2$ statistic ranged from 0.184 to 0.745 for the degradation rate of the potentially degradable CP fraction and the effective degradability of CP, respectively. Waters and Givens (1992) divided this limited data set according to classification of the sample as primary or regrowth and found that calibration statistics improved for all degradability parameters. These authors found that spectra in the regions of 2050 to 2190 and 2212 to 2342 nm which are each associated with sulphur and amide bonding (Miller, 2001) accounted for most of the variation in soluble and potentially degradable CP (Waters and Givens, 1992).

Halgerson et al. (1995) reported results of a study using a sample set of perennial grass (n=152) and alfalfa (n=100). Spectral data were collected using two types of scanning monochromators but wavelengths were not specified in the report. The spectral data were subjected to de-trending and samples for calibration were selected using the H statistic although its value was not reported. Approximately 50% of the samples of grass and alfalfa respectively were classified as spectrally different resulting in 67 grass and 49 alfalfa samples being used for calibration. Calibrations were developed using PLSR, a mathematical treatment of 1,4,4,1 and cross validation to avoid overfitting. While results were reported as CP degradability, the experimental method employed measured forage protein disappearance after 12 h (grasses) or 16 h (alfalfa) ruminal incubation. Results were reported on a DM and CP basis. Based on the 1-VR statistic, NIRS could account for 98 and 86% of the variation in CP disappearance (%DM) for alfalfa and grass respectively. However, calculation of the RPD statistic from the SECV and SD data reported showed that the calibration developed to predict ruminal disappearance of perennial grass CP (%DM) was adequate only for screening purposes. Performance was better for alfalfa CP disappearance (%DM)
as the RPD statistic was 6.6 versus that for grass of 2.72. However, when data were expressed on a %CP basis, the RPD statistic for both grass and alfalfa decreased to 1.06 and 4.05 respectively. Halgerson and co-workers (1995) postulated the decreased performance was due to increasing error contributed by the CP laboratory procedure. This study is noteworthy if consideration is given to replacing the multi-hour in situ procedure with a single time point incubation as suggested by Ørskov and McDonald (1979) who proposed that CP disappearance after a 24 h ruminal incubation could be used to predict CP degradability in the rumen.

De Boever et al. (1998) investigated the feasibility of using NIRS to predict the effective degradability of CP in grass and alfalfa differing in maturity. From the sample description reported by De Boever et al. (1988), it would appear that the grass (n=16) and alfalfa (n = 38) samples used in the study by Antoniewicz et al. (1995) discussed previously were incorporated into this study. Forty-five samples of non-ensiled grass and 35 samples of alfalfa were added to the sample set. De Boever and co-workers (1998) noted that the independent validation set of grass samples used for this study, was those used by Waters and Givens (1992) as discussed previously. This choice of validation set introduced bias into this study. The data used for calibration were derived from in situ studies using ruminally-cannulated cattle in which dried and ground samples were incubated for a total of 48 h. The data used for validation (Waters and Givens, 1992) were derived from in situ studies using cannulated sheep in which fresh chopped samples were incubated for 72 h. In both cases, effective degradability was calculated using a ruminal turnover rate of 4% h⁻¹.

Spectral data were collected by scanning dried, ground samples from 1100 to 2500 nm, at 4 nm intervals (De Boever et al., 1998). Calibrations were derived using either linear regression analysis (MLR) or PLSR. Spectral data used for MLR were transformed using first and second derivatives but were left as raw log 1/R values for PLSR. In addition to the validation set of grass samples supplied by Waters and Givens (1992), the samples were divided into nine subsets for cross
validation using multiple linear regression. Cross validation of the calibrations determined by PLSR was conducted using 20 sub-sets chosen by the computer software.

As expected, increasing the number of wavelengths used in MLR resulted in higher $R^2$ and lower SEC values for calibration equations predicting the effective degradability of CP in grass and alfalfa. Using MLR, NIRS could account for 92.4 to 93.1% and 91.4 to 93.9% of effectively degradable CP values of grass and alfalfa, respectively. The PLSR method could account for 85.2% and 93.3% for the effectively degradable CP of grass and alfalfa, respectively. De Boever et al. (1998) also developed regression equations based on maturity, CP and crude fiber (CF) to predict the effective protein degradability of grass and alfalfa. The NIRS calibration equations showed superior performance as the equations based on maturity, CP and CF could only account for 17.7 (maturity) to 79.5% (CP and CF) of the variation in the effective degradability of grass CP and 35 (maturity) to 82.3 % (CF and maturity) for alfalfa CP.

In regard to validation, De Boever et al. (1998) reported only the SEP obtained by predicting the effective degradability of grass silage CP in samples from Waters and Givens (1992). The SEP, which ranged from 5.1 (PLSR) to 19.6 (raw log 1/R data, MLR) is considerably greater than the SEC for all derivatives and regression techniques, which would be expected in light of the differences in the calibration and validation sets as described above. Unfortunately, De Boever and co-workers (1998) did not report SD of effectively degradable grass silage CP, making it impossible to calculate the RPD statistic. Therefore, no conclusion can be drawn as to the feasibility of using these calibrations in routine testing of grass forages.

1.3.2 Grass and alfalfa forage

The feasibility of using NIRS to predict ruminal degradability of forage CP was investigated by Antoniewicz et al. (1995) using samples of fresh grass ($n = 16$) and alfalfa ($n = 38$) which varied in stage of maturity within cutting. Effective degradability was calculated using CP disappearance.
data from 2, 4, 8, 16, 24 and 48 h ruminal incubations, and ruminal turnover rate of 4% h\(^{-1}\). Spectral data were collected on dry, ground samples over the range of 1445 to 2348 nm using a fixed filter instrument containing 19 filters. Stepwise regression was used to develop a calibration equation for effective degradability of CP. Antoniewicz et al. (1995) evaluated equations containing three or six wavelength terms for each of the grass and alfalfa sample sets and five wavelength terms for a combined sample set of grass and alfalfa. These equations were chosen based on the highest coefficient of determination (R\(^2\)) and lowest SEC. Cross validation was performed for the combined set of grass and alfalfa (n = 54) by forming six sub-sets of nine samples each. Each sub-set was predicted by an equation formed from the remaining five sub-sets. Differences between predicted and in situ reference values were collected for each of the six sub-calibrations and averaged to compute the final standard error of performance (SEP).

Calibrations developed for grass and alfalfa using six wavelength terms had higher values for R\(^2\) and lower SEC values than those developed using three wavelengths. However, as noted by Antoniewicz et al. (1995), this may be due to overfitting of the equation. Separate calibrations were developed for a primary growth (first cutting) sample set and combined (primary and regrowth) set for both grass and alfalfa samples. It is of interest to note that, contrary to the data of Waters and Givens (1992), there was little loss in prediction accuracy when the combined sample set was used for calibration as shown by similar R\(^2\) and SEC values to those obtained for the primary growth set of samples. NIRS accounted for 90% of the variation in effective degradability of forage CP when calibrations were developed using the combined sample set of grass and alfalfa, five wavelength terms and cross validation. The best combination of wavelengths to predict effective degradability of forage CP was 1445, 1759, 1818, 2270 and 2310 nm. Interestingly, these wavelengths are recognized as principal absorption bands for cellulose and oil, rather than protein (Osborne et al., 1993; Williams, 2001).
In a study by Hoffman et al. (1999a), 108 dried and ground samples of alfalfa silage were ruminally incubated for 24 h. Using the CENTER and SELECT procedures in WinISI software (Infrasoft International LLC, Port Matilda, PN, USA), 41 spectrally unique samples were identified and used to develop a calibration equation to predict the amount of alfalfa CP remaining after a 24 h ruminal incubation (CPR-24). The spectral data were transformed using a first derivative mathematical treatment. The NIRS model accounted for 94% of the variation in CPR-24 values for the samples contained in the external validation set of 67 samples.

In a follow-up study, Hoffman et al. (1999b) developed calibrations to predict in situ protein fractions using 32 samples of legume and grass silage. Spectral data were collected from 1100 to 2498 nm for each dried and ground sample and transformed using a 1,4,4,1 or a 2,10,10,1 derivative. The regression method employed for calibration development was PLSR. Cross validation was not employed in this study and therefore, best equations for each in situ protein fraction were determined based on highest $R^2$ and lowest SEC.

Hoffman et al. (1999b) reported data for the amount of soluble CP, potentially degradable CP, degradation rate of the potentially degradable CP, as well as the amount of protein remaining (CPR) after 3, 6, 12, 24, 48 and 72 h of ruminal incubation. For all equations except CPR-12 h, a mathematical treatment of 2,10,10,1 produced the best calibration equation based on highest $R^2$ and SEC. NIRS could account for 94 to 96% of the variability for the above protein fractions. Validation, either through cross validation or an external validation set, was not performed. Hoffman et al. (1999b) concluded that the results were encouraging but, as with many of the research reports reviewed thus far, did not provide any means to assess the applicability of these calibration equations for use in forage testing programs.

In the last paper of this trilogy, Hoffman et al. (1999c) reported the strong relationship between the measurement of CPR-24 (the amount of CP remaining after 24 h ruminal incubation) in legume and grass silage and ruminally undegraded CP (RUP) as determined by the full in situ
procedure using multiple incubation times \((r^2>0.90)\). The content of RUP was calculated using the equation of NRC, 1989. The strong relationship noted by Hoffman et al. (1999c) lends support to the work discussed previously by Halgerson et al. (1995) suggesting that ruminal incubation at a single time point is sufficient to estimate RUP. The ability to use a single time point incubation procedure to determine RUP would enable collection of data for a greater number of feedstuffs at a decreased cost. However, previous reports by Antoniewicz et al. (1995), Halgerson et al. (1995) and De Boever et al. (1998) show stronger calibration statistics for sample sets composed of alfalfa over those comprised of grass silage. Further research is required to support the conclusion that NIRS can accurately predict a measurement such as CPR-24 determined using forages other than alfalfa.

Dorhurst and Hoffman (2000) continued the work of Hoffman et al. (1999a, b, c) by investigating the feasibility of using NIRS to predict RUP in legume and grass hays as determined by the in situ method. NIRS experimental protocols were similar to those described previously (Hoffman et al., 1999a, b, c). Using PLSR and a second derivative mathematical treatment \((2, 10, 10, 1)\), NIRS could account for 83% of the variation in ruminally undegradable CP observed in 106 samples of legume and grass hay. Dorhurst and Hoffman (2000) reported the SD of RUP\% in the calibration data set and an SECV statistic, although it is not clear what method of cross validation was employed in the study. Using these SD and SECV statistics, an RPD of 2.53 was calculated, indicating this calibration would be of use to screen forage samples according to ruminally undegraded CP content.

1.3.3 Barley Forage

Hsu et al. (1998) collected 108 samples of barley hay and silage and 195 samples of barley straw and evaluated the feasibility of using NIRS to predict DM degradability determined by in situ incubation for 0, 4, 8, 12, 24, 72, 120 and 240 h. Spectral data were collected for dry ground samples between 400 and 2498 nm, scanning every 2 nm. Three calibration sets were formed using a
Mahalanobis (H) distance of 0.36, 0.60 (Shenk and Westerhaus, 1991) and 0.80. These sets contained 202, 113 and 65 samples, respectively. Samples not chosen as spectrally unique were used for validation. The goal of this exercise was to determine optimal size of the sample set required to accurately predict in situ degradability values. The final calibration was developed on all 302 samples and cross validation was used to prevent overfitting. Spectral data were subjected to detrending and SNV and a derivative mathematical treatment of 3,10,10,1 was used for calibration development. Several statistics including 1-VR, SEC, SECV and RPD were used to ascertain the best calibration for each constituent.

Hsu et al. (1998) did not provide information regarding mean or range values for soluble DM, potentially degradable DM, degradation rate of potentially degradable DM, or effective degradability of DM assuming a rumen turnover rate of 6% h⁻¹. The authors drew attention to data for soluble and effective degradability of DM, presented in graphical form that clearly showed two populations of samples. As noted by Hsu et al. (1998), the amount of soluble DM was much lower in barley straw than in barley greenfeed or silage. Values of soluble DM extrapolated from the graphs appear to range between 5 and 25% for barley straw and 40 and 55% for barley greenfeed/silage. Similarly, the effective degradability of DM appeared to range between 20 and 35% and 50 and 65% for straw and greenfeed/silage respectively. NIRS was able to account for 99 and 98% of the variability for soluble and effective degradable DM, respectively. It is not possible, however, to comment on the robustness or usefulness of these calibrations as information regarding SD of the original data set was not provided.

Of interest are the conclusions reported regarding the optimal sample size to be used in developing calibrations for predicting in situ parameters. Hsu et al. (1998) concluded that using an H value of 0.8 in the SELECT procedure could reduce the sample size. However, examination of the data shows that the highest RPD value was achieved using the recommended H value of 0.6 (Shenk and Westerhaus, 1991). The findings of this study are not strong enough to warrant changing the
recommendation of Shenk and Westerhaus (1991) especially, as Hsu et al. (1998) acknowledge previous work (Reeves et al., 1991) showing decreasing prediction accuracy if calibration sample size is reduced.

As discussed previously, Hsu et al. (1998) investigated the feasibility of using NIRS to predict degradability of barley straw, greenfeed and silage. These authors reported that the amount of DM that was soluble and was effectively degraded at a rumen turnover rate of 6% h\(^{-1}\) was distinctly different for straw versus greenfeed/silage populations. Mathison et al. (1999), using the same set of 195 straw samples, investigated the use of NIRS to predict chemical composition and DM degradability fractions and compared the results of this study to the original work of Hsu et al. (1998). Spectral data were collected between 400 and 2498 nm by scanning the dried and ground samples. The H statistic, set at 0.6, identified 110 spectrally unique samples that were used for calibration. The remaining 85 samples were used as an external validation set. Cross validation was used to prevent overfitting of the PLS developed equations. Spectral data were transformed using a third derivative (3,10,10,1) mathematical treatment, de-trending and SNV. In addition to developing NIRS calibrations, Mathison et al. (1999) used stepwise linear regression to determine relationships between chemical constituents and degradable fractions as determined by the in situ procedure.

Although Mathison et al. (1999) did not report range or SD values for each degradability fraction, the RPD statistic was reported based on the SEP obtained using the external validation set. In addition, these authors reported the 1-VR statistic, a measure of variance accounted for in calibration cross validation.

An RPD value of 2.5 and 2.9 was reported for soluble DM and effective DM degradability calculated using an outflow rate of 3% h\(^{-1}\), respectively indicating these calibrations may be used as a screening tool in forage testing programs (Williams, 2001). Interestingly, the RPD statistic fell to 2.03 when effective degradability was calculated using an outflow rate of 6% h\(^{-1}\) although no explanation was offered by Mathison et al. (1999) for this observation.
NIRS could only account for 69 and 18% of the variation found in the external validation set for the amount and rate of degradation of the potentially degradable DM respectively. The RPD statistic for each calibration was 1.77 and 1.10 respectively indicating that NIRS may have limited use for determining this fraction or rates of degradation. Likewise, Mathison et al. (1999) did not find a significant relationship between any chemical constituent and rate of degradation as the best multiple regression equation, based on CP and ADF, could only account for 5% of the variation in degradation rate measurements for samples in the external validation set. Similarly, the multiple regression equation based on chemical analyses for NDF, ADF and lignin contents did not perform statistically better than the calibration model developed for NIRS prediction of the potentially degradable DM fraction (Mathison et al. 1999).

1.3.4 Mixed Forages

Todorov et al. (1994) investigated the degradability of DM and CP in 34 ground samples of green grass, legume hay, haylage, dehydrated alfalfa and wheat and barley straw. Spectral data were collected between 1618 and 2318 nm. Calibration equations were derived for the soluble fraction, potentially degradable fraction, degradation rate, and effective degradability of DM and CP using a 1,4,4,1 mathematical treatment of spectral data. The $R^2$ values ranged from 0.60 to 0.93 and from 0.46 to 0.84 for degradability parameters of DM and CP respectively. According to Todorov and co-workers (1994), no validation of these equations was performed due to the small sample size and statistics required for computation of RPD were not reported. The relationship between NIRS data and CP degradability, as measured by effective degradability was weaker ($R^2 = 0.67$) than that between NIRS and DM degradability ($R^2 = 0.93$). Todorov et al. (1994) postulated this observation was due to microbial contamination of in situ residues. In light of the work by Waters and Givens (1992) showing stronger calibrations when samples were grouped to increase homogeneity, it could
be postulated that the weaker relationship between NIRS and CP degradability was due to the very diverse set of samples used by Todorov et al. (1994) for calibration. It would have been of interest for these authors to rebuild calibrations using only the 19 samples of grass and legumes to ascertain the effect of sample diversity on calibration success.

Swift et al. (1994) reported the feasibility of using NIRS to predict rumen soluble N content of 40 samples of corn silage (12), grass silage (18) and grass hay (10). This study used in situ reference data provided by von Keyserlingk (1994). Using the CENTER and SELECT procedures (WinISI version 1.05, Infrasoft International LLC, Port Matilda, PN, USA), 25 spectrally unique samples were used for calibration development. The best calibration equation as determined by a coefficient of determination ($R^2$) of 0.94 was derived using a second derivative mathematical transformation. The equation was tested using an external validation set formed from the remaining 15 samples. The SEP obtained was 12.6% and the authors concluded that additional samples were necessary in order to improve calibration performance (Swift et al., 1994). As discussed previously, the diversity of the sample set may have negatively impacted calibration success as measured by a high SEC value.

De la Roza et al. (1998) investigated the feasibility of using NIRS to predict DM and CP degradability, using in situ values as the reference data. The sample set of 53 corn and 77 grass silages was freeze-dried, ground and scanned over a wavelength range of 400 to 2500 nm. Samples exceeding the maximum H statistic of 3.0, were excluded from the data set, as recommended by Shenk and Westerhaus (1991). Scatter correction techniques such as SNV, detrend and a combination of SNV and detrend were evaluated as well as first and second derivative mathematical treatments. The regression method employed was PLSR and cross validation was used to avoid overfitting. Equations were evaluated according to lowest SECV and highest 1-VR. In addition, De la Roza et al. (1998) reported RPD statistics for each calibration developed.
Unacceptable RPD statistics were reported for all measures of DM degradability except for the sum of the soluble DM and potentially degradable DM where the RPD value of 3.27 would enable this equation to be used for screening of forage samples. Values of RPD were higher for all measures of CP degradability ranging from 1.76 (degradation rate of potentially degradable CP) to 3.00 for soluble CP. Except for the ability of NIRS to predict degradability rate of DM or CP, it was concluded that accuracy of the NIRS calibrations was high or promising, but was dependent on the accuracy of the reference data (De la Roza et al., 1998).

1.3.5 Corn

Recently, De Boever et al. (2002) reported in situ protein fractions for 26 samples of corn silage grown and harvested from 1991 to 1998. This study was unique in that the samples were not dried and ground before incubation in the rumen; rather, frozen samples were chopped, sealed in nylon bags and inserted into the rumen and residues collected at 12, 24, 48, 72 and 288 h. Potentially degradable CP was determined as the difference between 100 and the sum of the soluble and the undegradable CP (288 h residue CP). A passage rate of 4.5% h\(^{-1}\) was used to estimate effectively degradable CP. The amount of CP escaping the rumen (%EP) was calculated as the sum of 5% of the soluble CP, plus the portion of the degradable CP escaping the rumen due to ruminal turnover, plus the undegradable CP. Spectral data were collected for the dried and ground samples between 1100 and 2500 nm in 4 nm increments. Calibrations to predict %EP were developed using PLSR and cross validation.

NIRS could account for 69% of the variation in %EP determined for 26 samples of corn silage. A simple linear regression developed using soluble CP as the independent variable, could account for 97% of the variation in %EP for the 26 samples of corn silage. This is, however, not a valid comparison as NIRS was based on dried and ground samples whereas the in situ fractions used to calculate %EP and soluble CP were determined using intact chopped samples.
In summary, the review of these 14 research reports, spanning the last 10 years, show there is no definitive answer to the question as to whether NIRS can accurately predict soluble and degradable fractions of DM and CP as determined by the in situ technique. Unfortunately, many of these studies did not use validation procedures, or used external validation sets spectrally similar to those samples used for calibration. A statistic such as RPD can be used to evaluate the potential of a calibration model for accurately predicting a constituent in an unknown data set. Many of these studies did not report a RPD statistic, nor did they provide data that could be used to calculate the RPD.

Other than the studies reported by Waters and Givens (1992) and De Boever et al. (2002) which were based on 19 and 26 samples, respectively, there have been no studies using sample sets of only grass or corn silage (Table 1.1). While size of the sample set is not the determining factor, the sample set must incorporate all variation expected in analysis of unknown samples in order to develop robust calibrations (Shenk and Westerhaus, 1994). In addition to the relatively small sample size, the work of De Boever et al. (2002) was compromised by inconsistent experimental methods used to determine degradability of samples used for calibration versus those used in validation. In addition, there have been no studies investigating the development of NIRS calibration models to predict grass silage CP degradability. Based on these studies, no conclusions can be drawn as to the feasibility of using NIRS to predict DM and CP degradation fractions in corn or grass silage as determined by the in situ technique.

The review does indicate that NIRS could play a valuable role in forage quality programs, either by providing information to plant breeders for screening new varieties or to ruminant nutritionists, who at the very least, could screen samples according to high versus low ruminal DM and CP degradability. In view of the importance of grass and corn silage in ruminant rations worldwide, it would be beneficial to further investigate the possibility of using NIRS to predict ruminally degradable fractions of DM and CP, using large sample sets of these two forages. It would
also be of interest to further explore the concept introduced by Halgerson et al., (1995) and Hoffman et al., (1999c) of using single time point incubation for determination of ruminal degradability of CP in grass and corn silage.

1.4 NIRS – Predicting Forage DM and CP Intestinal Digestibility

A search of the scientific literature did not yield any publications regarding the use of NIRS to predict intestinal digestibility of forage DM and/or CP as determined by the mobile nylon bag technique.

1.5 NIRS – Predicting Forage Amino Acid Composition

The majority of published work regarding the use of NIRS for the prediction of AA composition of feedstuffs is with concentrates such as wheat and barley, rather than forage. One of the first papers published was by Rubenthaler and Bruinsma (1978) who reported successful calibration development for prediction of lysine in wheat. Subsequently, Williams et al. (1984, 1985) and Kaffka (1988) reported that NIRS could be used to rapidly determine the AA composition of homogenous sets of wheat, barley, peas and lupins.

More recently, Fontaine et al. (2001) investigated the applicability of using NIRS to predict AA composition of vegetable protein meals, field peas, and animal by-product meals. Sample set size ranged from 59 for poultry by-product meal to 333 for meat and bone meal. Material was ground through a 0.5 mm screen before spectral data were collected for the wavelength segment 1100 to 2500 nm. Data were subjected to de-trending and SNV, and transformed using first or second derivative mathematical treatments. The regression method was MPLSR using cross validation of five or six sub-sets of samples.

Calibration and validation statistics were inferior for Met and Cys as compared to Lys, Thr, Trp, Arg, Ile, Leu and Val (Fontaine et al. 2001). As explained by Fontaine et al. (2001), this was likely due to the error inherent in the analytical procedures used for the determination of these AA.
RPD statistics were presented in graphical form showing a range within each AA, dependent on feedstuff. For example, RPD statistics for Lys ranged from 2.5 to 4.0 for sunflower meal and meat meal respectively. The author’s conclusion that the RPD statistic ranges from 3 to 7 for all calibrations except sulphur AA in peas and cystine in fish and poultry meals is not supported by the graphical representation of these data (Fontaine et al. 2001).

Consideration of feedstuff AA composition in the formulation of ruminant diets is a relatively new phenomenon (O’Connor et al. 1993). Although there are several research reports detailing the AA composition of forages, there are few if any reports regarding the use of NIRS for the prediction of forage amino acid composition. In view of the mixed results using large sets of homogenous feedstuffs such as soybean meal (Fontaine et al.2001), it would be of interest to determine the feasibility of using NIRS to predict AA composition of very heterogeneous feedstuffs such as forage.

1.6 Objectives

The first objective of this thesis is to provide a definitive answer regarding the feasibility of using NIRS to predict degradable DM and CP fractions as determined by the in situ technique using sizeable sample sets for each of corn and grass silage. As part of this objective, the relationship between single time point incubations and degradability fractions and the ability of NIRS to predict this parameter will be explored.

The second objective is to ascertain the feasibility of using NIRS to predict intestinal digestibility of forage DM and/or CP using the mobile nylon bag technique as the reference method.

The third objective is to explore the feasibility of using NIRS to predict essential AA composition of corn and grass silage.
Table 1.1. Summary of 14 studies investigating feasibility of building NIRS calibration models to predict DM and CP degradability using in situ derived reference values.

<table>
<thead>
<tr>
<th>Forage Type</th>
<th>Author(s)</th>
<th>Type (#) Sample</th>
<th>In Situ Techniques</th>
<th>NIRS Techniques</th>
<th>Statistics</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Grass</td>
<td>Waters and Givens (1992)</td>
<td>Non-ensiled rye grass (19)</td>
<td>Sheep - 72 h incubation. Correction for MP - None Washing - NR Fractions - CP A, B, A+B, kd, ED (5)</td>
<td>Dried 1 mm ground samples WL = 1100 to 2500 nm MT = 1st Scatter = NR Regression - NR Validation - None</td>
<td>RSQ and SEC</td>
<td>Small sample set Preliminary work only. RSQ ranged from 0.18 to 0.75. No method of validation</td>
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<tr>
<td>Grass</td>
<td>HALGERSO N ET AL., 1995</td>
<td>Grass, non ensiled (152) - 67 spectrally unique used for calibration.</td>
<td>Steer - 12 h incubation. Correction for MP - ADIN. Washing - 45 min Fractions - CP disappearance after 12 h incubation.</td>
<td>Dried 1 mm ground samples WL - NR MT = 1, 4, 4, 1 Regression - MPLS Scatter - D Validation - Cross Validation</td>
<td>RSQ, SECV, 1-VR</td>
<td>Degraded CP is 100 minus 12 h incubation residue. RPD = 2.7. Single time point to predict RUP</td>
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<td>Grass and Alfalfa</td>
<td>Antoniewicz et al., 1995</td>
<td>Grass, non ensiled (16) Alfalfa, fresh (38)</td>
<td>Dry Cows – 48 h incubation Correction for MP – NR Washing – 18 min Fractions – CP A, B, A+B, kd, ED (5) NIRS only A+B, ED (5)</td>
<td>Dried 1 mm ground samples WL – 1445 to 2348 (19 filters) MT - NR Scatter – None Regression – Stepwise Validation – None</td>
<td>RSQ, SEC</td>
<td>Step wise regression can lead to overfitting inflating RSQ. Filters used in calibrations not detailed.</td>
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<tr>
<td>Hoffman et al. (1999a)</td>
<td>Grass and alfalfa (108) fermented in min-silo system</td>
<td>Cows – midlactation 24 h incubation Alfalfa hay used to standardize batches of in situ Correction for MP – NIRS calibration estimating RNA CP Washing – 4 min Fractions – CP remaining after 24 h incubation (CPR)</td>
<td>Dried 1 mm ground samples (41) WL – NR MT – NR Scatter – NR Regression – NR Validation – Prediction of CPR for 67 samples spectrally similar to calibration samples</td>
<td>RSQ, SEC</td>
<td>RSQ high but lack of information prevents assessment as to applicability in forage testing programs.</td>
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| Study | Grass and alfalfa silage (32) | Lactating cows – 72 h incubation Alfalfa hay used as standard Correction for MP – NIRS prediction used to estimate RNA CP Washing – 4 min Fractions – CP A, B, C, kd, RUP (6) and CPR for 3, 6, 12, 24, 28 and 72 h incubation | Dried, 1 mm ground samples WL – NR MT – 1, 4, 4, 1 or 2, 10, 10, 1 Scatter – NR Regression – MPLS Validation – None | RSQ, SEC | Small sample set of grass and legume silage combined. No validation procedures. Unable to assess applicability of equations to outside sample sets.

Hoffman et al. (1999c) | Grass and legume silage (121) | Cows – stage of lactation NR 24 h incubation Corrections for MP – NIRS calibration used to estimate RNA CP Washing – 4 min Fractions – CP, A, B, C, kd, RUP (6) and CPR after 24 h incubation | Dried, 1mm ground samples WL – NR MT – 1, 4, 4, 1 or 2, 10, 10, 1 Scatter – NR Regression – MPLS Validation – 61 samples spectrally similar to 60 samples in calibration set | RSQ, SEC | Collected another 300 samples and predicted RUP. Compared predicted values to NRC (1989) tabular values. Would have been of greater value to select spectrally different samples and conduct in situ analyses to assess performance of equations. |
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<th>Grass and Alfalfa hays (208) of which 106 selected for calibration</th>
<th>See Hoffman et al. (1999 c.)</th>
<th>Dried, 1 mm ground samples</th>
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<td>WL – NR</td>
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<td>MT – 1, 4, 4, 1 or 2, 10, 10, 1</td>
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<td>Regression – MPLS</td>
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<td>Scatter – NR, Validation</td>
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<td>Barley</td>
<td>Hsu et al. (1998)</td>
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<td>Barley, greenfeed and silage (108) and straw (195)</td>
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<td>Dried, 1 mm ground samples</td>
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<td>WL – 400 to 2498 nm</td>
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<td>MT – 3, 10, 10, 1</td>
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<td>Scatter – SNVD</td>
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<td>Regression – MPLS</td>
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<td>Validation – Cross validation</td>
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<td>RSQ, SECV, 1-VR, RPD</td>
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<td>Mixed Forages</td>
<td>Todorov et al. (1994)</td>
<td>Grass and Legume, non ensiled (19) Alfalfa and Meadow hays (5) Alfalfa haylage (4) Wheat/Barley Straw (4) Dehy Alfalfa (2) Used as one set of 34 samples</td>
<td>Cows – Stage of lactation NR 72 h incubation Correction for MP – NR Correction for MP – NR Washing – NR Fractions – DM and CP A, B, A+B, kd, ED (2, 5, 8)</td>
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<td>Swift et al. (1994)</td>
<td>Corn silage (12) Grass silage (18) Grass hay (10)</td>
<td>Dry cows – 96 h incubation Correction for MP – none Washing – 20 to 25 min Fractions – CP, A</td>
<td>Dried, 2 mm ground samples WL – 400 to 2498 nm MT – NR Scatter – SNVD Regression – MPLS Validation – samples not spectrally selected for calibration</td>
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<tr>
<td>De la Roza et al. (1998)</td>
<td>Corn, silage (53) Grass, silage (77)</td>
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<td>Rams – 1 group of 3 for corn silage, 1 group of 3 for grass silage Correction for MP – NR Washing – NR Fractions – DM and CP A, B, A+B, kd, ED (using ruminal outflow rate calculated using Ytterbium)</td>
<td>Dried, 2 mm ground samples WL – 400 to 2500 nm MT – 1&lt;sup&gt;st&lt;/sup&gt; or 2&lt;sup&gt;nd&lt;/sup&gt; D Scatter – Detrend, SNVC, MSC Regression – MPLS Validation – Cross validation</td>
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<tr>
<td>RSQ, SECV, 1-VR, RPD</td>
<td>More information required on the calibration sample set. No indication if corn silage and grass silage were two individual populations in terms of DM and CP degradability fractions. Graphical representation would have been helpful.</td>
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<td>CORN</td>
<td>DE BOEVER ET AL. (2002)</td>
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<td>SILAGE, (26)</td>
<td>Cows – lactating (12, 24, 48, 72 and 288 h incubation) Different replicates at different times and weight per bag doubled for 288 h incubation. Washing – 50 min Fractions – CP % EP with 4.5% passage rate.</td>
<td>Dried, 1 mm ground samples WL – 1100 to 2500 nm MT – NR Scatter – NR Regression – MPLS Validation – Cross validation</td>
<td>RSQ SECV</td>
</tr>
</tbody>
</table>
Figure 1.1. Disappearance of CP from one sample of grass silage (GS) incubated in the rumen for 0 to 96 h.
Figure 1.2 Infrared portion of the electromagnetic spectrum.
Figure 1.3. Average spectra of corn silage (CS) and grass silage (GS) collected by scanning dried, ground samples on a scanning monochromator. Average of two scans per sample. Log 1/R is equivalent to absorption.
Figure 1.4. Spectra of poultry manure ground through a fine, medium or coarse mesh screen. Log 1/R equivalent to absorption. Wavelengths measured in nm.

1.5. Spectra of poultry manure ground through a fine, medium or coarse mesh screen. Spectra were transformed using the standard normal variate (SNV) algorithm. Log 1/R equivalent to absorption. Wavelengths measured in nm.
Figure 1.6. Spectra of poultry manure ground through a fine, medium or coarse mesh screen. Spectra were transformed using the standard normal variate (SNV) and de-trending procedures. Log 1/R equivalent to absorption. Wavelengths measured in nm.

Figure 1.7. Plot of 10 samples of corn silage showing orientation in two-dimensional space as defined by log 1/R values at 1940 and 2280 nm.
Figure 1.8. Histogram of H (Mahalanobis) values for 77 samples of ground corn silage.
SAMPLE COLLECTION

SCAN SAMPLES, COLLECT SPECTRA
(Check for anomalies between duplicates)

MATHEMATICAL TREATMENT
(SNVD, Derivative) OF SPECTRA

PRINCIPAL COMPONENT ANALYSIS
Selection of spectrally different samples
by examination of H statistic. Reject
wild H outliers and redundant samples

FORMATION OF CALIBRATION SET
(consists of spectrally different samples)

EQUATION DEVELOPMENT

SELECTION OF 'BEST' EQUATION
BY EXAMINATION OF NIRS STATISTICS
SECV, 1-VR, RPD

Chemical Analyses

Combinations of Derivative, Spectral Pretreatment, and Wavelength segments

Figure 1.9. NIRS calibration flowchart.
1.7 REFERENCES


CHAPTER 2

Predicting Dry Matter and Crude Protein Degradability and Amino Acid Composition of Corn Silage by Near Infrared Reflectance Spectroscopy

2.0 Introduction

Corn silage (CS) provides a palatable source of energy to ruminant animals, and therefore plays an integral role in diets for lactating dairy cattle in many geographical regions around the world. However, CS, like all forages is inherently variable in nutrient value due to hybrid, climatic conditions, maturity upon harvest, and conservation methods.

In recent years, computer programs used to balance rations for lactating cattle have been revised to incorporate protein solubility and degradability (NRC, 2001; Boston et al., 2000). These programs are models that attempt to synchronize the supply of carbohydrate and protein to optimize microbial growth while providing the required amount of ruminal escape protein and AA. Successful implementation of these dynamic modeling systems requires the ability to measure or predict the degradation characteristics of CP in the rumen and, at the very least, the ability to measure or predict the AA composition of feedstuffs. Methods found in the literature for estimating degradability include in vivo (Rymer, 2000), in situ (Hvelplund and Weisbjerg, 2000) and in vitro (Weiss, 1994) techniques, chemical analyses (Cherney, 2000) and lastly, near infrared reflectance spectroscopy (NIRS) (Deaville and Flinn, 2000).

Earlier work from this laboratory (von Keyserlingk et al., 1996) reported substantial variation in values for in situ degradability and disappearance of DM and CP as well as for AA content of CS. Preliminary work using this set of reference data (Swift et al., 1994) indicated that NIRS could potentially be an effective tool for the prediction of soluble and degradable fractions of CP in forages. This finding has been confirmed in several studies using barley straw
(Mathison et al. 1999), alfalfa (Hsu et al. 1996) and a mixed set of grass silage (GS) and CS (De la Roza et al. 1998). To date, however, there has been no large-scale work with CS nor are there any reports regarding the use of NIRS to predict intestinal digestibility of DM or CP or AA composition of CS.

The first objective of this study was to investigate the feasibility of using NIRS to predict ruminal degradability and intestinal disappearance of DM and CP in CS. Ruminal degradability and intestinal disappearance values are compared to observations currently published in the scientific literature. The second objective was to determine the usefulness of NIRS to predict the content of essential AA in CS.

2.1 Materials and Methods

2.1.1 Sample Collection and Preparation

Samples were collected as part of a customer service program of a feed company located in the lower Fraser Valley region of British Columbia. In total, 191 samples of pre-ensiled (44) and ensiled (147) chopped whole plant corn harvested from 1992 to 1995 were collected from 108 farms and 11 experimental plots. Hybrids commonly grown in this geographical region as well as eight experimental hybrids being tested for yield and lodging were represented. Samples were collected from farms using agronomic, harvesting and storage practices representative of normal farming practice. Samples were immediately frozen in a chest freezer at -10°C until time of in-situ determinations. Upon thawing, DM was determined by drying the whole sample at 55°C in a forced air oven until constant weight was achieved. The dried samples were ground through a 2-mm screen prior to ruminal incubation and reground through a 0.5-mm screen prior to chemical analyses. Samples were analysed for N using a LECO FP-428 N analyzer (LECO Corp., St. Joseph, MI). The CP content of each sample was calculated as N x 6.25.
Determinations of ADF and NDF were undertaken using an ANKOM Fiber Analyser #F200 (Ankom Co., Fairport, NY) according to the procedures outlined by Goering and Van Soest (1970) as detailed in Komarek et al. (1994). The AA composition of each CS sample was determined using the method of Bidlingmeyer et al., (1984) adopted by Waters (Waters Corp., Milford Mass). Approximately 300 mg of forage dry matter was placed in a sample tube, which was gently flushed with N. While flushing, 15 ml of distilled, oxygen-free 6.6M HCl was added. The tube was capped, mixed well and placed into a heating oven at 110°C for 20 h. Primary and secondary amines were derivatized with phenyl isothiocyanate (PITC). Reagents were removed by vacuum and the derivatives were separated by HPLC on a reverse phase column. Samples were not subjected to a preoxidation step for the subsequent quantitation of methionine sulfone and cysteic acid.

2.1.2 Cows and Feeding

Six non-lactating Holstein cows, each fitted with a rumen cannula and a T-shaped duodenal cannula, were used in this experiment. Each cow was fed a daily diet consisting of 3.0 kg of a commercially prepared dairy concentrate (16% CP) and 5.5 kg of an alfalfa (60%) and grass hay (40%) mixture. The diet was fed in equal portions every 6 h to maintain a relatively stable rumen environment. Cows were cared for according to the Canadian Council on Animal Care (1993) guidelines.

2.1.3 In Situ Ruminal Incubation of Feeds

Dried ground CS (2 – 3 g) was placed in a nylon bag (5 x 10 cm; pore size, 52 μm; ANKOM Co., Fairport, NY) and incubated according to the procedure described by von Keyserlingk et al., (1996). Each sample was incubated in quadruplicate (two replicates in each of
two cows) in the rumen in reverse order for 96, 72, 48, 24, 12, 8, 4, 2 and 0 h. Upon removal from the rumen, the nylon bags, including the 0 h bags, were placed in a conventional washing machine filled with cold water. Four or five rinse cycles of five minutes each were required to ensure the rinse water was clear. To determine DM disappearance, residues were dried in a forced-air oven at 55°C until constant weight was achieved. Replicates within cows were pooled, ground through a 0.5-mm screen and analysed for N. No correction was made for possible contamination of the sample by microbial protein.

The difference between the DM and CP content of the original CS sample and the residue at each incubation time point was used to calculate percent disappearance. Using the equation developed by Ørskov and McDonald (1979), modified by Dhanoa (1988) and Denham et al. (1989), the amounts of the soluble (A) fraction, potentially degradable (B) fraction and the rate of disappearance of B (kd) was determined by non-linear regression for each sample. This was achieved using the Marquardt method, a linear iterative curve fitting process within the PROC NLIN procedure of SAS (SAS Institute Inc., Cary, NC). The effective degradability of DM and CP was calculated using the modified equation of McDonald (1981) and a hypothetical rumen outflow rate of 6% h⁻¹.

2.1.4 In Situ Intestinal Incubation of Feeds

Samples were incubated using the mobile bag technique of de Boer et al., (1987). Quadruplicate 1-g samples of CS were pre-incubated in the rumen of one cow for 12 h prior to being inserted into the intestine using a T-shape cannula. One bag was inserted into the duodenum every 30 min. Bags were collected from the feces and mechanically washed using a conventional washing machine until the rinse water was clear. The DM content of the residue was determined by drying in a forced air oven at 55°C until constant weight was achieved.
Replicates within cows were pooled and ground through a 0.5-mm screen prior to N analyses as outlined previously. Total tract disappearance of DM and CP was calculated as the difference in analyses between the residue and the initial sample. Disappearance of DM and CP in the intestinal tract was calculated by subtracting the amount that disappeared following 12 h incubation in the rumen from that which disappeared from the total tract.

2.1.5 Statistical Analyses

The statistical program, JMP version 4.04 (SAS Institute Inc, Cary, NC) was used to compute reported means, ranges, standard deviations and Pearson correlation co-efficients.

2.1.6 NIRS measurements and calibration

Duplicate samples of CS that had been dried and ground through a 2-mm screen were scanned using a NIRSystems 6500 (Foss NIRSystems Inc., Silver Spring MD) scanning monochromator. Samples were presented to the machine in a natural product cell (4.5 x 5.7 x 21 cm) and data collected every 2 nm from 400 nm to 2498 nm. The duplicate spectra, collected as log 1/R, were averaged for each sample.

The CENTER and SELECT options of WinISI statistical software Version 1.50 (Infrasoft International LLC, Port Matilda, PA, USA) were used to identify five unique calibration sample sets. These sample sets were used to develop equations for the prediction of in situ ruminal DM and CP degradability, DM and CP disappearance (ruminal, intestinal and total tract), and essential AA composition. All equations were developed using the modified partial least squares (MPLS) regression technique (Martens and Naes, 1989). Following the recommendations of WinISI Version 1.50, combinations of scatter correction and derivatization treatments were used to transform the spectra data. The scatter treatments were detrend (D) or standard normal variate
and detrend (SNVD) as described by Barnes et al., (1989). Three derivatization transformations (1,4,4,1; 2,6,4,1; 3,10,10,1; 4,10,10,1) and four wavelength segments (408 to 2498,8; 808 to 2498,8; 808 to 2498,4; 808 to 2498,8; 1108 to 2498,8) were utilized. In total, 32 different calibration equations were evaluated for each constituent.

In order to compare equations, statistics generated by the WinISI statistical software Version 1.50 were used. The first was the standard error of cross validation (SECV). In this procedure, the sample population to be used for calibration was randomly divided into several groups. One group was removed and a calibration equation developed using the remaining groups. This calibration equation was then used to predict the composition of the separated group. The procedure was repeated until all groups had been predicted. The SECV statistic was an average of the residuals between the predicted values and the actual laboratory values for each group of samples. Cross validation allowed all samples in the data set to be used for both calibration and validation. The second statistic was calculated as the difference between 1 and the variance ratio (1-VR). The variance ratio (VR) is computed as (SECV)/(SD)² where SD is the standard deviation of the reference data. The statistic, 1-VR, estimates the amount of the total variation in the reference set which is explained by the cross validation error (SECV).

The RPD (ratio of standard error of prediction/validation to standard deviation) statistic, as presented by Williams (2001), provides a basis for standardizing the SECV across equations. This statistic is calculated as SD of the calibration data divided by the SECV for each equation. The RPD should be as high as possible. Values greater than 2.3 indicate the equation is suitable for use in screening programs only whereas, values between 5 and 10 indicate the equation may be used in quality control purposes (Williams, 2001).
2.2 Results and Discussion

2.2.1 In Situ Determinations for DM and CP

The proximate composition of CS samples used in the in-situ and NIRS studies is presented in Table 2.1. The mean DM and CP content did not differ between pre-ensiled and ensiled CS and was similar to that published by von Keyserlingk et al. (1996). However, the present data set incorporated ensiled samples with a wider range of ADF and NDF content as shown by the larger standard deviations noted in this study versus that reported by von Keyserlingk et al. (1996) (3.0 vs. 1.8; 4.6 vs. 3.3, respectively).

In situ data for DM degradation of 139 samples of the 191 samples of CS collected are presented in Table 2.2. Soluble DM (DMA) ranged from 17.6 % to 48.3 % with a mean value of 30.8%. The mean value for the potentially degradable fraction of DM (DMB) was 52.7% with a range in values of 36.5 to 78.4%. Previous work from this laboratory (von Keyserlingk et al. 1996) showed a range in DMA from 43.8 to 56.9% with a mean value of 48.2% and a mean for DMB of 38.2% with a range of 30.7 and 42.1% respectively. These two studies were identical in terms of methodology but differed greatly in the sample sets used for in situ analysis. Whereas the work of von Keyserlingk et al. (1996) was based on 12 samples of unknown origin collected from one growing year, the present study incorporated 191 samples representing 34 hybrids grown over a range of environmental conditions and crop years. To demonstrate the effect of crop year on DMA, data were summarized for one hybrid, Pioneer 3957 (Pioneer Hybrid International Inc., Des Moines IA) within crop year. The DM content of these 23 samples ranged from 17.0 to 31.4% in 1993 and from 18.7 to 38.7% in 1995. Mean DMA in 1993 was 35.8% with a range from 26.9 to 41.9% while in 1995 it was 26.7% with a range from 20.5 to 34.2%.
Three studies, all conducted within the same geographical area as the current study, reported DMA and DMB values for CS. Mir et al. (1991) reported DMA and DMB values of 22 and 66% respectively for one sample of CS harvested at 35% DM. In a follow-up study, Mir et al. (1992) reported values of 18.6 and 23% for DMA and 55.1 and 66.3% for the DMB fraction of one hybrid harvested at early (26.3 %DM) and at late (33.9%DM) maturity. Johnson (2000) reported disappearance of DM at 0 h as 19.6 and 19.9% for one hybrid grown in two consecutive crop years. Values for DMA and DMB observed in these studies are within the ranges of values noted in the current study.

Effective degradability of DM, calculated at a theoretical rumen turnover rate of 6 % h^{-1} (DMED6) ranged from 33.4 to 63.2% with a mean value of 47.2% (Table 2). In contrast, von Keyserlingk et al. (1996) reported DMED6 values ranging from 56.8 to 68.4% with a mean value of 62.6%. As explained previously, the sample set used in the current study was much more diverse in terms of hybrid and maturity than that used by von Keyserlingk et al. (1996). The mean rate of DMB degradability (DMkd = 3.5 % h^{-1}) noted in this study was in agreement with that published by Mir et al. (1992) for CS harvested at similar DM but below the value of 4.70% h^{-1} reported by von Keyserlingk and co-authors (1996). In light of the higher DMED6 values reported by these authors, it could be expected that the DMkd would be higher than noted in the current study.

Data for soluble CP (CPA), potentially degradable CP (CPB), and the effective degradability of CP (CPED6) are presented in Table 2.2. There is paucity of data in the scientific literature reporting the fractions of CP in CS determined using the in-situ method. The samples in the current study exhibited a wide range in CPA (12.7 to 80.1%) with a mean value of 47.4%. The mean value of CPA found in this study is lower than the values noted by De Boever
et al. (2002) and von Keyserlingk et al. (1996) who reported narrower ranges in CPA observations. The sample set used in the current study incorporates a number of samples with a CPA content below that found in these reports. In part, this is due to a small number of pre-ensiled samples collected for this study. Hvelplund and Weisbjerg (2000) noted that ensiling forages increases the soluble CP fraction, and therefore, the inclusion of pre-ensiled samples would result in increasing the range in CPA as found in this study. In the recently published Nutrient Requirements of Dairy Cattle, (NRC, 2001) values for CPA ranged from 48.8 (mature CS) to 57.8% (immature CS). Johnson (2000) found CP disappearance at 0 h to be 54.3 and 54.9% for one hybrid grown in two consecutive crop years. Values for CPB obtained in the present study support those presented by Mir et al. (1992) and Susmel et al. (1990). The mean value for CPED6 (57.7%) is similar to that of Arieli et al. (1999), but below that reported by von Keyserlingk et al. (1996). There is close agreement between the current study and that of von Keyserlingk et al. (1996) in terms of the maximum value for CPED6 being approximately 80% (83.8 vs 80.1%). However, approximately 50% of the samples in this study had a CPED6 value below the minimum of 63.4% reported by von Keyserlingk and co-workers (1996). As with DM, this may be due to the wide range in hybrids and growing conditions represented in the current study.

In the current study, CPED6 provides an estimate of the amount of protein degraded in the rumen assuming an outflow rate of ruminal contents of 6% h\(^{-1}\). Conversely, CPED6 subtracted from 100 gives an estimate of the amount of protein escaping rumen degradation, commonly referred to as bypass protein. The mean content of bypass protein, calculated in this manner was 42.3% with a range of 16.0 to 71.0% (data not shown). Furthermore, there was a strong inverse relationship between CPA and bypass protein (Pearson correlation coefficient = -
0.95) in the samples used in this study. De Boever et al. (2002), using 26 samples of corn silage, reported that the washable protein fraction (WCP) best predicted escape protein as shown by the equation:

$$\% EP = 73.11 - 0.618 \text{ WCP} \quad (R^2 = 97\%) \quad \text{Equation 2.1}$$

where WCP was calculated as the difference between the CP content of the sample and the CP content of the residue remaining after rinsing 0 h nylon bags in a washing machine for 50 min using cold water.

In the current study, bypass or escape protein could be predicted by the equation:

$$\% EP = 78.04 - 0.75 \text{ CPA} \quad (R^2 = 90\%) \quad \text{Equation 2.2}$$

Hvelplund and Weisbjerg (2000) reported that CPA and CPED6 will be related in forages with high protein solubility values as the in situ method assumes that soluble protein will be immediately degraded in the rumen. These authors report average CPA and CPED6 values for five samples of CS as 79.8 and 84%, respectively.

The rate of CP degradation (CPkd) ranged from 1.0 to 11.1% h\(^{-1}\) with a mean value of 4.4% h\(^{-1}\) which concurs with previous work reported by von Keyserlingk et al. 1996. It was noted that for some samples, large differences existed between cows in the degradation rate of CP in the sample. Success in NIRS calibration for constituents measured in this study depend on the precision of the in situ data. Therefore, it was decided to arbitrarily eliminate data for the rate of CP degradation when a greater than two fold difference in values between cows within sample was observed.

Disappearance of DM after 12 h incubation in the rumen was 44.9% with a range from 24.7 to 63.4% (Table 2.3). Work reported by several laboratories (Andrae et al. 2001; Bal et al. 2000; Johnson 2000) support the findings of this study for 24 h (56.7%) and 48 h (68.4%)
disappearance of DM (Table 2.3). Pearson correlation coefficients relating 12 h, 24 h, and 48 h disappearance of DM with DMED6 were 0.95, 0.79 and 0.76 respectively. Susmel et al. (1990) reported that the rate of CS DM degradation in the rumen was positively correlated ($r=0.96$) with NDF content. In addition, these authors reported that potentially digestible DM (the sum of DMA and DMB) was positively correlated ($r=0.82$) with NDF content. Neither finding could be supported by the data collected in the current study. Pearson correlation coefficients between NDF content in CS and the rate of DM degradation (c) and potentially digestible DM (DMA + DMB) were 0.35 and 0.02 respectively. The NDF content of CS did have a stronger association with DM disappearance over time as shown by the Pearson correlation coefficients for 12 h (0.23), 24 h (0.33) and 48 h (0.38) DM disappearance. It can be theorized that this is due to increasing concentration of NDF over time as the more readily digestible fractions such as sugars, starches and pectins are degraded leaving the slowly degradable cellulose and hemicellulose and undegradable lignin fractions.

The amount of CP disappearing after 12 h incubation was 52.3% with a range of 19.0 to 84.5% (Table 2.3) which is in agreement with that observed by von Keyserlingk et al. (1996). After 24 h ruminal incubation, 62.8% of the CP had disappeared which is similar to values reported by Johnson (2000) who measured CP disappearance from CS harvested at 2/3 milkline.

Intestinal and total tract disappearance for DM and CP are reported in Table 2.4. Intestinal disappearance of both DM and CP were notably higher in this study than those reported by von Keyserlingk and co-workers (1996). This is not unexpected in view of the higher CPED6 values for DM and CP found by von Keyserlingk et al. (1996). Higher rates of degradability in the rumen would result in less material to disappear in the small intestine. De Boever et al. (2002) reported an average intestinal digestibility of escape protein in 26 corn
silage samples of 72.8% ranging from 56.4 to 84.8%. Intestinal CP digestibility in the current study ranged from 0.8 to 99.6% (data not shown) with a mean value of 54.6% when calculated as:

\[
\% \text{ CP Intestinal Digestible} = \frac{\% \text{ Intestinal CP Disappearance}}{100 - 12 \text{ h CP Disappearance}} \times 100 \quad \text{Equation 2.3}
\]

The wide range in CP digestibility in the intestinal tract noted in the current study can be attributed to the diversity in the sample set over that used by De Boever et al. (2002). Total tract disappearance for DM and CP of 59.6 and 79.7% respectively were in agreement with those found by von Keyserlingk et al., (1996).

2.1.2 Amino Acid Analysis

The AA composition (%DM), total AA content (%CP) and essential AA content expressed as a % total AA content, of CS are presented in Table 2.4. The range of total AA content expressed as a percent of total CP was very large (36.0 to 86.5%) with a mean of 61.6%. Samples of CS with the least amount of CP as AA (<45%) were high in CP (>10.5%), contained elevated amounts of CP in the soluble form (CPA>60%) and consequently, high levels of CPED6 (>65%). In two samples, this was due to the addition of other CP sources namely, urea and barley screenings. Another sample was harvested early (20.8% DM) and ensiled using a commercially available acid preservative whilst another sample was ensiled cannery corn waste. The samples of CS with greater than 80% of the total CP as AA were non-ensiled samples with less than 25% DM and less than 30% CPA.

Van Straalen et al. (1997) reported that essential AA represented 44.7% of the total AA content found in CS. This observation was confirmed in the current study, as essential AA accounted for 44% of the total AA content. The ratio of essential to total AA in CS would appear
to be relatively constant as the range between minimum and maximum values of essential AA as a proportion of total AA was narrow (40.3 to 47.9%).

The profile of AA (%DM) in CS (Table 2.4) observed in this study is similar to published values (Fickler et al. 2001; von Keyserlingk, 1994; Muscato et al. 1983) with the exception of Arg and Met. The Arg content ranged between 0.17 and 0.75% with a mean value of 0.32%, which is approximately, double that reported in the aforementioned studies. This may be due to the analytical method employed in this study. Williams (1994) reported that AA analysis using PITC derivatives might overestimate the amount of Arg due to incomplete resolution from methionine sulfoxide and dehydroalanine. It was of interest to note that the Arg content of CS samples exceeding that reported by Fickler et al., (2001) were all harvested in an immature state as determined by their low DM and high ADF content.

The mean Met content of 0.06 (% DM) agrees with reports by Muscato et al. (1983) and von Keyserlingk (1994) but is considerably lower than the value reported by Fickler et al. (2001). This difference may be explained by the methodology employed for Met determination. The omission of the preoxidation step during AA analysis has been reported (Williams, 1994) to decrease the recovery of Met by 25%. This step was not included in the analysis of AA in this study and is not reported in the studies of Muscato et al. (1993) or von Keyserlingk (1994).

2.3 NIRS

2.3.1 In Situ Determinations for DM and CP

The scatter and mathematical treatments, number of wavelengths and the statistical evaluation of calibration equations developed for DM and CP degradation and disappearance are presented in Table 2.5. For most constituents, the use of the SNVD scatter correction method in combination with a second derivative mathematical treatment produced the most acceptable
equations based on the SECV, 1-VR and RPD statistics.

The SECV, 1-VR and RPD values of the equation predicting DMA were 2.72, 0.87 and 2.8, respectively. These observations are supported by the work of De la Roza et al. (1998) who reported corresponding values of 1.80, 0.94 and 3.99, respectively for a sample set composed of 53 CS and 77 grass silages. Mathison et al. (1999) reported that NIRS could account for 84% of the variation in the soluble DM fraction found in barley straw which validates the 1-VR value of 0.87 found in the current study. Using a mixed set of 34 forages that included grass, legumes and straws, Todorov et al., (1994) reported a coefficient of determination value of 0.86 when predicting soluble DM with NIRS. Based on the RPD statistic for DMA observed in the current study (2.8), it may be concluded that NIRS provides a viable option for screening CS samples for soluble DM content.

The relationship between NIRS spectral data and the amount of potentially degradable DM (DMB) and the rate of degradation of this DM fraction (c) was less acceptable than for DMA. A review of the 1-VR values for each constituent revealed that NIRS could be expected to account for only 64% and 19% of the variation in DMB and in the rate of degradation (DMkd), respectively. In addition, the RPD statistic for each constituent was less than 2.3 (1.7 and 1.2, respectively) indicating that NIRS may not be a suitable method for predicting these indices. De la Roza et al. (1998) reported NIRS could be used to predict the DMED6 in CS based on their observations of 0.87 and 2.83 for 1-VR and RPD respectively. However, these authors also reported that the prediction of degradation rates did not meet the required minimum RPD specification of 2.3. The findings of Mathison et al. (1999) were similar to those of the current study as they reported coefficient of determination and RPD values for the potentially degradable DM in straw as 0.69 and 1.77 and for the DMkd as 0.18 and 1.10, respectively.
Therefore, it would appear that NIRS could be calibrated to predict the potentially degradable proportion of DM but that it is not an appropriate method for the prediction of the degradation rate of this fraction. This finding could be expected as NIRS is based on the measurement of light reflected from hydrogen containing bonds. Therefore, NIRS could predict a rate only if that rate was correlated to some constituent contained in the material being analysed, and if that constituent could be predicted by NIRS. Mathison and co-workers (1999) reported that rate of DM degradation was not correlated with any chemical entity commonly measured such as CP and ADF. Another important consideration in the development of NIRS equations, is the use of accurate reference methods. Hsu et al. (1998) reported a CV of 26.42% for the rate of degradation of DM in alfalfa used as a standard in 12 individual in-situ incubation studies. These authors also reported a low coefficient of determination (0.49) and 1-VR value (0.43) for the rate of DM degradation for a mixed set of barley greenfeed, silage and straw. The use of a standard between in-situ runs was not undertaken in this study. However, it may be speculated that variation between cows may have contributed to the inability to build an acceptable calibration equation for the prediction of the rate of DM degradability.

Todorov et al. (1994) and De la Roza et al. (1998) reported coefficient of determination values greater than 0.90 describing the relationship between NIRS and effective degradability of DM. Mathison et al. (1999) reported an acceptable RPD statistic of 2.90 when NIRS was used to predict DM degradability at a fractional outflow rate of 3% h⁻¹. However, when the outflow rate was changed to 6 % h⁻¹ and the effective DM degradability recalculated, the RPD statistic dropped to 2.03 although the coefficient of determination did not change substantially (0.77 to 0.75). The coefficient of determination value of 0.73 for DMED6 that was observed in the current study is similar to that reported by Mathison et al. (1999). However, the RPD statistic
(1.6) was well below the value of 2.3 indicating that further investigation is required before NIRS can be accepted as an appropriate method for the prediction of effective degradability of DM in CS.

The relationship between NIRS spectral data and in situ reference values for DM disappearance from the rumen (12 h), from the intestinal tract and from the total tract was weak. The RPD statistic for each of these equations was 1.6, 1.5 and 1.6, respectively. A search of the scientific literature failed to yield any reports that could support or refute these findings.

In general, the ability of NIRS to predict CP degradation and disappearance was superior to that for DM (Table 2.5). A comparison showed good agreement between these observations and those of De la Roza et al. (1998). Both studies found that NIRS could account for more than 90% of the variation in CSCPB and CPED6 and that the RPD statistic of the equation developed for each of these constituents approached or exceeded 2.3. This supports the conclusion that NIRS may be successfully employed to screen CS samples for degradable CP. Similar to DM, NIRS was not successful in predicting the rate (CPkd) of CP degradation as the 1-VR and RPD statistics were 0.25 and 1.4, respectively. Again, these values were similar to those of De la Roza et al. (1998). The calibration statistics for CPA were notably higher than those reported by De la Roza et al. (1998). This may be due to the greater range of CPA values found in the sample set used in the calibration process of the current study. As shown in Table 2.5, the relationship between in situ values for CP disappearance and NIRS spectral data was strong. The values for 1-VR were 0.75, 0.86 and 0.80 for CP disappearance from the rumen (12 h), intestine and total tract respectively. As in the case of DM, a search of the literature did not yield any scientific reports that could validate these findings.
2.3.2 Amino Acids

The statistical evaluation of calibration equations for each of the essential AA, except Met is presented in Table 2.6. As discussed previously, success in NIRS calibration relies heavily on accurate reference data, which was not available for Met in this study due to the omission of the pre-oxidation step during Met determination. NIRS could account for 53% (Val) to 83% (His, Phe) of the variation in the sample data set. However, if these calibration equations were used to predict essential AA composition of an unknown data set, the relationship between NIRS and analytical data is weak as demonstrated by the low 1-VR values. The RPD values were also below 2.3 indicating that the applicability of these equations would be limited. To the author's knowledge, there are no scientific reports documenting the feasibility of using NIRS to predict AA composition of CS. Recently, Fontaine et al., (2001) reported that NIRS could be used to predict the essential AA composition of vegetable and animal protein feedstuffs. The relationship between NIRS spectral data and wet chemistry values for each AA was much stronger than that observed in the current study. Several differences existed between the two studies such as the grind size of the sample (2 mm vs. 0.5 mm) and the analytical procedures used for AA determination. Another large difference was concentration of each AA to be analysed as the vegetable and animal protein feedstuffs contained, at least, three times as much of each AA as did the CS samples in the current study. Based on studies where NIRS has been used to determine AA composition (van Kempen and Bodin, 1998; Fontaine et al., 2001), it can only be concluded that further work is required to ascertain the feasibility of using NIRS to predict AA composition of CS.

2.3.3 Spectral Data

The interpretation of spectral data can provide information regarding the chemical
constituents of importance to DM and CP digestibility of forages (Deaville and Givens, 1998; De la Roza et al., 1998; Redshaw et al., 1986). In the current study, principal wavelengths important in equation development were selected by an examination of the correlation coefficients associated with each wavelength. In reviewing the equation developed for DMA, the two largest coefficients, both positive in nature, belonged to wavelengths (1508 and 1548 nm) linked to N-H bonding in primary amides (Murray and Williams, 1987). The wavelength segment from 1500 to 1508 nm was also of primary importance in equations developed for DMED6 and DM disappearance in the intestinal tract. The role of protein in DM degradability has been acknowledged in several reports beginning with Norris et al. (1976) who identified 1512 nm as the primary wavelength in an equation predicting in vivo digestibility of DM for a mixed set of forages. Deaville and Givens (1998) recognized the wavelength region of 1430 to 1620 nm as being associated with high DM degradability of CS. The wavelength 1468 nm, also linked with N-H bonding in amides (Murray and Williams, 1987), was the primary wavelength related to DM digestibility in cattle and sheep as reported by Redshaw et al. (1986). The two largest correlation coefficients in the equation developed to predict 12 h DM disappearance from the rumen belonged to wavelength segments 972 to 984 nm, and 1044 to 1048 nm which are related to N-H bonding (Osborne et al., 1993). These coefficients were negative in sign, indicating a depressing effect of these components on DM disappearance from the rumen.

The relationship between DM degradability and/or disappearance and CP is not surprising. The ratio of grain to stover (leaves and stalk) increases as the plant matures (Deinum and Struik, 1986). CS grown in the south coastal region of British Columbia typically contains 40% grain but grain content can vary between 27 and 45% depending on year, hybrid, stage of maturity and location (Hunt et al. 2001). De Smet et al., (1995) reported that the DM
degradability of corn grain is less than other grains such as barley due to the granular nature of cornstarch. Starch in corn grain is encased by a matrix of proteins, which serves to impede enzyme hydrolysis. Verbič et al. (1995) found that the rate of corn grain DM degradation differed among hybrids with dent corn being approximately 1.5 times as fast as flint corn. The in situ data from the current study do show a strong relationship between CPED6 and DMA and between CPED6 and DMED6. The Pearson correlation coefficients for each pair of constituents was 0.67 and 0.77, respectively.

The relationship between spectral data and CP degradation/disappearance was not as clearly defined as it was for DM degradation/disappearance. The wavelength segment of importance in calibrations developed for CPA and CPED6 was between 2050 and 2200 nm. According to Murray and Williams (1987), many chemical constituents are related to this area including long chain fatty acids (2100 to 2180 nm), pyrimidines (2050 to 2140 nm) and secondary amines (2110 to 2140 nm). Redshaw et al., (1986) reported that 2168 and 2174 nm were of importance for predicting digestible CP in cattle and sheep respectively. De La Roza et al., (1998) found that 2068 nm was the primary wavelength in equations developed for predicting soluble and degradable fractions of CP. These authors did not speculate about the relationships of these wavelengths to chemical constituents. The disappearance of CP after a 12 h-ruminal incubation and in the intestinal tract was related to protein structure. Primary and secondary wavelength segments of importance were 1572 to 1580 nm and 1546 to 1556 nm, respectively. These areas are related to N-H bonding found in secondary amides (Murray and Williams, 1987; Osborne et al. 1993).

In conclusion, in situ data obtained from this study corroborates previous work conducted in this laboratory (von Keyserlingk, 1994) showing substantial variation in soluble and
degradable DM and CP fractions as well as in AA composition of CS. Therefore, it is very important to obtain information regarding solubility and degradability of CP fractions before using a dynamic ration-balancing model such as the recently released NRC (2001).

For most constituents, the use of the SNVD scatter correction method in combination with a second derivative mathematical treatment produced the most acceptable equations based on the SECV, 1-VR and RPD statistics. Based on the RPD statistic used to evaluate calibration equations, NIRS provides a viable option for the prediction of soluble DM, soluble CP, degradable CP and CP disappearance from the intestinal tract and total tract. It was not possible to produce robust calibration equations to predict rates of DM or CP degradability from this data set. Further study is required to ascertain the usefulness of NIRS in predicting AA composition of CS.

Interpretation of spectral data shows that DM solubility and degradability of CS is linked to N-H bonding within the corn plant. There was a strong relationship between soluble DM, potentially degradable DM and the effective degradability of CP. A review of the major wavelengths used in each calibration did not indicate that cellulose, hemicellulose or lignin play a major role in DM digestibility of CS. The relationship between spectral data and CP degradation/disappearance was not well defined.

2.4 Acknowledgements

Financial support for this research provided by Pro Form Feeds Ltd. and the British Columbia Ministry of Agriculture, Fisheries and Food (Research Partnership Program) is gratefully acknowledged.
Table 2.1 Proximate composition of pre-ensiled and ensiled corn samples.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Ensiled (44)</th>
<th>Ensiled (147)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>DM %</td>
<td>18.2</td>
<td>35.9</td>
</tr>
<tr>
<td>CP %$^1$</td>
<td>6.9</td>
<td>10.1</td>
</tr>
<tr>
<td>ADF%$^1$</td>
<td>23.2</td>
<td>32.7</td>
</tr>
<tr>
<td>NDF %$^1$</td>
<td>39.4</td>
<td>58.5</td>
</tr>
</tbody>
</table>

$^1$ expressed as % of DM
Table 2.2 DM and CP degradation characteristics of CS in total and calibration sample sets.

<table>
<thead>
<tr>
<th></th>
<th>Total Sample Set</th>
<th>Calibration Sample Set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIN</td>
<td>MAX</td>
</tr>
<tr>
<td>DMA %³</td>
<td>17.6</td>
<td>48.3</td>
</tr>
<tr>
<td>DMB %³</td>
<td>36.5</td>
<td>78.4</td>
</tr>
<tr>
<td>DMkd % h⁻¹³</td>
<td>1.2</td>
<td>6.9</td>
</tr>
<tr>
<td>DMED6 %³</td>
<td>33.4</td>
<td>63.2</td>
</tr>
<tr>
<td>CPA ⁴</td>
<td>12.7</td>
<td>80.1</td>
</tr>
<tr>
<td>CPB %⁴</td>
<td>10.1</td>
<td>65.4</td>
</tr>
<tr>
<td>CPkd % h⁻¹⁴</td>
<td>0.1</td>
<td>11.1</td>
</tr>
<tr>
<td>CPED6 %⁴</td>
<td>29.0</td>
<td>83.8</td>
</tr>
</tbody>
</table>

¹ Number of samples in total sample set was 139 for DM and 101 for CP (except for DMkd/CPkd).
² Number of samples in calibrations sample set equals 64 for DM and 53 for CP (except for DMkd/CPkd).
³ DMA = % immediately soluble DM, DMB = % potentially degradable DM, DMkd = rate of degradation of DMB, DMED6 = effective degradability of DM calculated at a ruminal passage rate of 6 % h⁻¹.
⁴ CPA = % immediately soluble CP, CPB = % potentially degradable CP, CPkd = rate of degradation of CPB, CPED6 = effective degradability of CP calculated at a ruminal passage rate of 6 % h⁻¹.
Table 2.3 Ruminal disappearance, intestinal and total tract disappearance of DM and CP in total and calibration sets of CS.

<table>
<thead>
<tr>
<th></th>
<th>Total Sample Set 1</th>
<th></th>
<th>Calibration Sample Set 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rumen 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>24.7</td>
<td>63.4</td>
<td>44.9</td>
<td>7.2</td>
</tr>
<tr>
<td>24 h</td>
<td>37.0</td>
<td>75.5</td>
<td>56.7</td>
<td>7.8</td>
</tr>
<tr>
<td>48 h</td>
<td>42.7</td>
<td>82.7</td>
<td>68.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Intestine 4</td>
<td>3.2</td>
<td>31.7</td>
<td>15.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Total Tract 5</td>
<td>38.1</td>
<td>72.4</td>
<td>59.6</td>
<td>5.2</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rumen 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>19.0</td>
<td>84.5</td>
<td>52.3</td>
<td>15.7</td>
</tr>
<tr>
<td>24 h</td>
<td>27.1</td>
<td>87.3</td>
<td>62.8</td>
<td>14.1</td>
</tr>
<tr>
<td>48 h</td>
<td>42.9</td>
<td>91.2</td>
<td>72.8</td>
<td>10.4</td>
</tr>
<tr>
<td>Intestine 4</td>
<td>0.2</td>
<td>57.9</td>
<td>27.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Total Tract 5</td>
<td>49.8</td>
<td>99.8</td>
<td>79.7</td>
<td>8.6</td>
</tr>
</tbody>
</table>

1 Number of samples in total sample set was 138 for DM and 77 for CP.
2 Number of samples in calibration sample set was 74 for DM and 42 for CP.
3 Percent of total DM (or CP) that disappeared after ruminal incubation for 12 h, 24 h and 48 h.
4 Percent of total DM (or CP) which disappeared in the intestinal tract. Equal to total tract minus 12 h DM (or CP) disappearance value.
5 Disappearance following incubation in the rumen for 12 h and incubation in the intestines.
Table 2.4. Amino acid composition (%DM) of CS in total and calibration sample sets.

<table>
<thead>
<tr>
<th></th>
<th>Total Sample Set (n=101)</th>
<th>Calibration Sample Set&lt;sup&gt;1&lt;/sup&gt; (n= 76)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>His</td>
<td>0.06</td>
<td>0.17</td>
</tr>
<tr>
<td>Arg</td>
<td>0.17</td>
<td>0.75</td>
</tr>
<tr>
<td>Thr</td>
<td>0.10</td>
<td>0.38</td>
</tr>
<tr>
<td>Val</td>
<td>0.24</td>
<td>0.51</td>
</tr>
<tr>
<td>Met</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>Ile</td>
<td>0.17</td>
<td>0.39</td>
</tr>
<tr>
<td>Leu</td>
<td>0.34</td>
<td>0.94</td>
</tr>
<tr>
<td>Phe</td>
<td>0.16</td>
<td>0.43</td>
</tr>
<tr>
<td>Lys</td>
<td>0.07</td>
<td>0.32</td>
</tr>
<tr>
<td>Asp</td>
<td>0.22</td>
<td>0.70</td>
</tr>
<tr>
<td>Glu</td>
<td>0.50</td>
<td>1.33</td>
</tr>
<tr>
<td>Ser</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>Gly</td>
<td>0.20</td>
<td>0.43</td>
</tr>
<tr>
<td>Ala</td>
<td>0.33</td>
<td>0.99</td>
</tr>
<tr>
<td>Pro</td>
<td>0.27</td>
<td>0.69</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.03</td>
<td>0.29</td>
</tr>
<tr>
<td>TAA&lt;sup&gt;2&lt;/sup&gt;</td>
<td>36.0</td>
<td>86.5</td>
</tr>
<tr>
<td>EAA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>40.3</td>
<td>47.9</td>
</tr>
</tbody>
</table>

<sup>1</sup>Calibrations developed for essential amino acids only.

<sup>2</sup>TAA = Total amino acids expressed as a %CP.

<sup>3</sup>EAA = Essential amino acids expressed as % TAA.
Table 2-5. Statistical evaluation of calibration equations for ruminal DM and CP degradation and intestinal disappearance of DM and CP.

<table>
<thead>
<tr>
<th></th>
<th>SCATTER</th>
<th>MATH</th>
<th>WL</th>
<th>RSQ</th>
<th>SECV</th>
<th>1-VR</th>
<th>RPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM&lt;sup&gt;8&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A%</td>
<td>SNVD</td>
<td>2</td>
<td>171</td>
<td>0.94</td>
<td>2.72</td>
<td>0.87</td>
<td>2.8</td>
</tr>
<tr>
<td>B%</td>
<td>D</td>
<td>2</td>
<td>255</td>
<td>0.79</td>
<td>3.92</td>
<td>0.64</td>
<td>1.7</td>
</tr>
<tr>
<td>kd % h&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>SNVD</td>
<td>3</td>
<td>252</td>
<td>0.29</td>
<td>0.01</td>
<td>0.19</td>
<td>1.2</td>
</tr>
<tr>
<td>ED6%</td>
<td>D</td>
<td>2</td>
<td>171</td>
<td>0.73</td>
<td>3.80</td>
<td>0.60</td>
<td>1.6</td>
</tr>
<tr>
<td>12h</td>
<td>SNVD</td>
<td>4</td>
<td>387</td>
<td>0.71</td>
<td>4.71</td>
<td>0.62</td>
<td>1.6</td>
</tr>
<tr>
<td>Intestine</td>
<td>SNVD</td>
<td>2</td>
<td>171</td>
<td>0.65</td>
<td>4.88</td>
<td>0.55</td>
<td>1.5</td>
</tr>
<tr>
<td>Total Tract</td>
<td>SNVD</td>
<td>1</td>
<td>259</td>
<td>0.69</td>
<td>2.81</td>
<td>0.59</td>
<td>1.6</td>
</tr>
<tr>
<td>CP&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A%</td>
<td>SNVD</td>
<td>2</td>
<td>171</td>
<td>0.96</td>
<td>5.44</td>
<td>0.90</td>
<td>3.2</td>
</tr>
<tr>
<td>B%</td>
<td>SNVD</td>
<td>2</td>
<td>255</td>
<td>0.92</td>
<td>6.19</td>
<td>0.79</td>
<td>2.2</td>
</tr>
<tr>
<td>kd % h&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>SNVD</td>
<td>1</td>
<td>259</td>
<td>0.59</td>
<td>0.02</td>
<td>0.25</td>
<td>1.1</td>
</tr>
<tr>
<td>ED6%</td>
<td>SNVD</td>
<td>2</td>
<td>255</td>
<td>0.96</td>
<td>5.31</td>
<td>0.84</td>
<td>2.7</td>
</tr>
<tr>
<td>12h</td>
<td>SNVD</td>
<td>2</td>
<td>206</td>
<td>0.87</td>
<td>8.20</td>
<td>0.75</td>
<td>2.1</td>
</tr>
<tr>
<td>Intestine</td>
<td>SNVD</td>
<td>4</td>
<td>194</td>
<td>0.93</td>
<td>5.92</td>
<td>0.86</td>
<td>2.7</td>
</tr>
<tr>
<td>Total Tract</td>
<td>D</td>
<td>2</td>
<td>206</td>
<td>0.95</td>
<td>3.23</td>
<td>0.80</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<sup>1</sup> Scatter Procedure. SNVD = Standard Normal Variate and Detrend; D = Detrend
<sup>2</sup> Math Treatment. 1 = 1,4,4,1; 2 = 2,6,4,1; 3 = 3,10,10,1; 4 = 4,10,10,1.
<sup>3</sup> WL segment. 1 = 408-2498, 8; 2 = 808-2498, 8; 3 = 808-2498, 4; 4 = 1108-2498, 8.
<sup>4</sup> RSQ = coefficient of determination (R<sup>2</sup>).
<sup>5</sup> SECV = standard error of cross validation
<sup>6</sup> 1-VR = 1- variance ratio
<sup>7</sup> RPD = SD of calibration sample set / SECV
<sup>8</sup>/<sup>9</sup> DMA (CPA) = % immediately soluble DM (CP), DMB (CPB) = % potentially degradable DM (CP), DMkd (CPkd) = rate of degradation of DMB (CPB), DMED6 (CPED6) = effective
degradability of DM (CP) calculated at a ruminal passage rate of 6 % h$^{-1}$. DM (CP)12 h =
Percent of total DM (CP) that disappeared after ruminal incubation for 12 h. DM (CP) Intestine
= percent of total DM (CP) which disappeared in the intestinal tract. Equal to total tract minus
12 h DM (or CP) disappearance value. DM (CP) Total Tract = Disappearance following
incubation in the rumen for 12 h and incubation in the intestines.
Table 2-6. Statistical evaluation of calibration equations for AA composition (% DM).

<table>
<thead>
<tr>
<th></th>
<th>SCATTER</th>
<th>MATH</th>
<th>WL</th>
<th>RSQ</th>
<th>SECV</th>
<th>1-VR</th>
<th>RPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>His</td>
<td>D</td>
<td>4</td>
<td>4</td>
<td>0.83</td>
<td>0.013</td>
<td>0.74</td>
<td>2.0</td>
</tr>
<tr>
<td>Arg</td>
<td>SNVD</td>
<td>2</td>
<td>4</td>
<td>0.73</td>
<td>0.051</td>
<td>0.64</td>
<td>1.7</td>
</tr>
<tr>
<td>Thr</td>
<td>SNVD</td>
<td>4</td>
<td>3</td>
<td>0.78</td>
<td>0.031</td>
<td>0.61</td>
<td>1.6</td>
</tr>
<tr>
<td>Val</td>
<td>SNVD</td>
<td>2</td>
<td>1</td>
<td>0.53</td>
<td>0.033</td>
<td>0.35</td>
<td>1.2</td>
</tr>
<tr>
<td>Ile</td>
<td>D</td>
<td>4</td>
<td>3</td>
<td>0.72</td>
<td>0.026</td>
<td>0.44</td>
<td>1.3</td>
</tr>
<tr>
<td>Leu</td>
<td>SNVD</td>
<td>4</td>
<td>1</td>
<td>0.66</td>
<td>0.062</td>
<td>0.54</td>
<td>1.5</td>
</tr>
<tr>
<td>Phe</td>
<td>D</td>
<td>4</td>
<td>3</td>
<td>0.83</td>
<td>0.029</td>
<td>0.69</td>
<td>1.8</td>
</tr>
<tr>
<td>Lys</td>
<td>SNVD</td>
<td>4</td>
<td>3</td>
<td>0.78</td>
<td>0.027</td>
<td>0.57</td>
<td>1.5</td>
</tr>
</tbody>
</table>

1 Scatter Procedure. SNVD = Standard Normal Variate and Detrend; D = Detrend
2 Math Treatment. 1 = 1,4,4,1; 2 = 2,6,4,1; 3 = 3,10,10,1; 4 = 4,10,10,1.
3 WL segment. 1 = 408-2498, 8; 2 = 808-2498,8; 3 = 808-2498,4; 4 = 1108-2498,8.
4 RSQ = coefficient of determination ($R^2$).
5 SECV = standard error of cross validation
6 1-VR = 1- variance ration
7 RPD = SD of calibration sample set / SECV
2.5 REFERENCES


CHAPTER 3

Predicting Crude Protein Degradability and Amino Acid Composition of Grass Silage using Near Infrared Reflectance Spectroscopy (NIRS).

3.0 Introduction

Rations for dairy cattle are typically formulated using computer programs that allow the operator to balance a ration to simultaneously meet multiple nutrient requirements. One of the most critical requirements is that for N, usually expressed as crude protein (CP = N x 6.25). Unlike the monogastric animal, dietary protein consumed by the ruminant will first be subjected to degradation by ruminal microbes. Protein escaping ruminal degradation will undergo digestion processes similar to that found in the monogastric animal.

Two decades ago, the concept of degraded and undegraded dietary protein was introduced into computer models used to balance rations for dairy cattle (NRC, 1985; NRC, 1989). Since that time, research has been undertaken to refine these models by investigating the amount and degradation rates of protein fractions in a number of feedstuffs (Batajoo and Shaver, 1998; von Keyserlingk et al., 1996; Xu et al., 1996). These reports have shown that there is a large variation between and within feedstuffs in terms of amounts of soluble CP, rate of CP degradation within the rumen, intestinal digestibility of undegraded CP and AA composition.

Of particular interest to dairy producers who feed grass silage (GS) are reports in the scientific literature documenting the effect of maturity on the degradability of grass CP in the rumen (Antoniewicz et al., 1995; Hoffman et al., 1993; Balde et al., 1993). Generally, the data indicate that increasing maturity decreases the amount of CP degradable in the rumen and may or may not, depending on the stage of maturity, have an effect on the amount of soluble CP. One
group of authors (Balde et al., 1993) concluded that the use of fixed book values for defining CP degradability of forages was not appropriate.

The effect of maturity on the feeding value of forages was recognized in a recent publication, Nutrient Requirements of Dairy Cattle (NRC, 2001). The data presented for nutrient composition (NRC, 2001) were a compilation of published information which often does not include identification of feedstuffs according to traditional maturity classifications. Therefore, nutrient composition data were reported for cool season grasses (hay and silage), legumes (hay and silage), grass and legume mixtures (hay and silage) and corn silage on the basis of maturity as defined by NDF content. Neutral detergent fiber is comprised of cellulose, hemicellulose and lignin, which are components of the plant cell wall. As a plant matures, cell wall content and lignification increase providing the basis for the relationship between NDF content and plant maturity (Van Soest, 1982).

Crude protein solubility, ruminal degradability and AA values are given for immature (<55% NDF), mid-mature (55-59% NDF) and mature (>60% NDF) GS by NRC (2001). In general, the amount of soluble CP decreases with increasing maturity, as does the rate of CP degradability in the rumen. However, within each stage of maturity, there is a wide range in soluble and degradable CP values as shown by large standard deviation values. Likewise, wide ranges in soluble CP, degradable CP and AA composition of GS grown in the south coastal region of British Columbia have been reported (von Keyserlingk et al., 1996). However, there have been no values reported for CP solubility, CP degradability and AA composition of GS grown in this region and classified according to maturity.

In view of the wide range within each stage of maturity, successful implementation of the NRC (2001) dairy formulation system will require the ability to measure or predict the
degradation characteristics of GS CP in the rumen. Waters and Givens (1992) first indicated that near infrared reflectance spectroscopy (NIRS) may provide a rapid method of predicting CP solubility and degradability characteristics using a small sample set of 19 non-ensiled ryegrass herbage. Several other reports have followed (Hoffman et al., 1999a; De Boever et al., 1998; Antoniewicz et al., 1995) which support this finding, but again using relatively small sample sets, mixed sample sets of grass and alfalfa or non-ensiled samples.

The objective of this study was to investigate the feasibility of using NIRS to predict ruminal degradability and intestinal digestibility of GS CP as determined by the in situ and mobile bag techniques. The second objective of this study was to ascertain the feasibility of using NIRS to predict essential AA composition of GS. Values for CP fractions, intestinal digestibility of CP and AA composition of GS at three stages of maturity are compared to those reported by NRC (2001) to assess their applicability to the lower Fraser Valley region of British Columbia.

3.1 Materials and Methods

3.1.1 Sample Collection and Preparation

Samples were collected as part of a customer service program of a feed company located in the lower Fraser Valley region of British Columbia. Samples of GS (114) harvested in 1993 and 1996 were collected from 91 farms. Data were collected regarding species, cutting (primary or regrowth) and additive application (Table 3.1). Samples were immediately frozen and stored in a chest freezer at -10°C until time of in situ incubation.

Upon thawing, DM was determined by drying the whole sample, in a forced air oven at 55°C until constant weight was achieved. The dried samples were ground through a 2-mm screen prior to incubation and reground through a 0.5-mm screen prior to chemical analyses.
Samples were analysed for N using a LECO FP-428 N analyzer (LECO Corp., St. Joseph, MI). The CP content of each sample was calculated as N x 6.25. Determinations of ADF and NDF were undertaken using an ANKOM Fiber Analyser #F200 (Ankom Co., Fairport, NY) according to the procedures outlined by Goering and Van Soest (1970) as detailed in Komarek et al. (1994). The AA composition of each GS sample was determined using the method of Bidlingmeyer et al. (1984) adopted by Waters (Waters Corp., Milford Mass). In this method, primary and secondary amines were derivatized with phenyl isothiocyanate (PITC). Reagents were removed by vacuum and the derivatives were separated by HPLC on a reverse phase column. Samples were not subjected to a preoxidation step for the subsequent quantification of methionine sulfone and cysteic acid.

3.1.2 Cows and Feeding

Six non-lactating Holstein cows, each fitted with a rumen cannula and a T-shaped duodenal cannula, were used in this experiment. Each cow was fed a daily diet consisting of 3.0 kg of a commercially prepared dairy concentrate (16% CP) and 5.5 kg of an alfalfa (60%) and grass hay (40%) mixture. The diet was fed in equal portions every 6 h to maintain a relatively stable rumen environment. Cows were cared for according to the Canadian Council on Animal Care (1993) guidelines.

3.1.3 In Situ Ruminal Incubation of Feeds

Dried ground GS (2 – 3 g) was placed in a nylon bag (5 x 10 cm; pore size, 52 μm; ANKOM Co., Fairport, NY) and incubated according to the procedure described by von Keyserlingk et. al. (1996). Each sample was incubated in quadruplicate (two replicates in each of two cows) in the rumen in reverse order for 96, 72, 48, 24, 12, 8, 4, 2 and 0 h. Replicates within
cows were pooled, ground through a 0.5-mm screen and analysed for N as described previously. No correction was made for possible contamination of the residue by microbial protein.

The difference between the CP content of the original GS sample and the residue at each incubation time point was used to calculate percent disappearance. Using the equation developed by Ørskov and McDonald (1979), modified by Dhanoa (1988) and Denham et. al. (1989), the soluble A fraction (CPA), potentially degradable B fraction (CPB) and the rate of disappearance of the B fraction (CPkd) was determined by non-linear regression for each sample. This was achieved using the Marquardt method, a linear iterative curve fitting process within the PROC NLIN procedure of SAS (SAS Institute Inc., Cary, NC). In keeping with the terminology of NRC (2001), the C fraction (CPC) was determined as 100 minus the sum of CPA and CPB. Grass silage CP (%) escaping ruminal degradation (GSRUP) was calculated according to NRC (2001) as:

\[ RUP = \text{CPB} \times \frac{(kp/\text{CPkd}+kp)}{1} + \text{CPC} \]

Equation 3.1

A ruminal outflow rate (kp) of 6% h\(^{-1}\) was used for all samples.

3.1.4 In Situ Intestinal Incubation of Feeds

Samples were incubated using the mobile bag technique of de Boer et al. (1987). Quadruplicate 1-g samples of GS were pre-incubated in the rumen of one cow for 12 h prior to insertion in the intestine. Using a T-shaped cannula, one bag was inserted into the duodenum of one cow every 30 min. Bags were collected from the feces and mechanically washed until the rinse water was clear. The DM content of the residue was determined by drying at 55°C until constant weight was achieved. Replicates within cows were pooled and ground through a 0.5-mm screen prior to N analyses as described previously. Total tract disappearance of CP was calculated as the difference in analyses between the residue and the initial sample.
Disappearance in the intestinal tract (CPIT) was calculated by subtracting the amount of sample CP that disappeared following 12 h incubation in the rumen from the amount of CP which disappeared from the total tract.

Digestibility of ruminally undegraded CP (RUPDig, %) was calculated as:

$$RUPDig = \frac{(CPIT/100-CP12) \times 100}{Equation \ 3.2}$$

3.1.5 Statistical Analyses

All statistical analyses were performed using JMP (Version 4.04, SAS Institute, Cary, NC). The Univariate procedure was used to compute least square means and standard errors for DM, CP, ADF and NDF. Least square means, standard error of the mean and significant differences for CP degradability and AA composition were established using oneway ANOVA with NDF group as the main effect. Significant differences between pairs of means were identified using the Tukey-Kramer HSD procedure. Differences between NDF levels were partially confounded by animal and period effects. Analytical variability was included in the error variance.

3.1.6 NIRS measurements and calibration

Duplicate samples of GS that had been dried and ground through a 2-mm screen were scanned using a NIRSystems 6500 (Foss NIRSystems Inc., Silver Spring MD) scanning monochromator. Samples were presented to the machine in a natural product cell (4.5 x 5.7 x 21 cm) and data collected every 2 nm from 400 nm to 2498 nm. The duplicate spectra, collected as log 1/R, were averaged for each sample.

The statistical software WinISI Version 1.50 (Infrasoft International LLC, Port Matilda, PA, USA) was used to develop equations for the prediction of CPA, CPB, CPC, CPkd, RUP,
RUPDig and essential AA composition. Samples in the calibration set were selected using the CENTER and SELECT options of this software. All equations were developed using the modified partial least squares (MPLS) regression technique (Martens and Naes, 1989). Combinations of scatter correction and derivatization treatments were used to transform the spectral data. The scatter treatments were none (NONE) or standard normal variate and detrend (SNVD) as described by Barnes et al. (1989). Three derivatization transformations (1,4,4,1, 2,6,4,1, and 3,10,10,1) and three wavelength segments (408 to 2498,8; 808 to 2498,8; and 1108 to 2498,8) were utilized. In total, 18 different calibration equations were evaluated for each constituent.

In order to compare equations, statistics generated by the WinISI statistical software were used. The first was the standard error of cross validation (SECV). In this procedure, the sample population to be used for calibration was randomly divided into several groups. One group was removed and a calibration equation developed using the remaining groups. This calibration equation was then used to predict the composition of the separated group. The procedure was repeated until all groups had been predicted. The SECV statistic was an average of the residuals between the predicted values and the actual laboratory values for each group of samples. Cross validation allowed all samples in the data set to be used for both calibration and validation. The second statistic was calculated as the difference between 1 and the variance ratio (1-VR). The variance ratio (VR) is computed as \( \frac{(SECV)^2}{(SD)^2} \) where SD is the standard deviation of the reference data. The statistic, 1-VR, estimates the amount of the total variation in the reference set which is explained by the cross validation error (SECV).

The RPD (ratio of standard deviation in the reference data to the standard error of cross validation) statistic, as presented by Williams (2001), provides a basis for standardizing the
SECV across equations. This statistic is calculated as standard deviation of the calibration data divided by the SECV for each equation. The RPD should be as high as possible. Values greater than 2.3 but less than 5 indicate the equation is suitable for use in screening programs only whereas, values between 5 and 10 indicate the equation may be used in quality control purposes (Williams, 2001).

3.2 Results and Discussion

3.2.1 In Situ Determinations for CP

The proximate composition of immature, mid-mature and mature GS samples, used in the in situ and NIRS studies is presented in Table 3.2. Immature GS contained significantly (P<0.01) more protein than samples classified as mid-mature or mature (17.8 vs 14.9 and 13.1%, respectively). NRC (2001) reported CP content of immature, mid mature and mature of 16.8, 16.8 and 12.7%, respectively. The content of ADF and NDF significantly (P<0.01) increased with plant maturity (Table 3.1). In general, the chemical composition of GS used in this study was similar to that reported by NRC (2001).

CP degradation characteristics for GS are presented in 3.3. The CPA fraction for the entire sample set ranged from 16.0 to 76.5% with a mean value of 49.4% (data not shown). von Keyserlingk et al. (1996) reported a mean CPA value of 41.1% (28.3 to 59.3%) for 20 samples of GS collected from the same geographical area. Samples of GS classified as mature contained significantly (P<0.01) less CPA than immature or mid-mature GS (3.3). While NRC (2001) reported CPA values of 60.1 and 60.4% for immature and mid-maturity GS, corresponding values observed in the current study were 53.2 and 48%, respectively. Mature GS analyzed in the current study contained a mean value of 39.9% CPA versus that of 47.9% reported by NRC (2001).
Proteolysis due to plant enzymes and clostridial bacteria continues through the ensiling process until acidic conditions are established (McDonald, 1981). Woolford (1984) reported that wilting encouraged proteolysis while Petit and Tremblay (1992) reported that it was the bacterial fermentation process, which had the greatest influence on the amount of CPA found in timothy silage. There are few reports in the scientific literature detailing the effect of maturity on CPA content of ensiled grass. Kohn and Allen (1995) observed higher degradation of CP in ensiled forages over those preserved as hay. Interestingly, increasing maturity resulted in a significant \((P<0.0001)\) decrease in CPA content of bromegrass, but had no effect on CPA content of reed canarygrass (Kohn and Allen, 1995). Forage CPA that is not converted to microbial protein in the rumen must be excreted which can have negative effects on milk production, reproduction and the environment (Jonker, et al. 1998). Further research would be of benefit in ascertaining the effect of maturity on the CPA content of ensiled grass, and could lead to revised recommendations regarding harvesting of grass for inclusion in diets of high producing dairy cattle.

The amount of grass silage CP potentially degraded in the rumen (CPB) ranged from 14.5 to 73.8% (whole sample set, data not shown) with a mean value of 40.4% which was similar to the value of 43.2% (13.4 to 58.3%) reported by von Keyserlingk et al. (1996).

There was no significant \((P>0.05)\) difference in CPB content of immature, mid-mature and mature GS (39.6, 42.5, 43.3%, respectively, Table 3.3) NRC (2001) reported corresponding values of 31.8, 31.0 and 37.1% for each of the three stages of maturity, respectively. A direct relationship between maturity and CPB content of non-ensiled grass has been reported in the scientific literature (Antoniewicz et al., 1995; Hoffman et al., 1993; Balde et. al., 1993). Antoniewicz et al. (1995) also reported the CPB fraction of regrowths cut 5 to 10 weeks after...
initial cutting. The amount of CPB appeared to be greater in regrowths as values ranged from 44.7 to 63.2% whereas values for primary growths ranged from 18.9 to 47.6%. In the current study, second and third regrowth samples had significantly (P<0.05) higher CPB contents than primary, first or fourth regrowth samples (47.1 and 50.2% vs 39.3, 36.6 and 40.2%, respectively. Data not shown).

The mean value of CPC, calculated as 100 minus the sum of CPA and CPB, was 7.1, 10.2 and 16.5% for immature, mid-mature and mature GS respectively (Table 3.3). These values were similar to those reported by NRC (2001). In addition to significant (P<0.01) difference in the amount of CPC in GS of different maturity (Table 3.3), there was a significant (P<0.01) difference between the CPC content of the of primary growth samples (12.0%, data not shown) versus samples from regrowth cuttings.

The mean value for the degradation rate of the CPB fraction (CPkd,) was 5.6% h\(^{-1}\) (whole sample set, data not shown) which is similar to the value of 5.1% h\(^{-1}\) reported by von Keyserlingk et al. (1996). The mean values of 5.2 and 4.8% h\(^{-1}\) for mid-mature and mature GS, respectively, were similar to those of NRC (2001). However, there was a discrepancy in CPkd values between the current study and NRC (2001) for immature GS (5.7 vs 8.1% h\(^{-1}\), respectively). There was no significant (P<0.05) difference in CPkd values between different stages of maturity (Table 3.3). This is in contrast to Balde et al. (1993) who reported a mean value for CPkd of 6.0% h\(^{-1}\) and found that increasing maturity of primary growth cuttings significantly (P<0.05) reduced the rate of degradation of CPB. Other reports (Antoniewicz et al., 1995; Hoffman et al., 1993; Waters and Givens, 1992) report higher values for CPkd in the range of 11.5 to 18% h\(^{-1}\). One reason for the discrepancy between the studies may be in experimental method used to calculate CPkd. In the current study and that of von Keyserlingk et al., (1996)
and Balde et al., (1993), bags were incubated in the rumen for 96h and the Marquardt procedure in NLIN was used to solve for CPkd. Statistical procedures used in the studies of Hoffman et al., (1993), Water and Givens (1992) and Antoniewicz et al., (1995) were varied. In addition, the time of ruminal incubation was 72, 72 and 48 h, respectively, in these studies.

NRC (2001) reports values for RUP of 21, 25.2 and 35.2% for immature, mid-mature and mature GS, respectively. The authors stipulate that these values are appropriate for animals whose DMI is 4% of BW, and the diet contains 50% of the DM as forage. Corresponding values observed in the current study for immature, mid-mature and mature GS were 28.1, 32.7 and 41.6% RUP, respectively (Table 3.3). Hoffman et al. (1993) reported RUP values, corrected for microbial protein contamination, of primary-growth orchardgrass at three stages of maturity as 18.3, 24.8 and 28.6%, respectively. One explanation for the higher RUP values noted in the current study over those observed by Hoffman et al. (1993) could be the decision to omit correction for protein from microbial contamination in the residue material collected after ruminal incubation. This will result in an underestimation of CP degradability in the rumen. Microbial contamination can account for 8 to 13% of residue dry matter (Michalet-Doreau and Ould-Bah, 1992) and will vary inversely to the CP content of the original sample. Therefore, maturity will also affect the amount of microbial contamination as demonstrated by Xu et al., (1996) who observed microbial CP as a percentage of total CP as 0 and 30%, respectively, in residues from in situ incubation of GS samples cut 37 and 66 d after first cut.

NRC (2001) estimated intestinal digestibility of RUP to be 65, 60 and 55% for immature, mid-mature and mature GS, respectively. Corresponding values from the current study were 59.1, 48.6 and 47.9%, respectively (Table 3.3). There was no significant (P>0.05) difference in the intestinal digestibility of RUP according to stage of maturity (Table 3.3) which is in contrast
to the report of Vanhatalo et al. (1996) who observed a significant (P<0.001) decrease in intestinal digestibility (75.6 to 56.3%) with advancing maturity of non-ensiled timothy grass. However, these authors (Vanhatalo et al., 1996) also reported a significant (P<0.001) increase in the CPA fraction with advancing maturity (20 to 49.1%). Van Straalen et al. (1993) noted the inverse relationship between ruminal CP disappearance and intestinal disappearance of CP. Vanhatalo et al. (1996) noted the effect of microbial protein contamination of the ruminal incubation residue used to determine intestinal CP disappearance. Failure to correct for microbial protein contamination in this study may have masked differences in intestinal digestibility of RUP due to maturity. In view of the conflicting results regarding ruminal CP disappearance in the study of Vanhatalo et al. (1996) and the failure to correct for microbial CP contamination in the current study, further research is required to clarify the impact of advancing maturity on intestinal digestibility of grass silage CP.

Ørskov and McDonald (1979) reported that a ruminal incubation for 24 h could possibly provide a direct estimate of CP degradability for most feedstuffs. Hoffman et al. (1999b) reported a strong correlation (r = 0.94) between 24 h ruminal incubation and the content of RUP in grass and legume silages. Earlier work by Hoffman et al. (1993) had shown a strong negative relationship (r = -0.81) between NDF content and ruminally degraded CP in samples of non-ensiled grass and legumes. Similarly, von Keyserlingk et al. (1996) observed a negative correlation (r = -0.76) between NDF content and effective degradability of CP in grass silage. For samples of GS used in the current study, there was a moderate relationship (r = 0.54) between initial sample NDF content and the RUP fraction (Table 3.4). The amount of CP in the sample had no relationship to the amount of CPA (r = 0.01) which is similar to the finding of von Keyserlingk et al. (1996). Crude protein disappearance after 12 h and 24 h ruminal incubation
was inversely related to RUP although the relationship was not as strong as shown by Hoffman et al. (1999b) (Table 3.4). This may, in part, be due to the lack of correction for microbial CP contamination which was completed in the study of Hoffman et al. (1999b) using an NIRS calibration to predict purine content of the nylon bag residue.

3.2.2 Amino Acid Analyses

The essential AA composition of GS, expressed on a DM basis for the total data set, and on a CP basis for three stages of maturity is shown in Table 3.5. There is disparity in the values reported in the literature regarding the AA composition of GS. This is not unexpected in light of the documented effect of proteolysis on AA composition of GS (Woolford, 1984; McDonald, 1981). Deamination and decarboxylation of amino acids, particularly Lys, Arg and His, is due to clostridial fermentation during the ensiling process (Woolford, 1984). The extent of proteolysis and AA catabolism during the ensiling phase will depend on how quickly acidic conditions are established, as shown by Jacobs and McAllen (1992) who compared two additive treatments, namely, formic acid and enzyme inoculant. The grass treated with formic acid at ensiling had significantly (P<0.05) greater concentrations of Lys and Thr than grass treated with exogenous enzyme. Concentrations of all other amino acids, except for Ala, tended to be numerically but not significantly (P>0.05) higher in the formic acid treated silage.

Previous reports in the scientific literature have shown that approximately 60 to 70% of grass silage CP is comprised of AA (Van Straalen et al., 1997; Xu et al., 1996). In the current study, total AA comprised 52.7% of CP, with no appreciable differences between GS of increasing maturity. Essential AA comprised approximately 26% of CP regardless of stage of maturity, which is numerically lower than the values of 32.57 and 32.9% reported by NRC
These values include Cys and Trp, which were not measured in the current study. Xu et al. (1996), reported AA accounted for of 29.72 and 33.14% of CP in GS of advancing maturity.

The AA concentration of the GS samples used in this study, expressed on a CP basis, is lower, with the exception of Arg, than those reported in the scientific literature (Berthiaume et al., 2001; Xu et al., 1996; von Keyserlingk, 1994) and by NRC (2001). Differences noted in Arg and Met concentrations may be due to differences in analytical methodology. Williams (1994) reported that AA analysis using PITC derivatives could overestimate the amount of Arg due to incomplete resolution from methionine sulfoxide and dehydroalanine. The omission of the preoxidation step to stabilize Met prior to subsequent analysis could reduce the recovery of Met by 25% (Williams, 1994).

It is not readily apparent why the concentration of the remaining essential AA is lower than reported in the scientific literature. The average CP content of the sample set used in the current study is greater by some 3% units (16.2%, data not shown) than in the samples analysed by Berthiaume et al. (2001) and von Keyserlingk (1994). Balde et al. (1993) and Xu et al. (1996) reported an inverse relationship between essential AA concentration and CP content. The size and diversity of the current sample set in terms of grass species, inclusion of regrowth samples, maturity within cutting, and inoculant use is greater than observed in the other studies and may have contributed to differences noted in AA concentration. The content of Thr, Val, Ile, Leu, Phe and Lys was significantly (P<0.05) lower in samples of fifth cut GS although reasons for this observation are not readily apparent (data not shown).

As mentioned previously, Lys is subject to decarboxylation during the wilting and ensiling processes. The mean value for Lys expressed as % of essential AA is approximately 8 % (Table 3.5) and does not change with increasing NDF concentration of GS. There is a wide
range in values (3.8 to 11.8%, data not shown) which encompasses the average values of 10 and
11% reported by NRC (2001) and Xu et al. (1996), respectively. Met, expressed as a % of
essential AA is less than the value of 3.7% published by NRC (2001). As discussed previously,
this observation can be attributed to analytical methodology.

3.3 NIRS

3.3.1 In Situ Determinations for CP

The derivative and scatter treatments, number of wavelengths and the statistical
evaluation of calibration equations developed to predict degradation of GS CP and AA
composition are presented in Table 3.6. For most constituents, the use of a second derivative
mathematical treatment produced the most acceptable equations based on the SECV, 1-VR and
RPD statistics.

The coefficient of determination (RSQ) values (Table 3.6) indicate that NIRS accounted
for 79 and 92% of the variation in the amount of CPA and CPB, respectively. These values are
markedly improved over that of 42% reported by Waters and Givens (1992) using in situ
reference data from 19 samples of fresh ryegrass. Todorov et al., (1994) reported RSQ values
of 0.84 and 0.87 for CPA and CPB, respectively using a set of 34 mixed forages. Hoffman et al.
(1999a) found a strong relationship between NIRS spectral data and CPA and CPB as indicated
by RSQ values of 0.96, and 0.96 respectively for a set of 34 grass and legume silage. It would
appear, therefore, that NIRS could predict the content of CPA and CPB in grass forages.
However, evaluation of the SECV, 1-VR and RPD statistics for each equation in the current
study suggest NIRS is not a suitable technique for prediction of CPA and CPB in routine forage
analysis programs as the RPD statistic for each equation is less than 2.3. De la Roza et al. (1998)
reached a similar conclusion based on RPD statistics of 2.12 and 2.34 for CPA and CPB,
respectively for a mixed set of corn and GS. Park et al. (1998) reported an RPD value of 3.33 for predicting CPA in 136 samples of GS. In this study (Park et al., 1998), CPA was determined using wet chemistry (total N minus acid insoluble N), suggesting that in situ reference data may lack precision necessary to build robust NIRS equations.

Similarly, NIRS was not successful in predicting either the CPC fraction or the rate of degradation of CPB (CPkd). Hoffman et al. (1999a) reported NIRS could explain 87% of the variation in CPkd values for 32 samples of grass and legume silage. However, De La Roza et al. (1998), using a mixed sample set of corn and GS, noted that the RPD statistic for the equation predicting CPkd was 1.35 which is similar to the RPD value of 1.2 calculated in the current study.

To the author's knowledge, only one other report has appeared in the scientific literature regarding the ability of NIRS to predict RUP as determined by the in situ technique. Hoffman et al. (1999a), using a set of 32 non-ensiled grass samples reported a coefficient of determination of 0.94. The samples used to build this calibration set contained a narrow range of RUP values (9.0-26.7%) unlike the current sample set which ranged from 16.2 to 61.5% in RUP content (data not shown). Based on an RPD statistic of 1.8, the equation developed to predict RUP of GS is not acceptable for use in routine forage analysis. Likewise, NIRS was not capable of accurately predicting the intestinal digestibility of RUP as based on a RPD statistic of 1.4. No other reports could be found in the scientific literature in regards to NIRS and prediction of RUP digestibility in the intestine.

3.3.2. Amino Acids

NIRS could account for 51% (Met) to 92% (Phe) of the variation in AA composition of GS. For all AA, except Met and Lys, RPD statistics met or approached the value of 2.3
indicating that NIRS could be used in forage screening programs to determine AA composition of GS. There appear to be no other scientific reports documenting the feasibility of using NIRS to predict AA composition of forage. Fontaine et al. (2001) reported that NIRS could be used to predict essential AA composition of vegetable and animal protein feedstuffs. These authors point out the importance of accurate reference data, and indicate the problems encountered with the analysis of sulphur-containing AA such as Met.

In conclusion, soluble CP (CPA) and slowly degradable CP (CPB) values for GS at three stages of maturity were obtained using the in situ technique and compared to values published in NRC (2001). CPA values decreased with increasing maturity and were less than those reported by NRC (2001). There was a direct relationship between CPB and maturity. The degradation rate of CPB was less than reported by NRC (2001) for immature GS. Values for CP not degraded in the rumen (RUP) observed in this study was numerically greater than reported by NRC (2001). Further research is required to ascertain intestinal digestibility of RUP in view of the discrepancy between observations noted in the current study and those estimated by NRC (2001). The content of essential AA expressed, as a % of CP was less than reported by NRC (2001).

NIRS was not successful in predicting CP solubility or degradability fractions determined by the in situ technique as shown by an RPD statistic less than 2.3 for each equation. Prediction of essential AA content was promising as RPD statistics for each equation, except Met and Lys, approached 2.3.

3.4 Acknowledgements

Financial support of the British Columbia Ministry of Agriculture, Fisheries and Food (Research Partnership Program) and ProForm Feeds Ltd is gratefully acknowledged.
Table 3.1. Demographics of 114 grass silage samples collected for NIRS calibration.

<table>
<thead>
<tr>
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<th>n</th>
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</thead>
<tbody>
<tr>
<td><strong>Cuttings</strong></td>
<td></td>
</tr>
<tr>
<td>First (primary growth)</td>
<td>40</td>
</tr>
<tr>
<td>Second (first regrowth)</td>
<td>18</td>
</tr>
<tr>
<td>Third (second regrowth)</td>
<td>15</td>
</tr>
<tr>
<td>Fourth (third regrowth)</td>
<td>11</td>
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<tr>
<td>Fifth (fourth regrowth)</td>
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</tr>
<tr>
<td>Unknown ¹</td>
<td>25</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td></td>
</tr>
<tr>
<td>Orchardgrass – Ryegrass Mixtures</td>
<td>40</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>26</td>
</tr>
<tr>
<td>Orchardgrass – Other Grass Mixtures</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Unknown ¹</td>
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<tr>
<td><strong>Additives</strong></td>
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</tr>
<tr>
<td>None applied</td>
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</tr>
<tr>
<td>Bacterial inoculant</td>
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</tr>
<tr>
<td>Other (Acid, Enzyme)</td>
<td>9</td>
</tr>
<tr>
<td>Unknown</td>
<td>35</td>
</tr>
</tbody>
</table>

¹ Data not collected by feed company.
Table 3.2. Proximate composition of grass silage samples used for NIRS calibration (mean value ± standard error).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immature (^1) (n=60)</th>
<th>Mid-Mature (^1) (n=40)</th>
<th>Mature (^1) (n=14)</th>
<th>Sig (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM %</td>
<td>37.0±1.8</td>
<td>40.3±2.2</td>
<td>37.0±3.7</td>
<td>NS</td>
</tr>
<tr>
<td>CP % DM</td>
<td>17.8 (^c) ±0.4</td>
<td>14.9 (^b) ±0.5</td>
<td>13.1 (^a) ±0.9</td>
<td>**</td>
</tr>
<tr>
<td>ADF % DM</td>
<td>31.0 (^a) ±0.4</td>
<td>37.8 (^b) ±0.5</td>
<td>41.0 (^c) ±0.8</td>
<td>**</td>
</tr>
<tr>
<td>NDF % DM</td>
<td>48.8 (^a) ±0.4</td>
<td>57.6 (^b) ±0.5</td>
<td>63.0 (^c) ±0.9</td>
<td>**</td>
</tr>
</tbody>
</table>

\(^1\) Samples are divided into maturity groups as follows: Immature = NDF<55%DM, Mid Mature = NDF 55-60%DM, Mature = NDF>60%DM.

\(^2\) Means within row with different superscripts are significantly different (P<0.01).
Table 3.3. Protein degradation characteristics of grass silage samples used for NIRS calibration (mean value ± standard error).

<table>
<thead>
<tr>
<th></th>
<th>Immature$^1$ (n=60)</th>
<th>Mid-Mature$^1$ (n=40)</th>
<th>Mature$^1$ (n=14)</th>
<th>Sig$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA$^3$ % CP</td>
<td>53.2$^b$ ±1.7</td>
<td>48.0$^{ab}$ ±2.1</td>
<td>39.9$^a$ ±3.1</td>
<td>**</td>
</tr>
<tr>
<td>CPB$^3$ % CP</td>
<td>39.6±1.6</td>
<td>41.8±2.0</td>
<td>43.6±3.1</td>
<td>NS</td>
</tr>
<tr>
<td>CPC$^3$ % CP</td>
<td>7.2$^a$ ±0.7</td>
<td>10.2$^b$ ±0.8</td>
<td>16.5$^c$ ±1.2</td>
<td>**</td>
</tr>
<tr>
<td>CPkd$^3$ % h$^{-1}$</td>
<td>5.7 ±0.3</td>
<td>5.2±0.4</td>
<td>4.7±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>RUP$^3$ % CP</td>
<td>28.1$^a$ ±1.3</td>
<td>32.7$^b$ ±1.6</td>
<td>41.6$^c$ ±2.4</td>
<td>**</td>
</tr>
<tr>
<td>RUPDig$^3$ % RUP</td>
<td>59.4±2.8</td>
<td>52.5±4.8</td>
<td>47.9±7.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^1$Samples are divided into maturity groups as follows: Immature = NDF<55%DM, Mid Mature = NDF 55-60%DM, Mature = NDF>60%DM.

$^2$Means within row with different superscripts are significantly different (P<0.01).

$^3$CPA = soluble CP, CPB = potentially degradable CP, CPC = 100 minus the sum of CPA+CPB, CPkd = rate of degradation of CPB, RUP = ruminally undegraded protein, RUPDig = intestinal digestibility of RUP.
Table 3.4. Correlation coefficients (r) between proximate composition or single point ruminal incubation and CP degradability.

<table>
<thead>
<tr>
<th></th>
<th>CPA(^1)</th>
<th>CPB(^1)</th>
<th>CPkd(^1)</th>
<th>RUP(^1)</th>
<th>RUP(\text{Dig})(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP % DM</td>
<td>0.01</td>
<td>0.28</td>
<td>0.19</td>
<td>-0.25</td>
<td>0.36</td>
</tr>
<tr>
<td>NDF % DM</td>
<td>-0.46</td>
<td>0.24</td>
<td>-0.20</td>
<td>0.54</td>
<td>-0.34</td>
</tr>
<tr>
<td>12 h CPDis(^2)</td>
<td>0.81</td>
<td>-0.72</td>
<td>0.45</td>
<td>-0.83</td>
<td>0.04</td>
</tr>
<tr>
<td>24 h CPDis(^2)</td>
<td>0.71</td>
<td>-0.51</td>
<td>0.59</td>
<td>-0.86</td>
<td>0.14</td>
</tr>
</tbody>
</table>

\(^1\) CPA = soluble CP, CPB = potentially degradable CP, CPkd = rate of degradation of CPB, RUP = ruminally undegraded CP, RUP\(\text{Dig}\) = intestinal digestibility of RUP.

\(^2\) 12 h CPDis = the amount of CP that disappeared from the sample after 12 h ruminal incubation, 24 h CPDis = the amount of CP that disappeared from the sample after 24 h ruminal incubation.
Table 3.5. Amino acid composition of grass silage samples used for NIRS calibration (mean value ± standard error).

<table>
<thead>
<tr>
<th></th>
<th>Total Set (n=91)</th>
<th>Immature (^1) (n=50)</th>
<th>Mid-Mature (^1) (n=30)</th>
<th>Mature (^1) (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.80 ±0.028</td>
<td>5.03±0.204</td>
<td>5.08±0.263</td>
<td>5.24±0.434</td>
</tr>
<tr>
<td>His</td>
<td>0.16±0.005</td>
<td>1.03±0.036</td>
<td>0.95±0.046</td>
<td>0.96±0.076</td>
</tr>
<tr>
<td>Ile</td>
<td>0.42±0.012</td>
<td>2.71±0.068</td>
<td>2.68±0.088</td>
<td>2.52±0.145</td>
</tr>
<tr>
<td>Leu</td>
<td>0.75±0.023</td>
<td>4.81±0.120</td>
<td>4.63±0.155</td>
<td>4.62±0.256</td>
</tr>
<tr>
<td>Lys</td>
<td>0.35±0.014</td>
<td>2.24±0.095</td>
<td>2.14±0.123</td>
<td>2.05±0.203</td>
</tr>
<tr>
<td>Met</td>
<td>0.10±0.005</td>
<td>0.66±0.036</td>
<td>0.60±0.046</td>
<td>0.71±0.076</td>
</tr>
<tr>
<td>Phe</td>
<td>0.48±0.016</td>
<td>3.06±0.084</td>
<td>2.92±0.109</td>
<td>3.02±0.179</td>
</tr>
<tr>
<td>Thr</td>
<td>0.42±0.015</td>
<td>2.67±0.093</td>
<td>2.62±0.120</td>
<td>2.56±0.199</td>
</tr>
<tr>
<td>Val</td>
<td>0.60±0.017</td>
<td>3.79±0.093</td>
<td>3.80±0.121</td>
<td>3.50±0.199</td>
</tr>
<tr>
<td>TAA % CP</td>
<td></td>
<td>53.4±1.31</td>
<td>52.5±1.69</td>
<td>50.4±2.79</td>
</tr>
<tr>
<td>EAA % TAA</td>
<td></td>
<td>48.6±0.43</td>
<td>48.3±0.56</td>
<td>49.8±0.92</td>
</tr>
<tr>
<td>Lys % EAA</td>
<td></td>
<td>8.1±0.22</td>
<td>8.2±0.29</td>
<td>8.0±0.48</td>
</tr>
<tr>
<td>Met % EAA</td>
<td></td>
<td>2.5±0.13</td>
<td>2.3±0.17</td>
<td>2.8±0.28</td>
</tr>
</tbody>
</table>

\(^1\)Samples are divided into maturity groups as follows: Immature = NDF<55%DM, Mid Mature = NDF 55-60%DM, Mature = NDF>60%DM.
Table 3.6. NIRS calibration equation performance for grass silage CP degradability and AA composition.

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>S</th>
<th>WL</th>
<th>RSQ</th>
<th>SECV</th>
<th>1-VR</th>
<th>RPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP %DM</td>
<td>1</td>
<td>NONE</td>
<td>2</td>
<td>0.95</td>
<td>1.00</td>
<td>0.93</td>
<td>3.7</td>
</tr>
<tr>
<td>CPA</td>
<td>3</td>
<td>SNVD</td>
<td>3</td>
<td>0.79</td>
<td>6.43</td>
<td>0.74</td>
<td>2.0</td>
</tr>
<tr>
<td>CPB</td>
<td>3</td>
<td>NONE</td>
<td>1</td>
<td>0.92</td>
<td>5.65</td>
<td>0.77</td>
<td>2.1</td>
</tr>
<tr>
<td>CPC</td>
<td>2</td>
<td>SNVD</td>
<td>1</td>
<td>0.70</td>
<td>2.74</td>
<td>0.54</td>
<td>1.5</td>
</tr>
<tr>
<td>CPkd</td>
<td>2</td>
<td>SNVD</td>
<td>2</td>
<td>0.47</td>
<td>1.33</td>
<td>0.34</td>
<td>1.2</td>
</tr>
<tr>
<td>RUP</td>
<td>1</td>
<td>SNVD</td>
<td>3</td>
<td>0.78</td>
<td>4.98</td>
<td>0.71</td>
<td>1.8</td>
</tr>
<tr>
<td>RUPDig</td>
<td>3</td>
<td>NONE</td>
<td>2</td>
<td>0.86</td>
<td>8.39</td>
<td>0.46</td>
<td>1.4</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>NONE</td>
<td>1</td>
<td>0.82</td>
<td>0.122</td>
<td>0.77</td>
<td>2.1</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>NONE</td>
<td>3</td>
<td>0.90</td>
<td>0.020</td>
<td>0.81</td>
<td>2.3</td>
</tr>
<tr>
<td>Ile</td>
<td>2</td>
<td>NONE</td>
<td>3</td>
<td>0.90</td>
<td>0.054</td>
<td>0.80</td>
<td>2.2</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>NONE</td>
<td>1</td>
<td>0.89</td>
<td>0.092</td>
<td>0.84</td>
<td>2.4</td>
</tr>
<tr>
<td>Lys</td>
<td>2</td>
<td>NONE</td>
<td>1</td>
<td>0.78</td>
<td>0.069</td>
<td>0.72</td>
<td>1.8</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
<td>NONE</td>
<td>1</td>
<td>0.51</td>
<td>0.040</td>
<td>0.37</td>
<td>1.2</td>
</tr>
<tr>
<td>Phe</td>
<td>2</td>
<td>SNVD</td>
<td>1</td>
<td>0.92</td>
<td>0.064</td>
<td>0.83</td>
<td>2.4</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
<td>SNVD</td>
<td>1</td>
<td>0.88</td>
<td>0.064</td>
<td>0.81</td>
<td>2.3</td>
</tr>
<tr>
<td>Val</td>
<td>2</td>
<td>NONE</td>
<td>3</td>
<td>0.90</td>
<td>0.074</td>
<td>0.82</td>
<td>2.3</td>
</tr>
</tbody>
</table>

1 D = Derivative treatment. 1 = 1,4,4,1; 2 = 2,6,4,1; 3 = 3,10,10,1.
2 S = Scatter treatment. NONE = no scatter treatment. SNVD = Standard Normal Variate and Detrend.
3 WL = Wavelengths. 1 = 408-2498;8. 2 = 808-2498;8. 3 = 1108-2498;8.
4 RSQ = coefficient of determination.
5 SECV = standard error of cross validation.
6 1-VR = 1 minus the validation ration.
7 RPD = ratio of standard deviation to SECV.
3.5 REFERENCES


CHAPTER 4.

GENERAL DISCUSSION AND CONCLUSIONS

4.0 Introduction

This research program was designed to meet three objectives. The first was to ascertain the feasibility of using the NIRS technique to rapidly and accurately predict ruminal degradability of DM and CP in CS and CP degradability in GS as determined by the in situ technique. The second objective was to develop calibration models to predict intestinal disappearance of DM and CP in CS and intestinal digestibility of CP in GS as determined by the mobile nylon bag technique. The last objective was to investigate the feasibility of using NIRS to predict the essential AA composition of CS and GS. The following discussion will outline results for each objective as well as recommendations for future research in this area.

4.1 Ruminal Degradability and Intestinal Digestibility of DM and CP

4.1.1 Corn silage

Rations balanced for ruminant animals traditionally include CS as a source of energy. The corn plant differs from grass in that energy is provided through ruminal degradation of non-structural (starch) and structural (stalk) carbohydrates. In rations formulated for high-producing dairy cows located in the lower Fraser Valley region, corn silage contributes only 10 to 20% of the protein requirement. However, CS can comprise over 35% of the dry matter intake of the same high producing cow. Work by von Keyserlingk et al. (1996) indicates significant variation between samples of CS grown in the lower Fraser Valley region in terms of DM and CP degradability. This phenomenon is not limited to this geographic region as shown by the work of Deaville and Givens (1998). Therefore, the ability to accurately assess the feeding value of CS in terms of ruminal degradation of DM and CP is important. The NIRS technique provides a
means of rapidly and economically meeting this objective. However, a search of the scientific literature yielded only one report investigating the feasibility of using NIRS to investigate ruminal degradability of CS. This study (De Boever et al., 2002) which was based on 26 samples of frozen, chopped CS considered only the ability of NIRS to predict the amount of CP escaping ruminal degradation (EP). These authors (De Boever et al., 2002) did not investigate the ability of NIRS to predict DM degradability, which is important in provision of energy to the cow.

The results of this study show that NIRS can play a role in screening CS DM and CP that is immediately soluble in the rumen (DMA). However, application will be limited in terms of predicting the amount of DM that is slowly degradable (DMB) or the rate at which DMB is degraded in the rumen. NIRS was able to predict the amount of CS CP that was effectively degraded in the rumen assuming a ruminal outflow rate of 6 % h\(^{-1}\).

Valentin et al. (1999) reported that sample preparation played a significant role in determining ruminal DM degradability. In their study, samples were chopped or ground through a 3 mm screen preceding incubation. Grinding increased significantly (P<0.05) the amount of soluble DM and effective DM degradability. Similar to the results of the current study, these authors (Valentin et al., 1999) found a highly significant (P<0.01) relationship between effectively degraded DM and soluble DM but only for samples that had been ground through a 3 mm screen. Samples in the current study were ground through a 2 mm screen prior to ruminal incubation. Grinding may have artificially increased ruminal DM degradability, hence resulting in the poor correlation between animal and spectral data. Further research work in this area may be designed to investigate the applicability of using NIRS to predict ruminal DM degradability.
using reference values determined using chopped samples ruminally incubated using macro
nylon bags.

Cozzolino et al. (2000) recently reported excellent results in terms of NIRS predicting in
vivo organic matter digestibility (IVOMD) as determined using the technique of Tilley and
Terry (1963). A calibration model, based on 290 samples of non-ensiled whole plant corn, was
able to account for 98% of the variation in IVOMD. An RPD of 5.4 for this model indicates that
it can be used in routine forage analyses programs. Ash comprised 1.5 to 16% of the DM
content of the samples used by Cozzolino et al. (2000) but it is not known if this range existed in
samples used in the current study. Pure minerals in ionic form or mineral salts do not absorb
energy in the NIRS region (Shenk and Westerhaus, 1994). Consideration should be given to
development of a calibration model to predict organic matter digestibility in CS as determined by
the in situ technique.

Of interest in the current study, was use of NIRS to predict the disappearance of CP from
CS following a 12 h ruminal incubation, as well as the disappearance of corn silage CP in the
intestinal tract. Ørskov and McDonald (1979) indicated that a single time point ruminal
incubation (24 h) may be adequate for determining ruminally undegraded CP. Halgerson et al.
(1995) and Hoffman et al. (1999) have reported on the feasibility of using NIRS to predict 12 h
and 24 h ruminal disappearance, respectively, of grass silage CP. A search of the scientific
literature, indicates that the current study is unique in documenting the possibility of predicting
ruminal disappearance of corn silage CP following a 12 h ruminal incubation. There is also no
previous work reported regarding the use of NIRS to predict intestinal disappearance of corn
silage DM or CP.
Cozzolino et al. (2000) reported correlation coefficients between IVOMD and either NDF or CP in CS of –0.49 and 0.48, respectively. Also of interest was the reported high correlation between ash and either ADF or NDF content (0.72 and 0.64, respectively). In the current study, the relationship between NDF content and ruminal DM degradability was low, indicating that correction for ash content may be important in developing NIRS predictions for ruminal DM degradability. Evaluation of the spectral data used in development of models to predict DMA and effectively degraded DM identified wavelengths associated with N-H bonding in amide molecules, thereby supporting the relationship between IVOMD and CP reported by Cozzolino et al. (2000).

In summary, results from the current study indicate that NIRS may not be an effective tool for predicting the ruminal degradability of DM in CS as determined by the in situ technique. However, further research is needed to assess the feasibility of NIRS predicting ruminal DM degradability if samples are left in a coarse or chopped form and incubated in a macro nylon bag. In addition, consideration should be given to correcting for ash if samples vary widely in ash content. NIRS was able to adequately screen for soluble CP content in CS as determined by the in situ technique. In light of the in vitro methods for the determination of CP fractions in forage (Kohn and Allan, 1995), and the small contribution of corn silage CP towards the nutrient requirements of the high producing ruminant, future calibration models may not rely on data obtained using the in situ technique as the reference method.

4.1.2 Grass Silage

Unlike CS, the energy density of GS is related to maturity and can be estimated by determination of ADF or NDF content in a laboratory (Van Soest, 1982). However, inclusion of GS in a diet for a high producing cow can contribute significant quantities of CP. The quality of
this CP, in terms of ruminal solubility and degradability, is influenced by species, fertilization, harvesting and ensiling practises (Woolford, 1984). Recently, NRC (2001) published recommendations for the nutrient requirements for dairy cattle. In this publication, values for CPA, CPB, CPC, CPkd, RUP and RUPDig were given for each of three classes of GS, defined by increasing NDF content. These values are a compilation of values published in the scientific literature. Where values for a parameter were unknown, estimated values, such as those for RUPDig, were incorporated (NRC, 2001).

While sample sets composed of grass silage and alfalfa silage have been used in NIRS studies to predict CP degradability (Hoffman et al., 1999), the only studies reported in the scientific literature using grass alone, used non-ensiled grass (Table 1.1). The wilting and ensiling processes can cause wide variation in the proportions of soluble and slowly degradable CP fractions in GS (Woolford, 1984) and therefore, research was needed to assess the feasibility of using NIRS to accurately and rapidly predict CP fractions in ensiled grass.

Numerical differences between NRC (2001) and the current study were noted for soluble CP for GS of mid-maturity (60.4 versus 48.0%, respectively), CPkd for immature GS (8.1 versus 5.8%, respectively) and RUPDig of mid-mature GS (60 versus 48.6%, respectively). Values for RUP and RUPDig obtained in the current study may be overstated due to the lack of correction for microbial protein as discussed below. However, it should be noted that NRC (2001) considered in situ data from 170 experiments and it is unknown how many of these experiments corrected in situ data for microbial protein contamination. For example, uncorrected data from previous work reported from this laboratory (von Keyserlingk et al., 1996) was included in the database.
Based on the RPD statistic generated for each calibration model developed in this study, it would appear that NIRS is not a suitable method for predicting ruminal degradability of grass silage CP as determined by the in situ technique. The most promising models were developed for CPA and CPB where RPD statistics approached 2.3 (2.0 and 2.1, respectively) which would enable these equations to be used in forage quality screening programs. It is not clear why NIRS failed to predict ruminal degradability of grass silage CP. As discussed below, the development of robust NIRS models is very dependent on accurate reference data. Halgerson et al. (1995) also made an interesting observation in that NIRS calibration models for predicting CP disappearance after a 12 h ruminal incubation were less robust when developed on a CP basis than on a DM basis. The former model incorporates the error incurred during the analytical procedure to determine N.

A search of the scientific literature failed to yield any reports of studies investigating the use of NIRS to predict intestinal digestibility of ruminally undegraded GS protein. RUPDig was not correlated to proximate constituents such as CP and NDF that are successfully predicted by NIRS.

4.1.3 The in-situ procedure

Although the technique used to develop an NIRS calibration model is important, the reference method chosen to generate the data used in the multiple regression process is of equal or greater importance. The accuracy of NIRS calibration models is directly related to the accuracy of the methods used to produce the reference data (Shenk and Westerhaus, 1994). For this study the in situ technique was chosen as the reference method to generate ruminal degradation values for DM and CP in CS and CP in GS. Although considerable effort has been expended to develop in vitro methods to predict ruminal degradability, no method has been
found which can be used across feedstuffs (Hvelplund and Weisbjerg, 2000). Therefore, the in situ procedure remains as the reference method to validate in vitro or NIRS techniques (Hvelplund and Weisbjerg, 2000).

Several reviews (Hvelplund and Weisbjerg, 2000; Michalet-Doreanu and Ould-Bah, 1992; Nocek, 1988) have outlined the inherent problems with the in situ technique including bag characteristics, animal effects and microbial contamination of the residue. A review of the in situ technique used in this study suggests several areas in which the method could be modified for use in future research.

In this study, non-lactating cows were used for in situ incubations. These animals were fed maintenance-type diets consisting of grain, as well as alfalfa and grass hay. Samples were incubated in duplicate in each of two cows.

Hoffman et al. (1999) reported that ruminal environment was the most influential source of error for in situ studies undertaken in their laboratory. They initiated a study to evaluate how many ruminally-cannulated cows are required to effectively estimate the average ruminal environment (Hoffman et al., 1999). Thirty cows at different stages of lactation (including dry cows), consuming one of nine different diets were used in the study. Based on the results, Hoffman et al. (1999) decided to use eight cows for in situ studies that generated reference data used to develop NIRS calibration models to predict RUP.

A minimum of three animals should be used in the in situ procedure according to standardization protocols implemented in Europe (Hvelplund and Weisbjerg, 2000). One problem commonly encountered during the current study was the disparity in disappearance data between two cows (Figure 1). A difference of 20 to 30% in disappearance of DM or CP between cows at specific time points did not seem biologically reasonable, and hence, the data were
discarded. This resulted in the elimination of approximately 10% of the data collected from the in situ analysis. Therefore, it is strongly recommended that each feedstuff under test is incubated in a minimum of three cows in future experiments utilizing the in situ method. Review of the data collected in the current study would suggest reducing the repetitions per cow is more acceptable as there were very few incidences of disparity between the duplicate bags placed within one cow.

In order to complete the current study, in situ studies were conducted during three individual time periods (1993, 1995 and 1997). It would have been of interest to assess the difference in DM and CP degradability using year as the main effect. In a study such as this where the investigator may wish to assess the variation in forage quality by year, it is necessary to remove the variation due to animal. Unfortunately, in this study, no record was kept as to identification of cow in each of the successive years. For example, cow A in 1993 may not have been cow A in 1995 or in 1997. Therefore, the observation of Nocek (1988), is reiterated in that that details regarding cow identification, physiological status, diet, feeding and management must be well documented.

Nocek (1988) recommended that cannulated animals be fed diets containing the same ingredient sources that are under test. Cone et al. (1999) studied the adaptability of the ruminal microbial population of cannulated cows to the forages being consumed and suggested that factors such as forage maturity can affect repeatability of in situ results. In this study, the forages fed to the test animals were alfalfa and grass hay, and not the silages under test. The extent to which this diet may have affected the degradability of CS and GS samples in the current study is unknown.
Microbial colonization of the in situ residue will have a minor effect on DM degradability but can result in the underestimation of CP degradability particularly for high-fiber, low-protein feedstuffs (Hvelplund and Weisbjerg, 2000). Xu et al. (1996) reported that microbial N comprised over 30% of the total N in GS cut 66 d (CP = 12.7%) after first cut versus 0% for GS cut 37 d (CP = 17.6%) after first cut. Correction for microbial protein contamination was beyond the scope of the current study. The extent to which non-correction for microbial protein affected the ability to produce robust NIRS calibration models for the prediction of CP ruminal degradability and intestinal digestibility is unknown. Research published since the beginning of this study regarding the use of purine derivatives (Johnson et al. 1998) may simplify the process of correcting for microbial contamination.

4.1.4 The mobile nylon bag procedure

In the current study, the mobile bag procedure of de Boer et al. (1987) was used to collect information regarding the intestinal disappearance of DM and CP in CS and CP in GS. In contrast to their recommendation to incubate feedstuffs in duplicate, four bags per feedstuff per cow were used to ascertain intestinal disappearance. Approximately 25% of these bags were not recovered from the feces or ruptured during the washing procedure. Data were discarded if fewer than three mobile nylon bags were recovered per sample. Van Straalen et al. (1993) reported bag recoveries of 99 and 97% in each of two experiments and losses of 6 and 9%, respectively. In view of the high loss rate experienced in this study, and the resulting need to discard data, it may be prudent to follow the example of Van Straalen et al. (1993) and Vanhatalo et al. (1996) and incubate six to 10 bags per feedstuff.

The mobile bag technique relies on a pre-incubation period in the rumen. As discussed previously, microbial protein contamination is of concern when high-fiber, low-protein samples
are incubated in the rumen. It can be postulated that the reference data used in the current study to develop a NIRS calibration model to predict intestinal digestibility of CS and GS crude protein was compromised by the lack of correction for microbial CP.

4.2 Amino acid evaluation

Limited work has been published regarding the use of NIRS to predict AA composition of feedstuffs. A search of the scientific literature failed to find any reports of NIRS predicting AA composition of forage. In this study, NIRS showed moderate promise in the prediction of AA composition of CS and GS. The RPD statistic was less than 2.3 for all essential AA in CS although RSQ and 1-VR statistics were promising. The relationship between spectral data and essential AA composition of GS was stronger in that the RPD statistic for all NIRS calibration models, except for Lys and Met, met or exceeded 2.3.

Recommendations for further work in this area would include reducing the grind size of the dried material to be scanned as more recent work has shown this to be beneficial (Fontaine et al., 2001). Investigations should also involve combining forages such as CS and GS in order to expand the range of AA composition in the reference database. It is also strongly recommended that wet chemistry procedures incorporate the correction step for determination of Met.

4.3 Future research in NIRS and forage quality.

Increasing pressure to establish environmentally friendly farming practices will drive future research to develop rapid and accurate methods of determining forage-feeding value. NIRS has become a routine method for evaluating the chemical composition of forages. A limiting factor to expanding the role of NIRS in predicting degradability and digestibility of feedstuffs is the lack of repeatable reference methods. The development of in vitro methods such as those discussed by Kohn and Allen (1995) hold the most promise in terms of providing
accurate reference data for development of robust NIRS calibration models to predict forage CP fractions. However, the in situ procedure will remain, at least for the time being, as the standard method against which in vitro and NIRS methods are tested (Hvleplund and Weisjberg, 2000). Therefore, future work using the in situ technique should focus on strategies such as increased replication and correction for microbial CP contamination that will increase accuracy and precision of the reference data.

In view of the strong correlation between 12 h and/or 24 h DM and/or CP disappearance, and ruminal degradability of DM and CP, further consideration should be given to the use of single time point ruminal incubations to replace the full in situ procedure. This could serve as a screening technique if a large number of samples are to be evaluated and would allow efficient use of animal, technical and analytical resources.

Results from this study show a significant variation in the amount of grass and corn silage CP contained in the soluble fraction. Soluble CP can be utilized in the rumen for microbial growth, provided there is an adequate source of readily available carbohydrate. Excess protein in the soluble form that is not incorporated in microbial protein, will be absorbed through the rumen wall and must be excreted in the form of urea. This has direct consequences on the energy balance of the lactating cow as well as reproduction and the environment. Future work in NIRS should focus on development of calibration models to predict soluble CP fractions in forages, as current wet chemistry methods require days versus hours to complete. In many cases, the forage in question will have been fed out before the analysis is received.

Forages play an important and ever increasing role in feeding dairy cattle. As research continues to provide information regarding feedstuff digestibility, and formulation software continues to utilize this information to formulate environmentally and economically friendly
rations, accurate assessment of forage feeding value will be critical. NIRS can provide the means to predict feeding value quickly and with precision.
Figure 4.1. Ruminal DM disappearance from two cows (2 bags per cow).
4.4 References


